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Habilitation Thesis

Osmotic combined to ionic control of sperm motility in taxonomically distant fish models: relations with cryopreservation

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Statement

I hereby declare that I elaborated the present Habilitation thesis entitled “Osmotic combined to ionic control of sperm motility in taxonomically distant fish models: relations with cryopreservation” independently with using of my own work or collaborative work of me and colleagues and with the help of other publication resources, which are properly cited.

In Vodňany 1st November, 2016 Borys Dzyuba, M.Sc., Ph.D.

List of content

1. Introduction

As “fish” refers loosely to several classes of vertebrates widely differing in their mode and habitat of reproduction, it should be noted that in the current thesis, this term is used for fish species which are representatives of different orders of class Actinopterygii (ray-finned fishes) according taxonomy proposed by Nelson (2006). Also, “fish model” is used here in sense of a species that possesses a specific mode of spermatozoa motility activation among a group of species with similar reproductive characteristics.

1.1. General description of fish sperm biology

Fishes possess the cystic mode of spermatogenesis, meaning that, initially, mitotic division of single spermatogonia is followed by meiotic division of spermatocytes, which in turn ends with appearance of spermatids and finally spermatozoa occurring in specific sub-cellular structure called cysts. The cysts are embedded into a wall of spermatogenic tubules and are surrounded by specific Sertoli cells. In the end of spermatogenesis, fully formed spermatozoa are released from cysts into the lumen of spermatogenic tubules, and they remain immotile until spawning. For a few fish species, specific sperm maturation is known as the process that results in acquisition of the potential for motility and fertilization which occurs outside the testes, in male genital ducts (Schulz and Miura 2002). It was demonstrated that in salmonids and eels sperm maturation is operating via an increase of pH of the seminal plasma regulated by bicarbonate anions (Morisawa and Morisawa 1988; Ohta *et al.* 1997). However, it was shown recently that in salmonids sperm maturation can also occur without pH increase suggesting existence of alternative mechanisms for this process (Ciereszko *et al.* 2015). It is not clear yet if the phenomenon of sperm maturation out of the testes is frequent among fishes of different taxa, as high motility and fertilizing ability of testicular spermatozoa was also described in several species. As example, testicular spermatozoa are commonly used in artificial propagation of some teleost species, such as African catfish (Viveiros *et al.* 2000), Northern pike (Lahnsteiner *et al.* 1998), yellow perch (Blazer *et al.* 2013), loach (Kopeika *et al.* 2003) and rainbow trout (Nynca *et al.* 2012).

Generally, fertilization occurs externally in most fish, meaning that gametes are released into environment, which differs significantly from internal conditions. This external fertilization strategy is associated in many fish species with specific features of sperm (Cosson 2004): very quick spermatozoa motility activation followed by a very brief period (seconds to minutes) of sperm movement with high velocity required to reach egg micropyle. The “cost” of this high initial activity is a very rapid consumption of intracellular energy accumulated as high-energy phosphate bonds in macroergic phosphate molecules (ATP, ADP, CrP) (Dzyuba *et al.* 2016). It is important to recall that ATP hydrolysis is the only source for the flagellar mechano-chemical motion of fish sperm. Parameters of spermatozoa motility, such as percentage of motile cells, motility duration, and spermatozoa velocity are considered as indicators of sperm quality which is closely related to their fertilizing ability.

Cellular processes responsible for fish spermatozoa motility activation involve (1) membrane reception of the activation signal and its transduction through the spermatozoa plasma membrane via the external membrane components, ion channels, and aquaporins; (2) cytoplasmic trafficking of the activation signal; (3) final steps of the signaling, including signal transduction to the axonemal machinery, and activation of axonemal dyneins and regulation of their activity (Dzyuba and Cosson 2014). These processes are considered as necessary elements of the motility activation cascade. However, nowadays it is widely accepted that there is no general model that can describe this cascade because of essential taxa specific variation of its elements (Zilli et al. 2012; (Morisawa 2008). It is important to emphasize that change of spermatozoa surrounding media occurring during sperm release from testes into spawning environment is the natural trigger of the aforementioned activation processes. Current habilitation deals with environment factors such as ionic composition and osmolality involved in fish sperm motility regulation without consideration of intracellular events responsible of the activation cascade.

