



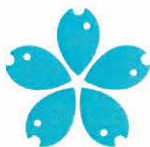
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Vodňany, 6.12.2016

Sdělení proděkana pro pedagogickou činnost č. 2/2016 Pravidla pro psaní disertačních prací na FROV JU

V návaznosti na Studijní a zkušební řád doktorských studijních programů na FROV JU (dále jen Studijní a zkušební řád DSP na FROV JU), článek 17 odst. 3 upravuje toto sdělení podrobná pravidla pro psaní disertačních prací na FROV JU.

- 1) Disertační práce se skládá z:
 - a) Obálky
 - b) Úvodní stránka
 - c) Prohlášení studenta
 - d) Stránka s obecnými informacemi (např. o školiteli; konzultantovi, laboratoři, kde student pracoval; děkanovi fakulty, fakultě, komisi obhajob práce apod.)
 - e) Obsahu disertační práce
 - f) Kapitoly 1 – General Introduction
 - g) Kapitol 2 až X – Publikované či akceptované články, popř. články odeslané do redakce k opublikování
 - h) Kapitoly X+1 – General Discussion, English Summary, Czech Summary, Acknowledgements, List of Publications, Training and Supervision Plan during Study, *Curriculum vitae*
- 2) Všechny kapitoly práce (s výjimkou českého souhrnu a českých názvů práce) musí být napsány v angličtině. Celkový rozsah práce by měl být 60–120 stran. Práce se zpracovává v programu Word (Microsoft Office), včetně tabulek, grafů a obrázků. Užívaný styl písma je Calibri. Grafy a obrázky (s výjimkou těch, které jsou součástí již publikovaných prací) musí být navíc dodány i v obrázkovém formátu (např. *.jpg, *.tiff, *.bmp, *.pdf) v dostatečném rozlišení.
- 3) Vlastní úprava disertační práce:
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- b) **Úvodní stránka** je prvním stránkou rukopisu odevzdávaného na studijní pracoviště před odesláním práce k jejímu oponování. Úvodní stránka obsahuje znak fakulty dle jednotného stylu v modré barvě umístěném v horní části stránky dle požadavků jednotného stylu JU. V horní třetině stránky je uveden název práce v Aj ve velikosti písma 24, tučně, řádkování 1, zarovnání do bloku. Pod Aj názvem je mezera (velikost písma 20, řádkování 1), za níž následuje název práce v Čj (velikost písma 14, tučně, řádkování 1, zarovnání do bloku). Mírně pod polovinou stránky je uvedeno celé jméno autora bez titulů práce v pořadí jméno (jména) a příjmení (velikost písma 14, kurzíva, zarovnání do leva). V zápatí se nachází text: Czech Republic, Vodňany, XXXX; kdy XXXX reprezentuje rok tisku práce (text je ve velikosti 14, zarovnání do leva). Okraje stránky a zarovnání textu vůči znaku fakulty jsou řízeny jednotným stylem JU.
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- e) **Obsah disertační práce** – Obsah práce zahrnuje všechny kapitoly počínaje kapitolou 1 (Obecný úvod) s jejich názvem a stránkováním. Podrozdělení v rámci jednotlivých kapitol na podkapitoly v obsahu je možné.
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V případě, že část nebo celá literární rešerše k tématu disertační práce byla opublikována v časopise s IF, je možno tuto kapitolu zkrátit a nahradit ji opublikovanou rešerší v časopise s IF. Písmo vlastního textu je Calibri, velikost písma 12, řádkování 1. Nadpisy kapitol jsou psány v písmu Calibri, tučně, velikost 12-14 dle úrovně kapitol. Doporučený rozsah této kapitoly je 10–20 stran. Další pokyny při psaní Kapitoly 1:

- Víceciferná čísla se neoddělují po 3 cifrách mezerou ani čárkou (Př. 6378 km, 2500000 x nikoliv 6 378 km; 6,378 km)
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- Obrázky – Označení a názvy obrázků jsou umístěny pod obrázkem. Např.: Figure 1. Map showing the locations of control (1, 2 and 9) and experimental (3–8) sections in the study area. Obrázky mohou být barevné, nicméně vlastní práce je kromě obálky tištěna černobíle. Barevné obrázky budou zahrnuty v pdf verzi práce, před vlastním tiskem budou ale barevné obrázky převedeny do stupně šedi. Tuto skutečnost je nutno vzít v úvahu, pokud by autor v obrázcích chtěl odkazovat na barevné odlišnosti apod.
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- *Př. citování periodika (časopisu)*
Bradford, M.M., 1976. Rapid and sensitive method for quantitation of mikrogram quantities of protein utilizing principle of protein dye binding. *Anal. Biochem.* 72, 248–254.
- *Př. citování knihy a ost. publikací*
Cheeseman, K.H., Slater, J.F., 1992. An introduction to free radical biochemistry. In: Cheeseman, K.H., Slater, T.S. (Eds.), *Free Radicals in Medicine*. Churchill Livingstone, New York, pp. 481–493.
Masopust, J., 2000. *Clinical Biochemistry*. Karolinum Praha, p. 429–429. (In Czech)
Kolarova, J., Velisek, J., Nepejchalova, L., Svobodova, Z., Kouril, J., Hamackova, J., Machova, J., Piackova, V., Hajslova, J., Holadova, K., Kocourek, V., Klimankova, E., Modra, H., Dobsikova, R., Groch, L., Novotny, L., 2007. *Anaesthetics for Fish*. RIFCH Vodnany, Methods, 77. 19 pp. (In Czech)
- *Př. citování příspěvků z konferencí*
Tveteras, R., Heshmati, A., 1998. Patterns of productivity growth and market conditions in the Norwegian salmon farming industry 1985–1993. In: Eide, A., Vassdal, T. (Eds.), *Proceedings of the 9th International Conference of the International Institute of Fisheries Economics and Trade*, Tromso, July 8–11, 1998, pp. 896–906.
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- **Poděkování (Acknowledgements)** – děkuje se především osobám, jež se podíleli na činnostech spojených s řešením tématu disertační práce a dále dalším osobám dle uvážení. Nedílnou součástí této kapitoly je i děkování projektům, z nichž byla činnost studenta na disertační práci podporována. Max. 1 strana.
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v Českých Budějovicích
University of South Bohemia
in České Budějovice
Czech Republic

Flagellar movement initiation, signaling and regulation of fish spermatozoa: physical and biochemical control

**Iniciace pohybu bičíku, signalizace a regulace pohyblivosti spermií ryb:
fyzikální a biochemické řízení**

Galina Prokopchuk

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Content

Chapter 1	8
General Introduction.....	10
Chapter 2	29
<i>In vitro</i> sperm maturation in sterlet, <i>Acipenser ruthenus</i>	441
Chapter 3	35
Volume changes during the motility period of fish spermatozoa: Interspecies differences	617
Chapter 4	697
Adaptations of semen characteristics and sperm motility to harsh salinity: extreme situations encountered by the euryhaline tilapia <i>Sarotherodon melanotheron heudelotii</i> (Dumeril, 1859)	49
Chapter 5	66
Control of sturgeon sperm motility: Antagonism between K ⁺ ions concentration and osmolality	68
Chyba! Záložka není definována.	
Chapter 6	76
Motility initiation of sterlet sturgeon (<i>Acipenser ruthenus</i>) spermatozoa: Describing the propagation of the first flagellar waves	78
Chapter 7	89
General Discussion	9182
English Summary	9504
Czech Summary	106
Acknowledgements	108
List of Publications	109
Training and Supervision Plan during Study	112
<i>Curriculum Vitae</i>	114

Chapter 1

General Introduction

1.1. Introduction

Fish has traditionally been, and remain, an important part of human nutrition. It is one of the essential sources of high-quality animal proteins, amino acids, minerals and healthy fats (Omega 3) for billions of people worldwide, particularly in low-income and food-deficit countries (Easterling, 2007; FAO, 2014; Rice and Garcia, 2011). Nowadays, fish account for about 17% of the global population's intake of animal protein and every year the consumption rate is growing in relation with expansion in the world's human population and economic development (FAO, 2014). The ever-increasing consumer demand for fish products has caused widespread overfishing of wild stocks and even extinction risk for some species (FAO, 2014). In turn, such large catches combined with the impacts of climate changes have led to the state that market can no longer be met by wild source alone. Fish farming is an alternative solution to supplement the growing commercial demand and reduce reliance on wild fisheries (Duarte et al., 2007). It involves raising fish in tanks, ponds, or ocean enclosures, providing a healthy, nutritional, high quality product (Li and Xu, 1995). Farming implies some forms of intervention in the rearing process (e.g., stocking, feeding, protection from predators); these management aspects result in a more stable and predictable supply than wild-catch fish. Like any actively developing production sector, successful and sustainable fish farming requires continuous improvement in various aspects of scientific knowledge and technological advances. For example, it is necessary to understand fish growth and reproduction, the size and age of spawning fish, mortality caused and rates, and many more (Bostock, 2011). In fact, a wide range of these issues have never received the proper amount of scientific attention and were studied only partially and in relatively few species. Accordingly, further innovative research is of strategic importance for solving practical problems that may arise during breeding manipulations under husbandry conditions.

One of the most important keys to efficient breeding management of farmed fish is control of the reproductive processes, namely, sexual maturation, spawning, and production of high quality gametes. These controls can be accomplished by environmental manipulations, such as photoperiod, water temperature or spawning substrate (Mylonas et al., 2010); however, sometimes it is impossible or at least impractical to regulate the reproductive performance, as the ecobiology of some fishes is not well known or is difficult to re-create (e.g., spawning migration, depth, riverine hydraulics, etc.) (Mylonas et al., 2010). Several assisted reproductive technologies are applied so as to overcome these problems, for example *in vitro* gametogenesis, artificial insemination, multiple ovulations, *in vitro* fertilization and embryo transfer (Pukazhenthil et al., 2006; Swanson, 2006). To date, the most common and widely used method in many breeding programs is artificial insemination. This technique involves the collection of sperm and ova and their mixing together in various media that maintain spermatozoan motility (Bellard, 1988; Suquet et al., 1995; Williot et al., 2005). The primary advantage of the artificial insemination is that the genetic potential of the best males can be transferred to a large number of offspring (Clark, 1950). Captive maintenance may alter natural spawning and may result in losing the capacity for spontaneous mating as well as some forms of reproductive dysfunctions of many commercially important species, so artificial insemination becomes a prerequisite for offspring production (Billard, 1978; Billard et al., 1974; Mylonas et al., 2010). In fish species such as sea bass, sea bream and Atlantic cod, the interest in artificial insemination is rising as the requirements for domestication and genetic selection increase (Billard, 1978; Moksness et al., 2004).

Successful insemination depends on metabolism and appropriate functioning of gametes (Bromage and Roberts, 1995; Kjørsvik et al., 1990; Mylonas et al., 2003). Up until now, the fish farming industry has been mostly concerned about the quality of eggs rather than that of sperm (Carral et al., 2003; Gorshkov et al., 2004). Nevertheless, poor sperm quality can also be a limiting factor that can affect the efficiency of artificial egg fertilization and thus, the total production of fingerlings (Bobe and Labbé, 2010). Semen characteristics – its quality, productivity, ejaculate volume and spermatozoan concentration – are highly variable between species, breeds, individuals and even portions of sperm from the same male obtained at different times (Detlaf et al., 1993). Numerous studies have revealed that males reared in captivity often produce milt of lower quantity or quality (Billard, 1986, 1989; Mylonas et al., 1998; Suquet et al., 1992). Repeated handling and stripping during the course of the spawning season may contribute to stress (Zohar and Mylonas, 2001). In addition, many other factors may be involved, e.g. biological characteristics of broodstock (Bezdicsek et al., 2010; Hanus et al., 2011; Štolc et al., 2009), rearing conditions (Cеровsky et al., 2009; Jacyno et al., 2009), environmental pollutants (Adeparusi et al., 2010), physiochemical properties of water (e.g. pH, salinity, temperature, oxygen concentration, turbidity, flow rate and photoperiod), hatchery practices (Pankhurst and Van der Kraak, 1997; Sumpter et al., 1994), artificial induction of spawning, seasonal changes or post stripping (Alavi et al., 2008b; Mylonas et al., 2003); all of these might affect semen quality at different levels of the broodstock husbandry or during collection (Dreanno et al., 1998; Linhart and Billard, 1994; Poupard et al., 1998) and storage of sperm *in vitro* (Hajirezaee et al., 2010). Hence, it is necessary to conduct sperm quality analysis before proceeding in a program of artificial insemination, especially when few males are used, so that the best samples can be selected (Bondari, 1983; Williot et al., 2000).

Quality and fertilizing potential of an ejaculate generally is assessed by several criteria, such as sperm morphology, motility profiles, concentration, seminal plasma osmolarity and pH, viability, and membrane integrity (Billard et al., 2007; Cosson, 2004; Krol et al., 2006; Suquet et al., 1994). Among these parameters, sperm motility is the most commonly used and appears to best correlate with fertility (Cosson, 2008a; Martinez-Pastor et al., 2008; Rurangwa et al., 2004). For a long time, movements of spermatozoa were subjectively estimated under low magnification (10-20×) of a phase contrast or a dark field microscope (Guest et al., 1976; McMaster et al., 1992). However, such visual evaluations provide only a coarse, inaccurate analysis, as it allows only approximate appraisal of overall percentage of motile spermatozoa (Levanduski and Cloud, 1988) and duration of progressive movement (Duplinsky, 1982). Later, Cosson et al. (1985) introduced the use of computerized sperm tracking systems, which considerably improved the accuracy and made motility analysis of fish sperm more reliable (Billard and Cosson, 1992). This method includes video recording of sperm movement via a microscope, followed by processing the recordings, either manually using image software, or automatically (Cabrita et al., 2009; Fauvel et al., 2010). Assessment of sperm motility with the computer-assisted methodology, is rapid and sensitive, allows to statistical analysis of a very large number of spermatozoan characteristics on which to quantify different sperm motility parameters, such as sperm velocity, head displacement, linearity and straightness of tracks, as well as precise percentage of motile cells and duration of motility; these data cannot be collected manually (Cosson, 2008a; Kime et al., 2001; Rurangwa et al., 2004). One limitation of this type of quality assessment is that wave propagations of sperm flagella previously could not be identified and determined. However, more recently, this issue was solved by application of different types of high-speed video recording and stroboscopic light sources, which allowed observing both the head displacement and the successive positions of sperm

flagellum. This advanced approach provides descriptions of flagella shape, beating frequency, variations in number and velocity of flagella waves, their length, amplitude and bend angle (Cosson, 2008a, 2010).

The appropriate evaluation of gamete quality cannot rely on one single approach, as none of them alone can accurately predict reproductive success. Development of novel biological technologies for improved gamete storage and effective quality assessment, dedicated fertilization procedures and more reliable predictors of fertility, as well as optimization of conservation programs have become one of the highest research priorities. Despite considerable progress achieved in understanding factors which are involved in the control of gamete quality, the picture of the cellular and molecular mechanisms responsible for the observed variability in quality remains largely incomplete. There is clearly still much to be done in the fields of reproductive strategy and fish breeding industry in general.

1.2. Fish reproductive system, spermatogenesis and sperm maturation

The reproductive system of male fish include testes, testicular gland, testicular main ducts (vas efferentia), sperm ducts (vas deferens) and blind pouches (Lahnsteiner et al., 1993a; Lahnsteiner et al., 1993b, 1994; Legendre et al., 1996). The morphometry of testes and accessory organs varies between species. For instance, in teleosts, the sperm duct originates from the posterior region of each testis and leads to the urinary papilla located in between the anus and urinary ducts, i.e. is not a part of the nephric or Wolffian duct (Alavi et al., 2008a; Coward et al., 2002). In contrast, the efferent ducts of chondrosteans, develop in close association with the kidneys, so the testicular sperm is supposedly mixed with urine during passage (Krayushkina and Semenova, 2006; Wrobel and Jouma, 2004). The testes serve two main functions: they support spermatogenesis and produce androgens that are important for the regulation of reproduction (Miura, 1998).

Reproductive cycle in fish is separated into two major phases, both controlled by the reproductive hormones of the brain, pituitary and gonad. It starts with spermatogenesis, when gametes proliferate mitotically, grow and differentiate, and continues with spermiogenesis; during the latter phase spermatozoa mature and are prepared for the release (Stanley, 1969; Stanley, 1971; Zirkin, 1975). In the course of the spermatogenesis, a few primordial germ cells are transformed into many highly differentiated spermatids carrying a haploid genome (Schulz et al., 2010). During the spermiogenesis, the spermatids proceed through a morphological metamorphosis, such as nuclear remodeling, organelle assembly and flagellum formation, and as a result, flagellated spermatozoa destined or capable of to contribute fertilization are produced (Billard, 1986; Pudney, 1995; Vizziano et al., 2008). Even though, fish spermatozoa in the testes are already morphologically developed, they may not be physiologically capable of becoming motile (Schulz et al., 2010). Following a species-specific reproductive cycle, spermatozoa are stored in the efferent duct system, where they meet the seminal fluid and mature, until spermiation and release occurs (Ciereszko, 2008; Lahnsteiner, 2003). As an example, the final process of spermatozoa maturation in salmonid fish occurs outside of testes, they acquire motility while passing along the sperm duct (Morisawa and Morisawa, 1986). Sperm maturation in this species and in mammals as well (Okamura et al., 1985) is mediated by extracellular concentrations of bicarbonate ions (HCO_3^-) and alkaline pH (7.8–8.15) of seminal plasma (Morisawa and Morisawa, 1988). It is postulated that these factors activate the adenylyl cyclase which leads

to an increase of intracellular cAMP concentration and thus to activation of sperm motility through phosphorylation of some flagellar proteins (Morisawa et al., 1993; Tash, 1990; Visconti et al., 1995). For acquisition of motility in Japanese eel spermatozoa, the presence of K^+ ions in addition to both external factors mentioned above is also required (Miura et al., 1995; Ohta et al., 1997). On the other hand, bicarbonate ions were shown to inhibit sperm motility in flatfish through the action of carbonic anhydrase (Inaba et al., 2003).

As seen above, maturing events are highly diversified between species and that is only a few species of teleost fish studied so far [rainbow trout, chum salmon, and Japanese eel (Miura and Miura, 2001; Morisawa and Morisawa, 1986)]. Further, chondrosteian fishes have a quite divergent excretory-reproductive system, the physiological process underlying sperm maturation in this phylogenetic group of species has not been described at all. As already noted, the sperm and urinary ducts in sturgeon are not completely separated and testicular sperm released into Wolffian duct seems to be diluted with urine (Alavi et al., 2012). Apparently, it is exactly the dilution with urine that would explain the low osmolality, ionic concentration, and protein content in thinned sturgeon's semen (Piros et al., 2002). In addition, it can be assumed that urine may play some role in the process of sturgeon spermatozoa maturation. Nevertheless, the existence of such a dilution process and moreover urine-involved sperm maturation remain to be established.

Accordingly, one of the objectives of this study was to improve the understanding of the mechanism of the acquisition of potential for sperm motility in sturgeon.

1.3. Activation of sperm motility

In almost all animal species, it is usual for spermatozoa to become motile either during or immediately following their release from storage within the male body. In species utilizing external fertilization, such as many echinoderms (Trimmer and Vacquier, 1986), fishes (Stoss, 1983) and amphibians (Hardy and Dent, 1986), spermatozoa, which are inactive in testis and seminal fluid, become motile once they are diluted into the surrounding water column at spawning.

It was shown that spermatozoan activation and subsequent motility are controlled by external conditions originating from the composition of the surrounding medium (Cosson, 2010; Morisawa, 1994). Among them are mainly environmental osmolality, ionic (K^+ , Ca^{2+} , Mg^{2+}) and gaseous components of external milieu and, in some cases, egg-derived sperm-activating substances or specific proteins from egg chorion (Alavi and Cosson, 2006; Inaba, 2007). For example, in salmon, as mentioned, the sperm activation results primarily from a combined regulation via an effect of external pH (Alavi and Cosson, 2005) and via an augmentation of the internal cAMP concentration (Morisawa et al., 1991). In trout, a large amount of data have demonstrated that K^+ concentration, combined with osmolality are both responsible for the extracellular signaling that triggers motility (Cosson, 2004; Morisawa, 1994). In herring, spermatozoa are not motile in seawater at spawning and activate only after sperm contact activating compounds released in the vicinity of eggs (Morisawa et al., 1992; Yanagimachi et al., 1992). In Nile tilapia, sperm motility is inhibited by a glycoprotein present in the seminal fluid and activation occurs when dilution is sufficient to significantly decrease seminal plasma concentration of this glycoprotein (Mochida et al., 1999). In other fish species, such as bitterling or fat minnow, it was revealed that initiation of sperm motility occurs after contacting activating chemicals generated by the micropyle area of the egg (Suzuki, 1958).

Recent data published by Yanagimachi et al. (2013) showed that sperm attraction by egg, especially by its micropyle, are quite common to many fish species. In a group of marine flatfishes, such as turbot, it was shown that motility activation is under control of dissolved CO₂ in equilibrium with HCO₃⁻ (Cosson et al., 2008b; Inaba et al., 2003). Another gas, NO, at very low concentrations induce motility of fathead minnow spermatozoa (Creech et al., 1998). It appears from the above list of examples that fish spermatozoa possess wide range of specific signaling pathways for motility activation, which are quite different in detail, even though most share a similar chain of events that involve specific activating molecules emanating from egg, ions fluxes, transduction of the signal inside the cell and/or activating response at the axonemal level (Darszon et al., 2008; Dzyuba and Cosson, 2014).

The activation process can be distinguished temporally as follow: due to difference of osmolality between the seminal fluid and the surrounding medium (fresh or sea water), an osmotic and/or ionic signal is perceived at the sperm membrane level (Alavi and Cosson, 2006; Morisawa and Suzuki, 1980; Perchec Poupard et al., 1997). Thereafter, flagellar waves start their propagation from head to tip at high beat frequency, leading to the forward displacement of the spermatozoa for hydrodynamic reasons (Boryshpolets et al., 2013; Cosson, 2008a).

1.3.1. Osmotic control of sperm motility activation

Osmolality constitutes a wide-spread controlling signal involved in activation of motility among many species (Morisawa et al., 1991). Reduction in ambient osmolality is the main factor in initiation of spermatozoa motility in cyprinid fishes (e.g. carp) as well as in other freshwater fishes (Alavi and Cosson, 2005, 2006; Perchec Poupard et al., 1997). In the case of marine species, it is opposite: an increase of the surrounding osmolality from seminal fluid to seawater is the main agent triggering flagellar motility (Cosson et al., 2008b), although in several species, changes in the phosphorylation state of some flagellar proteins also may be involved (Zilli et al., 2008a, b). Alteration of environmental osmolality leads to a water movement through the cell membrane to equilibrate the concentrations of solute from both sides, eventually activating a biochemical cascade, which leads to spermatozoa motility (Krasznai et al., 2003). Such water transfer may alter cytoplasmic volume of spermatozoa that is accompanied by a reorganization of the elastic membrane structure and by hyperpolarization of the cell membrane (Krasznai, 2003). For example, in case of carp spermatozoa that swell subsequent to activation conditions (Dzyuba et al., 2008; Dzyuba and Kopeika, 2002; Perchec et al., 1995), hypo-osmotic shock leads to membrane potential alteration that induces opening of the voltage-gated potassium channels and thus, decrease of intracellular K⁺ (Krasznai et al., 1995). The K⁺ efflux causes membrane potential changes, which provoke an activation of stretch-dependent Ca²⁺ channels and initiation of sperm motility through a calmodulin-dependent signaling cascade (Krasznai et al., 1995; Krasznai et al., 2003; Perchec Poupard et al., 1997). In general, water transport can take place according to two possible options: passive diffusion, in some cases facilitated through water channel proteins (aquaporins), or actively, through ion co-transporters (Goodman, 2002). As in case of carp spermatozoa the osmolality response is very fast; it might be suggested that sperm swelling at the moment of activation results from water transport mainly through aquaporins and after due to water diffusion that constitutes a much slower process (Verkman, 1992). Taken together, it may be proposed that the first step of membrane reception of the osmolality signal in carp spermatozoa is relayed by a rapid communication of this signal to the

sperm surface (Dzyuba and Cosson, 2014), due to involvement of aquaporins (Zilli et al., 2011), followed by a stretch-activated mechanism (Cosson, 2004; Cosson et al., 2008a; Zilli et al., 2012). Similar suggestion of activation events was previously suspected for turbot spermatozoa (Cosson et al., 2008b).

Some sub-populations of sperm having the ability to swell, were also reported in rainbow trout, but in this case, spermatozoa were incubated in hypotonic, non-activating solution (Cabrita et al., 1999). It is worth noting that in rainbow trout, spermatozoa do not possess aquaporins, and water transport is not a major process for spermatozoa activation (Bobe and Labbé, 2010). Hence, involvement of cell volume alteration in spermatozoa motility signaling pathways may be species-specific.

The osmolality activating signal is reversible after re-exposure to the initial osmolality environment (Cosson et al., 1999); a second motility period can be observed in marine species as well as in freshwater fish species (Cosson, 2010). Even though, osmolality surrounding sperm cells is considered as the main signalling activation for fish sperm, other factors also may be required for activation of fish sperm motility. The osmolality represents a combined effect of ions and/or molecules (so-called solute) that contribute non-specifically to the molar composition of the medium surrounding spermatozoa; therefore, it is difficult to separate osmolality effects on sperm cells from the effects of ionic and gaseous composition (Alavi and Cosson, 2006; Cosson, 2004; Morisawa and Yoshida, 2005). In salmonids (trout or salmon) and in chondrosteans (sturgeon or paddlefish), the regulation of sperm motility is mostly attributable to a downward shift of the K^+ ions concentration from seminal fluid to freshwater such that the accompanying osmotic pressure shift is not the most crucial (Morisawa, 1980; Morisawa, 1985, Cosson, 2004). These fish species possess a so-called ionic mode of sperm motility activation (Alavi and Cosson, 2006). Moreover, sturgeon spermatozoa have been shown to have their motility activated not only in hypotonic aquatic environment, but also in media isotonic, or even slightly hypertonic to the seminal fluid (Alavi et al., 2011; Cosson et al., 1999; Dzyuba et al., 2013). It seems that spermatozoa of fishes with ionic mode of motility activation are incapable of volume changes at their motility initiation and during their motility period. In this regard, also it is not clear, how these sperm cells maintain a constant volume under hypotonic conditions and if activation of stretch-dependent channels is involved. The present study attempts to verify some of the above suggestions and shed light on the mechanisms of volume changes in fish species with different modes of motility initiation.

1.3.2. Signaling for motility activation in euryhaline fishes

As mentioned above, motility activation of some fish spermatozoa is not so strictly dependent on environmental osmolality. This is also the particular case for euryhaline fish, such as medaka, where motility may be initiated in media with osmolality ranging from 25 to 686 mOsm.kg⁻¹ (Yang and Tiersch, 2009). Euryhaline fishes can acclimate to wide range of salinities, from freshwater to seawater or even higher (Laudet et al., 2012; Panfili et al., 2004) where they can reproduce and possess unique sperm osmotic sensitivity. In the tilapia, *Sarotherodon melanotheron heudelotii*, sperm can modulate their regulatory mechanism according to rearing salinity of the broodfish (Legendre et al., 2008). It was shown that reproductive success of this species under varying salinities is ensured by expression of testis genes (Avarre et al., 2014). Experiments using demembrated sperm of another euryhaline tilapia, *Oreochromis mossambicus*, revealed that Ca^{2+} ions play a key role in this adaptive ability and are necessary for activation of sperm motility (Linhart et al., 1999; Morita et al.,

2003, 2004). Here, motility activation requires an increase in the intracellular Ca^{2+} ions concentration, but signaling pathway by which Ca^{2+} mobilization and, ultimately, motility occurs is different in sperm of freshwater- and seawater-acclimatized tilapia (Morita et al., 2003, 2004). Under hypotonic conditions, such increase was found to occur via intracellular Ca^{2+} stores released by osmotic shock (Morita et al., 2003). Therefore, it is proposed that acclimation of motility regulatory mechanisms in tilapias takes place due to the modulation of the flow of Ca^{2+} supply. So far, the respective roles of osmolality and Ca^{2+} in the control of sperm activation of euryhaline fish are not fully clear. In the current work, the adaptive mechanisms enabling reproduction of euryhaline tilapia in a broad range of salinities has been explored.

1.3.3. Involvement of ionic components in the activation cascade

Rise of internal Ca^{2+} concentration is also known to regulate motility initiation in sperm of puffer fish (Oda and Morisawa, 1993), ascidians (Nomura et al., 2000; Yoshida et al., 1994; Yoshida et al., 2002), carp (Krasznai et al., 2000) and Salmonidae (Boitano and Omoto, 1992; Cosson et al., 1989; Kho et al., 2001). According to the present knowledge, the general signaling pathway controlling motility is viewed as a cascade of interactions between small molecules and catalysts leading to phosphorylation of protein controlling sperm flagella motility (Cosson, 2008b). At motility activation, the main signals responsible for the transfer of information from the membrane to the axoneme are involving membrane polarization, Ca^{2+} entry in the sperm cell, intracellular cAMP rise and phosphorylation of some specific protein components of the axoneme, depending on species (Cosson, 2008b; Dzyuba and Cosson, 2014).

The signaling pathway at motility activation was extensively studied in salmonid fishes, especially in trout (Morisawa et al., 1983). Motility activation of mature salmonid and chondrosteian spermatozoa is inhibited by low concentration of potassium ions and decrease in extracellular K^+ concentration triggers the initiation of flagellar motility (Cosson, 2004; Morisawa and Suzuki, 1980; Morisawa and Morisawa, 1986). The decrease in extracellular K^+ is the first signal, which induces K^+ efflux that in turn leads to hyper-polarization of the plasma membrane (Blaber and Hallett, 1988; Boitano and Omoto, 1991) and Ca^{2+} influx through dihydropyridine-sensitive calcium channel (Cosson et al., 1989). Subsequently, cAMP is produced (Morisawa and Okuno, 1982) that induces phosphorylation of axonemal proteins via a tyrosine-protein kinase, which in turn initiates flagellar motility (Hayashi et al., 1987).

Interestingly, the addition of extracellular Ca^{2+} promotes initiation of trout sperm motility, even in the presence of up to $10 \text{ mmol.l}^{-1} \text{ K}^+$ (Cosson et al., 1991; Tanimoto et al., 1994). In addition, motility can be suppressed by addition of Ca^{2+} channel blockers (Tanimoto et al., 1994). Thus, the increase in internal Ca^{2+} rather than efflux of K^+ was considered to play a major role in the initiation of motility. On the other hand, Boitano and Omoto (1991) showed that the membrane potential is associated with motility initiation. Therefore, this set of results suggests that membrane hyper-polarization and Ca^{2+} influx may contribute independently to an increase of the cAMP production. However, there are several species where cAMP is not needed in this process, such as striped bass spermatozoa for example (He et al., 2004). This proposed model has only speculative value, as some of steps remain to be elucidated, such as the involvement of water channels in salmonid sperm.

In some conditions, K^+ inhibition of salmon sperm motility can be by-passed (Morita et al., 2005) by its exposure to a hyper-osmotic shock prior to transfer into a K^+ -rich swimming

solution. More recently, such K^+ by-pass effect (osmolality dependent) was shown to be accompanied by a transient increase of intracellular Ca^{2+} concentration followed by protein phosphorylation steps leading to motility (Takei et al., 2012).

Despite the similarity between modes of sperm activation, cascade of signal transduction at motility initiation in chondrosteian spermatozoa in contrast to salmonids is not fully studied. There is only a hypothetical model based on observations for salmonids sperm (Alavi et al., 2011). However, there might be variance between these species. For example, it was recently discovered that sturgeon spermatozoa could remain in the quiescent stage even in a K^+ -free solution, just due to the hypertonicity of this solution (Judycka et al., 2015). The present study was designed to explore the process of motility initiation and its regulation in sturgeon spermatozoa.

1.3.4. Phenomenology of the initiation step of flagellar movement

Fish sperm motility is acquired under the control of many extrinsic and intrinsic factors and relies on the specialized structure of the sperm flagellum called "axoneme" (Cosson, 2010). Fish spermatozoa belong to a simple "aquasperm" consisting of a head that is comprised of a nucleus, mid-piece with centrioles and mitochondria, and a motility device, the axoneme of the flagellum (Cosson, 2008b; Jaspers et al., 1976; Lahnsteiner and Patzner, 2008). The behavior of the flagellum actually determines the guideline of a spermatozoon. The flagellar membrane of sperm in some fish, salmonid and sturgeon among them, have fins that extend along most of the length on each side of the flagellum (Billard, 1983; Cosson et al., 1999). It was recently shown that the presence of these lateral extensions contributes to improved swimming performance of fish spermatozoa (Gillies et al., 2013). The ultra-structure of a sperm flagellum comprises an axoneme, built as a scaffold of nine double microtubules constituting the periphery of a cylinder with two single microtubules in the center (Gibbons, 1981). The biochemical composition of the axoneme is a complex arrangement of at least 500 different protein subunits used by the flagellum for its operation (Diniz et al., 2012; Piperno, 1991). Each microtubule doublet includes a continuous alignment of molecular motors – dynein-ATPases, which are in charge of generating mechanochemical forces leading to sliding of microtubules relative to each other (Spungin, 1991). The functioning of the axonemal mechanics suggests that peptidic connections intervene in such a way that some intrinsic proteolytic enzymes transiently hydrolyze them (Gagnon et al., 1984). While knowledge has been largely accumulated on the motor components, little is known about the elements regulating the bending processes. Efficient forward propagation of spermatozoa relies on the capacity of flagella to generate waves that result from dynein dependent microtubule sliding resulting from dynein-ATPase activity. Each bend formed at the head-tail junction travels along the flagellum towards the tip, inducing a forward translation of the whole sperm cell in opposite direction (Cosson and Prokopchuk, 2014). The wave propagation is supported by a bending/relaxing cyclic mechanism that spread in the wave and transmits the powering action of the dynein-ATPase motors all along the axoneme (Cosson and Prokopchuk, 2014). Translational drive exercised on the spermatozoon is due to the thrust of its own flagellum on the milieu surrounding the sperm cell. During the movement of sperm of many different species, flagella generally describe a pseudo-sine wave shape.

At spawning, fish males shed sperm into surrounding water at the same time as females deliver ova and typically, spermatozoa must reach the egg within their lifetime – a very brief period (seconds to minutes). Therefore, highly efficient flagella must become fully active

immediately on contact with water and propel the sperm cell at high initial velocity. So, right after activation, fish spermatozoa beat with their flagella at very high frequency up to 70–100 Hz to achieve this goal. This function implies a fast consumption of the ATP stored within the spermatozoon (Cosson, 2010, 2013).

Taking into account such a rapid transition of sperm cells into a fully active state, the earliest and most significant steps of flagellar activation is almost impossible to capture by an observer. For instance, during experimental activation of sperm motility directly in a drop of swimming solution set on the glass slide of a microscope, it is necessary to achieve a homogenous suspension of spermatozoa quickly. However, in practice, the efficient mixing of sperm samples by an expert experimenter takes several seconds, and consequently flagellar wave initiation occurs exactly during such a procedure. Even with the very rapid mixing, it is difficult to obtain correct focus on the object (spermatozoon) immediately, when applying photo or video microscopy methods for evaluation of sperm quality, so usually, recording starts after a delay of 3–5 s. (Cosson, 2008a). In contrast to fish spermatozoa, some studies allowing description of flagella behavior at motility initiation were performed on sea urchin (Gibbons and Gibbons, 1980; Goldstein, 1979; Ohmuro et al., 2004) and arenicola (Pacey et al., 1994a; Pacey et al., 1994b) spermatozoa. It is worth mentioning that most of the knowledge about flagellar function and wave propagation comes from the use of sea urchin (Brokaw, 1990; Gibbons, 1972; Gibbons, 1986), or *Chlamydomonas* (a green unicellular algae) and its numerous motility mutants (Brokaw and Kamiya, 1987; Goldstein, 1982; Ringo, 1967).

As emphasized above, in many species, the main signal that activates fish sperm motility is osmotic. Previously, it was shown that an osmotic shock of extreme amplitude received by carp sperm cells could relieve the inhibition of movement and with delayed response (at 3–4 minutes after mixing), trigger their motility after incubation in media with an osmolality of 400 to 3200 mOsm.kg⁻¹ (Perche Poupard et al., 1997). Based on this pioneer study, a specific experimental situation was designed in the current work so as to induce a delay between mixing and sperm motility activation. This approach was applied to various fresh water species and allowed to investigate by high-speed video techniques, the detailed and quantitative description of the initiation of flagellar waves specifically in sturgeon spermatozoa.

1.4. Objectives of the thesis

The current study was devoted to the comprehensive investigation of the process of sperm motility initiation in fishes and pursuing the following objectives:

1. To study the processes underlying the spermatozoa maturation.
2. To investigate the coping mechanisms in fish spermatozoa with osmotic and ionic activating mode, as well as in spermatozoa of euryhaline fishes, to various osmotic conditions.
3. To describe the regulation and initiation of flagellar beating in chondrosteian spermatozoa.

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Chapter 2

In vitro sperm maturation in sterlet, *Acipenser ruthenus*

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Short Communication

In vitro sperm maturation in sterlet, *Acipenser ruthenus*



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ABSTRACT

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

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1. Introduction

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

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

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

2. Materials and methods

All experiments were performed according to the principles of the Ethical Committee for the Protection of Animals in Research of the University of South Bohemia, Research Institute of Fish Culture and Hydrobiology, Vodnany, Czech Republic. During the natural spawning season (April–May), six sterlet (*Acipenser ruthenus*) males (3–4 year-old, 0.6–1.0 kg body weight, bw) were transferred from fish-farming ponds (water temperature 8–10 °C) into the 0.8 m³ 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 (Ca²⁺) 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 CaCl₂). To investigate the influence of Ca²⁺ on sperm maturation, ethylene-bis(oxyethylenitrilo)tetraacetic acid (EGTA, Ca²⁺ 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 Ca²⁺) content were significantly lower in Wolffian duct sperm than

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

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

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

in testicular sperm (Table 1). Since testicular sperm and Wolffian duct sperm were collected before and after sperm passage through the kidney, respectively [6], the differences between both sperm types may result from dilution of testicular sperm. The testicular sperm/Wolffian duct sperm concentration ratio was used as an approximate estimation of testicular sperm dilution. In the current study, the dilution rate ranged from 26 to 262. It is possible that urine is the diluting factor since ion concentrations are similar in urine and seminal fluid of Wolffian duct sperm (Table 1). This supposition is also supported by previous reports, showing similarities in ionic composition of seminal fluid and urine in sterlet [8,9]. Extracellular K⁺ inhibits sturgeon flagellar activity in a concentration dependent manner [10,11], and this inhibition can be overcome by Ca²⁺ [12]. Thus, high K⁺ and low Ca²⁺ concentrations in seminal fluids from Wolffian duct sperm, conditions which may result from testicular sperm dilution with hypotonic urine [8,12], seem to be the factors that prevent sperm motility activation. We have observed significant differences in testicular sperm and Wolffian duct sperm responses to dilution with activating medium. The activation medium activated the Wolffian duct sperm motility, but was not able to initiate testicular sperm motility immediately after collection and at the end of the experiment (2 h later). The Wolffian duct sperm was characterized by high motility rate and VCL values (Table 2) typical for sterlet [13]. In our opinion, the difference in the ability to trigger motility may be an indicator of sperm maturity.

It is of interest that after pre-incubation of testicular sperm in urine or seminal fluids from Wolffian duct sperm, motility was initiated in AM (Table 2). This is consistent with the notion that sperm maturation occurs in the sturgeon. It also suggests that under *in vivo* conditions the maturation takes place within 10 min after the testicular sperm passage through the kidney and results from dilution of testicular sperm with urine. The

maximum level of motility rate was reached after 10 min of pre-incubation, while maximal VCL required 25 min of pre-incubation (Table 2). As sperm velocity is determined by flagellar energy-dependent activity [12], the differences in VCL observed for various testicular sperm incubation times may arise from differences in intracellular ATP concentration. It is possible that intracellular ATP level gradually increases during sperm maturation, but this hypothesis requires additional research.

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

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

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

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

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

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

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

Conflict of interest

None declared.

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Chapter 3

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

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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 *Acipenser 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.

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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

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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 either 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 (*Acipenser 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 Cryo-ALTO 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 Micro-Image 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:

$V = R^3 \times (4 \times \pi) / 3$ 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:

$V = \pi \times L \times (r_1^2 + r_1 \times r_2 + r_2^2) / 3$ 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 ($V/V_{0(\text{micro})}$) using the formula:

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

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 $V/V_{0(\text{micro})}$ was calculated using the formula:

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

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

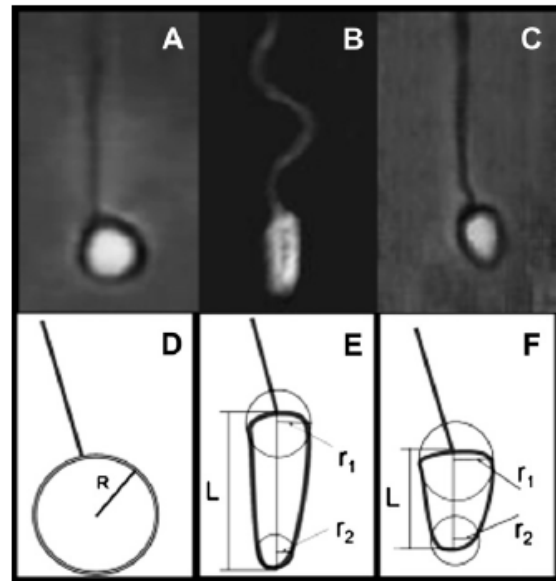


Fig. 1. Light microscopy images of spermatozoa (A–C) and graphic depictions of spermatozoa head parameter calculation (D–F): (A) and (D), carp; (B) and (E), sterlet; (C) and (F), brook trout. The head contour is shown as a thick line in each image. The limit of the head was considered to be the center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

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.

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. Spermatoctrit alteration measurements

Sperm of sterlet was concentrated using centrifugation for 30 minutes at $300\times g$ 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 $1500\times g$ in a hematocrit tube [7]. Relative sperm volume evaluated using the spermatoctrit alteration method was calculated using the formulas:

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

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

where SpC_m is spermatoctrit in the studied media and SpC_{100} and SpC_{300} are spermatoctrit 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 spermatoctrit 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 \pm 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

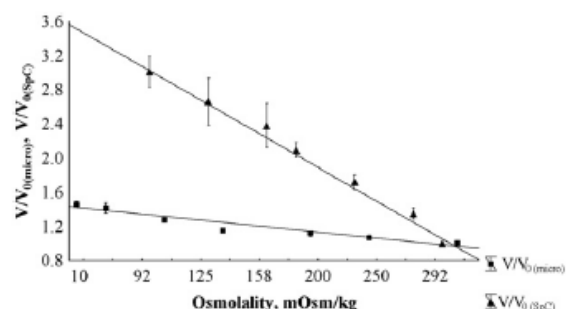


Fig. 2. Relative volume of carp spermatozoa measured using spermatoctrit and relative spermatozoa head volume measured using microscopy in media of differing osmolalities. Values are presented as mean \pm SD. $V/V_{0(SpC)}$; $R^2 = 0.9974$; $P < 0.001$; $V/V_{0(micro)}$; $R^2 = 0.9605$; $P < 0.001$.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

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, spermatoctrit, 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, spermatoctrit, and CryoSEM methods).

3. Results

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

The spermatoctrit 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 spermatoctrit ($V/V_{0(SpC)}$; $R^2 = 0.9974$; $P < 0.001$) 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 spermatoctrit alteration, with spermatozoa head volume, as measured using microscopy, was observed (Fig. 3).

Changes in spermatoctrit 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 spermatoctrit in solutions with different osmolalities (data not shown) were

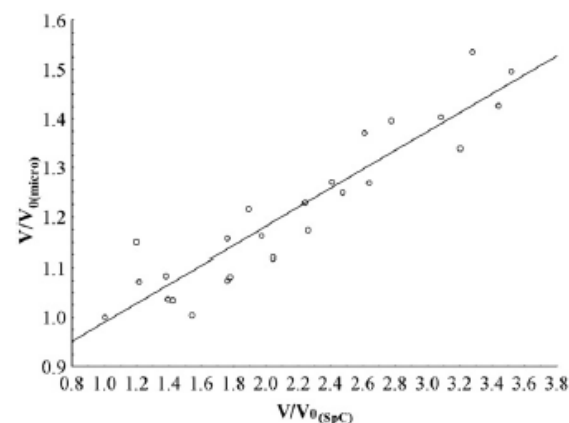


Fig. 3. Correlation of carp spermatozoa volume changes measured using microscopy and spermatoctrit alteration methods. $R^2 = 0.9475$; $P < 0.001$. $V/V_{0(micro)}$ relative spermatozoa head volume measured using microscopy; $V/V_{0(SpC)}$ relative volume of carp spermatozoa measured using spermatoctrit.

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 ($V/V_{0(\text{micro})}$: $R^2 = 0.835$; $P < 0.001$) 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 $V/V_{0(\text{micro})}$ 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 $V/V_{0(\text{micro})}$ ($P = 0.1153$) 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, $V/V_{0(\text{micro})}$ was calculated from CryoSEM micrographs (Fig. 8). Again, no significant differences ($P = 0.3946$) between $V/V_{0(\text{micro})}$ in hypotonic (10 mM TRIS-HCl) and

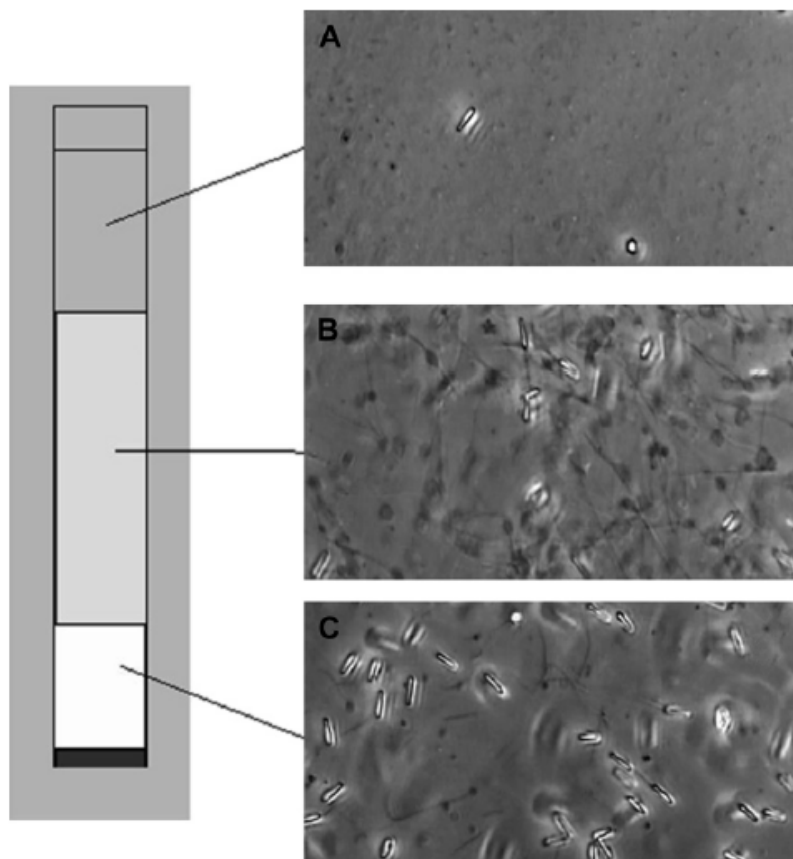


Fig. 4. Typical view of the layers in the hematocrit capillary after spermatocrit alteration measurements in sterlet sperm: (A), (B), and (C) represent supernatant, upper fraction of the pellet, and lower fraction of the pellet, respectively. (A) Fluid containing low numbers of heads, flagella, and whole 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.

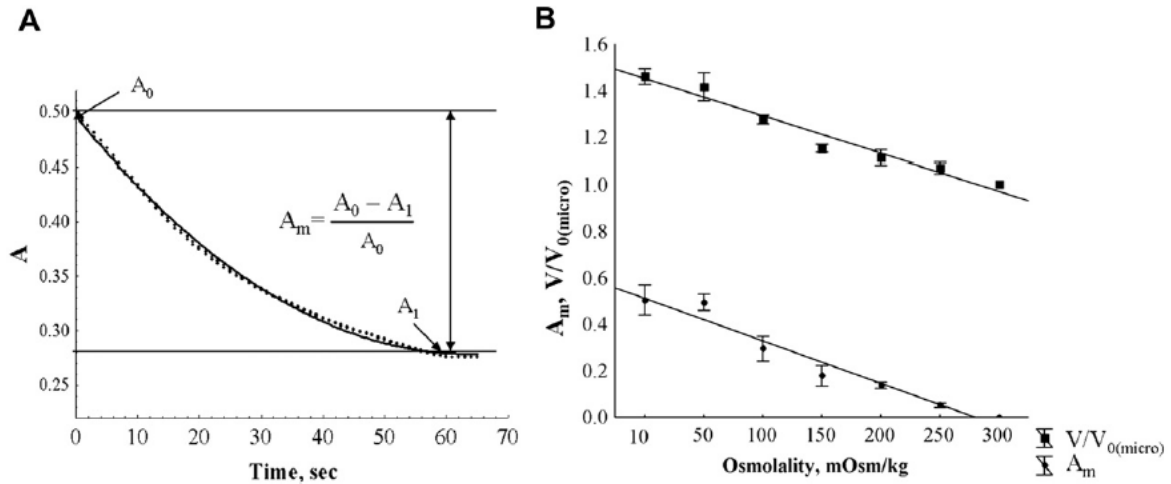


Fig. 5. (A) Schematic of dynamics of optical density in carp sperm suspended in hypotonic solution. Values A_0 , A_1 , and the formula for calculation of relative change in absorbance (A_m) are presented. (B) Relative volume of carp spermatozoa measured using microscopy and A_m in media of different osmolalities. Values are presented as mean \pm SD. A_m : $R^2 = 0.7084$; $P < 0.001$. $V/V_{0(\text{micro})}$: $R^2 = 0.835$; $P < 0.001$. $V/V_{0(\text{micro})}$, 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 $V/V_{0(\text{micro})}$ ($P = 0.1566$) 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.

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 spermatoctrit alteration and light microscopy produced a satisfactory explanation of the fish sperm swelling process.

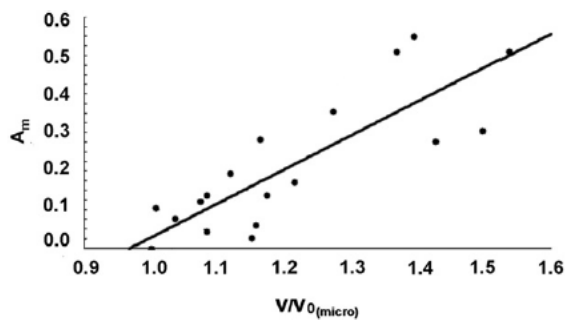


Fig. 6. Correlation between relative change in absorbance (A_m) and $V/V_{0(\text{micro})}$. $R^2 = 0.7341$; $P < 0.001$. $V/V_{0(\text{micro})}$, relative carp spermatozoa head volume measured using microscopy.

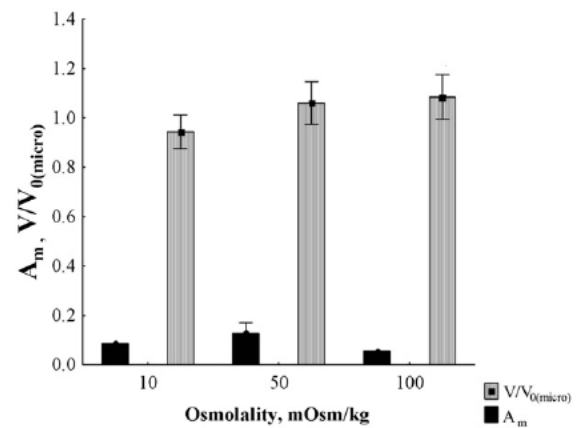


Fig. 7. Relative volume of sterlet spermatozoa measured using microscopy, and relative absorbance alteration (with respect to initial absorbance) methods in media of different osmolalities. Average values \pm SD are presented; no significant difference between values of A_m ($P = 0.3679$) and $V/V_{0(\text{micro})}$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

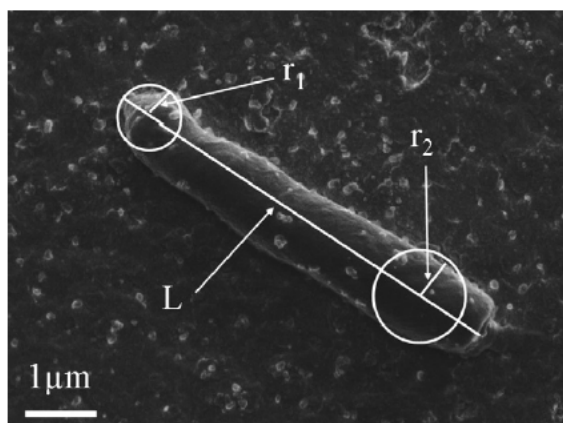


Fig. 8. Scanning electron micrograph (CryoSEM) of the sterlet spermatozoa head observed in a cryo-field emission microscope. L, r_1 , and r_2 are geometric parameters measured for head volume calculations. For more detailed description see 2. Materials and methods.

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

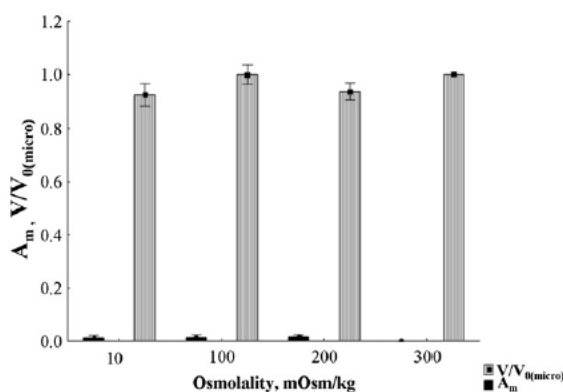


Fig. 9. Relative volume of brook trout spermatozoa measured by microscopy and A_m alteration in media of different osmolalities. Average values \pm SD are presented. No significant difference between values of A_m ($P = 0.6205$) and $V/V_{0(\text{micro})}$ ($P = 0.1566$) are shown. A_m , relative absorbance;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

solution can induce membrane damage, and that further centrifugation leads to partial sperm cell collapse.

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 $V/V_{0(\text{micro})}$ 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

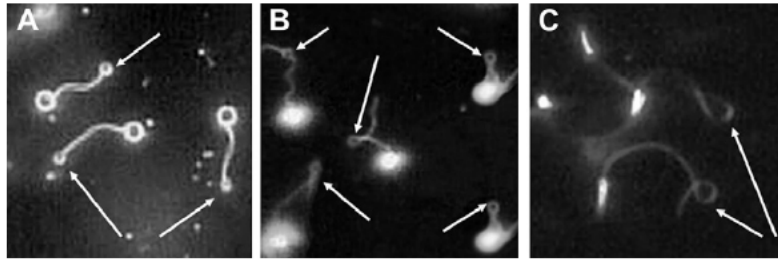


Fig. 10. Images of spermatozoa exposed for 40 seconds to hypotonic solution activating motility: (A) carp sperm; (B) trout sperm; and (C) sterlet sperm. Loops and/or bubbles are indicated by arrows.

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.

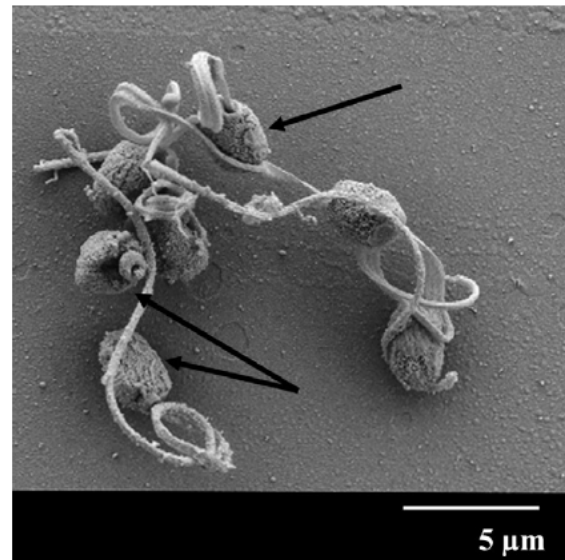


Fig. 12. Scanning electron micrograph of brook trout spermatozoa fixed in glutaraldehyde solution. Disc shape spermatozoa head is indicated by arrows. Loops and blebs are visible on flagella.

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 fresh-water 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 (aquaporins), or actively through ion cotransporters [27,28]. It was hypothesized 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.

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Chapter 4

Adaptations of semen characteristics and sperm motility to harsh salinity: extreme situations encountered by the euryhaline tilapia *Sarotherodon melanotheron heudelotii* (Dumeril, 1859)

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Adaptations of semen characteristics and sperm motility to harsh salinity: Extreme situations encountered by the euryhaline tilapia *Sarotherodon melanotheron heudelotii* (Dumeril, 1859)

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ABSTRACT

In most teleost fishes, sperm cells are quiescent in the seminal plasma and are activated by either a drop (fresh water fish) or an increase in osmolality (marine fish) when released in the water. It is most interesting to examine how the mechanisms of sperm motility activation can adapt to a broad range of salinities, as applies to some euryhaline species, and particularly to the tilapia *Sarotherodon melanotheron heudelotii*, which can reproduce at salinities from 0 up to 120 in the wild. Here, the gonado-somatic index, semen characteristics, and the osmotic and ionic requirements of sperm motility activation were compared in *S. m. heudelotii* reared in fresh water (FW), sea water (SW), or hypersaline water (HW; salinities of 0, 35, and 70, respectively). No salinity-dependent differences were found in gonado-somatic index or semen characteristics, except for an increase of seminal plasma osmolality with increasing salinity (from 318 to 349 mOsm kg⁻¹ in FW and HW fish, respectively). The osmolality range allowing the highest percentages of sperm activation broadened and shifted toward higher values with increasing fish ambient salinity (150–300, 300–800, and 500–1200 mOsm kg⁻¹, for FW, SW, and HW fish, respectively). Nevertheless, at the three fish rearing salinities, sperm could be activated in media that were hypotonic, isotonic, or hypertonic relative to the seminal plasma, at least when some calcium was present above a threshold concentration. The [Ca²⁺] required for the activation of *S. m. heudelotii* sperm is (1) higher in fish reared at a higher salinity (2) higher in hypertonic than that in hypotonic activation media, whatever the fish rearing salinity, and (3) higher in the presence of Na⁺ or K⁺, the negative effects of which increased with an increase in fish rearing salinity. The [Ca²⁺]/[Na⁺] ratios allowing for maximal sperm motility in SW or HW fish are close to those observed in natural environments, either in sea or hypersaline waters. In comparison to most teleosts with external fertilization, the total duration of sperm motility in *S. m. heudelotii* was exceptionally long (>2 hours regardless the fish rearing salinities). The decrease in sperm activity with increasing time since activation did not result from limiting energy reserves, as the addition of calcium in the activation medium caused most spermatozoa to become motile again. The comparison of sperm characteristics of *S. m. heudelotii* acclimated from FW to SW or HW with those of fish maintained all lifelongs at their native salinity showed that adaptive responses were completed within 2 months or less.

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1. Introduction

In most teleost fish species, spermatozoa are immotile while in the seminal plasma inside the testes. For species with external fertilization, the activation of sperm motility occurs after ejaculation and is triggered by signals from the surrounding medium [1,2]. Depending on the species, sperm motility activation occurs mostly in response to primary signals, which can be either of an osmotic or ionic nature, or a combination of both [3,4]. In most marine fish species, the main signal results from the passage from a medium with low osmolality (seminal fluid) to a medium with high osmolality (sea water) [5,6], whereas a reverse situation occurs for fresh water species (from high to low osmolality) [7]. In some species such as trout (more generally salmonids) [8] or sturgeons (chondrosteans) [9], the main signal for triggering sperm motility was identified as the switch from a high K^+ concentration in the seminal fluid to a low K^+ concentration in fresh water, and this K^+ signal occurs in conjunction with that observed for Ca^{2+} [10]. These traits further emphasize the interest to study fish species that are capable to reproduce in a broad range of osmolalities (environmental water salinities) and lead to the following question: what are the mechanisms by which sperm motility triggering can adapt to such a broad salinity range?

Because of its exceptional tolerance to a broad range of ambient salinity, the euryhaline tilapia *Sarotherodon melanotheron* Rüppell, 1852 (Cichlidae, Perciformes), is a most valuable fish species to tackle this question. This estuarine species naturally occurs in West African lagoons and estuaries from Senegal to Congo [11], where it has an economic importance for both capture fisheries and aquaculture [12,13]. The subspecies *Sarotherodon melanotheron heudelotii* (Dumeril, 1859) exclusively occurs from Senegal to Guinea. It has been found to reproduce successfully at salinities ranging from about 0 (e.g., Guiers lake) up to 120 in the Saloum estuary, where high salinity results from reduced fresh water inputs and high evaporation [14–16]. Our first observations on sperm motility in *S. m. heudelotii* showed that the osmolality-enabling sperm activation increased significantly with the rearing salinity of the broodfish [17]. In another euryhaline tilapia, *Oreochromis mossambicus*, observations on fish that were acclimated to fresh or sea water indicated a major role of Ca^{2+} ions in this adaptive ability [18–20]. In the different works on *O. mossambicus*, fish sperm was examined at different times after their acclimation to fresh or sea water to sea water (after about 1 month [20–22], 5 months [23], or up to 1 year [18]), and it is uncertain whether acclimation was completed by then or if some results referred to a transitional stage. The issue of acclimation dynamics might even be more relevant when acclimating fish to hypersaline water.

The present study aimed to better understand the mechanisms enabling reproduction of *S. m. heudelotii* in environments with contrasting salinities. Here, the effects of ambient osmolality and ions on the motility parameters of spermatozoa were examined in fish reared under controlled conditions in fresh (FW), sea (SW) or hypersaline waters (HW, i.e., 70, twice as much as the salinity of full-strength sea water). It also aimed to further investigate

the respective roles of osmolality and Ca^{2+} in the control of sperm activation. Testis growth and semen characteristics were also evaluated in the fish reared at the three salinities. To compare contrasting situations as regards the duration of acclimation, we studied sperm characteristics and motility parameters in fish acclimated from FW to SW or HW over a few weeks period and in their progeny hatched and reared all their lifelong at the same salinity. To our knowledge, except for an abstract on this topic [17], this is the first study investigating the osmolality and ions dependence of sperm motility activation in cichlid fish acclimated to water salinity higher than that of sea water.

2. Material and methods

2.1. Biological material

The fish used here were the descendants of a Senegalese population (Niayes natural ponds, Dakar area) of *S. m. heudelotii* maintained in fresh water since 1998 at the ISE-M experimental facility in Montpellier (France).

During the first part of the experiments, male and female broodfish were aged 2 to 3 years. About one third of the broodstock was maintained in fresh water throughout. The remaining fish were acclimated from fresh to sea water (salinity 35; about 1100 mOsm kg^{-1}) over 5 weeks by the progressive addition of synthetic sea salt ("Instant Ocean", Aquarium system, Sarrebourg, France; about 1 g per L of salt day^{-1}). Thereafter, about half of the fish acclimated to sea water were progressively acclimated at the same rate (over 5 weeks) to hypersaline waters (salinity 70; about 2200 mOsm kg^{-1}), whereas others were maintained at salinity 35 throughout. After acclimation to salinity, broodfish were maintained for 2 to 10 months at their respective salinities before sperm collection.

The *S. m. heudelotii* broodfish previously acclimated at the three salinities (0, 35, and 70) reproduced spontaneously in the rearing tanks. Fertilized eggs were collected from the mouths of mouthbrooding males, incubated in McDonald jars until the start of exogenous feeding, then, fish were reared on formulated feed. Throughout their life, these fish were maintained at their native salinity. At the three salinities, males became sexually mature at about 6 to 7 months of age. Testes and sperm characteristics were analyzed when fish were aged 14 to 39 months. This second data set permitted the comparison of gonad characteristics and physiological response of sperm between fish hatched and reared at the same salinity (0, 35, or 70) throughout and fish acclimated from fresh water to salinity 35 or 70. In the rest of the article, for the sake of simplicity, we refer to these two categories of fish as "native" and "acclimated", respectively. In total, 137 males (65–403 g) were examined in the present study, of which 76 acclimated fish (18 FW, 30 SW, and 28 HW) and 61 native fish (22 FW, 20 SW, and 19 HW).