1.2. Osmotic combined to ionic control of sperm motility in taxonomically distant fish models

Fish spawn in aquatic environments with a wide range of salinities (osmolality), ionic composition, and temperature, therefore, it is not surprising that their spermatozoa possess environment-dependent mechanisms of motility activation. Historically, “simple”

categorization of fish as sea- and fresh-water spawning species distinguished two main ways by which sperm motility is regulated by the environment: 1) osmolality and 2) ionic composition (Morisawa and Suzuki 1980). There are numerous examples of spermatozoon activation by hypotonic (by reference to seminal fluid) or hypertonic signals in fresh- and sea-water spawning fish, respectively (Cosson 2010). For these species, the general mechanism of spermatozoa activation includes water movement across the sperm membrane as a primary factor leading to changes in both membrane properties (as a result of osmotic cell swelling/shrinkage) and internal ion concentration. However, there are numerous fish species, in which ionic composition of environment plays the role of determinant for motility initiation even though, the role of ions is masked during natural activation where both ionic and osmotic conditions change simultaneously occur at spawning. Frequently, the environment concentration of Ca^{2+} or K^{+} ions is a key factor determining sperm motility activation in these species, provided that the osmotic environment be adequate (Alavi and Cosson 2006). Even though both environmental osmolality and ionic composition change during sperm release into environment, each of them could be differentially involved in motility activation, indicating a species-specific hierarchy of environmental signals. In the current , “osmotic” or “ionic” mode of sperm motility activation are defined according to the following sense: in case of "osmotic" mode, it is only the change in environment osmolality that is enough to activate spermatozoa motility, while in "ionic" mode, the change of environment ionic composition is the mandatory condition: in this last case, it is a decrease of concentration of an ion that is crucial to release the inhibition.

Based on available data, species used for studies incorporated into the thesis can be assigned to different fish models. Silver carp *Hypophthalmichthys molitrix*, common carp *Cyprinus carpio* (Poupard *et al.* 1997), Eurasian perch *Perca fluviatilis* (Boryshpolets *et al.* 2009) are considered as freshwater model species which possess a hypotonic mode of motility activation, as a reduction of osmotic pressure itself, but not environment ion composition, determines sperm motility activation. Sterlet *Acipenser ruthenus* (Alavi *et al.* 2011), rainbow trout *Oncorhynchus mykiss* (Morisawa 2008) are examples of freshwater spawning fish, which can be considered as model species characteristic of an ionic mode of sperm motility activation as their sperm motility can be activated in isotonic media of specific ion composition. Blackchin tilapia *Sarotherodon melanotheron heudelotii*, blackstripe pipefish *Syngnathus abaster*, haarder

Mugil soiuy are euryhaline species for which the mode of sperm motility activation has not been completely described until now.

Northern pike *Esox Lucius* (Alavi *et al.* 2009) cannot be defined as representative of any defined models because its sperm can be activated in isotonic conditions where no specific ionic environment is required. In addition, the mating process in blackstripe pipefish *Syngnathus abaster* is unusual because the female transfers eggs into special male reproductive organ – the brood-pouch – where fertilization takes place. The role of environment in sperm motility activation in that case cannot be predicted because of the absence of available information about fertilization process.

Simple organization of fish subclasses and orders according to Nelson (2006) is presented on Fig. 1, from which it is clear that species used in the present study belongs to taxonomically distant orders (Acipenseriformes, Cypriniformes, Salmoniformes, Esociformes, Mugiliformes, Gasterosteiformes, Perciformes). Generally, the proposed classification reflects the evolutionary relationships among orders recently supported by molecular phylogeny study in fishes (Betancur *et al.* 2013).

Elucidation of reasons for existing different spermatozoa motility activation modes in taxonomically distant fishes requires future studies.