2.2. Rearing conditions

In all situations, broodfish of *S. m. heudelotii* were reared under a 12L:12D photoperiod, at a stocking density of 25 to 30 fish m^{-3} and a sex-ratio of 1:1, in six 400-L indoor troughs (two at each of the three maintenance salinities)

connected in pairs to three independent water recirculation systems at salinities set at 0 (FW), 35 (SW), and 70 (HW). Every water recirculation system was equipped with mechanical and biological filters (about 90 L each) allowing the maintenance of water quality. Every 2 days, water salinity was controlled with a refractometer and adjusted accordingly by addition of synthetic sea salt or fresh water. In all instances, water temperature was maintained at 26 °C to 29 °C by 300-W submersed heaters connected to a thermostat (Biotherm 2000), and oxygen was maintained near saturation with air stones. All fish were fed commercial pelleted feeds *ad libitum* (32% and 41% crude proteins, for adults and juveniles, respectively).

2.3. Sperm collection and characteristics

Sarotherodon melanotheron is an oligospermic species [24]. Here, not all males of *S. m. heudelotii* were emitting sperm after abdominal stripping, and when they did, semen was generally heavily polluted by urine, which resulted in sperm activation. It was therefore decided to work exclusively with intratesticular sperm, in which spermatozoa were immotile. The fish were anesthetized (Eugenol, 0.05 mL L⁻¹) and then killed by an overdose of anesthetic (Eugenol, 0.5 mL L⁻¹) in ice (in accordance with the EU Directive 2010/63/EU) for dissection and testis collection. After dissection, blood remnants were removed and the surface of the testes was dried with absorbent paper. Fish and testes were weighed (nearest 0.1 g) for calculation of the gonado-somatic index (GSI, % = 100 gonad mass/fish body mass). Sperm was collected by means of slicing the testicular tissue, and the aspirated sperm were transferred in 1.5 mL Eppendorf tubes (Flip-Tube, Gemü GmbH, Switzerland). The total volume of sperm collected in every male fish was estimated with a micropipette (nearest µL). Collected sperm was stored at 4 °C until subsequent motility measurements in different activation media (see Section 2.4). When in sufficient amount after motility trials, the remaining sperm was centrifuged (3000 × g, 10 minutes). The osmolality of the seminal plasma was measured with a micro-osmometer (Type 13–Autocal, Roebing, Berlin, Germany). At each experimental salinity, the concentrations of spermatozoa were determined for at least five individual fish after fixation of individual sperm samples in a 155 mM NaCl solution containing 1% formalin and count under the microscope (× 200), using a Thoma hemocytometer (Marienfeld-Superior, Germany).

The ionic composition of seminal plasma was determined in 3 to 5 individual male for every salinity condition. Concentrations of anions (Cl⁻) and cations (Na⁺, K⁺, Mg²⁺, and Ca²⁺) in the seminal plasma were measured by ionic chromatography and inductively coupled plasma mass spectrometry (ICP-MS), respectively, at the regional technical platform A.E.T.E. (UM, Montpellier, France).

2.4. Sperm motility activation

Sperm motility parameters were assessed using video images recorded on a digital camcorder (Sony DCR-TRV10) through a microscope with a dark field condenser lens

(DMLB100, Leica Microsystem, Wetzlar, Germany) and × 20 objective lenses, equipped with a stroboscopic illuminator (Strobex, Chadwick-Helmuth, El Monte, CA, USA) and a CCD camera (KP-M1, Hitachi Denshi Ltd, Tokyo, Japan). At all three salinities, sperm motility was initiated by dilution of intratesticular sperm in a drop of activation medium (AM, see in the following section) on a microscope slide at ambient temperature (21 °C–25 °C). In every situation, a volume of 0.5 µL of sperm was added to 50 µL of AM and immediately mixed before observation and video recording. In some situations, it turned out that the sperm of *S. melanotheron* could be motile for over 30 minutes. The actual duration of sperm motility in this species could not be measured for such long periods on the microscope slides because of the progressive evaporation of AM. Therefore, the total duration of sperm motility in each salinity condition was determined in larger volumes, after mixing in a plastic tube 6 µL of sperm of an individual male fish and 300 µL of appropriate AM (diluted artificial sea water at 300 mOsm kg⁻¹ for FW fish or 600 mOsm kg⁻¹ for SW and HW fish; see Section 3). Sperm activity was estimated from 10 µL aliquots examined under the microscope at various time periods after activation. This operation was repeated for two to three males at each of the three salinities under study.

The effects of ambient osmolality and ions on sperm motility were tested using different activation media: (1) synthetic sea salt as the reference ionic medium; (2) sucrose for testing the effect of osmolality in absence of ions, and (3) sucrose supplemented with Ca²⁺ for a first evaluation of the specific effect of this ion. All three media were evaluated over a broad range of osmolalities (20–3300 mOsm kg⁻¹). In the osmolality ranges that were found optimal for sperm motility (see Section 3), different electrolytic solutions (NaCl, KCl, CaCl₂, and MgCl₂) were used to evaluate the effects of specific ions. Distilled water, sucrose, and electrolytic solutions were buffered with 10 mM Tris adjusted to pH 8.2. In all cases, 0.1% (w:v) BSA was added to the AM to prevent sperm adhesion to the surface of the microscope glass slide. The osmolality of every activation media was measured with the Roebing micro-osmometer. Some media (see Section 3) were supplemented with ethylene glycol tetraacetic acid (EGTA) or CaCl₂ at various concentrations for evaluating the Ca²⁺ requirements for the activation of spermatozoa. The free Ca²⁺ concentration ([Ca²⁺]) of AM was calculated by using a specific software [25] using total concentration values of the three components involved in the Ca²⁺ equilibrium (Ca²⁺, EGTA, and H⁺). The effects of replacing Ca²⁺ by Mg²⁺ were also evaluated.

At the three salinities under study, undiluted intratesticular sperms stored at 4 °C were found to maintain over a long period (>24 hours in most cases) their ability to be activated in an appropriate AM. Henceforth, long series of observations could be performed on the sperm of a particular individual fish without any loss in sperm motility potential. Controls at the end of the observation series showed motility scores similar to those observed in freshly collected sperm, thereby attesting that the low motility scores observed in some media did not originate from sperm aging.

For each situation of sperm activation (composition of AM, osmolality, and time from activation), observations

were made on two to four sperm samples collected from 2 to 6 males at each rearing salinity condition.

2.5. Sperm movement analysis

During analyses of the maximal duration of sperm activity, percentages of motility were visually estimated in terms of percentages of motility by two experienced observers (Jacky Cosson and Marc Legendre). In all other observations, the motility of *S. m. heudelotii* sperm after activation was assessed from video records at the beginning of the swimming phase (15 seconds after sperm activation) and after 60 seconds of activity. Sperm velocity (velocity curvilinear) and percentage of motile spermatozoa were obtained from the analyses of the positions of each sperm cell on successive video frames, using a CASA system and image analysis software (Olympus Micro Image 4.0.1. for Windows) [26,27].

Detailed images of moving spermatozoa were obtained using $\times 100$ phase contrast optics (Zeiss Ph 3 NeoFluar 100x, Oil; Olympus BX50 microscope) and recorded with a high-speed video camera (Olympus i-speed TR), providing a high (848 \times 688 pixel) spatial resolution at 1000 frames per second. This enabled the analysis of three wave parameters of the flagella of motile sperm cells (Fig. 1): (1) the wave amplitude was measured as the distance from a reference line (set as the midline of the flagellum) to the crest of the corresponding wave; (2) the wavelength was calculated as twice the distance between two consecutive intersections of the flagellum and the reference line; measurements were made on a half sine wave because of the short length of the sperm cell flagellum in the case of *S. m. heudelotii* (see Section 3); and (3) the third measurement evaluated here was the wave velocity corresponding to the movement of the wave crest over one full-beat cycle. These three parameters were characterized on sperm activated in diluted artificial sea water at osmolalities within the range of values identified as optimal for sperm activation (300 mOsm kg⁻¹ for FW fish, 600 mOsm kg⁻¹ for SW, or 900 mOsm kg⁻¹ HW fish; see Section 3) and were related to the velocity of sperm cells during the same motility sequences.

2.6. Statistical analyses

Unless unspecified otherwise, values are means \pm standard error (SE). Two-way ANOVA was used to compare body mass, GSI, and semen characteristics between fish from the two generations (acclimated and native) at the three salinities. One-way ANOVA followed by Duncan's multiple range posthoc tests was used for comparison of means between

fish reared at different salinities. Repeated measures ANOVA on the whole data set and paired *t* tests at each experimental salinity were used for comparing the motility (%) of the same sperms activated in diluted sea water and in sucrose solutions of appropriate osmolalities. When necessary, ANOVA were made after angular transformation of data to stabilize residual variance. The relationships between sperm motility (%) and free calcium concentration (expressed as $pCa = \log(1/[Ca^{2+}]))$ displayed a sigmoid shape, which is usual for dose-response relationships. These relationships were linearized after transformation of motility (M, %) using the formula $y = \ln(M\%/ (100 - M\%))$. The linear relationships between sperm motility (after transformation) and *pCa* or sperm velocity were compared between FW, SW, and HW sperm by analysis of covariance analyses followed by Duncan's posthoc tests. Tests were done using Statistica (10) software. Null hypotheses were rejected at $P < 0.05$.

3. Results

3.1. Male and sperm characteristics

The mean fish body mass did not differ as a function of fish generation (acclimated vs. native) or ambient salinity (0 vs. 35 vs. 70; ANOVA 2, *P* of 0.213 and 0.174, respectively). The gonad and semen characteristics did not show significant difference between males acclimated in SW or HW for duration of 2 to 10 months and those having spent their whole life at the corresponding salinities (*P* of 0.699 for GSI, 0.633 for sperm volume, 0.053 for osmolality of seminal plasma, and 0.069 for sperm concentration). Therefore, the data from acclimated and native males were pooled for further analyses of salinity effects.

Gonado-somatic index, sperm volume, and spermatozoa concentration in the intratesticular sperm did not vary significantly between fish maintained at different salinities (0, 35, or 70; Table 1). Globally, the volume of intratesticular sperm collected per fish varied between 1 μ L and 220 μ L, and mean values were very low (<60 μ L) at all salinities. Similarly, the mean GSI never exceeded 0.3% whatever the water salinity. The osmolality of the seminal plasma tended to increase with the fish maintenance salinity and was significantly higher for HW males (349 \pm 5 mOsm kg⁻¹) than for FW or SW males (318 \pm 6 and 330 \pm 4 mOsm kg⁻¹, respectively; ANOVA, $F_{2,61} = 8.31$, $P < 0.001$). No significant correlation was found between individual GSI and fish body mass, between sperm volume and spermatozoa concentration, or between sperm volume and seminal plasma osmolality. By contrast, the sperm volume was positively correlated to fish GSI ($r^2 = 0.225$, $P < 0.001$).

The electrolytic composition of the seminal plasma did not differ significantly between males raised at different salinities, as regards Cl⁻ (102–149 mM), Na⁺ (112–130 mM), K⁺ (17–23 mM), Mg²⁺ (1.2–1.9 mM), or Ca²⁺ (0.2–0.9 mM) (Table 2). Nevertheless, the Ca²⁺ content in HW sperm (0.2 \pm 0.2 mM) tended to be lower than that in FW sperm (0.9 \pm 0.2 mM), with an intermediate situation being observed for SW sperm (0.5 \pm 0.2 mM).

The spermatozoa of *S. m. heudelotii* have a relatively small size and short flagellum. Whatever the rearing

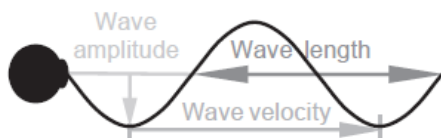


Fig. 1. Schematic representation showing measurements carried out on active sperm flagellum (see text for details).

Table 1

Body mass, gonado-somatic index (GSI), volume of intratesticular sperm collected, osmolality of seminal plasma, and sperm concentration (n spermatozoa per mL of sperm) in males of *Sarotherodon melanotheron heudelotii* maintained in fresh water (FW), sea water (SW), or hypersaline water (HW).

Water salinity	Fish body mass (g)	GSI (%)	Sperm volume (μL)	Seminal plasma osmolality (mOsm kg^{-1})	Sperm concentration ($\times 10^9$)
0 (FW)	221 \pm 11 ^a (40)	0.23 \pm 0.02 ^a (40)	37 \pm 8 ^a (40)	318 \pm 6 ^a (14)	5.1 \pm 1.5 ^a (5)
35 (SW)	227 \pm 10 ^a (50)	0.26 \pm 0.02 ^a (50)	59 \pm 7 ^a (50)	330 \pm 4 ^a (30)	4.6 \pm 0.8 ^a (18)
70 (HW)	205 \pm 10 ^a (47)	0.22 \pm 0.02 ^a (47)	48 \pm 7 ^a (47)	349 \pm 5 ^a (20)	5.6 \pm 1.0 ^a (11)

Mean \pm SE. Within a column, values sharing one superscript in common do not differ at $P < 0.05$.

In each cell, values between parentheses refer to the number of observations (combining acclimated and native fish).

salinity, they do not exceed about 17 to 20 μm in total length with a flagellar length of about 15 to 18 μm (Fig. 2).

3.2. Effects of osmolality of ionic or nonionic media on sperm activation

3.2.1. Sperm motility

When maintained in their seminal fluid, before any further dilution in appropriate media, the intratesticular sperm of *S. melanotheron* were systematically immotile. The effects of osmolality of different activation media (AM; synthetic sea salt solutions, sucrose, or sucrose complemented with 1 mM CaCl_2) on sperm activation were examined for acclimated and native fish.

In acclimated fish, the range of osmolality of activation media that enabled sperm motility activation shifted to higher values and broadened as the ambient salinity of fish increased (Fig. 3). In synthetic sea salt solutions, no significant differences in initial sperm motility values (15 seconds after activation) could be found over a broad range of osmolalities: 1 to 450, 100 to 1100, and 300 to 1900 mOsm kg^{-1} for fish from FW, SW, and HW, respectively. At the three rearing salinities, sperm motility in sea salt solutions did not decline between 15 seconds and 60 seconds after activation, except at relatively low osmolalities (≤ 100 – 150 mOsm kg^{-1}) where the percentage of motile sperm dropped rapidly, partly as a consequence of sperm flagella alteration (curling) because of osmotic constraints. The ranges of osmolalities at which the highest percentages of motile spermatozoa ($>70\%$) were still observed 60 seconds after activation were 150 to 300, 300 to 800 and 500 to 1200 mOsm kg^{-1} , for fish in FW, SW, and HW, respectively, (Fig. 3A–C). These osmolality ranges were therefore considered as optimal for activating the sperm of *S. m. heudelotii* at the corresponding fish rearing salinities. It is worth noting that at all salinities, the osmolality of the fish rearing water was outside of these optimal osmolality ranges for sperm motility: it was lower in FW fish (about 20 mOsm kg^{-1}), and higher in SW and HW fish (about 1100 and 2200 mOsm kg^{-1} , respectively).

In sucrose solutions, the general patterns of sperm response of acclimated fish to osmolality were similar to those observed in sea salt solutions, except at high osmolalities (above the optimal range) at which sperm activation was lower (Fig. 3D–F). Interestingly, the motility scores in sucrose of some SW or HW sperm (two of six males examined at each rearing salinity) were very low at all osmolalities and particularly at those higher than in the seminal fluid (Fig. 3E and F). By contrast, the very same sperms presented an excellent motility either in sea salt solution or in sucrose added with 1 mM Ca^{2+} (Fig. 3H and I). This indicates that these individual sperms were lacking calcium for activation in nonsupplemented sucrose solutions. Analyses of the ionic composition of the activation media showed that 0.5-M sucrose solutions were not totally devoid of electrolytes, as they contained 11 to 18 μM of Ca^{2+} . Some calcium also came into the activation medium by the addition of BSA (estimated to 1–10 μM) and from the sperm seminal fluid (see Table 2). Obviously, this amount of free Ca^{2+} in sucrose solutions (order of magnitude of 10^{-5} M) did not suffice for activating the sperms of some SW or HW males, whereas it was enough for others, at least within the optimal ranges of osmolality. By contrast, when sperm movement was analyzed in activation media with very high osmolality, this amount of calcium never sufficed to trigger sperm activation in any male under study in SW or HW (Fig. 3B vs. Fig. 3E for SW sperm; Fig. 3C vs. Fig. 3F for HW sperm). As shown in Figure 3H, I, this shortage was partly compensated by the addition of 1 mM Ca^{2+} to sucrose.

Motility percentages in sea salt and sucrose solutions at osmolalities within the range of optimal values for sperm activation (150–300 mOsm kg^{-1} for FW fish, 600–900 mOsm kg^{-1} for SW and HW fish) were compared for several acclimated fish in FW ($n = 12$), SW ($n = 14$), and HW ($n = 20$). Although considering exclusively the individuals exhibiting high sperm activation scores (motility $>60\%$) in synthetic sea salt, the corresponding motilities in sucrose varied significantly depending on the rearing salinity of male fish (repeated measures

Table 2

Electrolyte components of the seminal plasma in males of *Sarotherodon melanotheron heudelotii* maintained in fresh water (FW), sea water (SW), or hypersaline water (HW).

Water salinity	n Fish	Concentration (mM)				
		Cl^-	Na^+	K^+	Mg^{2+}	Ca^{2+}
0 (FW)	3	99.3 \pm 24.6 ^a	127.1 \pm 14.9 ^a	19.8 \pm 4.0 ^a	1.1 \pm 0.4 ^a	0.9 \pm 0.2 ^a
35 (SW)	5	149.0 \pm 18.0 ^a	112.2 \pm 11.0 ^a	23.1 \pm 3.0 ^a	1.9 \pm 0.3 ^a	0.5 \pm 0.2 ^a
70 (HW)	3	110.9 \pm 23.3 ^a	130.4 \pm 14.1 ^a	16.6 \pm 3.8 ^a	1.2 \pm 0.3 ^a	0.2 \pm 0.2 ^a

Mean \pm SE. Within a column, values sharing one superscript in common do not differ at $P < 0.05$.

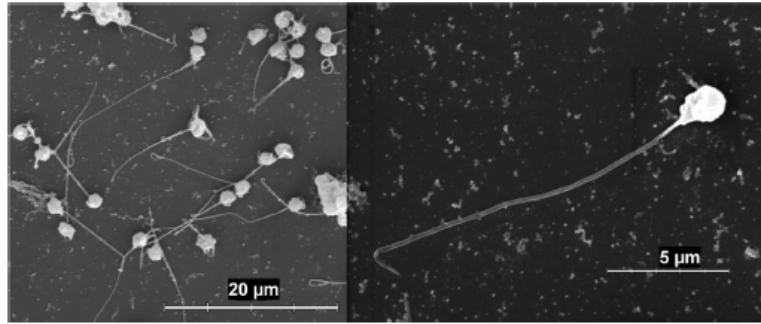


Fig. 2. General morphology of *Sarotherodon melanotheron heudelotii* sperm using scanning electron microscopy after sperm fixation in PBS buffer with 2.5% glutaraldehyde (example from fish reared in sea water).

ANOVA, $F_{4,84} = 2.97$, $P < 0.024$; Fig. 4). For FW fish, sperm motility was high and varied little between individual fish in both activation media. This indicates that in both situations the Ca^{2+} requirements of spermatozoa were

fulfilled. The addition of 1 mM EGTA to 0.1 M sucrose AM almost prevented sperm movements for FW fish (results not shown). In contrast to the situation for FW fish, the sperms of SW and HW fish were significantly less active

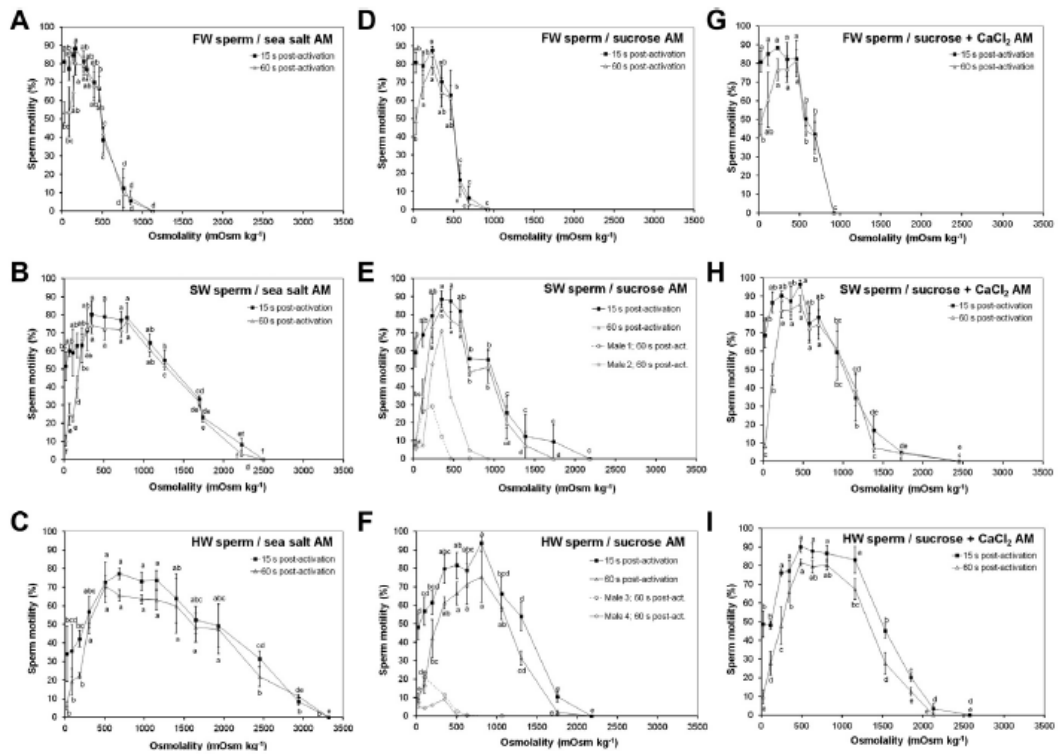


Fig. 3. Percentage of motile spermatozoa (mean \pm SE) 15 seconds and 60 seconds after activation in different media (AM) at various osmolalities (left column: synthetic sea salt; central column: sucrose; and right column: sucrose added with 1 mM Ca^{2+}) for *Sarotherodon melanotheron heudelotii* reared in fresh water (FW, upper line), sea water (SW, central line), or hypersaline water (HW, lower line). Within a same series, values sharing one superscript in common do not differ at $P < 0.05$. For SW and HW sperm in sucrose AM (E and F), responses of two individual males with very low motility scores are not included in the calculation of mean and are presented individually. See text for details.

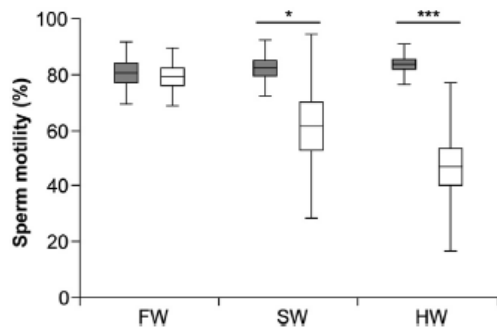


Fig. 4. Motility of sperm activated in diluted sea water (gray) or in sucrose (white) solutions for males of *Sarotherodon melanothon heudelotii* reared in fresh water (FW, $n = 12$), sea water (SW, $n = 14$), or hypersaline water (HW, $n = 20$). The osmolality of swimming medium was 300 mOsm kg^{-1} for FW fish and 600 mOsm kg^{-1} for SW and HW fish. Only fish with sperm motility $\geq 60\%$ in diluted sea water were retained for this analysis (repeated measures ANOVA, $F_{4,84} = 2.97$, $P < 0.024$ and paired t tests at each rearing salinity; *: $P < 0.05$; ***: $P < 0.0001$). Boxes represent mean \pm SE and whiskers are \pm standard deviation.

in sucrose than those in synthetic sea salt, with substantial differences between individual fish (Fig. 4). It is worth noting that the responses of SW or HW fish sperm in sucrose, when compared to those in synthetic sea salt, were independent from the time since the end of the salinity acclimation process of fish (i.e., 2–10 months after the nominal salinity was attained). Furthermore, the motility of sperm from HW males in sucrose solutions was totally inhibited after addition of 1 mM EGTA.

Figure 5 compares the percentages of motile sperm cells from native and acclimated fish in synthetic salt at different osmolalities. At the three rearing salinities, the sperm responses of native and acclimated fish were almost identical. These comparisons indicate that the salinity-dependent shift in the osmolality range that maximizes sperm activation could be achieved rapidly (<2 months) after fish acclimation to salinity.

3.2.2. Sperm velocity

In acclimated fish, the osmolality ranges producing the highest sperm velocities in diluted sea salt or sucrose solutions systematically encompassed those in which the percentages of motile sperm cells were maximal (Fig. 6 vs. Fig. 3). In diluted sea salt, the initial (15 seconds after activation) sperm velocity was higher in the sperm collected from FW males ($60\text{--}75 \mu\text{m second}^{-1}$) than in those from SW or HW males ($40\text{--}45 \mu\text{m second}^{-1}$). Sixty seconds after activation, sperm velocity was similar to that observed after 15 seconds within the optimal osmolality range for sperm activation, whereas it tended to be reduced at lower osmolalities, particularly for FW and SW fish (Fig. 6), probably as a consequence of osmotic damages to the flagella. Comparisons between activation media indicated that the maximal velocity of *S. m. heudelotii* sperm cells were similar in all media, except for the sperm of FW fish, which exhibited a lower initial velocity in sucrose solution ($40\text{--}50 \mu\text{m second}^{-1}$) than those in diluted sea water ($60\text{--}75 \mu\text{m second}^{-1}$).

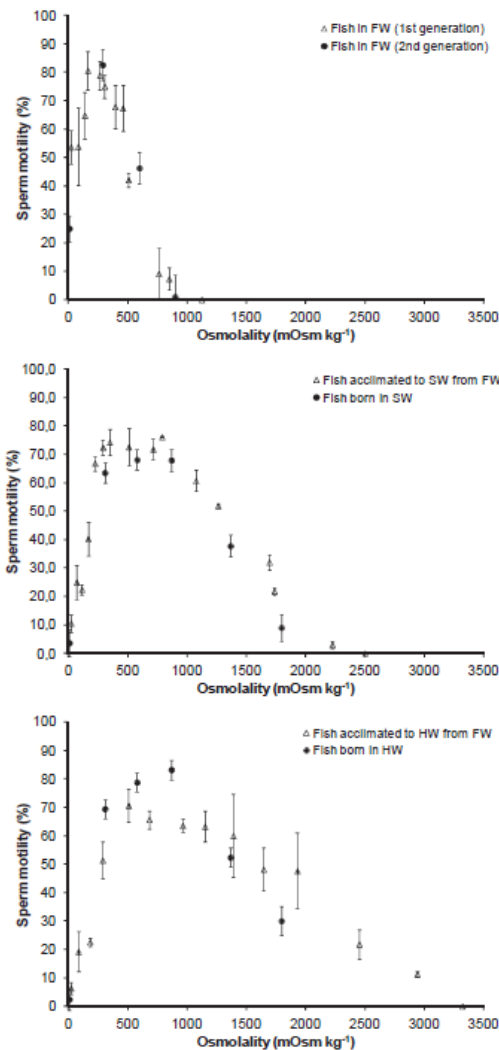


Fig. 5. Percentage of motile spermatozoa (mean \pm SE) as a function of osmolality, salinity of the fish rearing environment (FW: 0; SW: 35; and HW: 70), and fish generation (i.e., fish acclimated from FW to SW or HW vs. fish hatched and maintained all lifelong at the same salinity). Data correspond to motility 60 seconds after activation in solutions of synthetic sea salt. Data from acclimated fish correspond to those presented in Fig. 3. FW, fresh water; SW, sea water; HW, hypersaline water.

The movement of sperm flagella and the resulting sperm velocity were analyzed in greater detail using a high-speed camera. For logistic reasons pertaining to the availability of the high-speed camera, these observations were carried out exclusively on the sperm of native fish. At the three fish rearing salinities, the waves of sperm flagella exhibited relatively small amplitudes (<3.6 μm), and sperm cells were rotating around the longitudinal flagellar axis while moving forward (Fig. 7). Several differences

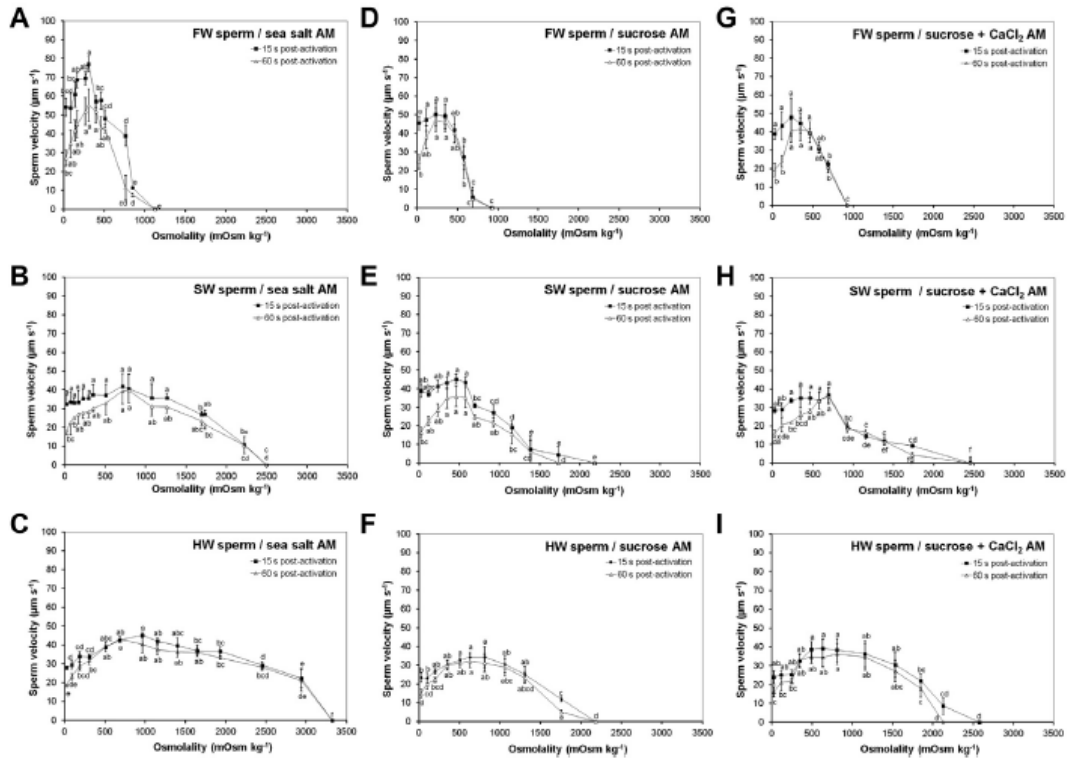


Fig. 6. Average velocity of motile sperm (mean \pm SE) 15 seconds and 60 seconds after activation in different media (AM) of various osmolalities (left column: synthetic sea salt; central column: sucrose; and right column: sucrose added with 1 mM CaCl_2) for *Sarotherodon melanotheron heudelotii* reared in fresh water (FW, upper line), sea water (SW, central line), or hypersaline water (HW, lower line). Within a same series, values sharing one superscript in common do not differ at $P < 0.05$.

were found in characteristics of sperm movements depending on fish-rearing salinity (Fig. 8). The wave amplitudes of the flagella were higher in the sperm of SW fish than in those of FW or HW fish (Fig. 8A). The flagellar wavelengths were about six times as long as wave amplitudes. They were significantly longer in the sperms of SW and HW fish than in those of FW fish. The velocity of the flagellar waves was significantly lower in HW sperm ($337.7 \pm 13.8 \mu\text{m second}^{-1}$, $n = 60$) than that in FW or SW sperm ($419.6 \pm 20.6 \mu\text{m second}^{-1}$, $n = 27$ and $456.6 \pm 15.8 \mu\text{m second}^{-1}$, $n = 46$, respectively; ANOVA, $F_{2,130} = 16.90$, $P < 0.0001$). The initial beating frequency of

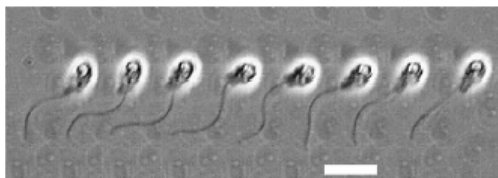


Fig. 7. Illustration of flagellar movements in sperm of *Sarotherodon melanotheron heudelotii*, as observed using high-speed camera images (white bar = 10 μm).

flagella (15 seconds after sperm activation) was much higher in FW sperm ($40.0 \pm 1.5 \text{ Hz}$) than that in SW or HW sperm ($31.2 \pm 0.8 \text{ Hz}$ and $30.9 \pm 0.8 \text{ Hz}$, respectively; Fig. 8B). As a result, the sperm velocities differed significantly between FW ($53.5 \pm 1.7 \mu\text{m second}^{-1}$), SW ($36.9 \pm 1.8 \mu\text{m second}^{-1}$), and HW fish ($31.0 \pm 1.5 \mu\text{m second}^{-1}$; ANOVA, $F_{2,129} = 35.35$, $P < 0.0001$; Fig. 8B).

3.3. Effects of ions on sperm motility

The effects of the ionic composition of the activation media were tested in acclimated fish, at experimental osmolalities falling in the optimal ranges for sperm activation ($100\text{--}150 \text{ mOsm kg}^{-1}$ for FW fish, $500\text{--}600 \text{ mOsm kg}^{-1}$ for SW and HW fish).

Activation media made of 50 mM (FW) or 250 mM (SW and HW) NaCl solutions always had a significant negative effect on sperm motility in comparison to sea salt solutions (Fig. 9). This effect was moderate in FW sperm (motility decreasing from 87.5% to 72.0%), whereas it was very strong in SW fish (from 80.0% to 3.7%), and the sperm of HW fish were not activated in NaCl. A solution of 250 mM KCl was also ineffective for activating the sperms of SW and HW

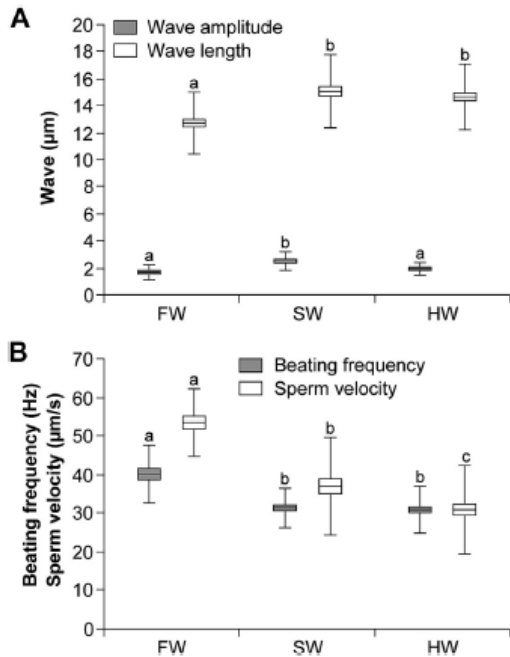


Fig. 8. Average wavelength and amplitude of flagellar movement (A) and flagellar beating frequency and sperm velocity (B) 15 seconds after activation in diluted sea water at 300, 600, and 900 mOsm kg⁻¹ for *Sarotherodon melanotheron heudelotii* reared in FW (n = 27), SW (n = 46), or HW (n = 60), respectively. Boxes represent mean \pm SE and whiskers are \pm standard deviation. For a same parameter, values sharing one superscript in common do not differ at P < 0.05. FW, fresh water; HW, hypersaline water; SW, sea water.

fish, whereas the motility of FW sperm in 50 mM KCl was as high as in the control sea salt solution (Fig. 9).

In presence of either Na⁺ or K⁺, the addition of Ca²⁺ to the activation medium restored sperm motility to the level observed in the control in sea salt solution. It appeared however that the amount of Ca²⁺ necessary to fully restore sperm motility was higher for HW fish (10 mM Ca²⁺) than that for FW or SW fish (1 mM Ca²⁺; Fig. 9).

In hyperosmotic media (about 500 mOsm kg⁻¹) made of 250 mM NaCl or KCl, almost no activation of FW sperm motility could be observed, in contrast to the high motility exhibited in sucrose or diluted sea water of equivalent osmolality (Fig. 3A, D). However, full motility of FW sperm could be restored by adding 10 mM Ca²⁺ to 250 mM NaCl or KCl AM (data not shown).

As expounded previously (see previous comments for Figs. 3 and 4), the low amount of Ca²⁺ present as a contaminant in sucrose solutions generally sufficed for activating the sperms of SW and HW fish, yet with substantial variations between individual fish. As was demonstrated by the inactivation of sperm after the addition of a chelating agent (1 mM EGTA), the presence of Ca²⁺ was strictly necessary for activating sperm motility. Adding a limited (50 mM) amount of NaCl to a nonelectrolytic activation medium (500 mM sucrose) resulted in a five-fold

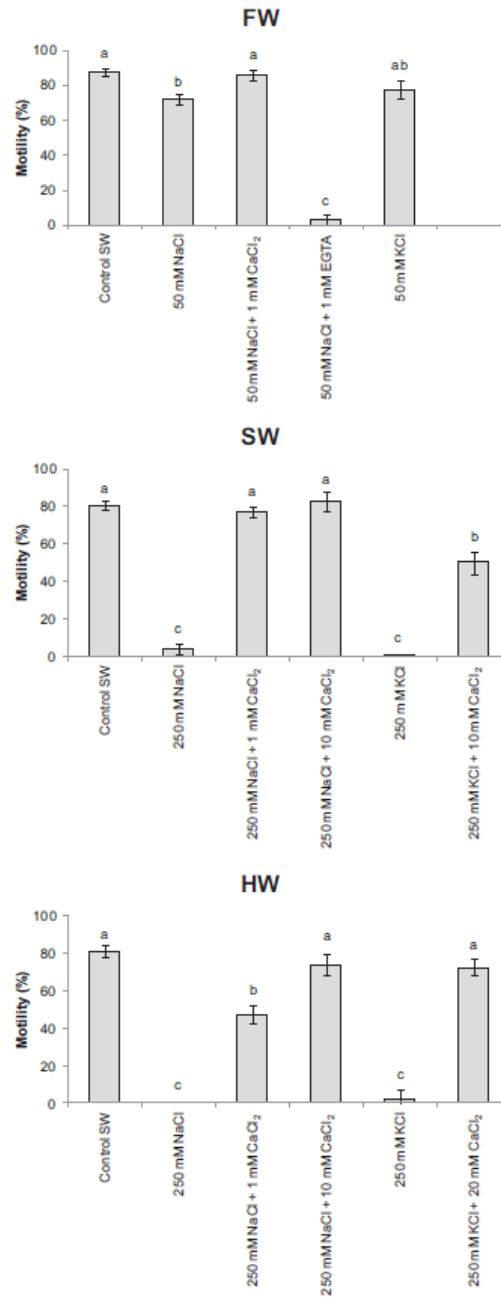


Fig. 9. Effects of electrolytic activation media containing Na⁺, Ca²⁺, or K⁺ on the percentage of motile sperm cells (mean \pm SE) from *Sarotherodon melanotheron heudelotii* males reared in fresh water (FW), sea water (SW), or hypersaline water (HW). Control (sea salt solution) and other media tested are at osmolalities of 100 to 150 mOsm kg⁻¹ for FW sperm and 500 to 600 mOsm kg⁻¹ for SW or HW sperm. Data sharing one superscript in common do not differ at P < 0.05.

decrease (from 76.4% to 15.7%) in the motility of SW fish sperm (Fig. 10). The addition of 10 mM Ca^{2+} to this medium almost restored sperm motility to the level observed in sucrose (Fig. 10). Similar observations were made for the sperm of HW fish after addition of 50 mM Na^+ or K^+ to sucrose AM (not illustrated).

These results indicate that for fish reared in SW or HW waters, the amount of external Ca^{2+} necessary for activating sperm motility is much higher in presence of Na^+ or K^+ than that in a nonelectrolytic medium. In contrast to the situation for Ca^{2+} , no positive effect was observed after the addition of Mg^{2+} to any activation medium at any of the three rearing salinities (not illustrated). Furthermore, in HW fish, no sperm activation was observed when 10 mM Mg^{2+} was added to sucrose solutions.

3.4. Effects of external calcium concentration on sperm motility

The effects of free Ca^{2+} concentration ($[\text{Ca}^{2+}]$) on sperm motility in acclimated fish were examined in NaCl activation media at osmolalities within the optimal range for sperm activation (100–150 mOsm kg^{-1} for FW fish and 500–550 mOsm kg^{-1} for SW and HW fish).

In all situations, the percentage of motility and sperm velocity was strongly and negatively correlated to pCa. At the three fish ambient salinities, the relationships between

sperm motility (%) and pCa were best fitted by sigmoidal curves, whereas those between sperm velocity and pCa were linear (Fig. 11). The equations corresponding to these relationships for FW, SW, and HW fish are given in Tables 3 and 4. After linearization, the relationships between sperm motility and pCa in FW, SW, and HW fish significantly differed from each other (analysis of covariance, $F_{(2,52)} = 71.98$, $P < 0.0001$). The $[\text{Ca}^{2+}]$ needed to obtain a particular motility score for the sperm of HW fish were 100 (80% motility) to 4000 (10% motility) times higher than those needed for FW fish, with an intermediate situation for SW fish. As an example, for obtaining 80% motility, the modeled $[\text{Ca}^{2+}]$ were of 0.16 mM, 7.9 mM, and 15.8 mM, for the sperms of FW, SW, and HW fish, respectively (Table 3). Differences between fish rearing salinities were also found for the relationships between sperm velocity and pCa (ANCOVA, $F_{(2,53)} = 79.61$, $P < 0.0001$), but they were significant only for the comparisons between HW and FW or SW fish and not for those between FW and SW fish.

3.5. Total duration of sperm motility

The total duration of sperm motility was analyzed in large volumes of sea salt solutions at optimal osmolality (see Section 2), for the sperm of native FW, SW, and HW fish. In all situations, sperm motility (%) decreased in a curvilinear way, but less rapidly in SW fish than that in FW or HW fish (Fig. 12). The time needed to pass from maximal sperm motility to 50% motility was about 60 minutes for SW fish, about 20 minutes for HW fish, and about 15 minutes for FW fish. Some sperm cells were still active 2 hours after activation for FW and HW fish, and up to 3 hours for SW fish.

The effect of Ca^{2+} supplementation (10 mM) about 1 and 60 minutes after sperm activation was examined in HW fish, which were found to be those with the highest requirements for Ca^{2+} (Fig. 13). One minute after sperm activation in diluted sea salt or in 0.5-M sucrose solution (about 600 mOsm kg^{-1}), the addition of Ca^{2+} slightly improved sperm motility. However, this tendency was not significant, probably because the mean percentages of active sperm cells were already high (>70%) before the addition of Ca^{2+} . One hour after sperm activation, sperm motility in diluted sea water and sucrose solutions had dropped considerably (to 1%–5%), whereas it still averaged about 30% in sucrose + 10 mM Ca^{2+} . At this moment, an addition of 10 mM Ca^{2+} significantly boosted sperm motility for sperm activated in diluted sea water or sucrose solution (from about 1%–5% to 50%–60%; $P < 0.05$). By contrast, the same addition to sperm activated in sucrose + 10 mM Ca^{2+} did not improve sperm motility, suggesting that in this case the external Ca^{2+} concentration was not limiting.

4. Discussion

4.1. Gonad and sperm characteristics

Testis growth in *S m heudelotii* was similar at the three experimental rearing salinities (GSI < 0.3%). These GSI values fall in the range that is generally reported for this

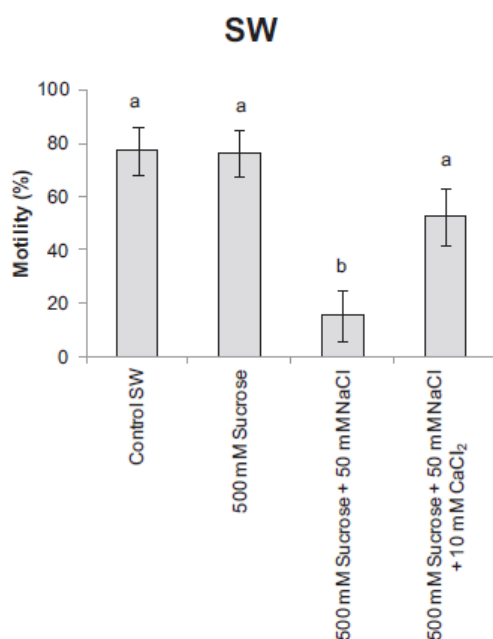


Fig. 10. Effects of Na^+ and Ca^{2+} in sucrose activation medium on the percentage of motile sperm cells (mean \pm SE) from *Sarotherodon melanocheilus* males reared in sea water (SW). Control (sea salt solution) and other media tested are at osmolalities of 500 to 600 mOsm kg^{-1} . Data sharing one superscript in common do not differ at $P < 0.05$.

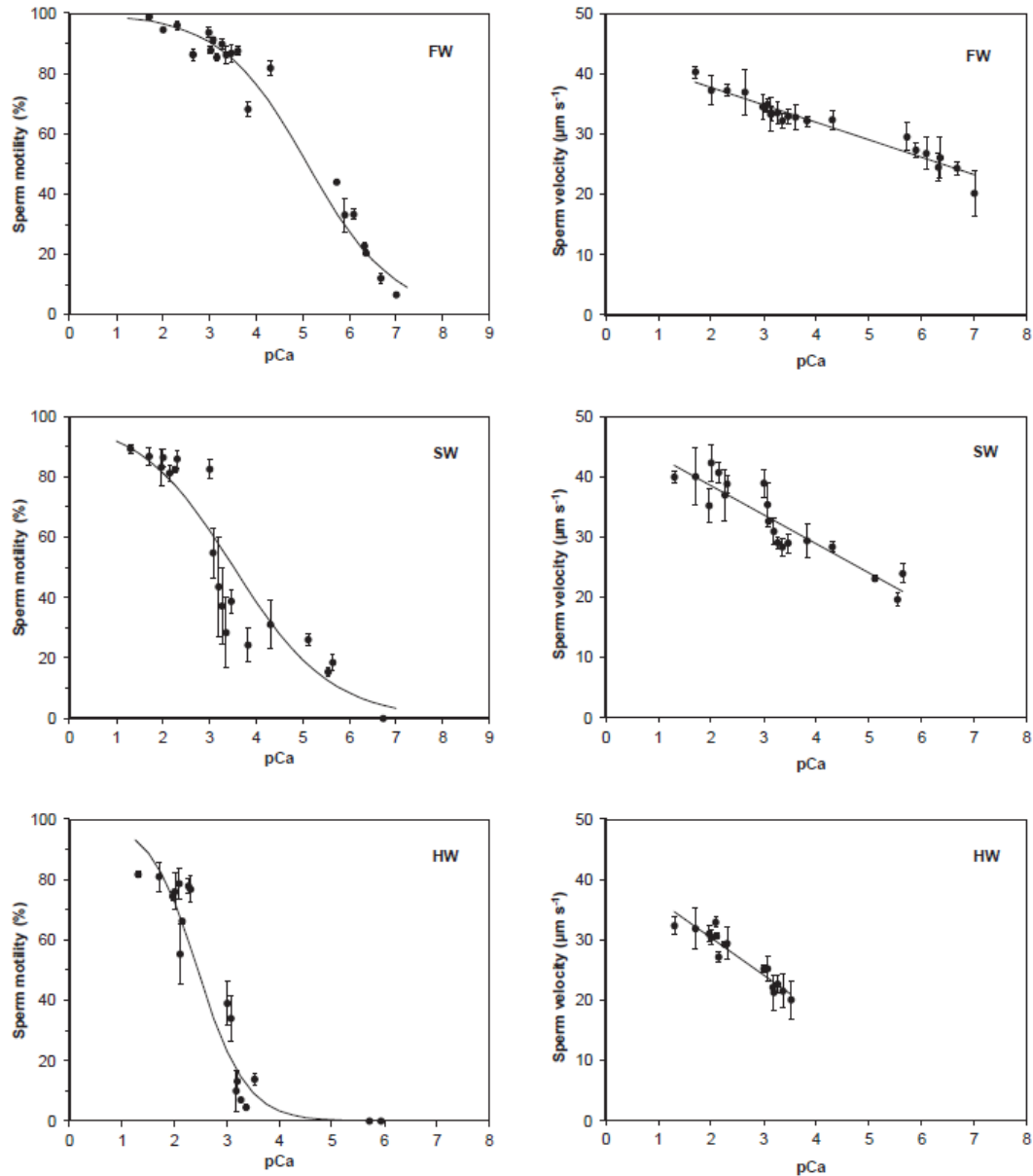


Fig. 11. Effects of free Ca^{2+} concentration ($\text{pCa} = \lg(1/[\text{Ca}^{2+}])$) on sperm motility (left column) and sperm velocity (right column) at 15 seconds after activation for *Sarotherodon melanotheron heudelotii* acclimated in fresh water (FW), sea water (SW), or hypersaline water (HW). Activation media were solutions of 50 mM NaCl for FW sperm or 250 mM NaCl for SW and HW sperm, to which various concentration of Ca^{2+} or EGTA were added. The final osmolalities of the activation media were 100 to 150 mOsm kg^{-1} for FW sperm and 500 to 550 mOsm kg^{-1} for SW or HW sperm. Model equations and statistics are given in Tables 3 and 4.

species either in cultured conditions or in the wild [24,28]. The volume of sperm that could be collected directly from the testes was small (30–60 μL) and was not influenced by ambient salinity. Sperm concentration did not vary either as a function of ambient salinity and averaged about 5×10^9

spermatozoa mL^{-1} . This absence of salinity-dependent variation contrasts with the situation for *O. mossambicus*, in which the concentration of testicular sperm in FW fish was about twice as high as in SW fish (9.9×10^9 vs. 4.6×10^9 spermatozoa mL^{-1}) [18].

Table 3

Coefficients (a, b), R^2 , and probability (P) of the relationships between sperm motility (M%) and pCa for *Sarotherodon melanotheron heudelotii* maintained in fresh water (FW), sea water (SW), or hypersaline water (HW).

Water salinity	n	a	B	R^2	P	pCa ₈₀	pCa ₅₀	pCa ₁₀
FW (0)	22	-1.0728	5.4677	0.939	<0.0001	3.8	5.1	7.1
SW (35)	19	-0.9663	3.4035	0.825	<0.0001	2.1	3.5	5.8
HW (70)	17	-2.1761	5.3222	0.833	<0.0001	1.8	2.4	3.5

The relationships are of the form: $M\% = 100 \frac{e^{(a + b \cdot pCa)}}{1 + e^{(a + b \cdot pCa)}}$ (see Fig. 11). The coefficient of determination R^2 and probability P are those of the corresponding linear model after data transformation $y = \ln(M\%/ (100 - M\%))$. The pCa values "pCa₁₀₋₈₀" are given by the models for sperm motility of 10%, 50%, and 80%, respectively (maximal motility observed in HW series).

The mean osmolality of *S m heudelotii* seminal plasma increased with ambient salinity, from 318 mOsm kg^{-1} in FW sperm to 330 mOsm kg^{-1} and 349 mOsm kg^{-1} in SW and HW sperm, respectively. In *O mossambicus*, the osmolality of the seminal plasma was also lower in FW fish than that in SW fish (283 vs. 330 mOsm kg^{-1} , [23]; 337 vs. 350 mOsm kg^{-1} , [18]). The ionic composition of the seminal plasma of *S m heudelotii* did not vary significantly as a function of fish rearing salinity and stood as 112 to 130 mM Na^+ , 17 to 23 mM K^+ , 1.1 to 1.9 mM Mg^{2+} , and 0.2 to 0.9 mM Ca^{2+} . Except for Na^+ , these values differ substantially from those documented for the intratesticular seminal plasma of *O mossambicus* acclimated to fresh water, i.e., 142 mM Na^+ , 50 mM K^+ , 0.18 mM Mg^{2+} , and 2.0 mM Ca^{2+} [19]. Proportionally, the greatest variations observed in the ionic composition of the seminal plasma in *S m heudelotii* at different salinities concerned Ca^{2+} , the concentration of which tended to decrease as ambient salinity increased. This particular tendency would need to be tested in larger fish samples.

The spermatozoa of *S m heudelotii* possess a relatively short flagellum (about 15–18 μm) in comparison to most other teleost species (20–100 μm) [29]. However, possessing a short flagellum is a sperm character shared by several other tilapia species, including *Oreochromis niloticus* and *O mossambicus* [18,30,31].

4.2. Effects of osmolality on sperm motility

When using synthetic sea salt solutions as an activation media, the motility of *S m heudelotii* sperm could be activated over a very wide range of osmolalities. The range of osmolality that enabled sperm activation shifted and broadened as the ambient salinity of broodfish increased,

Table 4

Coefficients (a, b), R^2 , and probability (P) of the linear relationships between sperm velocity (SV) and pCa for *Sarotherodon melanotheron heudelotii* maintained in fresh water (FW), sea water (SW), or hypersaline water (HW) ($SV = a \cdot pCa + b$; see Fig. 11).

Water salinity	n	a	b	R^2	P
FW (0)	22	-2.908	43.622	0.939	<0.0001
SW (35)	19	-4.844	48.218	0.846	<0.0001
HW (70)	16	-6.179	42.667	0.898	<0.0001

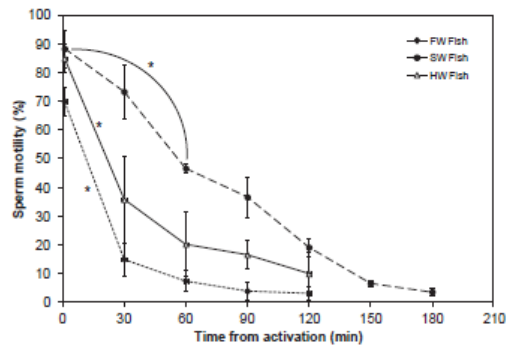


Fig. 12. Sperm motility (mean \pm SE) as a function of time since activation in diluted sea water for male *Sarotherodon melanotheron heudelotii* reared in fresh water (FW, n = 3), sea water (SW, n = 3), or hypersaline water (HW, n = 3). Sperms were maintained at constant room temperature (21 °C–22 °C) from the time of activation until the final observation. The osmolality of swimming medium was 300 mOsm kg^{-1} for FW fish and 600 mOsm kg^{-1} for SW and HW fish. *P < 0.05.

from 24 to 800 mOsm kg^{-1} for FW sperm to 24 to 2200 mOsm kg^{-1} and 24 to 3000 mOsm kg^{-1} in SW and HW sperm, respectively. Therefore, at the three rearing salinities under study, sperm motility could be activated in hypotonic, isotonic, and hypertonic conditions relative to the tonicity of the seminal plasma. The optimal ranges of osmolalities allowing the highest percentages of sperm activation were 150 to 300 mOsm kg^{-1} in fish reared in FW, and 300 to 800 and 500 to 1200 mOsm kg^{-1} in those acclimated to SW and HW, respectively. Similarly in *O mossambicus*, the optimal osmolality range was 70 to 333 mOsm kg^{-1} for the sperm of FW fish and 333 to 645 mOsm kg^{-1} for the sperm of SW acclimated fish [18]. No observations were made for the latter species at any osmolality and/or salinity above that of sea water. To our knowledge, except for these two euryhaline tilapia species, shifts in the optimal osmolalities for sperm activation in regards to environmental salinity have only been reported in three other aquatic animals with external fertilization: the sea-urchin, *Lytechinus pictus*, maintained in full or hypoosmotic sea water (diluted to 75%) [32]; the Gulf killifish, *Fundulus grandis* (Cyprinodontidae), acclimated to salinity between 0 and 50 [33]; and different wild populations of the Anurian amphibian, *Crinia signifera*, living at various environmental osmolalities (15–30 mOsm kg^{-1}) [34].

The present study demonstrates that salinity-dependent shifts in optimal osmolality for sperm activation in *S m heudelotii* can take place during the acclimation of individual animals from a same population. Several observations herein showed that this shift could occur rapidly, within a few weeks, after the acclimation of fish to a new level of environmental salinity. In *O mossambicus*, these changes had already started after a few days [23] and were clearly marked 1 month after the acclimation of fish to fresh or sea water [19,20]. Tiersh and Yang [33] also reported that environmental salinity influences sperm behavior in males of *F grandis*, with substantial changes

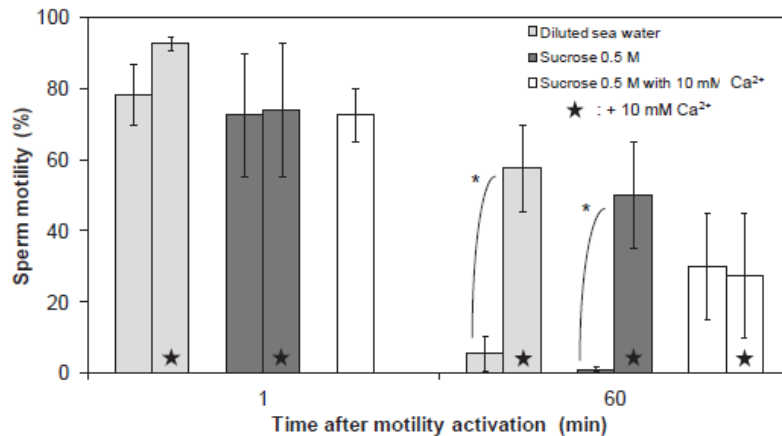


Fig. 13. Effect of calcium on sperm motility (mean \pm SE) in different swimming media (diluted sea water, sucrose 0.5 M, or sucrose 0.5 M containing 10 mM CaCl_2 ; osmolality close to 600 mOsm kg^{-1} in all situations) for *Sarotherodon melanotheron heudelotii* reared in hypersaline water. Sperm is activated in a tube containing the swimming medium. One and 60 minutes after sperm activation, 10 mM Ca^{2+} was added on the microscope slide to a drop of swimming medium containing the activated sperm, and its effect on sperm motility was compared to a control situation (i.e., without additional Ca^{2+}). *: $P < 0.05$.

after only 30 days of acclimation. In *S. m. heudelotii*, these changes were clearly marked after 2 months (present study). In the latter species, a weekly follow-up of sperm motility in the same individuals during the acclimation period from fresh to sea water indicated that the shift of optimal osmolality occurred progressively during the increase of water salinity, and noticeable changes were already observed after 3 to 4 weeks of acclimation (J. Cosson and M. Legendre, unpublished observations). This dynamic process raises the question of whether the osmolality shifts described here after acclimation to salinity referred to a transient situation, especially for HW fish, in which the osmolality tolerance was extremely broad (24–3000 mOsm kg^{-1}). However, the close similarity between the sperm responses of acclimated and native fish (Fig. 5) indicates that this osmolality shift was completed less than 2 months after fish acclimation to salinity. The minimal delay for achieving this shift in *S. m. heudelotii* and its between-individual variability remain to be determined.

Interestingly, both for acclimated and native fish, the ranges of optimal osmolality for sperm activation never encompassed the environmental osmolalities at which the fish were maintained, i.e., 150 to 300 versus less than 30 mOsm kg^{-1} in FW fish, 300 to 800 versus 1100 mOsm kg^{-1} in SW fish and 500 to 1200 versus 2200 mOsm kg^{-1} in HW fish. At ambient water osmolality, sperm motility was thus not maximal, but this obviously sufficed for an efficient fertilization, as numerous fertilized spawns were regularly found in the mouth of incubating males at the three salinities under study [35].

As in most other teleost fishes, the spermatozoa of *S. m. heudelotii* are quiescent in their seminal plasma. The factors that prevent the activation of mature spermatozoa in the testis vary depending on species. In Salmonidae and Acipenseridae, potassium is a key ion controlling sperm motility, in combination with osmotic pressure [36]. In most other fishes, sperm motility is triggered by a change in

osmotic pressure between the seminal fluid and the ambient water, the trigger being an increase in osmolality for marine fishes and a decrease in osmolality in Cyprinidae [37]. In *S. m. heudelotii*, at the three salinities under study, sperm motility could be induced in media of osmolality equivalent to that of the seminal plasma (300–350 mOsm kg^{-1} , see Table 1). Therefore, osmolality is clearly not the factor that inhibits sperm motility inside the testis in this species, and further investigations are needed to identify its nature. In the Tanganyikan Cichlid, *Astatotilapia burtoni*, sperm motility was induced by a decrease in the ratio of Na^+/K^+ concentrations or by a decrease in Na^+ concentration only, depending on the populations under study [38]. In the Nile tilapia, *O. niloticus*, the presence of a sperm immobilizing factor in the seminal plasma was reported [39]. This factor appeared to be a high molecular weight glycoprotein that is secreted by Sertoli cells and epithelial cells of the sperm duct and is also bound to the head of the spermatozoon. This glycoprotein is present in seminal plasma as a homopolymer creating a high viscoelastic environment that could prevent sperm motility. It is thus possible that the seminal plasma of *S. m. melanotheron* also contains some similar immobilizing factor: motility activation would be induced simply by mechanical release, provided that the osmolality and ionic composition of the activation medium are appropriate.

After activation in diluted sea water of optimal osmolality, the characteristics of sperm motility (flagellar wave amplitude, wavelength, and beating frequency) in *S. m. heudelotii* varied between fish reared at different salinities. The flagellar beating frequency was higher in FW sperm (around 40 Hz) than that in SW and HW sperm (around 30 Hz). Initial sperm velocity was also higher in FW sperm (55–75 $\mu\text{m second}^{-1}$) than that in SW or HW sperm (about 40 $\mu\text{m second}^{-1}$). These velocities are close to those reported for the sperm of *O. mossambicus* acclimated to FW or SW (40–70 and 35–50 $\mu\text{m second}^{-1}$, respectively) [18].

The sperm velocities in these two euryhaline tilapia species appear very low in comparison to those documented in most other teleosts studied so far, both in marine and fresh water species ($140 \mu\text{m second}^{-1}$ and $135 \mu\text{m second}^{-1}$ on average, respectively) [40]. The relatively small lengths of sperm flagella in these euryhaline cichlids and their low beating frequencies certainly contribute to limit sperm velocity.

4.3. Effects of ions on sperm motility

The variations of sperm motility against osmolality in *S. m. heudelotii* were very similar in synthetic sea salt and in nonelectrolytic sucrose solutions, except for the sperms of SW and HW fish in hyperosmotic situations. At first sight, this result might suggest that osmolality is probably a major factor regulating sperm motility. However, it was shown here that, in FW, SW, or HW fish, the removal of free Ca^{2+} by addition of 1 mM EGTA almost totally inhibited sperm motility, either in NaCl or in sucrose solutions. This highlights the importance of external Ca^{2+} in the process of sperm activation in *S. m. heudelotii*, even in the case of fish reared in fresh water. These findings contrast with the observations on the sperm of FW acclimated *O. mossambicus*, for which the addition of 5 mM EGTA to 50 mM NaCl (as in the present study) still permitted high motility scores [19].

In the present study, it was shown that sucrose solutions that were supposedly devoid of any electrolyte actually contained some free Ca^{2+} (about 10^{-5} M Ca^{2+} in 500 mM sucrose). In sucrose solutions at optimal osmolalities, this amount sufficed for fully activating sperm motility in FW and SW fish and to a lesser extent in HW fish. By contrast, the sperm motility of SW and HW fish in sucrose solutions at osmolalities greater than $1500 \text{ mOsm kg}^{-1}$ was clearly lower than that in synthetic sea salt of similar osmolality, and Ca^{2+} supplementation was necessary to restore sperm motility (Fig. 3). The present study showed strong between-individual variability in the ability of *S. m. heudelotii* sperm to reach high motility scores in sucrose solutions of optimal osmolality. This variability was low in FW fish, substantial in SW fish, and even greater in HW fish. Its extent was not related to the duration of fish acclimation to salinity, as similar ranges of individual variations were observed in acclimated and native fish. It is possible that this individual variability reflects different minimal requirements for external Ca^{2+} . The following finding supports such hypothesis: the addition of Ca^{2+} restored the motility of sperm showing intermediate or low motility in a plain sucrose solution. So far, the mechanisms behind these individual variations have not been identified. Avare et al. [41], who analyzed gene expression in the testes of some of the individual fish used in the present study, reported differential gene expression as a function of rearing salinities. However, no correlation was evidenced between gene expression and sperm requirement for external Ca^{2+} (J.C. Avare and M. Legendre, unpublished data). Further observations focusing more accurately on gene expression in fish sperm, rather than in testis, would be useful to better understand the nature of these between-individual variations in sperm motility activation.