1.3. Specific features of sturgeon urogenital system in relation to sperm biology

The morphology of the reproductive system of Acipenseriformes as representatives of chondrostei is quite different from that in teleostei because of specific connection between testes and kidneys (Lahnsteiner and Patzner 2009) (Fig. 2). This connection should reduce osmolality of sturgeon milt because of the mixing with urine occurring during its passage through the kidney (Alavi *et al.* 2012b). Furthermore, it is proposed that an ionic mode of sperm motility activation is required to keep spermatozoa immotile (urine osmolality is lower than that of testicular sperm) before entering the environment at spawning. However, until recently, this speculation had not been proved and elucidation of the processes accompanying this unusual natural mixture of sperm with urine is required.

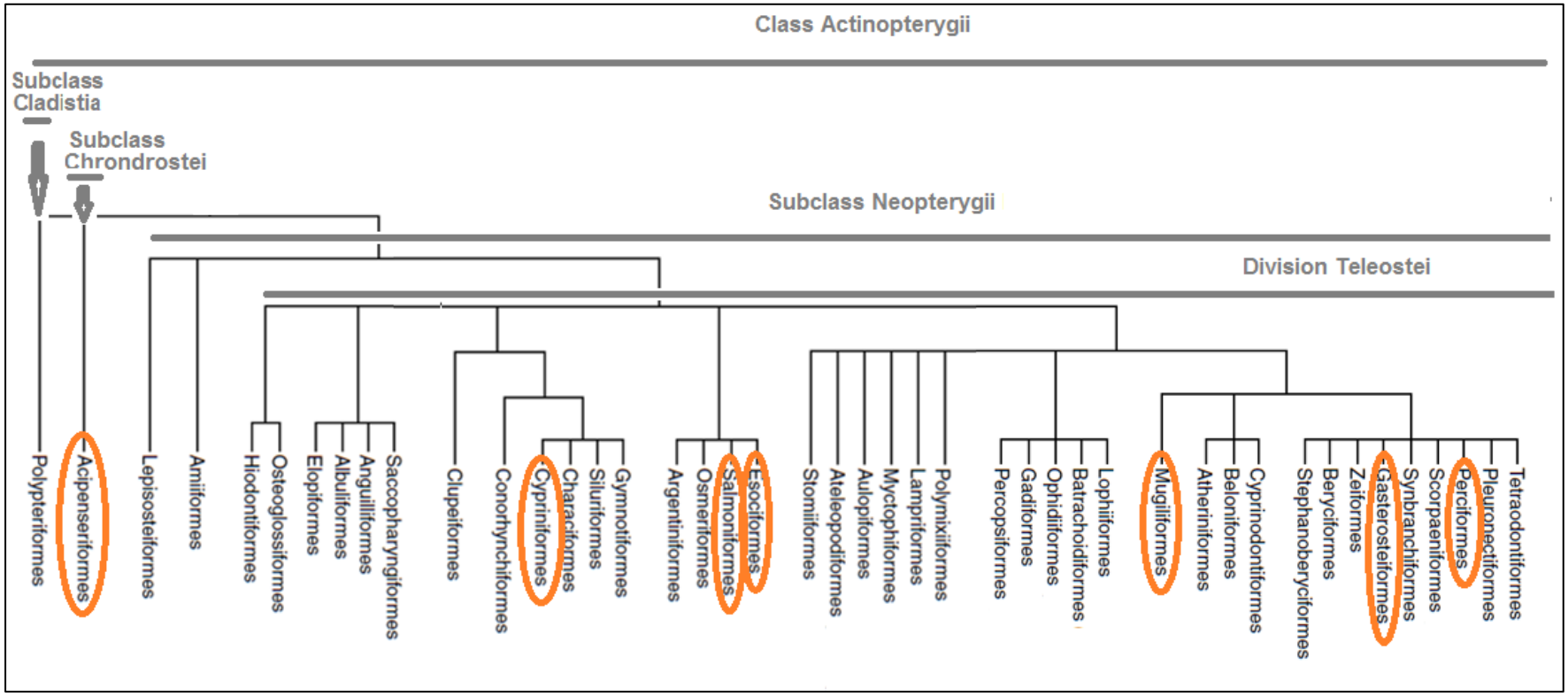

Fig. 1. Fish taxa included in class Actinopterigii (Nelson 2006). Orders to which belong species used in the studies included to the thesis are marked by red oval.