Our results also show that the presence of Na^+ or K^+ in the activation medium has a negative effect on the motility

of *S. m. heudelotii* sperm. This negative effect was low in FW sperm and high in SW or HW sperm. In all situations (FW, SW or HW fish), the decrease in sperm motility resulting from the presence of Na^+ or K^+ ions in AM could be compensated for by the addition of Ca^{2+} . The presence of Ca^{2+} also allowed sperm motility to occur in solutions of higher osmolalities, as was also observed in *O. mossambicus* [18,20]. We provided evidence (see previous section) that the limited concentration of Ca^{2+} (about 10^{-5} M) present as a contaminant in an activation medium composed of 500 mM sucrose + 0.1% BSA was sufficient (although with individual variations) and necessary for activating sperm motility. The addition of 50 mM NaCl to such sucrose solution strongly depressed the motility of SW fish sperm and further addition of 10 mM Ca^{2+} almost restored motility to its initial level (Fig. 10). Thus the external Ca^{2+} concentration required to fully activate sperm motility is clearly higher when Na^+ or K^+ are present in the activation medium. These results are in agreement with those of Linhart et al. [18] stating that, in *O. mossambicus* acclimated to FW or SW, sperm motility is controlled both by the osmotic pressure and by Ca^{2+} ions combined with Na^+ ions.

It is possible that, in the range of permissive osmolalities, the ratio between Ca^{2+} and Na^+ or K^+ concentrations in the AM represents a determinant of sperm activation in *S. m. heudelotii*. This feature should deserve more attention in future investigations particularly in relation to the osmolality of activation media. The type of artificial sea water used as activation medium in the present study contains 462 mM Na^+ , 9.4 mM K^+ , and 10.3 mM Ca^{2+} [42], thus the $[\text{Ca}^{2+}]/[\text{Na}^+]$ ratio (0.022) is constant whatever the osmolality. In NaCl solutions with different Ca^{2+} contents, we showed that the amount of external Ca^{2+} needed for activating sperm motility with an equivalent efficiency was dependent on the fish rearing salinity (for 80% sperm motility, 0.16 mM, 7.9 mM, and 15.8 mM Ca^{2+} , for FW, SW, and HW fish, respectively, see Table 3, Fig. 11). The resulting $(\text{Ca}^{2+})/(\text{Na}^+)$ ratios in these efficient activation media were 0.003, 0.03, and 0.06 for the sperm of FW, SW, and HW fish, respectively. The $[\text{Ca}^{2+}]/[\text{Na}^+]$ ratio allowing high sperm motility in FW fish was therefore about 10 to 20 times lower than that in SW and HW fish. This effective $[\text{Ca}^{2+}]/[\text{Na}^+]$ ratio for the sperm of SW fish (0.03) is very close to that in synthetic sea salt (0.022). Similar effects of the $[\text{Ca}^{2+}]/[\text{Na}^+]$ ratio were also observed for trout sperm [43].

In their work on fresh water and sea water acclimated *O. mossambicus*, Morita et al. [19,20] found that sperm motility was associated with an increase of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). In sperm of FW fish activated in hypotonic conditions, the increase of $[\text{Ca}^{2+}]_i$ is independent of extracellular $[\text{Ca}^{2+}]$ and seems to result from the mobilization of intracellular Ca^{2+} stores, which are probably located in the neck region of the sperm head (also known as the sleeve structure). By contrast, $[\text{Ca}^{2+}]_i$ in the sperm of *O. mossambicus* acclimated to sea water increased only in the presence of extracellular Ca^{2+} . Morita et al. [44] suggested that Ca^{2+} binds to flagellar proteins to activate flagellar mobility. They proposed that an increase in $[\text{Ca}^{2+}]_i$ induces Ca^{2+} /Calmodulin kinase-dependent regulation, including protein phosphorylation for activation or regulation of dynein activity in the flagellar axoneme. The

present study did not examine the molecular bases of sperm motility regulation in *S. m. heudelotii*. Nevertheless, we observed that a supplementation of the activation medium with 200- μ M W-7, a calmodulin antagonist, strongly and systematically decreased the motility of *S. m. heudelotii* sperm (Alavi, Cosson and Legendre, unpublished observations), as is the case in *O. mossambicus* [44].

Sperm motility regulation in *S. m. heudelotii* therefore shares many traits in common with that of *O. mossambicus*. Nevertheless, some differences were observed between the two species, as extracellular Ca^{2+} appeared to be necessary, even in hypotonic conditions, for activating the sperm of *S. m. heudelotii* reared in fresh water. In these conditions, the motility of *S. m. heudelotii* sperm was reduced to almost zero when extracellular Ca^{2+} levels were nominally depleted by EGTA, in contrast to the situation in *O. mossambicus* [19,20].

4.4. Total duration of sperm motility

Once activated, fish sperm generally have a short period of motility (a few tens to a few hundreds of seconds), and in general, the sperms of marine fishes swim over longer periods than those of fresh water fishes (on average, about 550 seconds vs. about 150 seconds) [2,40]. So far, total sperm motility duration has never been reported to last more than one hour in tilapia species, with the longest durations (30–60 minutes) observed in the euryhaline *O. mossambicus* [18,19,21], whereas in other tilapia species, the sperm is motile for less than 15 minutes [30]. In *S. m. heudelotii*, the sperm of SW fish activated in diluted sea salt within the optimal osmolality range showed a slow and progressive decrease of motility over time; with some (5%–10%) sperm cells remaining motile 3 hours after activation. This activation period is thus much longer than in most other fish species. During the first hour after the activation of *S. m. heudelotii* sperm, the decrease in sperm motility was steeper in FW and HW fish than that in SW fish. In *O. mossambicus*, the total duration of sperm motility was also longer in fish acclimated to sea water than in those acclimated to fresh water (60 vs. 40 minutes) [21].

As far as we know, the longest durations (3–7 days) of sperm motility in fish have been reported for internal fertilizing species. These species display unusual characteristics. In some of them (the spotted wolffish, *Anarhichas minor* [45]; the ocean pout, *Zoarces americanus* [46]), sperm cells are already motile in the seminal plasma. In some other species, sperm cells gain motility after the breakdown of spermatozeugmata inside the female genital tract (e.g., the guppy, *Poecilia reticulata* [47]). To our knowledge, the medaka, *Oryzias latipes*, another euryhaline species, is the only teleost with external fertilization in which the duration of sperm motility was reported to exceed that of *S. m. heudelotii*. Yang and Tiersch [48] reported that medaka sperm could remain motile for as long as 1 week from activation. Nevertheless, this exceptionally long duration for a fish with external fertilization might have been probably overestimated, as in this study activated sperm samples were stored at 4 °C between successive observations. The medaka is a subtropical species and such low storage temperatures may have reduced sperm activity in a substantial way [49]. In the present study, activated sperm

samples were constantly kept at room temperature (21 °C–25 °C) ensuring continuous sperm activity.

It is interesting to notice that sperm of fishes with internal fertilization and that of euryhaline fishes with external fertilization (namely tilapias, *S. m. melanotheron*, present study, and *O. mossambicus* [19,20]; the medaka, *O. latipes* [48]; and the golf killifish, *F. grandis* [33]) share in common the ability of being activated over an osmolality range including that of their seminal plasma. Fish species that are capable to reproduce successfully over a broad range of water salinities in their natural environment also seem to share particularly long durations of sperm motility in comparison to most other fishes. Tiersch and Yang [33] hypothesized that these traits of euryhaline species might be preadaptations or initial steps in the processes that led to the evolution of internal fertilization in fresh water fishes.

In *S. m. heudelotii*, the reduced sperm activity at the end of the motility period did not result from limiting energy reserves, as the addition of calcium in the activation medium caused most spermatozoa to become motile again. In the case of *S. m. heudelotii*, such very long duration of motility is indicative of high ATP production rate to sustain its consumption by the flagella machinery [50]. Detailed studies would be necessary to analyze the energetic aspects of sperm motility and the mechanisms by which calcium is involved in the maintenance and restoration of sperm movement in this euryhaline tilapia. In *O. mossambicus*, demembrated spermatozoa could be ATP-reactivated in the presence of Ca^{2+} (10^{-4} M) and absence of cAMP, whereas cAMP, cGMP or ATP alone failed to reactivate [19], thereby showing the importance of internal Ca^{2+} availability for sperm motility in this species.

4.5. Conclusions

The present experimental study provided evidence that the mechanism of sperm activation in the euryhaline tilapia *S. m. heudelotii* remains globally the same whatever the fish ambient salinity. As evidenced here, the sperm of fish reared in FW, SW, or HW conditions can be activated in hypotonic, isotonic, or hypertonic activation media, provided that $[\text{Ca}^{2+}]$ suffices, and this minimal concentration is definitely salinity-dependent. However, the optimal osmolality range for sperm activation in *S. m. heudelotii* was found here to shift toward hypertonicity when the fish rearing salinity was increased. In the present study, we provide evidence that the external $[\text{Ca}^{2+}]$ required for the activation of *S. m. heudelotii* sperm is (1) higher in fish reared at a higher ambient salinity (2) higher in hypertonic than that in hypotonic activation media, whatever the fish rearing salinity, and (3) higher in the presence of Na^+ or K^+ . The negative effect of Na^+ or K^+ on the motility of *S. m. heudelotii* sperm increases with increasing fish rearing salinity. The $[\text{Ca}^{2+}]/[\text{Na}^+]$ ratios allowing maximal sperm motility in SW or HW fish are close to those observed in natural environments, either in sea water or in hypersaline waters, as a result of evaporation. These results contrast with those reported in another euryhaline tilapia, *O. mossambicus*, in which external Ca^{2+} was not necessary to activate sperm motility in fish acclimated to fresh water. The very long duration of sperm motility is another

distinctive characteristic of *S. m. heudelotii* sperm in comparison to most other oviparous teleosts. At the end of a swimming period, sperm motility could be reactivated by addition of Ca^{2+} . Further studies on this euryhaline tilapia will be necessary to characterize sperm bioenergetics and the role of Ca^{2+} in the long duration of sperm motility.

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Chapter 5

Control of sturgeon sperm motility: Antagonism between K⁺ ions concentration and osmolality

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Control of sturgeon sperm motility: Antagonism between K^+ ions concentration and osmolality



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ABSTRACT

Spermatozoa are stored in a quiescent state in the male reproductive tract and motility is induced in response to various environmental stimuli, such as change of osmolality (general case) and a decrease of extracellular K^+ in fish from Acipenseridae family. This study was aimed to investigate the relationship between osmolality and extracellular K^+ concentration in controlling sperm motility in sturgeon. Pre-incubation of sturgeon sperm for 5 s in hypertonic solutions of glycerol, NaCl, or sucrose (each of 335 mOsm/kg osmolality) prepares sturgeon spermatozoa to become fully motile in presence of high concentration of K^+ ions (15 mM), which has previously been demonstrated to fully repress motility. Furthermore, presence of 0.5 mM KCl during the high osmolality pre-incubation exposure completely prevented subsequent spermatozoa activation in a K^+ -rich media. Manipulating the transport of K^+ ions by the presence of K^+ ionophore (valinomycin), it was concluded that once an efflux of K^+ ions, the precursor of sturgeon sperm motility activation, is taking place, spermatozoa then become insensitive to a large extracellular K^+ concentration.

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1. Introduction

According to the IUCN, over 85% of sturgeon species are at risk of extinction (IUCN, 2015). Wild sturgeon populations show steady decline due to overfishing for commercial production, poaching, habitat destruction, and environmental pollution (Birstein et al., 1997; Havelka et al., 2011). Such severe depletion of worldwide stocks of sturgeon is exacerbated by their late maturation and low reproduction rate. As an example, females and males of one of the most commercially valuable sturgeon species, beluga (*Huso huso*), reproduce for the first time at 15–18 and 10–15 years, respectively (Gesner et al., 2010). In order to meet intensive sturgeon demand and to ensure their survival and

preservation, special strategies for aquacultural restocking, which relies on artificial reproduction and rearing of offspring, have been introduced as a solution to mitigate fishing pressures (Bronzi et al., 2011). One of the most essential elements in the technologies for reproductive efficiency is artificial insemination. In this regard, successful insemination directly depends on quality, metabolism, and the appropriate functioning of male gametes (among other factors). Therefore, an understanding of sperm biochemistry, physiology, and signaling mechanisms for motility activation and regulation are essential for artificial fertilization procedures in sturgeon.

Spermatozoa of sturgeon, like that in most other fish species with external fertilization, are immotile in the genital tract prior to release into the aquatic media (Cosson, 2010; Cosson et al., 2000; Morisawa and Suzuki, 1980). This immotility is maintained both by high concentrations of potassium (K^+) ions and osmolality of seminal fluid (Alavi

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et al., 2004; Gallis et al., 1991). Abrupt changes of these physiological factors during spawning immediately triggers sperm motility (Cosson, 2010).

Numerous studies revealed that osmolality of seminal plasma of different sturgeon species ranges from 25 to 112 mOsm/kg, which is relatively lower than in teleost fishes (Alavi and Cosson, 2006; Alavi et al., 2004, 2008, 2012). This phenomenon is associated with the specific anatomy of the sturgeon urogenital system; i.e., efferent ducts coming from the testes directly contact the kidneys. Hence, sperm when released from the testes is diluted by hypotonic urine in the Wolffian ducts, resulting in a decrease in osmolality and ionic concentration of the milt (Dzyuba et al., 2014). The activation of sturgeon spermatozoa is initiated upon contact with a hypotonic aquatic environment, but also can be induced in media, which has an osmolality that is isotonic, or even slightly hypertonic to the seminal fluid (Alavi et al., 2004, 2011; Cosson et al., 1999; Dzyuba et al., 2013). Studies indicated that alteration of osmolality alone would not activate sperm if high concentrations of K^+ ions were present (Alavi et al., 2004; Psenicka et al., 2008). It was therefore suggested that for sturgeon sperm (Alavi et al., 2004; Gallis et al., 1991; Toth et al., 1997), as in salmonids (Bondarenko et al., 2014a, 2014b; Linhart et al., 2002; Morisawa and Suzuki, 1980), a key signal for the initiation of motility is the decrease in extracellular K^+ , while a change of only osmotic pressure seems to play less of a role for regulatory processes. The lowest concentrations of extracellular K^+ ions found to prevent activation of sturgeon sperm range between 0.1 and 2 mM (Alavi and Cosson, 2006; Alavi et al., 2004; Cosson and Linhart, 1996). To date, despite the general assumption of ionic and osmotic contribution, the signal transduction pathway for motility initiation of sturgeon spermatozoa is still not fully understood.

Recent investigations discovered that sturgeon spermatozoa could remain in the quiescent stage even in K^+ -free solution, just due to the hypertonicity of this solution (Judycka et al., 2015). In addition, exposure of sturgeon sperm to a hyper-osmotic stress induced by organic solvents results in a significant delay in motility activation (Prokopchuk et al., 2015). Based on these findings, it may be hypothesized that hyperosmolality can alter the sensitivity of sturgeon spermatozoa to external K^+ ions.

This study was therefore designed to investigate the relationship between osmolality and extracellular K^+ concentration in controlling sperm motility in sturgeon. More specifically, we explored the motility of sturgeon spermatozoa treated with hypertonic electrolyte (NaCl) and nonelectrolytes (glycerol or sucrose) solutions having similar osmolality, both in the presence and absence of K^+ ions.

2. Materials and methods

2.1. Sperm sampling

All experimental procedures were approved by the Animal Research Committee of Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of

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European sturgeon (beluga; *H. huso*) from the sturgeon farm of Fischzucht Rhonforelle GmbH & Co Company (Marjoss, Germany) was used as a model fish. Five sexually mature males (19 years old) with average weight of 48–51 kg were intramuscularly treated with homogenized carp pituitary suspension at a dose of 4 mg/kg of body weight. Milt from each male was obtained 30 h after hormonal injection by a catheter from the urogenital papilla and collected directly into a sealable plastic bag, taking special care to avoid contamination with feces, blood, mucus, or water. Fresh sperm samples were kept dry on ice until use.

2.2. Experimental procedure

Spermatozoa were activated in 10 mM Tris-HCl, pH 8.5 to test sperm motility and to serve as the experimental control. To study the effects of hyperosmotic pretreatment on triggering spermatozoa motility in presence of high K^+ , the milt was diluted 10-fold in one of the three different hypertonic solutions (all at 335 mOsm/kg): glycerol (non-ionic), sucrose (non-ionic), and NaCl (ionic), each buffered with 10 mM Tris-HCl at pH 8.5. After 5 s incubation at room temperature (22 °C) in one of these hypertonic media, 5 μ L of sperm suspension was transferred to a glass slide into a 50 μ L drop of pure 10 mM Tris-HCl, pH 8.5 (for a control) or 10 mM Tris-HCl, pH 8.5 containing 15 mM KCl, to observe motility activation.

In order to evaluate motility inhibition by extracellular K^+ , intact spermatozoa were activated in 10 mM Tris-HCl, pH 8.5 supplemented with 0.125, 0.25, 0.5, 1, or 15 mM KCl. The same concentrations of KCl were tested in all hyperosmotic solutions, as pre-incubation treatments, to verify the possible blocking of a subsequent motility activation in pure or K^+ -comprising 10 mM Tris-HCl, pH 8.5 due to the presence of K^+ ions during the sperm pre-incubation.

To investigate spermatozoa sensitivity to external K^+ after hyperosmotic pretreatment, 0.4 μ M valinomycin (a K^+ selective ionophore, affecting the transmembrane transport of K^+ ions) was added to the activators: pure or K^+ -comprising 10 mM Tris-HCl, pH 8.5.

Osmolalities of each hyperosmotic media were measured (mOsm/kg) in triplicate using a vapor pressure osmometer (model 5520, Wescor, Logan, Utah, USA).

2.3. Sperm motion assessment

Sperm swimming was recorded immediately after activation using a CCD analog video camera (SSC-DC50AP, Sony, Japan) at 50 half frames per seconds (fps) (25 fps interlaced, PAL standard) mounted on a dark-field microscope (Olympus BX41, Tokyo, Japan) with a 20 \times objective lens and illuminated with a stroboscopic lamp (ExposureScope, Vodnany, Czech Republic) set to a flash frequency of 50 Hz (Rodina et al., 2008). All video records were analyzed to estimate percent of motile spermatozoa

(motility %), curvilinear velocity (VCL, $\mu\text{m/s}$) and motility duration (s) (Linhart et al., 2000; Rodina et al., 2008). VCL was defined at 15 s after activation using a macro in Olympus Micro Image software (V. 4.0.1. for Windows, Japan). Five consecutive video frames were overlapped to create an image with successive positions of sperm heads. VCL was calculated as length of spermatozoa head track divided by the time elapsed between the first and fifth video frame (Rodina et al., 2008). The total duration of motility was evaluated until forward propulsion of spermatozoa stopped.

2.4. Statistical analysis

Data were analyzed using STATISTICA (StatSoft, Inc. version 12). Results were compared using the Kruskal–Wallis test, followed by the multiple comparisons of mean ranks (Siegel and Castellan, 1988). Alpha was set at 0.05. Data are presented as mean \pm S.D.

3. Results

Motility activation of intact beluga sperm was totally suppressed when a minimum of 1 mM KCl was present in 10 mM Tris-HCl, pH 8.5 and *a fortiori* when 15 mM KCl concentration was used (Fig. 1). Similarly, spermatozoa remained completely immotile in all 335 mOsm/kg solutions: glycerol, or NaCl or sucrose. However, when sperm was incubated in one of these hypertonic media for 5 s and then immediately transferred into a supplemented (15 mM KCl) low osmotic solution (10 mM Tris-HCl, pH 8.5), spermatozoa became motile after a brief delay (Fig. 1). The motility of cells pre-treated by glycerol or sucrose (high osmolality) and activated in 15 mM KCl did not differ significantly ($p > 0.05$) from those activated after pre-treatment in 10 mM Tris-HCl (pH 8.5) or from the experimental control (spermatozoa activated in pure 10 mM Tris-HCl, pH 8.5). However, motility was significantly different ($p < 0.05$) for sperm incubation in NaCl followed by activation in 15 mM KCl compared to the controls and the other treatments (Fig. 1a). On the other hand, VCL and motility durations were significantly ($p < 0.05$) influenced by transient hyperosmotic shocks with subsequent dilution in K^+ -rich Tris-HCl (Fig. 1b, c). It is worth noting that spermatozoa pre-incubated in high glycerol, NaCl, or sucrose concentration and then triggered by 10 mM Tris-HCl (pH 8.5) showed significantly longer motility duration than those activated in pure 10 mM Tris-HCl (pH 8.5) without any pre-incubations.

We tested the influence on the capacity of post-treated spermatozoa to become motile in K^+ -rich solution, if K^+ ions (KCl at 0.125, 0.25, 0.5, 1, or 15 mM) were added during pre-incubation. When pre-treatment media were supplemented with 0.125 or 0.25 mM KCl, total motility of spermatozoa subsequently activated in 15 mM KCl was notably lower ($p < 0.05$) in comparison to those triggered by Tris-HCl (Fig. 2a, d and g). Further, when the concentration of KCl was increased to 0.5 mM and higher (for 1 and 15 mM results not shown), the sperm movement in the 15 mM KCl supplemented swimming solution was completely inhibited. Whereas, in control K^+ -free swimming solution motility remained either similar to the

conditions when 0.125 or 0.25 mM KCl were present during the sucrose pre-treatment (Fig. 2d), or was only slightly affected ($p < 0.05$) when pre-incubation proceeded in glycerol or NaCl (Fig. 2a and g). Together with the percentage of motile cells, the motility duration and VCL of spermatozoa swimming in K^+ -rich solution were greatly ($p < 0.05$) influenced by the presence of 0.125 or 0.25 mM KCl in all pre-treatment media (Fig. 2). It is remarkable that sperm cells activated in 15 mM KCl after pre-incubation in hyperosmotic media containing 0.25 mM KCl showed VCL values similar to those pre-incubated with 0.125 mM KCl (Fig. 2b, e and h). The same was observed for motility duration (Fig. 2c and i), except in the case of sucrose pre-treatment (Fig. 2f), where spermatozoa pre-treated with 0.25 mM KCl swam for a two-fold shorter period ($p < 0.05$).

To target the transmembrane transport of K^+ ions after the hyperosmotic treatment, a pharmacological reagent selectively modifying the membrane permeability to K^+ ions, valinomycin (K^+ selective ionophore) at 0.4 μM concentration was added into all activation media. The addition of valinomycin did not prevent pre-treated sperm from becoming motile in 10 mM Tris-HCl (pH 8.5) (Fig. 3). However, the presence of this compound in K^+ -rich activating solution, totally suppressed the activation of sucrose or NaCl pre-treated sperm. In case of glycerol pre-treatment, a small proportion (Fig. 3a) of spermatozoa showed a brief period of motility in the 15 mM KCl (Fig. 3c) swimming solution but at a low velocity (Fig. 3b).

4. Discussion

In sturgeon species, including beluga, sperm motility is under joint control by K^+ ions concentrations as well as osmolality of the seminal plasma (Alavi et al., 2004, 2011; Cosson, 2010; Gallis et al., 1991). However, sperm cells remain quiescent when transferred from seminal fluid (about 1 mM K^+) into a hypotonic media if the K^+ concentration is equivalent to that of seminal fluid (or higher) (Alavi et al., 2004; Psenicka et al., 2008). At appropriate osmolality, a significant decrease or removal of extracellular K^+ ions allows motility initiation of sturgeon sperm (Alavi et al., 2011; Cosson et al., 2000, Cosson, 2010; Linhart et al., 1995), which is similar to salmonid spermatozoa, where motility activation mechanisms have been well studied (Morisawa and Suzuki, 1980; Morisawa et al., 1983; Tanimoto et al., 1994). Based on observations for salmonid sperm and taking into account the similarity between Salmonidae and Acipenseridae modes of sperm activation, Alavi et al. (2011) proposed a hypothetical model of sperm motility initiation in sturgeons. According to their model, external factors, such as a hypo-osmotic signal or a decrease of environmental K^+ ions at appropriate external osmolality, may provoke the opening of specific membrane K^+ channels causing an efflux of K^+ . This efflux would induce the hyperpolarization of the cell membrane, thus leading to a Ca^{2+} influx through Ca^{2+} channels subsequently followed by membrane depolarization. Consequently, an increase of intracellular Ca^{2+} concentration stimulates flagellar motility via Ca^{2+} /calmodulin activated phosphodiesterase similar to the cascade of events described for Salmonidae (Morita et al., 2004). Although this theoretical

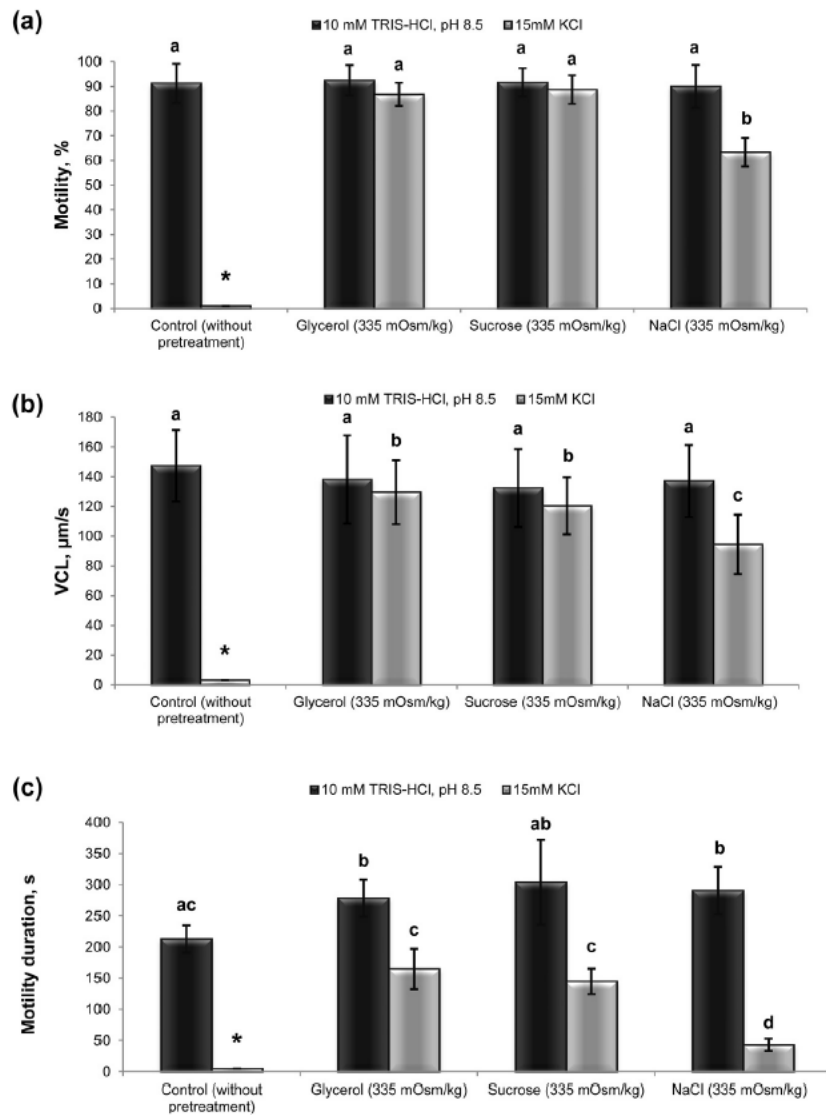


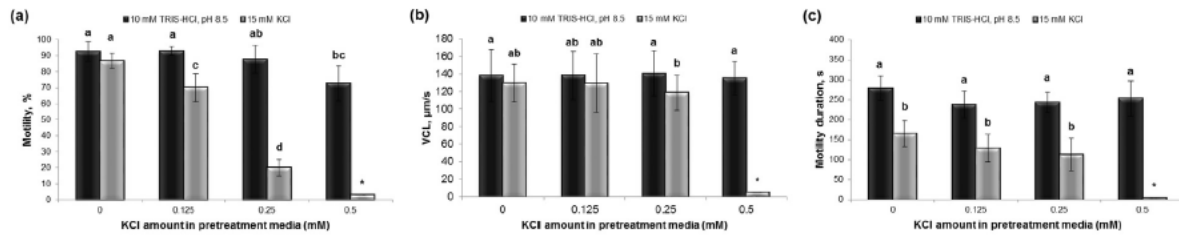
Fig. 1. Beluga sperm motility (a), VCL (b), and duration of motion (c) after 5 s pre-incubation in hypertonic (335 mOsm/kg glycerol, 335 mOsm/kg sucrose, or 335 mOsm/kg NaCl) media followed by activation in either 10 mM TRIS-HCl (pH 8.5) alone or supplemented with 15 mM KCl. Data are presented as mean \pm SD; ($n=5$). Values with the same superscripts are not significantly different ($p>0.05$). Samples showing no motility (*) were not included in the statistical analysis.

model attempts to elucidate the processes of motility initiation in sturgeon spermatozoa, a detailed mechanism of sturgeon sperm activation remains unclear and requires further studies.

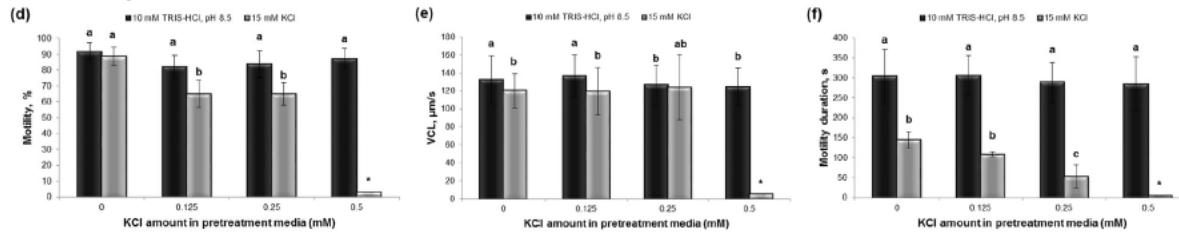
As shown in the present study, sturgeon spermatozoa remain motionless even in the absence of additional external K^+ ions, when transferred into an ionic (NaCl) or non-ionic (glycerol or sucrose) solution having osmolality (335 mOsm/kg), which is higher than that of the seminal plasma (≤ 100 mOsm/kg). These observations are in agreement with previous reports, which suggest that osmolality is not the principal factor prohibiting sperm activation in the seminal fluid (Alavi et al., 2004, 2011; Bondarenko et al., 2013; Dzyuba et al., 2013). Nevertheless,

recent studies showed that freshwater fish spermatozoa (i.e., carp, sturgeon, trout, tench, and pangasius) are able to exhibit motility in very high osmotic conditions created by organic compounds such as dimethyl sulfoxide, which rapidly penetrate membranes (Perchec-Poupard et al., 1997; Prokopchuk et al., 2015). The presence of these compounds permits a quick balancing of the osmotic pressure difference between both sides of the plasma membrane, thus leading to sperm activation. Similarly rapid osmoregulation may not occur under conditions of hyperosmolality that is caused by glycerol (depending on a cell type; Steinbach, 1966), NaCl or sucrose, as these components may penetrate the cell membrane much more slowly or may be even non-permeant (sucrose molecules).

A 335 mOsm/kg Glycerol



B 335 mOsm/kg Sucrose



C 335 mOsm/kg NaCl

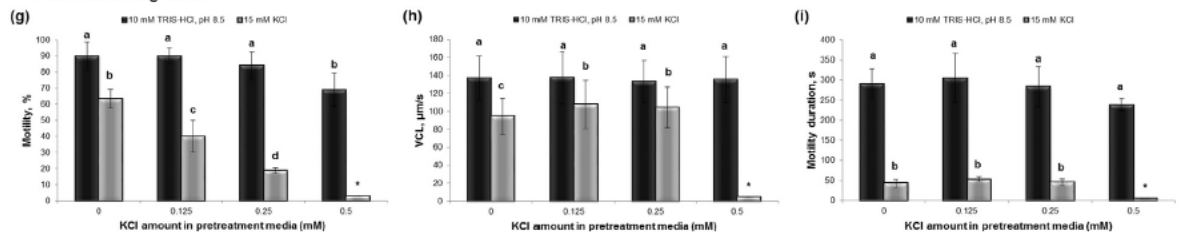


Fig. 2. Effects of hyperosmotic treatment by 335 mOsm/kg glycerol (A), 335 mOsm/kg sucrose (B), or 335 mOsm/kg NaCl (C) supplemented with different K^+ concentrations (0.125, 0.25, or 0.5 mM) on motility percentage (a, d and g), velocity (b, e, and h) and motility duration (c, f, and i) of beluga sperm in either 10 mM TRIS-HCl (pH 8.5) alone or supplemented with 15 mM KCl. Data are presented as mean \pm SD; ($n = 5$). Values with the same superscripts are not significantly different ($p > 0.05$). Samples showing no motility (*) were not included in the statistical analysis.

Thus, in order to re-form the osmotic equilibrium, intracellular water molecules may exit the sperm cells along the concentration gradient and, as a result, spermatozoa will shrink to a critical minimal volume and their membrane will become more rigid, so as to impede the transport processes and the dehydrated cell will irreversibly collapse (Lucke et al., 1935; Miermont et al., 2011; Pedersen et al., 2001; Rasmussen et al., 2008). In this regard, sperm incubation in hyperosmotic solution was limited to 5 s in our present assay.

On the other hand, when high osmolality surrounding sperm cells was decreased by successive dilution in a low-osmotic media, such as 10 mM Tris-HCl (pH 8.5), spermatozoa showed regular motility. Moreover, after a short incubation in hyperosmotic media, sperm cells were able to swim in the presence of high K^+ concentration previously established as fully repressive for motility. Most likely, a pressure surge caused by the transfer of spermatozoa from isotonic to hypertonic conditions triggers an efflux of K^+ ions out of the cells, which presents the sturgeon sperm with a first signal for motility initiation. It was assumed that the osmotic shock would cause a modification of the membrane permeability (especially regarding K^+ ions) due to reorganization of the lipid bilayer (Marian et al.,

1993). In the same line of evidence, Takai and Morisawa (1995) suggested that alteration of external osmolality may be converted into lowering of the intracellular K^+ concentration and that this K^+_{ext} decrease would constitute the signal that initiates sperm flagellar motility.

From our results, it appears that once an efflux of K^+ is taking place, beluga spermatozoa become insensitive to extracellular K^+ ions and probably to other environmental ions. Similarly, published results reveal that trout spermatozoa continue to move even though the surrounding K^+ ions concentration is increased within 1 s after activation up to an inhibitory level (Boitano and Omoto, 1991). Manipulating the transport of K^+ ions through channels by the presence of valinomycin, an ionophore that equilibrates K^+ ions concentration across biological membranes (Pressman et al., 1967), we confirmed that such insensitivity to K^+ actually occurs. When adding valinomycin into hypotonic K^+ -rich solution, K^+ becomes membrane-permeant, so that K^+ ions readily influx back into the post-treated sperm cells as to equilibrate K^+ ions concentration between intracellular and extracellular compartments, thereby resulting in spermatozoa quiescence. Though, after pre-treatment with glycerol hyperosmotic solution, a small amount of cells was still briefly motile,

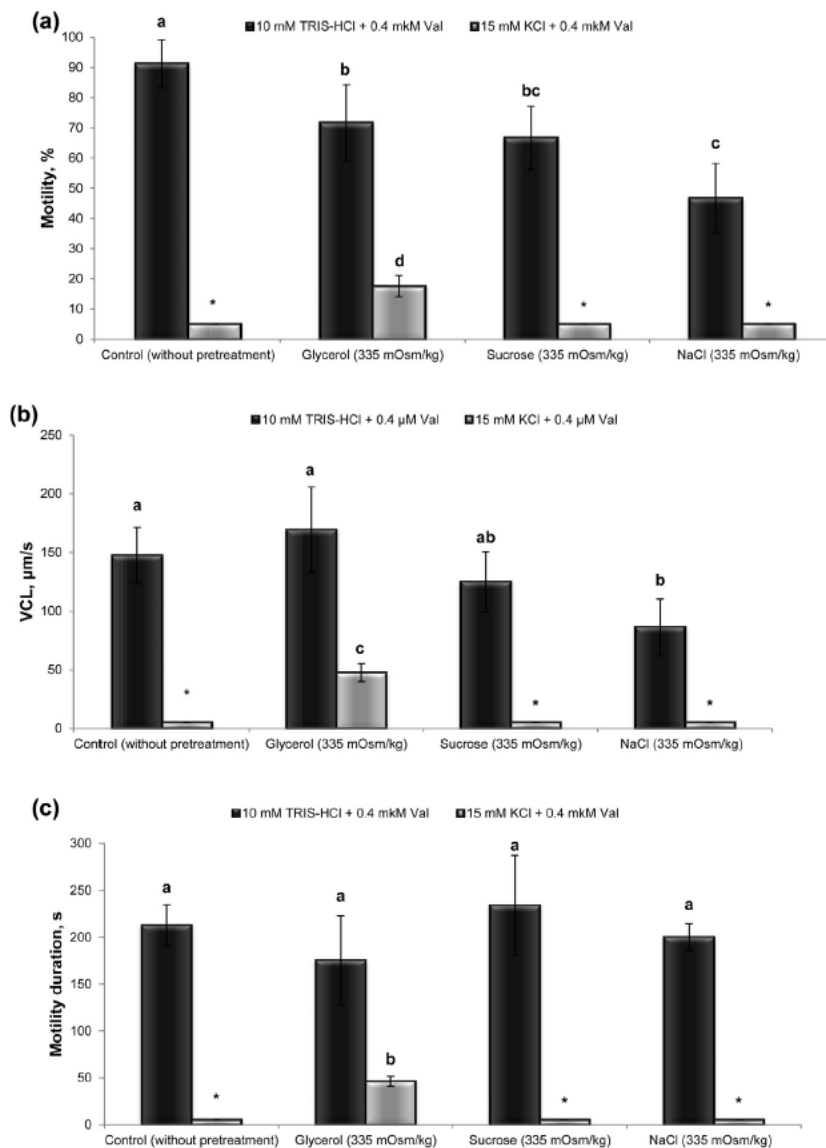


Fig. 3. Effects of hyperosmotic treatment by 335 mOsm/kg glycerol, 335 mOsm/kg sucrose, or 335 mOsm/kg NaCl on motility percentage (a), velocity (b), and motility duration (c) of beluga sperm activation. $0.4 \mu\text{M}$ valinomycin was added into the activating media composed of either 10 mM TRIS-HCl (pH 8.5) alone or supplemented with 15 mM KCl. Data are presented as mean \pm SD; ($n = 5$). Values with the same superscripts are not significantly different ($p > 0.05$). Samples showing no motility (*) were not included into statistical analysis.

a feature that may be specifically associated with the influence of glycerol itself. It was demonstrated that glycerol molecules might cause the fall in membrane electrical capacity (Gage and Eisenberg, 1969) and modify the membrane by inducing higher stiffening (Pocivavsek et al., 2011), hence in our case, an increase in membrane ionic permeability due to the ionophore may occur slowly.

Considering findings of this study, it may be suggested that an osmotic shock prior to exposure to motility activation allows the cells to by-pass the inhibitory effect of high K^+ ions concentration. Similarly, Morita et al. (2005) succeeded to unlock the movement inhibitory effect of K^+ by pre-treatments of intact salmonid sperm with glycerol or

erythritol at high osmolality. Later the same research group found that treatment with not only organic alcohol but also compounds such as NaCl or KCl at high concentration lead to similar effects (Takei et al., 2012). They suggested that, in salmonids, there are two parallel pathways, one ionic and another osmotic-dependent path, both leading to activation and both controlling phosphorylation of crucial proteins for motility triggering. Apparently, a similar duality in activation pathways would occur in sturgeon sperm. In contrast to salmonids (Takei et al., 2012), sturgeon spermatozoa pretreated by KCl hyperosmotic solution did not initiate their motility in low-osmotic K^+ -comprising conditions (results not shown). Moreover, the presence

of 0.5 mM KCl during the high osmolality pre-incubation step completely prevented further spermatozoa activation in K⁺-rich hypotonic media. Approximately same level of extracellular K⁺ concentration (between 0.1 and 2 mM depending on sturgeon species) was shown to inhibit activation of intact sturgeon's sperm (Alavi and Cosson, 2006; Cosson and Linhart, 1996; Toth et al., 1997). Such a blocking effect of external K⁺ supports our assumption that the efflux of K⁺ ions out of the cells occurs exactly during sperm pre-incubation.

In summary, our study has revealed that sturgeon spermatozoa may be activated by use of an unexpected signaling pathway, independent from ionic stimulation. This alternative regulation mechanism involves shock due to the osmotic difference (application of a hyperosmotic treatment immediately followed by dilution into a low-osmotic media) and eliminates dependence on the previously described blocking effect of K⁺ ions. Further detailed investigations are required for better clarification of molecular mechanisms that regulate motility in sturgeon sperm.

Conflicts of interest

We declare no actual or potential conflict of interest regarding the submitting manuscript.

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Chapter 6

Motility initiation of sterlet sturgeon (*Acipenser ruthenus*) spermatozoa: Describing the propagation of the first flagellar waves

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Motility initiation of sterlet sturgeon (*Acipenser ruthenus*) spermatozoa: Describing the propagation of the first flagellar waves



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ABSTRACT

In the present study, for the first time in fish spermatozoa, we describe the precise chronology of motility initiation of sterlet (sturgeon) sperm from completely immotile flagella to regular full wave propagation. The successive activation steps were investigated by high-speed video microscopy, using specific experimental situation, where sperm motility initiation was delayed in time up to several seconds (10 ± 2.68 seconds). Starting from fully immotile, the flagellum shows some trembling for a brief period, soon followed by appearance of the first real bend (so-called "principal bend") with a large wave amplitude $4.28 \pm 0.65 \mu\text{m}$, then by the "reverse bend," the latter presenting a lower ($P < 0.05$) wave amplitude ($1.14 \pm 0.32 \mu\text{m}$). This couple of first bends formed at the basal region begins to propagate toward the flagellar tip but gradually fades when reaching the midflagellum, wherein consequently the sperm cell remains nonprogressive. This behavior repeats several times until a stage where the amplitude of the reverse bend gradually reaches a value similar that of the principal bend: The larger amplitude of this couple of bends finally leads to sustain a real "takeoff" of the sperm cell characterized by a full flagellar wave propagation generating an active forward displacement similar to that occurring during regular steady state motility (several seconds after activation). Starting from the earliest stages of motility initiation, the wave propagation along the flagellum and formation of new waves proceeded in a helical manner leading to a 3-dimensional rotation of the whole spermatozoon. Eventually, we estimated that the time period needed from the activation signal (contact with fresh water) to full wave propagation ranges from 0.4 to 1.2 seconds.

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1. Introduction

Most endangered and commercially valuable fish species, such as sturgeons [1–5], are in a great need of aquacultural restocking, which relies on artificial reproduction [6–10]. Successful insemination directly depends on

metabolism and the appropriate functioning of male gametes. Movement is one of the crucial functions of spermatozoa, allowing them to actively reach and penetrate the female gamete, as such monitoring a sperm velocity, the percentage of motile cells, and the duration of motility becomes essential for evaluating a male's fertilization potential [11–13]. All above characteristics are based on studies of the sperm head displacement [14,15]; however, the observation of behavior of sperm flagellum is much more suitable for predictive description of the sperm

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quality, as this organelle forces sperm cells to propel through an aquatic environment [16,17]. In addition to the practical applications, estimation of different parameters specific to flagellar beating may also lead to a better understanding of the internal mechanics which explain how the motion of flagella is controlled [18].

To date, one of the first and probably most studied flagella, in terms of movement characteristics, is that of sperm cells of sea urchins (echinoids) [16,19–21]. On the contrary, little is known about the operation of fish sperm flagella, which also represent a unique object of investigation. In contrast to reptiles or mammals [22], spermatozoa of most fish species remain immotile in the testes [23–26], but upon release into an aquatic environment (fresh or salt water depending on species), all cells become immediately and synchronously motile within a very brief period (supposedly, a fraction of second). In addition, fish sperm motility initiation is easily controlled at will by experimenters by selecting specific ionic or osmotic solutions to which sperm cells are exposed [27].

In most fish species, the activation process is generally initiated by alteration of enviroing osmotic pressure except in few cases where concentration of specific ions is crucial [28–31]; in acipenseridae and salmonidae, the main factor responsible for sperm motility activation is the decrease in extracellular K^+ concentration [25,32–35]. As a response to the osmotic and/or ionic primary signal, second messengers such as Ca^{2+} , cAMP, and protein phosphorylation will transfer this signal to the flagellar machinery [36], more specifically to molecular motors (dynein ATPase of flagella) [37,38].

One should take into account the fact that sperm cells obey to a rapid transition from an inactive to a fully active state, when proceeding to the experimental activation of sperm motility directly in a drop of swimming solution on the glass slide, meaning, it is necessary to achieve a homogenous suspension of spermatozoa quickly [39]. However, in a practical situation, the efficient mixing of sperm samples by the experimenter takes a few seconds, and consequently, flagellar wave initiation occurs exactly during that mixing procedure, referred to as the “blind period”. Furthermore, when applying high-magnification video microscopy methods for evaluation of sperm quality, it is difficult to focus on the object immediately. Therefore, video recording can be initiated only after a delay of 3 to 5 seconds. Thus, the earliest and most significant time frame during the evolution of sperm motility (i.e., first seconds) becomes impossible to observe, as previously emphasized.

While exploring effects of organic solvents (such as DMSO) on fish sperm motility, we observed that the presence of these components in the swimming solution alters motility activation processes. When DMSO at a concentration of 10% is included into the swimming medium, a few seconds delay elapses between mixing and sperm activation, as compared to a fraction of second suspected to occur in regular activation medium. In the present study, we take an advantage of generating such artificial delay in motility activation by application of an osmotic shock, so as to allow the detailed observation and quantification of flagella shape parameters precisely at the motility initiation step of sterlet sturgeon spermatozoa.

2. Materials and methods

All experimental procedures were approved by the Animal Research Committee of Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Research Institute of Fish Culture and Hydrobiology, Vodňany, Czech Republic.

2.1. Sperm samples preparation

Four mature Sterlet sturgeon (*Acipenser ruthenus*) males were treated with carp pituitary suspension at 4 mg/kg, and milt samples were obtained by catheterization of urogenital tract within 24 hours after hormonal treatment. Fresh sperm samples were stored on ice and used for motility recording within 5 hours. Spermatozoa motility was triggered by dilution into an activating medium (AM) composed of 1-mM $CaCl_2$, 10-mM NaCl, 10-mM Tris-HCl, pH 8.5 [40] and supplemented with DMSO at a final concentration of 10% or by AM as the experimental control. All solutions contained 0.25% Pluronic (Sigma-Aldrich, Steinheim, Germany) to prevent sperm stickiness to the surface of glass and create a flat drop by reducing the superficial tension of the water.

2.2. Video microscopy

2.2.1. Low-magnification microscopy and Computer-assisted sperm analysis (CASA)

To evaluate sperm motility parameters, dry sperm using the tip of a hypodermic needle was mixed in a 20 μ L drop of AM on the glass slide without a coverslip. Immediately thereafter, sperm movements were recorded through a dark-field microscope (Olympus BX41, Tokyo, Japan) with a $\times 20$ objective lens using a CCD analog video camera (SSC-DC50AP, Sony, Japan) at 50 half frames per second (fps) (25 fps interlaced, PAL standard) under stroboscopic illumination by Exposure Scope (Vodňany, Czech Republic) [41]. The percentage of motile cells was estimated in duplicate per sperm sample and expressed as the average number of all spermatozoa observed in the field of view at a particular time point. From video records, sperm velocity (curvilinear velocity [VCL]) was measured by evaluating the head positions of spermatozoa on five successive frames using Olympus Micro Image software (V. 4.0.1. for Windows, Japan) [41].

2.2.2. High-magnification microscopy and image analysis of flagella

Bending forms of sperm flagella were observed using an Olympus BX50F microscope (Tokyo, Japan) equipped with standard halogen illumination and with either a $\times 50$ dark-field or a $\times 100$ positive phase-contrast objective. Observations were recorded using a high-speed video camera (model i-SPEED TR from Olympus, UK) with spatial resolution of 848 \times 688 pixels and time resolution up to 1000 fps in routine records to obtain sharp images of flagella [17]. From several series of images covering one or several successive beat cycles, the detailed analysis of the flagellar beating behavior was performed by use of image analysis software (Olympus Micro Image 4.0.1. for Windows, Japan).

Different flagella beating parameters, such as beat frequency of waves, wavelength, wave amplitude, wave velocity, and bend angle, were used to evaluate variation of flagella wave patterns during the sperm motility initiation (Fig. 1). Flagellar beat frequency was calculated either from the time interval between two successive wave initiations occurring at the head–tail junction of a spermatozoon or according to Gibbons and Gibbons [42]. The wavelength (λ) was measured as the distance between the two inflexion points adjacent to a wave. The wave amplitude (a) was determined as the perpendicular distance between the peak of a wave and the reference line drawn from the head of a spermatozoon to the tip of a flagellum through mid-points of bent regions. The velocity of flagellar wave was defined by the change in distance (S) between corresponding wave crest points followed along the flagellum in successive images over time. The local bend angle (α) was determined as the angle between tangents to the two inflexion points adjacent to the bend. Sperm velocity from high-speed video records was measured as described previously (see Section 2.2.1). Because spermatozoa activation was not homogeneous and motility initiation for each spermatozoon occurred individually, the measurements of aforementioned characteristics were performed on the first two consecutive beat cycles immediately after entering each of the stages of flagellar motility initiation and additionally at 5 seconds after motility initiation both in AM and AM including 10% DMSO. Average acceleration over a period of time between the start of sperm propagation until entering into the full-beating phase was measured using the formula: $\bar{a} = \Delta V / \Delta t$ where ΔV is the change in velocity and Δt is the duration of the period of acceleration.

Description of the process of flagella triggering in fish sperm was divided into several stages by analogy with a similar situation described for sea urchin spermatozoa [43].

2.3. Statistical analysis

Data were analyzed using STATISTICA (version 12; StatSoft, Inc.). Results were tested for normality using the Shapiro–Wilk test, and homogeneity of variances was tested using a Levene's test. Normally distributed data were analyzed by one-way ANOVA followed by the Tukey test. In the case, the data violated ANOVA assumptions, a nonparametric Mann–Whitney U test was used. The significance

level was set at 0.05 for main effects and interactions. Results are presented as mean \pm standard deviation.

Changes in spermatozoa VCL and motility percentage over the postactivation time were subjected to polynomial regression analysis according to McDonald [44].

3. Results

3.1. Delay in motility initiation

Without inclusion of DMSO in the AM (regular activation conditions), motility initiation of sterlet sperm was occurring immediately after the contact with swimming medium. In contrast, dispersion of sperm into the same activating solution supplemented with 10% of DMSO led to a retardation of motility initiation for 6 to 14 seconds (10 ± 2.68 seconds) between dilution of spermatozoa and the start of motility (Fig. 2); such a delay allowed us to record details of successive flagellar activation events of several individual spermatozoa. Figure 2 shows that in DMSO-free medium, the percentage of motile cells reached a maximal value immediately after mixing, whereas in a media containing 10% DMSO, the maximum number of motile cells was reached 40 to 55 seconds later. In addition, duration of motility period lasted slightly longer when DMSO was present in the swimming solution.

CASA analysis shows that VCL of sterlet spermatozoa diluted in AM–DMSO solution increased gradually, whereas for sperm activated in pure AM, VCL curve has a descending character. At 10 seconds after sperm mixing with the swimming solution, spermatozoa that were diluted in AM without DMSO swam already at a maximum rate and their velocity values were higher ($P < 0.05$) than in those which have been activated by AM–DMSO solution (Fig. 3). Nevertheless, VCL of sperm triggered by AM with DMSO gained its maximal value at 60 seconds postactivation, and this value was equivalent to a VCL obtained at the same time point for spermatozoa activated in pure AM. However, when comparing maximal VCL values for both groups, in case of sperm activation by AM–DMSO solution, the maximal VCL (at 60 seconds after mixing) was notably lower ($P < 0.05$) than the maximal VCL (at 10 seconds after mixing) in spermatozoa triggered by DMSO-free AM.

3.2. Stages of motility initiation

A delay in sperm activation due to the presence of DMSO allowed us to particularize the flagellar bending shapes at

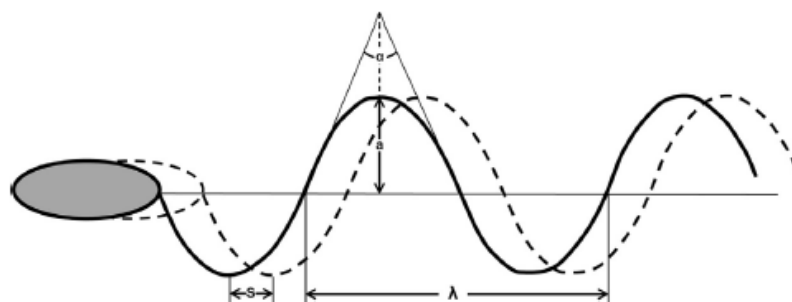


Fig. 1. Graphic depiction of sturgeon sperm illustrating the measurements of the flagellar wave parameters: wavelength (λ), wave amplitude (a), distance between corresponding wave crest points (S), and local bend angle (α). Further explanation is given in the text.

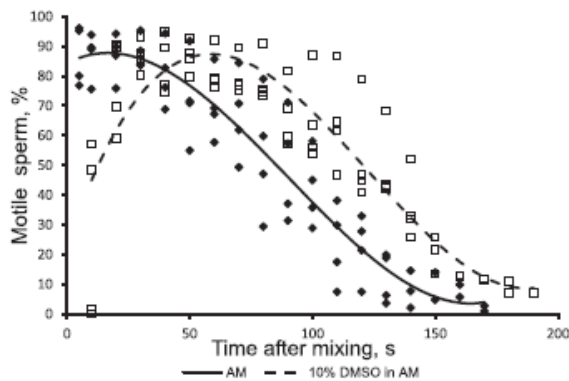


Fig. 2. Motility percentage of sterlet sturgeon (*Acipenser ruthenus*) sperm during swimming period after dilution in activating medium (AM; 1-mM CaCl_2 , 10-mM NaCl, 10-mM Tris-HCl at pH 8.5; $n = 4$; closed symbols) and same AM containing 10% DMSO ($n = 4$; open symbols). Lines are fitted using cubic polynomial regression: $y = 5 \times 10^{-5}X^3 - 0.01X^2 + 0.42X + 84.31$, $r^2 = 0.886$, $P < 0.05$ for AM and $y = 7 \times 10^{-5}X^3 - 0.03X^2 + 2.5X + 23.05$, $r^2 = 0.763$, $P < 0.05$ for AM containing 10% DMSO. Values are means of each semen specimen.

motility initiation. Before activation, spermatozoa were immotile, revealing either a straight flagella shape or a curved form with slightly tilted head (so-called immotile stage, Fig. 4A, B). As mentioned previously, this stage lasted 6 to 14 seconds, and then, spermatozoa started to shiver at the low amplitude (so-called shivering stage, Fig. 4C).

At this stage, sperm flagella initiated its first movements, namely bendings in one big curvature, but without real wave development, and then straightening. Such twitching process occurred irregularly and very slowly, so sometimes it was hardly recognizable.

In most cases (85%), the very first bend was initiated at the head–tail junction along 25% to 30% of a flagellum with localization of the wave covering a distance about 7 to 9 μm from the head (Fig. 4D, E). In a smaller proportion of cells

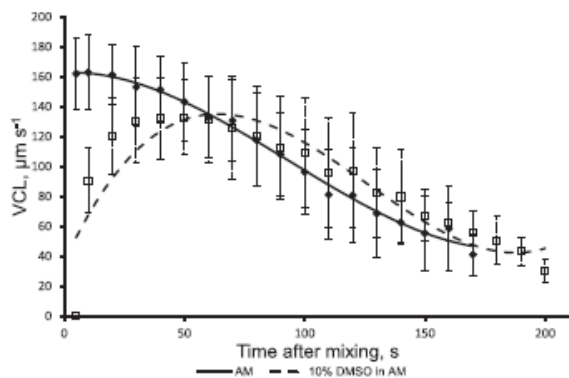


Fig. 3. Curvilinear velocity (VCL) of motile sperm of sterlet sturgeon (*Acipenser ruthenus*) during their swimming period after dilution in either activating medium (AM; 1-mM CaCl_2 , 10-mM NaCl, 10-mM Tris-HCl at pH 8.5; $n = 4$; closed symbols) or same AM containing 10% DMSO ($n = 4$; open symbols). Lines are fitted using cubic equations: $y = 4 \times 10^{-5}X^3 - 0.01X^2 + 0.1X + 162.7$, $r^2 = 0.994$, $P < 0.05$ for AM and $y = 1 \times 10^{-4}X^3 - 0.04X^2 + 3.5X + 35.9$, $r^2 = 0.809$, $P < 0.05$ for AM containing 10% DMSO. Values are expressed as mean \pm standard deviation.

(15%), the origination of the wave at the middle or tip of the flagella could be also documented. When the angle subtended by this first bend reached approximately 1.5 rad, the bend started to progress toward the flagellum tip. In most cases, this angle increased in amplitude and bend angle while traveling to the tip of the sperm. However, the introductory bend (as well as several subsequent P bends) almost never succeeded to reach the tail tip but rather faded at the midflagellum region. On the basis of assumption of the position of the sperm head in Figure 4D, E, which in fact determines geometrically the path of each sperm cell, this bend was referred to as a principal bend (P bend) as proposed by Gibbons and Gibbons [45]. By definition, a newly appearing bend is generated in the direction opposite to the P bend and thus considered as the reverse bend (R bend). However, early in the flagella initiation process, the R bend appeared with a far lower wave amplitude than P bend ($P < 0.05$), whereas the next P bend was launched with a larger amplitude than the previous one. Bend amplitudes of first and second P bends were 4.28 ± 0.65 and 5.37 ± 1.3 μm , while at the same time, bend angles of these bends were 1.57 ± 0.42 rad and 1.34 ± 0.38 rad, respectively. In contrast, bend amplitudes of the first and second R bends were 1.14 ± 0.32 μm and 2.01 ± 0.63 μm , respectively. Altogether, the 2 to 5 following waves showed similar processes; thus accordingly, this motility initiation step was called P-bend propagating stage (Fig. 5). These series of initial waves presented a low beat frequency of 9.16 ± 2.6 Hz and then propagated at a velocity of 89.34 ± 22.18 $\mu\text{m}/\text{s}$. Depending on the total number of P bends and pauses between births of the successive waves, this stage lasted from 0.1 to 0.6 seconds. This period was shorter in cases where the very first deep bend appearing close to the head traveled to the tip of the flagella right away leading the spermatozoon to enter immediately the next stage. Commonly, along with the formation of the very first bend near the head, a broad reverse curve showed up at the distal tip of the flagellum (Fig. 5).

Although flagella produced the first successive P bends in one direction, as seen from the Figure 5, the wave propagation along the flagellum and formation of new waves at later stages gave rise to nonplanar (of mainly helical shape) waves, in such a manner that they lead to a 3-dimensional rotation of whole spermatozoon. At this stage of motility initiation, the spermatozoon rotation (every 360° turn) occurred once in 3 to 5 beating cycles, but later, one full head rotation was accomplished during one single beat cycle.

At the P-bend propagating stage, sperm cells were not propelling. Forward motion of spermatozoon occurred only provided the amplitude of R bend progressively increased up to a value close to the associated P bend, so that, finally, the P + R couple of bends formed a real full wave localized at the proximal part of the flagellum (Fig. 5, latest positions). This stage represents the beginning of the next phase of motility initiation; i.e., the transition from P-bend propagating to full-beating stage. The wave formed at the basal region during its promotion still did not reach the distal tip of the flagellum, and as occurred for earlier P bends, it faded somewhere in the middle of the flagellum, whereas the distal part of the tail appeared rigidly straight

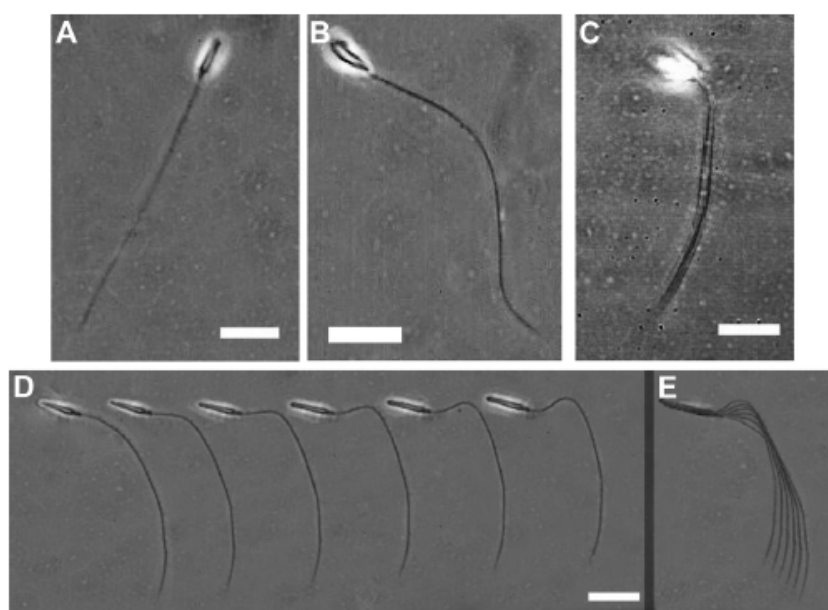


Fig. 4. Flagellar bending patterns of nonpropagative sterlet sturgeon (*Acipenser ruthenus*) spermatozoa at different stages leading to motility initiation. Representative sperm flagella with straight (A) and curved (B) form before its activation are shown. In C panel, superimposed images of a sterlet spermatozoon illustrate the initial shiver of flagella. Successive snapshots were obtained every 0.01 ms (D), and overlapping images (E) of the same cell show detail of the appearance of a first bend in the vicinity of the head–tail junction. Scale bar = 10 μm .

(Fig. 6), in such a way that spermatozoa moved as if dragging the distal half of the flagellum as a passive trailer. It is only when subsequent beat cycles set up that the waves were gradually extending to the entire length of the flagellum. The number of beat cycles required for this result varied among sperm cells, but, overall, this process lasted about 200 to 450 ms, wherein the beating frequency was not significantly different from that during the previous phase (Table 1). However, during that period, the value of the wave velocity was twice higher than that during the P-bend propagation stage (Table 1).

During this activation step, only two, rarely three, curvatures were present along the proximal portion of the flagellum (Fig. 6). Although the amplitudes of P and R bends seemed to be quite similar, the Mann–Whitney U test ($P < 0.05$) shows that they were different, whereas the curvature values of these bends were not significantly different between the bend angles (Table 1). As clearly seen from Figure 5, sperm flagella continued to generate helical

waves, but from this stage, sperm rotation occurred at a higher frequency: once per every 1 to 2 beat cycles or one full rotation (360°) generated every 2.0 to 2.5 beat cycles.

When eventually the wave was spread out over the flagellum length, the spermatozoon entered into its final step of motility initiation, i.e., the full-beating stage (Fig. 7) where the flagellum acquired the usual symmetrical wave shape, with the characteristic feature of R-bend amplitude being as large as that of the associated P bend (Table 1). By this moment, the amplitude of both bends increased ($P < 0.05$) in comparison to the transitional phase and moreover continued to grow gradually, but insignificantly, over the next 5 seconds (Table 1). Along with this amplitude rise, there observed a decrease in bend angle values (Table 1). During this process, the number of curvatures traversing the flagellar length did not change significantly in comparison with the previous phase; therefore, taking into account that the wave had expanded over the whole length of flagellum, the length of the waves generated

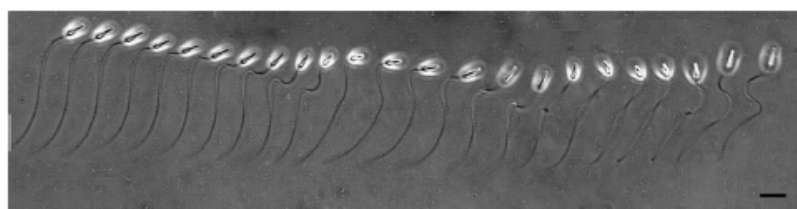


Fig. 5. Successive images of a sterlet sturgeon (*Acipenser ruthenus*) spermatozoon captured at its P-bend then R-bend propagating stage of motility initiation, as observed in this example, at 12 seconds after contact by dispersion in activating medium (1-mM CaCl_2 , 10-mM NaCl, 10-mM Tris–HCl at pH 8.5) supplemented with 10% DMSO. Frames obtained by high-speed video microscopy using phase-contrast optic with $\times 100$ magnification lens. Selected successive images are every 15 ms in this panel. Scale bar = 10 μm .