Fishes used in the selected papers (see attachments) and considered as a model species are Acipenseriformes: *Acipenser ruthenus;* Cypriniformes: *Cyprinus carpio, Hypophthalmichthys molitrix;* Esociformes: *Esox lucius,* Salmoniformes: *Oncorhynchus mykiss;* Perciformes: *Sarotherodon melanotheron heudelotii, Perca fluviatilis;* Gasterosteiformes: *Syngnathus abaster*; Mugiliformes: *Mugil soiuy (Liza haematocheila).*

The event of final physiological stage of spermatogenesis resulting in the acquisition of the potential for motility and fertilization in morphologically fully developed spermatozoa has been described for few teleostei (salmonids and eels). It has been shown that sperm maturation takes place in rainbow trout, chum salmon (Morisawa and Morisawa 1988) and Japanese eel (Miura and Miura 2001), when spermatozoa reach the main sperm ducts after passage through efferent ducts. In these three species, the natural hormonal regulation of seminal fluid pH was proposed as a key physiological mechanism in fish sperm final maturation (Morisawa and Okuno 1982; Miura and Miura 2001). Moreover, as urine in freshwater fish species is hypotonic to semen, contamination by urine in teleostean fish species is usually considered to be detrimental (Perchec *et al.* 1995), because of potential induction of motility which occurs untimely to fertilization (Alavi and Cosson 2006). In sturgeon species, direct evidence for a physiological maturation mechanism occurring at the end of spermiogenesis similar to that described in salmonids has not been previously obtained (Dettlaff *et al.* 1993; Chebanov and Galich 2013).

Establishment of relationships between urogenital system anatomy, sperm maturation and sperm motility activation mode is one of the topics of interest of the current habilitation.