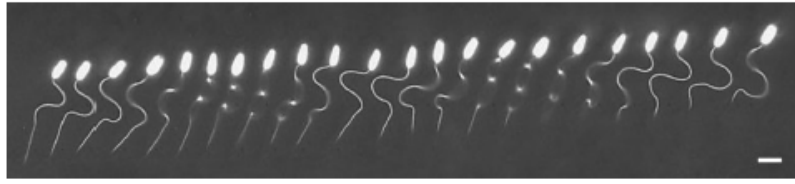


Fig. 6. Flagellar images of sterlet sturgeon (*Acipenser ruthenus*) spermatozoon captured during the transition from P-bend propagating stage to full-beating stage. Successive snapshots (every 13 ms) of the same spermatozoon, which was activated by dispersion into activating medium (1-mM CaCl₂, 10-mM NaCl, 10-mM Tris-HCl at pH 8.5) supplemented by 10% DMSO, were obtained by high-speed video microscopy using dark-field optic with $\times 50$ magnification lens. Scale bar = 10 μ m.

during the full-beating stage alike their amplitudes significantly increased (Table 1). However, one can remark that the wave characteristics measured at 5 seconds post-activation time point in sperm cells activated in the absence of DMSO were significantly lower than those activated in presence of DMSO (Table 1). In addition, at 5 seconds in AM alone, mainly three to four curvatures along the flagellum were observed. On the other hand, bend angles of these curvatures were larger ($P < 0.05$) than those of the cells exposed to AM with 10% DMSO (Table 1), which could be explained by amplitude differences between both situations. Visually, the spermatozoa diluted in AM with 10% DMSO look to swim similarly those in AM alone, but the former had lower propagation velocity ($P < 0.05$; Table 1) as a result of poorer performance of their flagella, as values of beating frequency and wave propagation here were both lower than those in cells activated by AM.

In total, for completely immotile spermatozoa, it took about 0.4 to 1.2 seconds (depending on the duration of the stages) to reach the full-beating state of flagella. Wherein, the average acceleration of all sperm cells for a time interval between the start of sperm propagation until the entering into full-beating phase was about $163 \pm 17 \text{ m/s}^2$.

4. Discussion

Under normal conditions, spermatozoa of most fishes, in particular, those of sterlet sturgeon start their motility

immediately after being released into the aquatic environment; in contrast, addition of 10% DMSO into a swimming medium inflicts a delay to the onset of motion of 6 to 9 seconds. Experimentally, this delay offers, as exploited in this paper, a very useful tool for observation of flagellum activation in real time, namely allowing obtaining of detailed record by high-speed video microscopy of the initial flagellar waves of individual spermatozoa. As stated earlier, such situation cannot be reached in regular swimming solutions because this event occurs precisely during the period that the experimenter uses to obtain dispersion of sperm cells in the observation drop of AM.

It was previously shown that in carp sperm, DMSO in a range of 1% to 20% can relieve the inhibition of movement due to the high osmolality and with delayed response (at 3–4 minutes after mixing) trigger their motility after incubation in media with an osmolality of 400 to 3200 mOsm/kg [46]. In the same way, according to our results, a delay due to a high DMSO (5%–15%) concentration in swimming media was observed on sperm of most freshwater fishes (i.e., sturgeon, trout, tench, pangasius), whereas this effect was not confirmed in the case of euryhaline and marine fish sperm (i.e., tilapia, seabass). Besides, taking into account the data from Percec-Poupard et al. [46] and our own observations, it appears that the latency period depends directly on the concentrations of DMSO added and thus the final osmolality of the solution; the higher the concentration, the longer a delay and vice

Table 1

Motion parameters of sterlet sturgeon (*Acipenser ruthenus*) sperm in the initial stages after dilution either in activating medium (AM; 1-mM CaCl₂, 10-mM NaCl, 10-mM Tris-HCl at pH 8.5) or AM containing 10% DMSO.

Stages	Transition from	Full beating		
	P bend to full beating	AM with 10% DMSO	AM with 10% DMSO	AM (at 5 s after
Swimming solution	AM with 10% DMSO	(immediately after	(at 5 s after motility	motility initiation)
		entering the stage)	initiation)	
Propagation velocity obtained by high-speed video ($\mu\text{m/s}$)	50 ± 11	103 ± 14	127 ± 18	177 ± 35
Beat frequency (Hz)	12 ± 3	18 ± 3	34 ± 7	78 ± 8
Wave velocity ($\mu\text{m/s}$)	169 ± 19	295 ± 43^a	320 ± 59^a	621 ± 83
Wavelength (μm)	17.5 ± 1.9^b	20.2 ± 2.5^a	20.2 ± 1.5^a	16.7 ± 1.2^b
Wave amplitude (μm)				
P bend	4.3 ± 0.7^b	5.1 ± 1.1^c	5.4 ± 0.6^c	4.4 ± 0.5^b
R bend	3.5 ± 0.4	5 ± 1.1^c	5.6 ± 0.7^c	4.1 ± 0.6
Bend angle (rad)				
P bend	1 ± 0.3^c	0.7 ± 0.4^{bc}	0.5 ± 0.1^c	0.6 ± 0.2^a
R bend	1.2 ± 0.3^c	0.9 ± 0.3^{bc}	0.6 ± 0.2^c	0.8 ± 0.2^a

Values are mean \pm standard deviation. For each parameter, values with similar superscript letters are not statistically significant ($P > 0.05$).

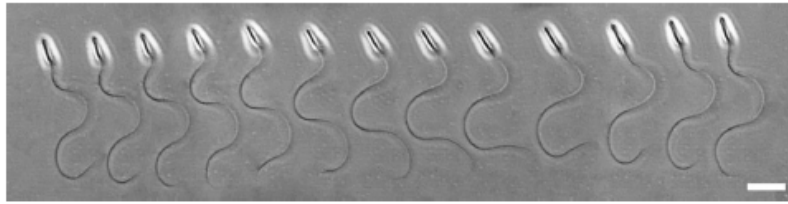


Fig. 7. Flagella images of sterlet sturgeon (*Acipenser ruthenus*) spermatozoa in their full-beating stage resulting from activation when 10% DMSO was included in activating medium (1-mM CaCl₂, 10-mM NaCl, 10-mM Tris-HCl at pH 8.5). Successive images (every 13 ms) were obtained by high-speed video microscopy using phase-contrast optic with $\times 100$ magnification lens. Scale bar = 10 μ m.

versa. Although there were variations in time of retardation or phase durations, in general, the process of flagellar motility initiation looked similar in all freshwater species.

Most likely, the fact that delays due to 10% DMSO were observed only in freshwater fishes is related to their mode of osmolality dependence for motility activation [31]. Similar effects on sperm cells of freshwater fish species were caused by other organic compounds, e.g., ethylene glycol, propylene glycol, or glycerol (Prokopchuk, unpublished data). Adding of these compounds into swimming media instantly switches the apparent tonicity of these solutions from hypotonic to hypertonic with respect to spermatozoa internal space; e.g., the osmolality of AM supplemented with 10% DMSO reaches 1538 mOsm/L, leading to such a high value of external osmotic pressure that any motility activation in freshwater fish sperm, including sturgeons, is fully impossible [25,31,47]. In such conditions of hyperosmolarity, an osmotic pressure gradient is induced between the intracellular and extracellular compartments, which drives the small molecules such as DMSO, rendering easier penetration through the cell membrane [48,49]. Such processes allow the DMSO concentration to become rapidly balanced between both sides of the plasma membrane. Complementarily, the intrusion of DMSO into cell membranes is known to affect their structure. In this respect, several studies report that DMSO molecules may, in a dose-dependent manner, interact with phospholipids and membrane proteins [50–52]. Regarding the mechanism of action of DMSO, Barnett [53] and Larsen [54] suggested that DMSO causes an increase in membrane fluidity, a feature that was confirmed by atomic-scale molecular dynamics simulations by Gurtovenko and Anwar [49], for low concentrations of DMSO (2.5 mol%–7.5 mol%). As a complement, these authors established that in the concentration range used in our present study, DMSO is able to induce formation of transient water pores into the membrane [49]. Such modification of the lipid bilayer structure of cell membrane also alters the transmembrane signaling [55], which is an essential aspect of the fish sperm activation mechanism. It has been reported by Jourdon [56] that DMSO can reversibly block the conductance of ionic currents through Na⁺ and Ca²⁺ channels and slightly reduce K⁺ currents in human neuroblastoma-glioma hybrid cells. The later results suggest that DMSO would directly affect Na⁺ and K⁺ ions transport through the fish sperm membrane, as it was previously shown that transmembrane movements of these ions are crucially involved in the osmotic signal

transduction of carp spermatozoa [46,57]. It is worth noting that according to Larsen [54], DMSO does not inhibit the Na⁺/K⁺-ATPase completely. Consequently, changes in external osmolality affect the intracellular K⁺ concentration, and this constitutes a crucial signal to initiate sperm motility in salmon and sturgeons at the flagellar axoneme level [35,58].

In contrast to freshwater fishes, sperm motility is induced in most marine species by hyperosmotic pressure, and therefore, the osmolality gradient must be largely directed from outside to inside the cell [31]: This allows to predict that the presence of DMSO in swimming media, although it highly increases external osmolality, does not bring any significant change in their mode of motility activation; this prediction is confirmed by our present observations. Apart from fish spermatozoa, DMSO has been shown to enhance the motility of sea urchin sperm cells [59]. As a confirmation of our hypothesis explaining the osmotic effect of DMSO as previously mentioned, a delay in motility activation similar to that described for native freshwater fish spermatozoa is not occurring in demembrated spermatozoa of freshwater fish species (Prokopchuk, unpublished data) because of the absence of transmembrane osmolality gradient. Therefore, we suspect that the delay duration induced by DMSO represents the period of time needed for reaching equilibrium concentration between both sides of the sperm membrane. Furthermore, along with the osmotic effect of DMSO, the deceleration of the ion channels mentioned previously could be the additional factor leading to a retardation of sperm motility activation of freshwater fishes.

To our knowledge, this paper is the first one that describes the flagellum waveforms during the successive steps of motility initiation in fish spermatozoa. However, several well-detailed studies on sperm flagella activation have been reported in sea urchin sperm [42,43,60]. Similarities occur between the latter authors' results and those presented in our present work, the process of motility initiation of sterlet sperm presents several successive phases, although they proceeded slightly differently than in sea urchin. Before activation, the motionless flagella appeared as straight (rigor shape) or presenting a single large bend; according to Gibbons [61], this may indicate possible formation of stable crossbridges between the dynein arms and the doublet microtubules in the axonemal structure of flagella. As it has been shown in many publications during the past decades, the sliding of the adjusted microtubules along each other's length in the flagellar

axoneme induces the basic movement of flagella in agreement with the initial Satir's [62] hypothesis. This sliding is supported by the activity of dyneins, which are attached transiently to some microtubule doublets to generate local sliding between each other, which results from individual dynein ATPase molecule movement powered by the hydrolysis of ATP as suggested by Gibbons and Rowe [63]. It is predicted from our present observations that the active synchronous sliding of microtubules starts after cross-bridges are formed. Yoshimura [21] suggested that organization of the crossbridges relates to high ATP concentration, what may mean that the hydrolysis of ATP occurs during the early period of sliding. This step would correspond to the shivering stage of sperm motility initiation, when spermatozoa disruptively tremble with only low amplitudes. In case of sterlet sturgeon sperm, this phase is less pronounced than in other freshwater fish sperm tested and in sea urchin, whose flagella curled up with a markedly bigger amplitude and the shivering stage lasting for much longer periods [43]. Altogether, in case of sterlet sturgeon and other freshwater fish spermatozoa, all stages of flagella motility initiation lasted significantly shorter than in sea urchin sperm, which is expected considering the total motility duration of sterlet sturgeon sperm. Eventually, a brief microtubule sliding results into the appearance of a first curvature [64]. There are several competing hypotheses attempting to explain the beating mechanism of axoneme, in particular the switching on the bend initiation [65]. Usually the initial bend is formed in the axoneme close to its junction with head: This might be suggested by the localization of highest ATP concentration because of its generation by mitochondria themselves located in the midpiece tail and its diffusion down the flagellum. However, Goldstein [60] challenged this hypothesis by showing that substantial ATP is stored within the entire flagellum before its activation, and our observations of beating commencement at the distal part of flagellum are consistent with Goldstein's results. The author also suggested that probably the forces responsible for the bend formation are not produced uniformly along a flagellum.

The very first bend develops quite slowly by increasing its amplitude, length, velocity of propagation and at the same time decreasing the subtended angle. Once a bend formed, it begins to propagate along the flagellum; this corresponds to a P-bend propagating stage. It has been shown that the bend formation derives from active microtubule sliding in one direction [66] and the propagation of this bend toward the distal tip is promoted by active sliding in the opposite direction [60,62]. However, beating initially occurs only near the head–tail junction, while the distal part of flagellum remains rigidly straight, in "rigor shape," or shows a broad curvature in its reverse bend: It is not clear if any microtubule sliding is responsible in those cases. In addition, the fact that initial first bends damped in the middle of flagellum during the progress of the motility period leads to question whether the sliding process within these bends is constant without failures or whether the phosphorylation signal of axonemal components (radial spokes or central pair) is complete. Nevertheless, whereas the P-bend propagation of the whole sterlet spermatozoon flagella remains nonefficient for

forwardly progression of the cell, the sea urchin spermatozoa at such stage were observed to be already moving forwardly [43]. Moreover, these authors stated that in the P-bend propagating stage, the P bend had the same characteristic features as during the full-beating, whereas our data obtained for fish sperm show significant increment in P-bend beating parameters. Evidently, along with this increase, the microtubule sliding amplitude or velocity also rises, as a result more and more active work of dynein ATPases. Denehy [67] and Gibbons [16] showed that the beat frequency is directly related to the ATPase activity of dynein motors, namely the rate of ATP hydrolysis. Oppositely to sea urchin sperm, the P-bend propagating phase is very brief in fish spermatozoa, whereas the transition from P-bend stage to full-beating stage needs a larger number of beat cycles than that in sea urchin. Remarkably, it is exactly at this step, when spermatozoa gradually developed principal and reverse bends with similar amplitudes, the forward propagation of sperm cells actually begins; apparently, beating of a single P bend even with high amplitudes is not sufficient for it. It has been reported that commencement of beating in live sea urchin spermatozoa is somewhat asymmetrically [60,68], whereas this is not the case for sterlet spermatozoa, as their propagation occurred only when the ratio between the principal and reverse bends was about 1. During the transition one after another, new successive bends progressively occupy larger fractions of the flagellum, wherein, according to suggestions of Goldstein [69], a new bend is initiated by the shear forces originating in the preceding bend.

Starting from the P-bend propagating stage of motility initiation, the wave propagation along the flagellum and formation of new waves proceeded in helical manner leading to a rotation of whole spermatozoon around its longitudinal axis. It is not a unique property of fish spermatozoa but rather behavior of sperm in many species, such as sea urchin, eel, starfish, medaka, human, golden hamster, *Xenopus*, and bull spermatozoa, which also rotate as they swim freely [70–79]. Although the helical motion is often seen, the physiological sense of spermatozoa rotation and the mechanism controlling the beat plane are still unclear [75]. Some theoretical models consider that flagellar helical waves may be generated either by localized sliding between doublet microtubules (sliding which propagates from the base of the flagellum toward the tip and at the same time spreads about the axoneme) [80–82] or by outer doublet sliding together with the continuous rotation of the central pair [83]. The last consideration made for eukaryotic flagellum is not valid for the sperm flagella; in addition, some of sperm flagella (e.g., spermatozoa of the eel [84] or the horseshoe crabs of the Asian species [85]) lack the pair complex but still produce helical bending waves [86].

From studies on the swimming patterns of sea urchin spermatozoa, Shingyoji et al. [87] concluded that the nine outer doublets are potentially equivalent in their ability to originate and propagate bends in any plane. Nevertheless, it was shown that the production of helical waves is much easier for a flagellum than formation of planar waves [86]. Moreover, in some cases (e.g., chemotaxis), the beating orientations may be changed during the flagellar motion.

For example, sea urchin and tunicate sperm flagella, which normally produce planar bending waves, under certain high viscosity conditions, may switch their beating plane to helical bending [77,86]. Similar feature was also observed in mammalian sperm during the progesterone-induced hyperactivated motility [88] or *Xenopus* sperm flagella, where it occurs independently without any changes in medium viscosity or chemical stimulation [79].

On the earliest stages of motility initiation, sterlet spermatozoa rotate once in a 3 to 5 beat cycles, but later, such rotation occurred faster. Apparently, together with a progress of the motility initiation, there is an evolution of flagellar waveform from the almost planar beat when spermatozoa rotate very slowly, to a “flattened helicoid” when the rotation occurs a bit quickly, and finally to a fully helical waveform when cells rotate most rapidly [89]. According to Gray [70], the helical motion of sea urchin spermatozoa in free fluid is a consequence of asymmetry in the flagellar bending waves and alteration of the asymmetry amount may regulate spermatozoa rotation [90]. It was shown that the degree of asymmetry in the flagellar beat pattern in sea urchin sperm can be modulated by changing the concentration of free Ca^{2+} [91], and changes in the degree of Ca-mediated asymmetry are believed to constitute the most important basis for sperm chemotaxis [87,92].

Recently, Boryshpolets [93] showed that the vicinity of a surface can be associated with rotation of sterlet sturgeon spermatozoa around their longitudinal axis, but the quality of the surface (supple vs. rigid) is of crucial importance. The local viscosity surrounding the micropyle area may also be influential [77]. The turning behavior of sperm (360° turn) could act as a “drilling” force, which would make the penetration of the egg micropyle mechanically favored. Similar changes in movements and behavior are described for mammalian sperm, which move in a specific manner when becoming hyperactivated [94].

From our set of observations, it appears that DMSO affects the motility of fish sperm in a species-specific manner, mostly according to the habitat of those species groups, either salt or fresh water adapted. The experimental design described in the present paper can provide an opportunity to investigate flagellar motility initiation in sturgeon spermatozoa and can lead to a basic understanding of natural sperm activation of freshwater fishes.

Acknowledgments

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Chapter 7

General discussion

English summary

Czech summary

Acknowledgements

List of publications

Training and supervision plan during study

Curriculum vitae

General Discussion

Prior to becoming an actively fertilizing unit, fish spermatozoon formed in testes pass a chain of manifold events: from maturation, when sperm cell acquires the potential for activation (Morisawa and Morisawa, 1986), to first flagellum beating in spawning environment. So far, the mechanisms underlying this progression and capacity of fish spermatozoa to adapt to external changes are poorly understood. The current study attempted to shed light on the regulatory processes and response arrangements of fish spermatozoa during the course of maturation and motility initiation.

The reproductive cycle in fish males is separated in the spermatogenesis and maturation phase (spermiation), which are controlled by the reproductive hormones of the brain, pituitary and testes. Spermatogenesis presents a series of differentiation changes during which spermatozoa are formed in the testes. Even though these cells are completely morphologically formed, they are non-functional and need to acquire the capacity to initiate flagellar motility (Schulz et al., 2010). This maturational step involves various biochemical and cellular events, which vary among species; also there is an anatomical diversity among taxa as to the anatomical localities where maturation occurs. Sperm maturation can be controlled a hormonally and this mechanism is widely used to stimulate final physiological maturation of fish spermatozoa *in vitro* (Redondo-Muller et al., 1991).

The process of sperm maturation has been well investigated in many animal species, especially in humans and domestic mammals (de Lamirande et al., 1997; Marengo, 2008; Sostaric et al., 2008). Mammal spermatozoa are produced in the testis, then transported to the proximal cauda epididymis; during this epididymal transit and storage, they come into contact with seminal plasma and thereby acquire the functional competence after a series of morphological, biochemical and physiological changes (Darszon et al., 2011; Mortimer, 1997), especially regarding flagellar and acrosomal functions. It was shown that fluid homeostasis during spermatogenesis and sperm maturation is critical for male fertility (Huang et al., 2006). Similarly, in fish spermatozoa, seminal plasma components protect mature sperm viability during storage (Ciereszko, 2008). In the cauda epididymis, sperm acquires the ability to move in a propulsive manner, but the functional maturation of spermatozoa is still not completed as they are unable to fertilize eggs (Eddy, 2006). It is only after ejaculation into the female reproductive tract, the spermatozoa go through several physiological changes, called capacitation, that render them competent for fertilization (Visconti et al., 2002). The regulation of human sperm capacitation includes participation of bicarbonate, calcium ions, reactive oxygen species, cyclic AMP, different protein kinases and protein phosphatases, and the extracellular signal-regulated protein kinase (de Lamirande et al., 1997; O'Flaherty et al., 2006).

For a long time, maturation of fish spermatozoa has not been considered as a limiting factor for success in fish reproduction, therefore, it was described only in a few teleost species, such as rainbow trout, chum salmon, and Japanese eel (Miura et al., 1995; Miura and Miura, 2001; Morisawa and Morisawa, 1986, 1988). Acquisition of sperm motility in these species occurs in response to an increase in environmental pH and/or concentrations of bicarbonate ions, indicating that substances in the sperm duct and ionic constituents of seminal fluid are involved in the acquisition of motility of teleostean sperm (Table 1). This feature was also confirmed in carp, where spermatozoa maturation can be mimicked *in vitro* by incubation in specific ions solutions (Redondo-Muller et al., 1991).

Up to the present study, the processes of spermatozoa maturation in sturgeon was not understood. There were only suggestions in literature that a mechanism of sperm maturation in sturgeon occurs via increase of pH via eSRS22 (a homologue of carbonic anhydrase regulation of ion and acid-base balance) or cAMP similar to teleost (Alavi et al., 2012). However, the current study on sturgeons showed that in contrast to salmonids, extracellular pH does not initiate sperm maturation (Chapter 2). This work described for the first time the sperm maturation process in sturgeons, establishing its localization and main components.

Sturgeons possess a primitive organization of excretory system that is different from teleostean species: the efferent ducts from the testes come in direct contact with the kidneys. Sperm maturation occurs outside the testes after dilution with urine (Chapter 2), and involves the participation of high molecular weight substances as well as calcium ions present in seminal fluid and/or urine (Table 1). Significant differences were observed between testicular sperm and sperm collected from Wolffian ducts, where sperm naturally mixes with urine. Testicular spermatozoa did not become motile in a swimming environment, but did acquire this capacity after *in vitro* incubation in urine or seminal fluid from Wolffian duct in a temperature and time-dependent manner. In addition, removal of high molecular weight substances from Wolffian duct seminal fluid, as well as addition of soybean trypsin inhibitor prevented maturation of testicular sperm in such seminal fluid (Chapter 2).

The present study revealed that during the maturation process, sperm motility is affected in its sensitivity to calcium ions. Later, Bondarenko et al. (2016) showed that motility of sturgeon testicular spermatozoa could be activated only when the intra- and extra-cellular Ca^{2+} concentration are high, suggesting the regulation of sperm maturation by Ca^{2+} influx into the cell and subsequent loading of some Ca^{2+} stores. Altogether, it provides the first evidence for the presence of Ca^{2+} stores in sturgeon spermatozoa. In a similar fashion, at least two Ca^{2+} stores have been detected in mammalian spermatozoa: one localized in the acrosome and another in the redundant nuclear envelope (Costello et al., 2009; Darszon et al., 2011). Sturgeon spermatozoa possess an acrosome, which is atypical compared to teleost fish, who lost their acrosome during evolution (Psenicka et al., 2011). It is a single membrane-delimited specialized organelle that is necessary for sperm/egg fusion at fertilization. In mammalian spermatozoa, the formation of the acrosome take place during maturation phase (Darszon et al., 2011). For sturgeons, there are no data on this account, though it can be assumed that acquisition of acrosomal competence could also occur during the sperm maturation. The modification of acrosome during sperm maturation is of great interest and requires further detailed investigation, furthermore, sturgeon sperm due to its specific features constitutes a perfect model for these studies.

The ability for spermatozoa to swim and penetrate the egg is dependent on their degree of maturation (Blandau and Rumery, 1964). Insufficient sperm maturation may also result in defective embryonic development (Fournier-Delpech et al., 1979). This study is a pioneering work in the field of sturgeon sperm maturation and there are plenty of questions that should be answered for better understanding of spermatozoa maturation process at cellular and molecular levels.

Following the maturation steps, ejaculation from the spermatid ducts expose gametes to the external environment during spawning where they encounter various biological and physical conditions, which regulate their activation and motility. In the first place, it is osmotic constituent of the external environment. In freshwater fish species, spawning occurs in low osmotic conditions, while in marine fishes, sperm is diluted in hypertonic seawater (Table 1). The present study showed that alteration of environmental osmolality affects the fish sperm

in different ways, depending on the species and modes of spermatozoan motility activation, either by an osmotic or ionic mode. Following activation by ambient water, it is crucial for cells to control their internal osmolality, membrane tension and hydrostatic pressure (Zonia and Munnik, 2007). Accordingly, in response to osmotic stress caused by hypotonicity, spermatozoa of freshwater fishes regulate the flow of water across their cell membrane and increase their cytoplasmic volume during the short motility period. However, the tendency of spermatozoa to swell due to environmental osmolality change during the motility period is species-specific (Chapter 3) and occurs only in spermatozoa having elastic cell membrane, as in carp sperm. Spermatozoa of this species increase their volume several-fold as a result of water influx (Perchec Poupard et al., 1997). In contrast, no sperm volume changes were observed in sterlet or in brook trout spermatozoa, both of which having an ionic mode of motility activation. From these observations, it may be concluded that spermatozoa of salmonid and sturgeon quickly react to the osmolality by initiating their flagellar motility, but also very efficiently control the volume changes concomitantly engendered by osmotic stress. In addition, there might be some differences in the water influx rates.

In carp as well as in puffer fish, a marine species, osmoregulation functions have been attributed to stretch-activated channels present in the sperm membrane, the latter being considered as prerequisite for water diffusion and motility activation (Krasznai, 2003). Similar mechanosensitive channels were also shown to be active in bacteria, where very slight changes in external osmolality are sufficient to shift the osmotic pressure across the plasma membrane that generate stretch and compression forces along the plane of the lipid bilayer (Chaumont et al., 2005; Kung, 2005). However, water diffusion is a very slow process (Chen and Duan, 2011) relatively to sperm processes discussed in this thesis, so in the present study it was hypothesized that sperm swelling could result from water influx through membrane water channels proteins, called aquaporins, with further activation of stretch-dependent channels and motility activation (Chapter 3). When such aquaporins are involved, the control of the osmolality response is highly accelerated and this process was recently demonstrated to be active in marine fish spermatozoa (Zilli et al., 2011). Later, several molecular species of aquaporins were characterized in fish spermatozoa, revealing that some aquaporins may re-localize at initiation or during the motility period (Chauvigné et al., 2015). In the recent past, the presence of aquaporins was also established in salmonid sperm cells (Takei et al., 2015). Likewise, aquaporins were shown to be important in sperm of other species (Martinac, 2004) and that they might function as osmosensors and volume regulators in cells of animals, plants, fungi and bacteria (Hill et al., 2004).

Due to the absence of stretch indications during activation and the motility period, activation of stretch-dependent ion channels do not seem to intervene in case of sturgeon and salmon spermatozoa. Thus, it remains unclear how sperm of these species can maintain a constant volume under hypotonic conditions. One of the possible explanations could be the involvement of ion co-transporters, as suggested in Chapter 3. Similar regulation of swelling by means of regulatory volume decrease was proposed for mammalian sperm (Yeung et al., 2006). This topic, however, needs further investigation.

It is known that long-term exposure of cells to a low osmotic condition may have negative effects on membrane (Gao and Critser, 2000). During the motility period in hypotonic conditions, flagellar morphology was strongly affected in spermatozoa of all three species studied and discussed in Chapter 3. Due to the osmotic shock received by sperm cells, their flagella undergo serious modifications developing bubbles along the flagellum membrane or loops at the distal part of the flagellum (Chapter 3). Formation of such loops and blebs on

membranes were previously described in carp (Perchec et al., 1996; Perchec Poupard et al., 1997) and pike (Hulak et al., 2008) spermatozoa. Obviously, these defects represent a serious handicap for flagellar motility, which contributes to a lower swimming efficiency and a reduction in the duration of the motility. Alteration of extracellular osmolality detected by spermatozoa causes a rapid change in intracellular ionic concentration and, consequently, flagellar axoneme waves are firstly reduced and then fully stop. Nevertheless, Dreanno et al. (1999) and Cosson et al. (1999) showed that this process is reversible. Based on the examination of other cell types (Langridge and Kay, 2006), it can be suggested that spermatozoa might use formation of blebs as a physiological condition to expand their total cell surface and increase their intracellular volume without bursting.

Sperm motility initiation is a very complex and highly organized process that involves the reception of external activating signal, its transduction to axonemal machinery and, ultimately, the activation of axonemal structures. The nature of an external signal inducing the sperm motility is highly dependent on the reproductive environment and behavior of fishes. In most freshwater fish species, regulation occurs mostly due to a decrease of environmental osmolality (Perchec Poupard et al., 1997); therefore, such hypotonic induction is accompanied by a reorganization of the membrane structure and hyperpolarization of the cell membrane (Krasznai, 2003). In other freshwater species, such as salmonid and sturgeon, the regulation of sperm motility activation is mostly attributed to a decrease of the extracellular K^+ concentration at sperm transfer from seminal fluid into freshwater (Table 1); here osmolality as well as K^+ concentration are much lower (Cosson, 2004; Morisawa and Suzuki, 1980). In some conditions, K^+ inhibition can be by-passed (Chapter 5) by pre-exposing sturgeon sperm to a high osmolality shock prior to its transfer to K^+ -rich swimming media. It is suggested that these spermatozoa may be activated by use of an unexpected signaling pathway, independent from regular ionic stimulation (Chapter 5). However, this issue remains to be further clarified. Similar outcomes were also obtained for salmon spermatozoa (Morita et al., 2005). In case of salmonids, it was shown that such K^+ by-pass effect (osmolality dependent) is accompanied by a transient intracellular Ca^{2+} concentration rise (Takei et al., 2012) followed by protein phosphorylation cascade leading to sperm motility. More recent results demonstrated that for the maintenance of salmonid fish sperm motility under normal conditions, the water influx across the plasma membrane is crucial, whereas water influx resulted from osmotic shock-induced membrane permeation is critical for the motility initiation of pre-treated sperm (Takei et al., 2015). Altogether, the K^+ by-pass effect described above, as well as general cascade of events occurring at motility activation, have been studied in more detail in salmonid than in sturgeon. Most of main ideas about the signaling and regulation of motility process in sturgeon species are stated by analogy to knowledge acquired in salmonids, such as, for example, the model of signaling pathway for sperm motility activation in sturgeons that was proposed by Alavi et al. (2011). However, as the current study clearly demonstrated, sturgeons and their spermatozoa, have original and specific features that definitely stimulate great scientific interest and therefore need a deeper investigation.

As mentioned above, in freshwater teleosts, the release of spermatozoa into the hypotonic external milieu induces a membrane hyperpolarization (Krasznai, 2003), which occurs mainly because of variation in the internal K^+ concentration combined with variation in Ca^{2+} concentration (Alavi and Cosson, 2006). According to numerous studies performed on trout spermatozoa (Morisawa et al., 1983; Morisawa and Suzuki, 1980; Tanimoto et al., 1994), internal response to these external triggers show the following sequence: a hypo-osmotic signal or a decrease of environmental K^+ ions provoke the opening of specific membrane K^+

channels and induce K^+ efflux, which eventually leads to hyper-polarization of the plasma membrane (Blaber and Hallett, 1988; Boitano and Omoto, 1991). These result in a Ca^{2+} influx (Cosson et al., 1989) through dihydropyridine-sensitive calcium channels and a transient increase of cAMP (Table 1), which induces phosphorylation of axonemal proteins. This subsequent cAMP-dependent phosphorylation of axonemal proteins and dynein light chain triggers the initiation of flagellar motility (Hayashi et al., 1987; Inaba et al., 1999). It should be noted that in cyprinids, the intracellular signaling is cAMP-independent (Table 1; Krasznai et al., 2000), therefore, a different cascade of events is probably involved.