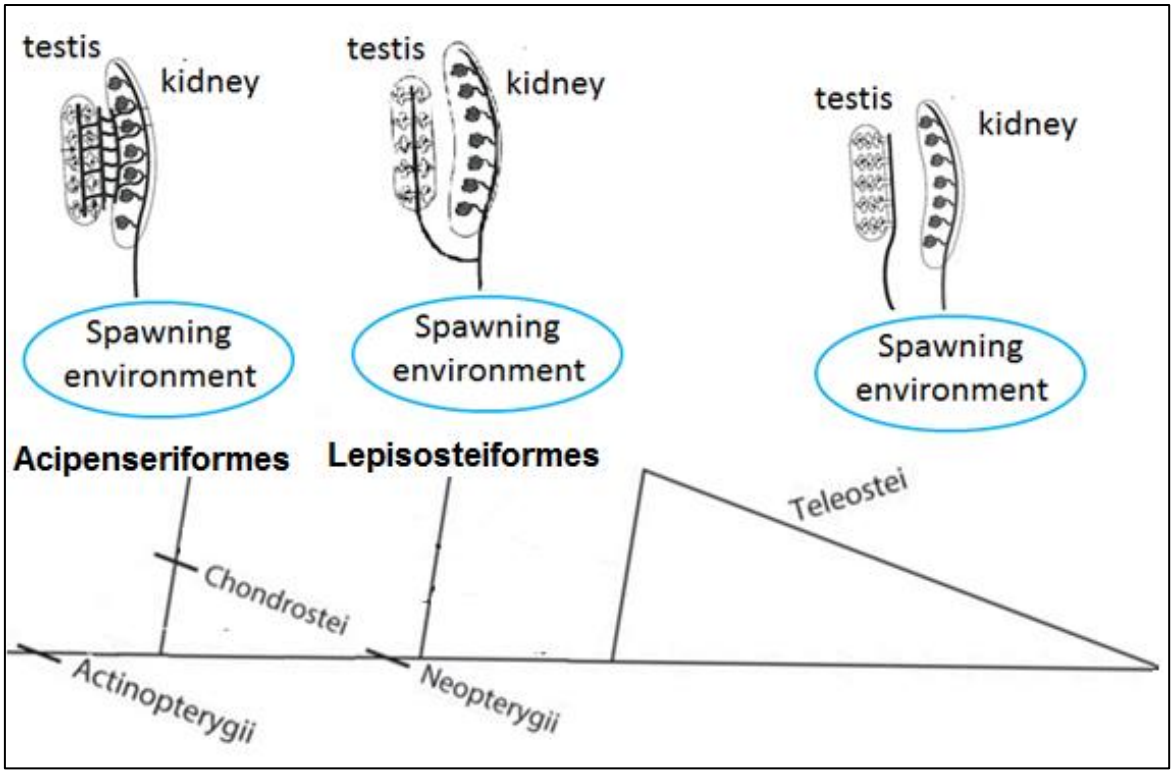

Fig. 2. Phylogeny of the main extant groups of Actinopterygii in relation to urogenital system anatomy. Modified from (Nelson 2006; Lahnsteiner and Patzner 2009).

1.4. Osmotic factor of cryo-damage in fish spermatozoa

Application of sub-zero temperature during sperm storage (cryo-banking) is currently considered as a potentially powerful tool in animal selective breeding and conservation biology. In 2010, FAO indicated that cryo-banking is considered as complementary to *in situ/vivo* conservation of fish genetic resources. Recent studies from FAO have determined that preservation of aquatic genetic diversity, future breed improvement and maintenance of good strains for production are the main objectives of cryo-banking programs in aquaculture (http://www.fao.org/3/a-mq711e.pdf). Sperm cryo-banking in fish gametes presents a big potential to be applied (Cabrita *et al.* 2010) and is also important because cryopreservation of eggs and embryos has not been so far possible due to their specific biological properties (Tsai and Lin 2012), but restoration of individuals is possible by androgenesis in fish using cryopreserved sperm (Zhang 2004).

However, drastic changes of osmolality during freezing/thawing of biological object occur because of ice crystal formation (Mazur *et al.* 2008), thus, indicating that increased environment osmolality is the main component of cryo-damage. While fish sperm cryopreservation has been studied since a long time, the elucidation of relationships between sperm cryo- and osmoresistance is needed especially because of unclear reasons of intra- and inter-species heterogeneity of sperm cryoresistance (Kopeika *et al.* 2007; Kopeika and Kopeika 2008). The study of possibility of sperm cryoresistance increase by manipulation *in vitro* is an additional interest of modern cryobiology but nowadays there is no general recipe for fish sperm (Müller *et al.* 2008).

That is why the studies focused on understanding the relationships between sperm motility activation mode and cryoresistance are incorporated into the present habilitation.

1.5. The aim of the present study

The main aim of the present habilitation is a compilation of author's published results that illustrate the physiological importance of species-specific mode of fish spermatozoa motility activation in relation to reproductive organ anatomy, spawning environment and spermatozoa cryopreservation outcome.

2. Results and discussion of the included papers

2.1. The role of environment osmolality and ionic composition in spermatozoa motility in taxonomically distant fish models

2.1.1.1. Ionic mode of motility activation in euryhaline fish species

Based on the studies of fish sperm motility relative to spawning environment, it has been demonstrated that some euryhaline species (blackchin tilapia, attachment 1; blackstripe pipefish, attachment 2) sperm are activated by an ionic mode. Sperm motility can be activated in media that are hypotonic, isotonic, or hypertonic relative to the seminal plasma (case of blackchin tilapia), and in media that are hypotonic or isotonic (case of blackstripe pipefish).

In case of blackchin tilapia, it has been demonstrated that motility activation mechanism involves external Ca^{2+} ions. While some evidence for such mechanism was already known, the novelty is related to the following aspects.

For blackchin tilapia, it was shown (attachment 1) that the salinity at which fish are living and osmolality of activating medium can be twice higher than previously described for another euryhaline fish, tilapia *Oreochromis mossambicus* (Linhart *et al.* 1999). The mechanism of sperm activation in the blackchin tilapia remains consistently the same (ionic mode requiring the presence of Ca^{2+} ions) whatever the rearing environmental salinity. 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 (Morita *et al.* 2003). The sperm of fish reared in salinities ranging from fresh water (0) up to hypersaline water (70) can be activated in hypotonic, isotonic, or hypertonic activation media, provided that the external Ca^{2+} concentration suffices, while the value of minimally needed concentration of Ca^{2+} is definitely salinity-dependent. External Ca^{2+} concentration required for the spermatozoa activation is higher if fish are acclimated to a higher ambient salinity, and if hypertonic activation media are used whatever the fish rearing salinity. Motility suppressing effects of Na^{+} or K^{+} ions were also determined. Additionally, Ca^{2+}/Na^{+} ratios that resulted in maximal sperm motility were found to correspond to those of sea water or hypersaline water rearing conditions. After cessation of motility of sperm that have been activated by isotonic or

hypertonic treatment, motility can be immediately restored by an excessive Ca^{2+} treatment, suggesting fundamental role of this ion in motility regulation.

In male-brooding blackstripe pipefish we have found that sperm motility is closely dependent on fertilization process itself, in which spermatozoa are maintained in close contact with the eggs by being constrained within a small volume of ovarian fluid. This observation is similar to one made in taxonomically close yellow seahorse, a marine species (Van Look *et al.* 2007). In case of blackstripe pipefish, ovarian fluid, considered as isotonic solution, was found to be the best activating medium, which suggests that blackstripe pipefish spermatozoa motility is activated according to an ionic mode.

While limited information about spermatozoa motility activation is available for species of family Syngnathidae (seahorses and pipefishes), euryhaline tilapias have been more widely studied. Our studies are in good accordance with previously published ones; it is clear that a concentration increase of internal Ca^{2+} is responsible for motility activation, that in turn leads to protein phosphorylation events which are believed to be the final step of signaling in motility activation (Morita *et al.* 2003). Recent studies also suggest that testis-specific gene expression is likely involved in the acclimation to salinity changes in blackchin tilapia (Avarre *et al.* 2014).

From these results, we suppose that it is an ionic mode of spermatozoa motility activation that is the essential component of euryhaline fish reproductive system that allows successful reproduction in wide range of environment salinities. At the same time, the precise molecular mechanisms of its operation are still a topic for future studies.

2.1.1.2. Ionic mode of motility activation in sterlet as freshwater spawning fish model

Biological role of ionic mode of sperm motility activation in some freshwater spawning fish species (such as salmonids and sturgeons) is not clear. Involvement of external Ca^{2+} and K^{+} ions but not osmolality as main regulators of sperm motility in these species can be speculated based on their anadromous status. If sperm motility activation was based on an osmotic mechanism, changes in body fluid osmolality could complicate the maintenance of sperm immotility in the testes during fish migration from marine to freshwater environment. However, sturgeon seminal fluid possesses an extremely low osmolality, thus ionic mode of sperm motility activation could exist. Understanding the relationship between osmotic and ionic

factors in sturgeon sperm motility is of interest because both mechanisms have been proposed as important components of the motility signaling cascade (Alavi *et al.* 2011).

We demonstrated (attachement 3) that sterlet sperm could be activated under all three environmental tonicities, hyper-, iso-, and hypo-tonic in relation to seminal fluid. In all three conditions, when motility is initiated by exposure to low external Ca^{2+}, it is possible to repeatedly activate motility by decreasing of osmolality or increasing Ca^{2+} concentration. The latter is similar to phenomenon observed in tilapia (attachment 1) suggesting the similarity of Ca^{2+} involvement in motility regulation in tilapia and sterlet, although those species are taxonomically very distant (see Fig. 1). It should be underlined that when motility stops (at the end of motility period), an immediate motility re-activation occurs by increase of external Ca^{2+} concentration. This strongly suggests that, in some environmental conditions, spermatozoa of fishes possessing ionic mode of motility activation stop their motility not because of energy source depletion but rather because of changes of intracellular composition. Precise mechanism supporting this process should be further investigated.