In contrast, sperm motility of marine fishes is activated by hyper-osmotic shock (Table 1) caused by an increase in osmolality from seminal fluid to sea water (Cosson et al., 2008a). Similar to what happens in freshwater fish, the hyperosmotic signal firstly increases intracellular Ca^{2+} concentration (Oda and Morisawa, 1993) due to a Ca^{2+} influx across the plasma membrane, or Ca^{2+} release from intracellular stores, or as a result of cytosol concentration following massive water efflux by aquaporins (Zilli et al., 2012). Although in some species, changes in the phosphorylation state of some flagellar proteins may be involved (Zilli et al., 2008a, b). The activation of the axonemal machinery in such case is a direct result of the increase of Ca^{2+} and other ions (Cosson et al., 2008a, b), or by Ca^{2+} /calmodulin- (Krasznai et al., 2003; Morita et al., 2004) or cAMP-dependent (Zilli et al., 2008b) protein phosphorylation of structural components of axonemal dyneins, kinases, and phosphatases anchored in the axoneme and in the radial spoke proteins (Table 1; Zilli et al., 2012).

As an example, influx of Ca^{2+} ions across the plasma membranes as a prerequisite for motility activation in hypertonic media, has been demonstrated in spermatozoa of seawater-acclimated euryhaline tilapia, *Oreochromis mossambicus* (Linhart et al., 1999; Morita et al., 2004), sea bass and tuna (Cosson et al., 2008a, b). The Ca^{2+} ions apparently bind to flagellar proteins in order to activate motility (Morita et al., 2010). It was proposed that an increase in internal Ca^{2+} concentration induces Ca^{2+} /calmodulin-kinase-dependent regulation, including protein phosphorylation for activation and control of dynein activity in the flagellar axoneme (Morita et al., 2010). At the same time, even in the presence of high external Ca^{2+} concentration, spermatozoa of freshwater-acclimated tilapia do not swim in hypertonic conditions (Morita et al., 2003). It seems that the increase in internal Ca^{2+} concentration here is independent from extracellular Ca^{2+} ions and results from the mobilization of intracellular Ca^{2+} stores, which are quite probably located in the neck region of the sperm head (Morita et al., 2010). Altogether, it was assumed that during acclimation of tilapia from fresh- to seawater, their sperm adapt to conditions of high salinity by losing Ca^{2+} -independent pathways of motility activation (Linhart et al., 1999).

On the other hand, the present experimental study performed on a closely related tilapia species, *Sarotherodon melanotheron heudelotii*, led to the discovery that extracellular Ca^{2+} appears to be necessary for activating the sperm motility of tilapia reared in fresh water even in hypotonic conditions (Chapter 4). Moreover, the higher the salinity or the more hypertonic ambient media at spermatozoa activation, the higher extracellular concentration of Ca^{2+} ions is required. It is worth noting that in this tilapia species, the sperm of fish reared in either fresh-, sea- or hypersaline water can be activated in swimming milieus whatever their tonicity, provided that a sufficient Ca^{2+} concentration is present (Chapter 4); this specificity appears to contradict the observations of Morita's group (Morita et al., 2003) on a related species. Hence, the results obtained in the present study indicate that osmolality is not the factor inhibiting sperm motility inside the testis in the *Sarotherodon melanotheron heudelotii*, but further investigations are needed to identify the precise nature of that factor.

Table 1. Triggers of sperm maturation and motility initiation in different fish species. ND – not determined; FWF – freshwater fish; MF – marine fish.

Family	Maturation mediator	Motility activation			References	
		Motility activation factor	Osmolality (mOsm/kg)	[Ca ²⁺]-ion dependency		cAMP dependency
Freshwater						
Cyprinidae	isotonic ionic (K ⁺ or Na ⁺) milieu	hypo-osmolality	<300	+	-	Krasznai et al., 2000 Krasznai, 2003; Redondo-Muller et al., 1991
Salmonidae	increase in environmental pH and/or concentrations of bicarbonate ions	decrease of extracellular K ⁺ and hypo-osmolality	<300	increase	+	Morisawa and Morisawa, 1986, 1988
Acipenseridae	high molecular weight substances of urine and Ca ²⁺	decrease of extracellular K ⁺ concentration	≤300	+	-	Chapter 2; Linhart et al., 1999
Anguillidae	increase in environmental pH	hyper-osmolality	>300	ND	ND	Miura et al., 1995
Marine water						
Scophthalmidae	ND	hyper-osmolality	≥300	+	-	Chauvaud et al., 1995
Serranidae	ND	hyper-osmolality	>300	+	-	Billard, 1978
Moronidae	ND	hyper-osmolality	>300	+	-	He et al., 2004
Herring	ND	egg molecules and extracellular K ⁺ in the micropyle area	>300	+	-	Cherr et al., 2008 Morisawa et al., 1992; Yanagimachi et al., 1992
Euryhaline						
Cichlidae	ND	changes of osmolality and extracellular Ca ²⁺ (higher with higher maintenance salinity)	<300 (FWF) >300 (MF)	weakly dependent +	- -	Linhart et al., 1999; Legendre et al., 2008 Chapter 4

In contrast to other externally fertilizing fish species (Cosson, 2010), tilapia spermatozoa possess quite long durations of motility (>2 hours regardless the fish rearing salinities) (Chapter 3). Similar feature has been also reported in case of medaka (*Oryzias latipes*), another euryhaline fish species, where swimming duration after sperm activation was observed to last as long as 7 days, whatever the osmolality of the swimming medium (from 25 to 686 mOsm/kg) (Yang and Tiersch, 2009). Furthermore, sperm motility in tilapia could be reactivated at the end of a swimming period simply by addition of extra Ca^{2+} (Chapter 4). Thus, it appears that reduced or stopped sperm activity at the end of the motility period do not result from limiting energy reserves; in contrast, such “exhausted” spermatozoa might exhibit significantly high production rate of ATP that would be enough to sustain its consumption by the flagella machinery as shown in several other species (Cosson, 2013). Overall, the mechanisms by which calcium is involved in the maintenance and restoration of sperm movement in this euryhaline tilapia (*S. melanotheron heudelotii*) remain to be elucidated.

Reaction of flagella with respect to osmotic signal has been quite well documented regarding its biochemical aspects, but this event has only recently been examined in detail (Chapter 6). Fish sperm activation is a very rapid phenomenon occurring immediately when sperm cells are dispersed in a swimming medium for microscopic observation (Cosson, 2010). Technically, it is very challenging to capture this brief event for detailed analysis (Cosson, 2008). Nevertheless, experimental situations explained in Chapter 6 allow inducing a delay (up to several seconds period) between mixing and activation of fish sperm. In such condition, it was possible to investigate the appearance of the initial flagellar bends, their development and position along the flagellum (Chapter 6).

Previously, detailed description of flagellar behavior at motility initiation were published only for spermatozoa of a few species, such as sea urchin (Gibbons and Gibbons, 1980; Goldstein, 1979; Ohmuro et al., 2004) and arenicola (Pacey et al., 1994a; Pacey et al., 1994b). The present work describes for the first time the precise chronology of motility initiation in fish spermatozoa (Chapter 6). In most cases, appearance of the first bend occurs in the proximal region of the flagellum close to the head, yet this event was rarely observed in the very distal part (Chapter 6). In all cases, this first bend progresses toward the flagellar tip and is immediately followed by a second but reverse bend on the opposite side of the flagellum, and so forth, although the sperm cell remains non-progressive. It is only when a number of bends propagated along the flagellum length become numerous that the cumulative thrust of these bends develop the real progressive displacement of the sperm cell. Apparently, a minimal level of flagellar propulsive power is required for spermatozoa to be propelled forward. This assumption was indirectly confirmed by measurements of beat frequency in spermatozoa showing that right after motility triggering, sperm flagella beat at a low frequency but beat frequency rises very rapidly, so that after a few seconds, the value is two-fold higher (Chapter 5). The total period needed for the flagellum to switch from immobility with rigid shape to full activity with regular propagating bends ranges from 0.4 to 1.2 seconds (Chapter 6).

In sturgeon, starting from the earliest stages of motility initiation, the wave propagation along the flagellum and new wave formation proceed in a helical manner leading to a 3-dimensional rotation of the whole spermatozoon (Chapter 6). It was previously shown that the flatness of waves of a fully motile flagellum is not perfect and that waves slightly deviate out of plane of fish sperm (Cosson, 2010). Such slight distortion was related to the ability of sperm cells to remain swimming in the vicinity of any surface (Cosson et al., 2003). Development of helical waves was also described in tunicate and sea urchins sperm flagella

(Ishijima, 2012), though according to Gray (1953) such helical propulsion has lower efficiency in terms of forward velocity of the spermatozoa, while swimming in a single plane is more advantageous, as it partly prevents dispersion of spermatozoa far from the egg.

Right after activation and during the first phase of motility period, waves that are propagate along the whole length of the sperm flagellum, generally describe a pseudo-sine wave shape. Wave shape of fish spermatozoa also partly results from the physical constraints imposed by the external milieu. One such constraint is viscosity, which affects sperm cells near the egg surface, i.e. ovary fluids or jelly-like layers that surround eggs in some fish species (Cosson, 2010).

In-depth understanding of the ultrastructure and biochemical aspects as well as the mechanisms of functioning of sperm flagella is of great interest, primarily for medical and zoo-technical fields but also from a fundamental point of view.

Conclusions

This thesis includes five publications where the processes of maturation of fish spermatozoa, their adaptability to different physical and biochemical circumstances, the extra- and intra-cellular signaling as well as the regulatory mechanisms of motility activation in fish spermatozoa were investigated.

The main conclusions from these studies are:

1. Sperm maturation in sturgeons occur outside the testes via dilution of sperm by urine at its release into Wolffian duct. This process involves the participation of high molecular weight substances as well as calcium ions that are present in seminal fluid and/or urine. This study also provides the first evidence for the presence of Ca^{2+} stores in sturgeon spermatozoa.
2. Spermatozoa of freshwater fishes with ionic and osmotic modes of motility activation respond in a species-specific manner to the ambient osmotic changes. In hypotonic condition, spermatozoa with osmotic mode change their volume during the activation and motility period.
3. In euryhaline tilapia, spermatozoa of fish reared in fresh-, sea- or hypersaline water can be activated in hypotonic, isotonic or hypertonic conditions of swimming milieu, provided that Ca^{2+} ions are present at various levels. In general, regardless the fish rearing salinity, the mechanism of sperm activation in this euryhaline tilapia remains the same.
4. In sturgeon spermatozoa, motility can be prevented by a sufficient K^+ concentration, but this inhibition can be by-passed by a pre-application of a hyperosmotic treatment immediately followed by hypo-osmotic shock: in such conditions, spermatozoa might be activated by an unexpected signaling pathway, independent from ionic stimulation. In addition, after such treatment, sturgeon spermatozoa motility becomes insensitive to a large extracellular K^+ concentration.
5. At motility initiation in sturgeon spermatozoa, the first few bends formed at the basal region are propagated toward the flagellar tip, but gradually subside when reaching the mid-flagellum. This behavior is repeated several times until the bend amplitude gradually reaches a similar value, eventually leading to sperm progressive displacement. Estimated period needed to switch from resting to full efficient forward motility is very short, ranging 0.4–1.2 s.

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English Summary

Flagellar movement initiation, signaling and regulation of fish spermatozoa: physical and biochemical control

Galina Prokopchuk

One of the most important keys to effective breeding management of farmed fish is control of reproductive processes: sexual maturation of broodstock, spawning procedures, and production of high quality gametes. Successful insemination depends on metabolism and appropriate functioning of gametes, in particularly of sperm. Semen characteristics – its quality, productivity, ejaculate volume and concentration – are highly variable between species, breeds, individuals and even portions of sperm from the same male obtained at different times. Despite considerable progress in understanding of factors involved in the control of gamete quality, the picture of the cellular and molecular mechanisms responsible for the observed variability in quality remains largely incomplete.

Prior to become an actively fertilizing unit, fish spermatozoon formed in testes undergo a sequence of manifold events: from maturation, when sperm cells acquire the potential for activation, to first flagellar beat in spawning environment. So far, the mechanisms underlying this progression and abilities of fish spermatozoa to adapt to external changes are poorly understood. The current study attempted to explain the regulatory processes and responses of fish spermatozoa during maturation and motility initiation.

The first objective of this study was to improve the understanding of the mechanism underlying the acquisition of potential for sperm motility in sturgeon. Sturgeons have a primitive organization of the excretory system that is unique to this fish group: the efferent ducts coming from the testes directly contact the kidneys. The physiological process underlying sperm maturation in this species previously has not been described. Our results showed that sperm maturation in sturgeons occur outside the testes because of dilution of sperm by urine (Chapter 2) and involves the participation of high molecular weight substances as well as calcium ions present in seminal fluid and/or urine. We demonstrated that testicular spermatozoa motility was not activated in a swimming environment, but acquired this capacity after *in vitro* incubation in urine or seminal fluid from Wolffian duct in a temperature and time-dependent manner. The present study also revealed that during the maturation process, sperm motility is affected in its sensitivity to calcium ions.

Following the maturation steps and the release from storage within the male, spermatozoa of fish species with external fertilization encounter at their activation various biological and physical circumstances, which in some cases constitute an external signal for motility initiation, but also may influence their motility period. Thence, the second aim of the present study was to investigate the coping mechanisms in fish spermatozoa with osmotic and ionic activating mode, as well as in spermatozoa of euryhaline fishes, to various environmental conditions.

We showed that alteration of environmental osmolality affects the sperm in different ways, depending on fish species and modes of spermatozoan motility activation, either osmotic or ionic mode (Chapter 3). In response to osmotic stress caused by hypotonicity, carp spermatozoa regulated the flow of water across their cell membrane and increased their cytoplasmic volume during their short motility period. In contrast, no sperm volume changes were observed in sterlet or in brook trout spermatozoa, both of which have an ionic mode of

motility activation (Chapter 3). From these observations, it is assumed that spermatozoa of salmonid and sturgeon quickly react to the osmolality signal by initiating their flagellar motility, but also very efficiently control the volume changes concomitantly engendered by osmotic stress.

Euryhaline fishes can acclimate to wide range of salinities, from freshwater to seawater or even higher, and spermatozoa of these species can modulate their regulatory mechanism according to rearing salinity of the broodfish. In the present study, we examined the mechanism by which sperm motility triggering can adapt to such a broad range of environmental salinity. Our results demonstrated that spermatozoa of euryhaline tilapia, *Sarotherodon melanotheron heudelotii*, reared in fresh-, sea- or hypersaline water can be activated in hypotonic, isotonic or hypertonic conditions of swimming milieu, provided Ca^{2+} ions are present at various levels (Chapter 4). It was established that the higher the fish rearing salinity or the more hypertonic ambient media at spermatozoa activation, the higher extracellular concentration of Ca^{2+} ions is required. We have also found that regardless of the fish rearing salinity, the mechanism of sperm activation in this euryhaline tilapia remains the same. The results obtained in the present study suggest that osmolality is not the main factor inhibiting sperm motility inside the testis in the *S. melanotheron heudelotii*.

A third aim of this study was to investigate the regulation of motility initiation and describe flagellar beating initiation in chondrosteian spermatozoa. In salmonids and sturgeons, the regulation of sperm motility activation is mostly attributed to a decrease of the extracellular K^+ concentration at sperm transfer from seminal fluid into freshwater, where osmolality as well as K^+ concentration are much lower. We detected that in sturgeon spermatozoa, K^+ inhibition can be by-passed (Chapter 5) if sperm cells are exposed to a high osmolality shock prior to being transferred to K^+ -rich swimming media. In addition, after such treatment, sturgeon spermatozoa motility becomes insensitive to a large extracellular K^+ concentration. Thus, we hypothesized that sturgeon spermatozoa may be activated by use of an unexpected signaling pathway, independent from regular ionic stimulation (Chapter 5).

The successive steps in activation of sturgeon spermatozoa were investigated by high-speed video microscopy, using specific experimental situation, where sperm motility initiation was delayed in time up to several seconds (Chapter 6). At motility initiation, the first couple of bends formed at the basal region are propagated toward the flagellar tip, but amplitude gradually diminishes upon reaching the mid-flagellum. This behavior is repeated several times, until a stage where the amplitudes of bends gradually reach similar value, which eventually leads to sperm progression. Starting from the earliest stages of motility initiation, the wave propagation along the flagellum and formation of new waves proceeds in a helical manner leading to a 3-dimensional rotation of the whole spermatozoon of sturgeon. The total period needed for the flagellum to switch from immobility with rigid shape to full activity with regular propagating bends ranges from 0.4 to 1.2 seconds (Chapter 6).

In conclusion, the results of the current study bring valuable pieces of information into the general understanding of the processes of maturation of fish spermatozoa, their adaptability to different physical and biochemical circumstances, the extra- and intra-cellular signaling as well as the regulatory mechanisms of motility activation in fish spermatozoa. These data present a great interest from a fundamental scientific point of view, as well as for practical applications as *in vitro* maturation of testicular sturgeon sperm can be used as practical tool for artificial reproduction in case of the death of the male donor.

Czech Summary

Iniciace pohybu bičíku, signalizace a regulace pohyblivosti spermií ryb: fyzikální a biochemické řízení

Galina Prokopchuk

Jedním z klíčových faktorů pro úspěšný chov ryb je možnost řízení reprodukčních procesů: pohlavního dospívání generačních ryb, výtěru ryb a tvorby vysoce kvalitních pohlavních produktů. Úspěšné oplození závisí na metabolismu a příslušné funkčnosti gamet zejména spermatu. Vlastnosti spermatu – jeho kvalita, oplozeníschopnost, objem a koncentrace spermií jsou velmi proměnlivé mezi druhy, plemeny, jedinci a dokonce i dávkami jedince v různých obdobích. I přes značný pokrok v porozumění faktorů účastnících se řízení kvality gamet zůstává celkový obrázek buněčných a molekulárních mechanismů odpovědných za pozorovanou variabilitu kvality z velké části neúplný.

Před tím než se spermie vznikající ve varlatech stane oplozeníschopnou, prochází celou řadou jednotlivých procesů od zrání, kdy spermie získává potenciál pro svoji aktivaci, až k prvním pohybům bičíku spermie v místě výtěru ryb. Dosud zůstává mechanismus těchto procesů, včetně schopnosti adaptability na okolní změny prostředí, z velké části neobjasněn. Cílem této práce je tedy objasnit některé tyto řídicí procesy a odezvu rybích spermií na tyto procesy během jejich dozrávání a počátku aktivace jejich pohybu.

Prvním cílem bylo lépe porozumět mechanismům, které umožňují získat potenciál pohyblivosti spermií u jeseterů. Jeseteři mají poměrně jednoduchou organizaci testikulárního vylučovacího systému, který je pro tuto skupinu ryb jedinečný: semenné kanálky jdoucí z varlat ústí přímo do ledvin. Fyziologický proces zodpovědný za dozrávání spermií u této skupiny ryb nebyl dosud znám. Naše výsledky ukazují, že k dozrávání spermií u jeseterů dochází mimo varlata po naředění spermatu močí (kapitola 2) za účasti sloučenin s vysokou molekulovou hmotností a za účasti vápníkových iontů přítomných v seminální plazmě a/nebo moči. Prokázali jsme, že pohyblivost testikulárních spermií nebyla stimulována v aktivačním médiu, ale stalo se tak po in vitro inkubaci těchto spermií v moči nebo seminální plazmě z Wolffova kanálku v závislosti na teplotě médií a čase inkubace. Bylo rovněž prokázáno, že schopnost pohyblivosti testikulárního spermatu během dozrávání je ovlivňována citlivostí na koncentraci vápníkových iontů.

Po procesu dozrávání a uvolnění spermií ze sběrného místa uvnitř těla samce jsou spermie ryb s vnějším oplozením při jejich aktivaci ovlivňovány celou řadou dalších biologických a fyzikálních jevů, které v některých případech slouží jako vnější signál pro aktivaci pohyblivosti, ale také mohou ovlivnit délku této pohyblivosti. Dalším cílem této práce bylo tedy porozumět aktivačním mechanismům u spermií ryb s iontově osmotickým způsobem aktivace i ryb euryhalních při různých podmínkách prostředí. Prokázali jsme, že změna okolní osmolality ovlivňuje spermie různým způsobem v závislosti na druhu ryb a mechanismu aktivace pohyblivosti spermií – osmotickém nebo iontovém (kapitola 3). V reakci na osmotický stres způsobený hypotonickým prostředím je u spermií kapra obecného regulován tok vody přes jejich buněčné membrány a zvyšován jejich cytoplazmatický objem v průběhu krátkého období jejich pohyblivosti. Na druhou stranu změna v objemu cytoplazmy nebyla pozorována u spermií u jesetera malého nebo sivena amerického, u kterých je iontový způsob aktivace pohyblivosti spermií (kapitola 3). Na základě těchto pozorování je předpokládáno, že spermie lososovitých a jeseterovitých ryb rychle reagují na změnu osmolality počátkem pohyblivosti

bičíku, ale velmi účinně řídí změny v objemu cytoplazmy současně vyvolané osmotickým stresem.

Euryhalní ryby jsou takové, které jsou schopny pobývat v širokém spektru salinit vodního prostředí od sladkovodního po mořské i vyšší a spermie těchto druhů mohou upravovat regulační mechanismy podle aktuální salinity, ve které se generační ryby nacházejí. V této práci jsme zkoumali, jakým způsobem se spouštěcí mechanismus pohyblivosti spermií může adaptovat na tak široké spektrum salinit okolního prostředí. Naše výsledky ukazují, že spermie euryhalní tilápie, *Sarotherodon melanocheilus*, chovaná ve sladké, mořské nebo hyperslané vodě může být aktivována hypotonických, isotonických nebo hypertonických podmínkách za předpokladu přítomnosti vápníkových iontů (Ca^{2+}) o různých koncentracích (kapitola 4). Bylo zjištěno, že čím vyšší je salinita prostředí, kde jsou ryby chovány, nebo čím vyšší jsou okolní hypertonické podmínky, tím je požadována vyšší mimobuněčná koncentrace Ca^{2+} iontů. Bylo rovněž zjištěno, že bez ohledu na salinitu, ve které jsou ryby chovány, zůstává mechanismus aktivace spermií u této euryhalní tilápie stejný. Výsledky naznačují, že osmolalita není hlavním faktorem inhibujícím pohyblivost spermií uvnitř varlat této tilápie.

Třetím cílem v této práci bylo objasnit regulaci počátku pohyblivosti spermií a popsat počátek pohybu bičíku u chrupavčitých ryb. U lososovitých a jeseterovitých ryb je regulace aktivace pohyblivosti spermií přisuzována snížení koncentrace iontů mimobuněčného draslíku (K^+) při přechodu spermií ze seminální plazmy do sladké vody, kde je koncentrace draselných iontů mnohem nižší. Zaznamenali jsme, že u spermií jeseterů může být inhibice draselnými ionty K^+ překlenuta (kapitola 5) při pre-expozici spermií vyššímu osmotickému šoku před jejich přechodem do prostředí média bohatého na K^+ . Po takovém ošetření se navíc aktivace pohyblivosti spermií jeseterovitých stává necitlivá k vysoké koncentraci mimobuněčných iontů draslíku. Domníváme se, že spermie jeseterovitých mohou být aktivovány pomocí neočekávaných signálních drah, a to nezávisle na normální iontové stimulaci (kapitola 5).

Postupné kroky aktivace spermií jeseterů byly sledovány s pomocí vysokorychlostní video mikroskopie užitím specifické experimentální situace, kdy počátek pohyblivosti spermií byl zpožděn v čase až o několik sekund (kapitola 6). Při počátku pohybu spermie se první vlny vznikající v bazální části bičíku začínají šířit směrem ke špičce bičíku, ale amplituda postupně slábne směrem ke středu bičíku. Toto chování se několikrát opakuje až do doby, kdy amplitudy vln postupně dosáhnou podobných hodnot, což případně vede k posunu spermie. Od počátečních fází pohybu bičíku postupuje šíření vln napříč bičíkem a vytváření nových vln probíhá šroubovitým způsobem vedoucím k třídimenzionální rotaci celé spermie jesetera. Celkový čas potřebný pro přechod bičíku z imobilního stavu s tuhým tvarem do plné aktivity s pravidelným šířením vln se pohybuje v rozmezí 0,4 až 1,2 sekund (kapitola 6).

Je možné konstatovat, že výsledky této práce přinášejí cenné informace pro obecné porozumění procesům dozrávání rybích spermií, jejich schopnosti přizpůsobit se různým fyzikálním a biochemickým okolnostem, extra i intra celulární signalizaci i regulačním mechanismům aktivace pohybu. Tyto informace jsou velkým přínosem pro základní výzkum i pro možnost praktického využití, neboť in vitro dozrávání testikulárního spermatu jeseterů lze využít při umělé reprodukci v případě úhynu samčích dárců.

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- GAJU (114/2013/Z), New methods and biotechnological approaches in fish reproduction and genetics (2013-2015, responsible leader Prof. Otomar Linhart)
- GACR P502/11/0090, Maturation and ageing of fish spermatozoa: A comparative study between teleostean and chondrosteian fish species as taxonomically distant models (2011–2015, responsible leader Marek Rodina, Ph.D.)
- GACR P502/12/1973, Characterization of swimming fish sperm flagella: biophysical quantification (2012–2015, responsible leader Jacky Cosson, Ph.D., Dr.h.c.)
- GACR P502/15/12034S, Ancient mechanism of sturgeon sperm for acquisition of ability to fertilize (2015–2017, responsible leader Jacky Cosson, Ph.D., Dr.h.c.)
- COST-STSM-FA1205-14644
- COST-STSM-FA1205-17049

List of publications

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Dzyuba, B., Bondarenko, O., Fedorov, P., Gazo, I., **Prokopchuk, G.**, Cosson, J., 2016. Energetics of fish spermatozoa: The proven and the possible. *Aquaculture*. In Press. doi:10.1016/j.aquaculture.2016.05.038. (IF 2015 = 1.893)

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Bondarenko, O., Dzyuba, B., Cosson, J., **Prokopchuk, G.**, Dzyuba, V., Rodina, M., Linhart, O., 2013. The role of Ca^{2+} in sterlet (*Acipenser ruthenus*) spermatozoa *in vitro* maturation. In: Book of abstracts “4th International Workshop of The Biology of Fish Gametes”, 17-20 September 2013, Albufeira, Portugal, P. 24–25.

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Training and Supervision Plan during Study

Name	Galina Prokopchuk	
Research department	2011–2016 – Laboratory of Reproductive Physiology of FFPW	
Supervisor	Jacky Cosson Ph.D., Dr.h.c.	
Period	25 th October 2011 until 15 th September 2016	
Ph.D. courses		Year
Basic of scientific communication		2012
Pond aquaculture		2012
Applied hydrobiology		2013
Ichthyology and Fish Taxonomy		2014
English Language		2014
Scientific seminars		Year
Seminar days of RIFCH and FFPW		2012
		2013
		2014
		2014
		2015
International conferences		Year
Prokopchuk, G., Dzyuba, B., Rodina, M., Cosson, J., 2015. Flagellar behavior of fish sperm at motility initiation. In: Book of abstracts “The 5 th International Workshop on the Biology of Fish Gametes”, 7–11 September 2015, Ancona, Italy, pp. 121-122.		2015
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Prokopchuk, G., Cosson, J., Dzyuba, B., Bondarenko, O., Rodina, M., Linhart, O., 2013. Control mechanisms of fish sperm activation: Breaking some rules. In: Book of abstracts “4 th International Workshop of The Biology of Fish Gametes”, 17–20 September 2013, Albufeira, Portugal, pp. 52–53.		2013
Prokopchuk, G., Cosson, J., Dzyuba, B., Boryshpolets, S., Rodina, M., Bondarenko, O., Linhart, O., 2012. Species-specific influence of cryoprotectant DMSO on sperm motility in fish. In: Book of abstracts “AQUA 2012. Global Aquaculture Securing Our Future”, 1–5 September 2012, Prague, Czech Republic, P. 899.		2012
Prokopchuk, G., Cosson, J., Dzyuba, B., Rodina, M., Linhart, O., 2012. Influence of DMSO and ethylene glycol on sperm motility in sterlet, <i>Acipenser ruthenus</i>		2012

(L.). In: Book of abstracts "Domestication in Finfish Aquaculture", 23–25 October 2012, Olsztyn-Mragowo, Poland, P. 80.	
Foreign stays during Ph.D. study at RIFCH and FFPW	Year
Peter and Udo Gross, Fischzucht Rhönforelle GmbH & Co, Marjoss, Germany (3 days, performing a series of experiments on sperm of European (<i>Huso huso</i>) and Siberian (<i>Acipenser baerii</i>) sturgeons)	2014
Prof. Carles Soler, Proiser Projectes i Serveis R+D S.L. Valencia, Spain. (2 weeks, co-working on development of a computer assisted flagella shape analyzer adapted to specificities of fish sperm motility)	2014
Prof. Eamonn Gaffney, Centre for Mathematical Biology within The Mathematical Institute of The University of Oxford, UK. (1 month, learning MatLab environment for elaboration of descriptive methods of the mechanics of wave initiation and propagation in fish sperm flagella)	2013
Prof. Pascal Fontaine, University of Lorraine, UR AFPA, Vandoeuvre-les-Nancy; and Dr. Marc Suquet, IFREMER, Argenton, France. (15 days, application of high-speed video microscopy technique, image (MicroImage, ImageJ) and CASA analyses for determination of motility characteristics of oysters spermatozoa)	2013
Biology Professor Dr. Trevor Pitcher, Department of Biology, Great Lakes Institute for Environmental Research, University of Windsor, Canada. (1 month, studying the effect of alternative reproductive tactic and ovarian fluid concentration on flagellar beating patterns of Chinook salmon spermatozoa using a high-speed video microscopy)	2012
Dr. Marc Legendre, IRD, Montpellier; IFREMER, Palavas Les Flots, France (1 week, application of high-speed video microscopy method for evaluation of flagellar movement of sperm of euryhaline tilapia (<i>Sarotherodon melanotheron heudelotii</i>))	2012
Pedagogical activities	
• Leading of project entitled Sperm motility in common carp at Summer school	2014
• Announcing the project entitled Sperm velocity and flagellar beat in trout at Summer school	2015
• Lecturing of students of bachelor study, discipline Fishery at USB FFPW in range of 50 teaching hours	2013–2015
• Training of students in measuring the sperm velocity using high speed camera in range of 40 teaching hours	2014–2016

Curriculum Vitae

PERSONAL INFORMATION

Name: Galina
Surname: Prokopchuk
Title: M.Sc.
Born: 15th January, 1992, Kiev, Ukraine
Nationality: Ukrainian
Languages: English (B2 level – FCE certificate), Ukrainian, Russian, Czech
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EDUCATION

2011 – present Ph.D. student in Fishery, Faculty of Fisheries and Protection of Waters, University of South Bohemia, Ceske Budejovice, Czech Republic
2010–2011 M.Sc., Faculty of Radiophysics, Department of Biological and Medical Physics, V. N. Karazin Kharkiv National University, Kharkiv, Ukraine
2006–2010 B.Sc., Faculty of Radiophysics, Department of Biological and Medical Physics, V. N. Karazin Kharkiv National University, Kharkiv, Ukraine
1996–2006 Pokotylyivskiy Lyceum “Promin”, Pokotylyivka, Kharkiv region, Ukraine

COMPLETED COURSES

05/04–07/04 2016 Andor Academy Prague - Biocev, Vestec, Czech Republic
16/06–20/06 2014 Course on Biological Specimen Preparation for Electron Microscopy – Biology Centre, Academy of Sciences Laboratory of Electron Microscopy, Ceske Budejovice, Czech Republic
05/03–09/03 2012 Basics of scientific communication Faculty of Fisheries and Protection of Waters, University of South Bohemia, Vodnany, Czech Republic

TRAINING

16/03–20/03 2015 Oral presentation and training – The 4th Training School of the AQUAGAMETE COST Action “Optical Microscopy and Image Analysis” at the FFPW, University of Ceske Budejovice, Vodnany, Czech Republic

RESEARCH STAY AND COLLABORATIONS

31/03–07/04 2014 Prof. Carles Soler, Proiser Projectes i Serveis R+D S.L. Valencia, Spain
12/10–12/11 2013 Prof. Eamonn Gaffney, Centre for Mathematical Biology within The Mathematical Institute of The University of Oxford, UK
15/05–21/05 2013 Prof. Pascal Fontaine, University of Lorraine, UR AFPA, Vandoeuvre-les-Nancy, France
22/05–21/05 2013 Dr. Marc Suquet, IFREMER, Argenton, France
25/09–14/10 2012 Dr. Trevor Pitcher, Department of Biology, Great Lakes Institute for Environmental Research, University of Windsor, Canada
21/01–29/01 2012 Dr. Marc Legendre, IRD, Montpellier