Potassium ion (K^+) is another participant of sperm motility initiation in sturgeons (Cosson and Linhart 1996; Alavi *et al.* 2012a): motility is prevented in external media containing millimolar K^+ concentration. It is believed that it is the internal K^+ concentration that keeps spermatozoa immotile (Linhart *et al.* 2002). To initiate sperm motility, spermatozoa should release the excess of K^+, therefore, activation media for sturgeon sperm should contain a very low K^+ concentration. We demonstrated (attachement 4) that following a treatment with hypertonic media, spermatozoa become non-sensitive to usual motility inhibiting concentrations of external K^+. Considering the findings in 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^+ 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. It was further indicated (attachment 4) that a similar duality in activation pathways would occur in sturgeon sperm: in contrast to salmonids, sturgeon spermatozoa pretreated by KCl hyperosmotic solution did not initiate their

motility in low-osmotic K^+-comprising conditions. 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 the 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 (Cosson and Linhart 1996; Toth *et al.* 1997; Alavi and Cosson 2006). 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. Therefore, our study 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.

These results expand our knowledge about the sequence of ion movement via spermatozoa membrane that is required for motility activation. Nowadays, application of this knowledge to better understanding of the basic parameters of flagellar activity and in relation to artificial sturgeon reproduction is in progress.

2.2. Roles of osmolality and ionic composition of seminal fluid in sturgeon spermatozoa maturation

In this chapter, the recent findings on relationships between specific regulation of sperm motility and male urogenital anatomy are described. The morphology of the reproductive system of acipenseriform fishes is quite different from that in teleostean species. Sperm of sturgeons pass through the kidneys rather than directly from the testes into the environment (Fig. 2). This statement is supported by several studies and reviews (Hoar 1969; Wrobel and Jouma 2004). Urine in sterlet was found to be hypotonic in comparison to blood plasma (Krayushkina and Semenova 2006). The mixing of sperm with hypotonic excretory product has been found to be a prerequisite for the capacity to be activated (attachment 5) rather than being a contaminant as classically considered in teleosts (Alavi *et al.* 2012b). We name this process "sperm maturation" in accordance to a similar one already described for few teleost species. Testicular spermatozoa, collected from any part of testes or efferent ducts, are not able

to be activated after dilution by different activating media, whatever the ionic composition, pH or osmolality. Pre-incubation of testicular sperm at room temperature for 10 minutes in seminal fluid collected from Wolffian ducts (*in vitro* maturation) will permit subsequent motility activation. After such *in vitro* maturation, sperm motility parameters were not significantly different from those of control sperm. Moreover, we demonstrated that spermatozoa 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 step takes place outside the testes, and that proteolytic activity of seminal fluids is involved in the process. Those observations were supported by detailed studies performed in our laboratory indicating that sperm maturation does exist in other aciperiformes species (Bondarenko 2015; Dzyuba 2015). Next, we described that sperm maturation in sturgeon: 1) is an energy consuming process, 2) involves respiration, and 3) is supported by a function of creatine-phosphate shuttle (aatachment 6). The last three observations demonstrate the intensive bioenergetic process that couples mitochondrial respiration with synthesis of ATP followed by accumulation of creatine-phosphate required for mature sperm motility. These findings are in good agreement with already known information about bioenergetics of fish sperm (Ingerman 2008; Dzyuba *et al.* 2016).

Finally, we confirm that *in vitro* matured testicular spermatozoa can be successively cryopreserved, and that embryo development rates using post-thaw matured testicular sperm and control sperm were not significantly different (attachment 7). So, the sperm maturation process in sturgeons is an extremely specific biological event and, thanks to its understanding, we were able to use sperm from sturgeon *post-mortem* for cryobanking purposes. As in teleostei this possibility was applied in several species (Viveiros *et al.* 2000; Routray *et al.* 2006; Nynca *et al.* 2012; Blazer *et al.* 2013) in sturgeons it has been proposed in only one reference (Horváth *et al.* 2002). However, the verification of this possibility was required till now.

Altogether, these findings demonstrate the novelty of studies performed in our laboratory on sperm maturation process in relation to both basic and applied aspect of sturgeon spermatology.

2.3. Consequences of fish spermatozoa sensitivity to environment osmolality and ionic composition: interspecific consideration of cryoresistance

Generally, cryopreservation outcomes of cell depend on its osmoresistance (Mazur *et al.* 1972). In fish, resistance to environment osmolality was evaluated as the ability to keep spermatozoa cellular volume unchanged under non-isotonic conditions, and species-specific osmoresistance is well known nowadays (Bondarenko *et al.* 2013). As spermatozoa of marine fishes naturally are adopted for motility initiation in high environment osmolality, their spermatozoa possess high cryoresistance in comparison to freshwater species (Suquet *et al.* 2000; Kopeika *et al.* 2007; Kopeika and Kopeika 2008). This properties of marine species is explained by specific lipid composition of spermatozoa membrane (Drokin *et al.* 1989).

Mentioned above properties of spermatozoa of marine fishes can speculatively be attributed to euryhaline species. We showed (attachment 8) that the spermatozoa of euryhaline haarder can be successfully activated across a wide range of environment osmolality (from isotonic to hypertonic level), which suggests ionic mode of motility activation, while spermatozoa of freshwater silver carp are activated by a hypotonic mode typical for freshwater fish. After freeze-thawing, up to 30 % of motile cells were found in silver carp samples, while up to 90 % of motile cells were observed in samples from the haarder. Additionally, haarder spermatozoa showed no changes in cell volume after dilution in activating or cryoprotective media, while the silver carp spermatozoa swelled and the cell was eventually disrupted. As cell environment is shifted to high hypertonicity during freezing (Mazur 2004), cryoresistance of silver carp and haarder spermatozoa may be determined by the ability to preserve cellular volume under wide range of non-isotonic conditions. This explanation is also in accordance to finding on high osmoresistance of sturgeon spermatozoa (Bondarenko *et al.* 2013) possessing also high cryoresistance (Billard *et al.* 2004; Horvath *et al.* 2011).

It should be also mentioned that in this study the relationship between ability for swelling (osmoresistance) and cryoresistance between individuals of silver carp was not found but higher hypotonic swelling rate was found in samples after cryopreservation in comparison with value before freezing. Altogether these observations suggest that 1) intra-species variation of cryoresistance is not determined by individual osmoresistance and 2) freezing/thawing process itself leads to decrease in spermatozoa osmoresistance. These data are in good agreement with

modern knowledge on fish sperm cryoresistance and existence of individual cryoresistance phenomenon (Kopeika and Kopeika 2008).

The existence of species-specific motility activation phenomenon by freezing/thawing process itself (cryo-activation) was found (attachment 9). As sperm cryo-activation precedes mixing with eggs, the fertilization rate can be compromised. This phenomenon was associated with spermatozoa motility activation mode. It was shown that sperm of fishes activated by osmotic mode (common carp and Eurasian perch) could be cryo-activated. In contrast, cryo-activation was not observed in sperm of rainbow trout and sterlet possessing ionic mode of motility activation. Quite probably this difference is determined by the presence of K^+ ions of inhibiting concentration in activating medium.

While recently described, sperm cryo-activation itself is scientifically interesting phenomenon, it should be taking into account during sperm cryopreservation practice.

2.4. Applied aspects of fish sperm cryopreservation

Sperm cryopreservation is considered as useful component of fish breeding programs. Its outcomes are well recognized especially in cases, when excess of sperm is available (Zhang 2004). That is exactly the case of sturgeons, in which quite big volume of sperm can be collected from each fish individuals several days after sperm is collected for *in vitro* egg fertilization (Alavi *et al*. 2012b). This multiple sperm collection method can enhance artificial propagation for sturgeons if storage by cryobanking is used, assuming that sequential samples are of good quality.

We described (attachement 10) characteristics of sperm from sequential collections in sterlet, *Acipenser ruthenus*, following a single dose of carp pituitary extract (CPE). Sperm production and spermatozoa fertilizing ability, percent motility, and velocity were investigated in fresh and frozen/thawed sperm. Sperm was collected by two procedures: (A) stripping 3 times per 24 h at 3 h intervals on 3 consecutive days beginning 12 h after CPE treatment, and (B) stripping 3 times over 6 h beginning 36 h after CPE treatment. Sperm samples were frozen by a conventional freezing procedure in a cryoprotective medium containing 10 % methanol. Both sequential stripping procedures yielded larger volumes of viable and fertile spermatozoa than did a single collection. Sperm parameters such as density and volume varied widely depending on collection time. Fertilization rates obtained with frozen/thawed spermatozoa

were 13–76 % (median value). So, finally it was concluded that sequential stripping and spermatozoa cryopreservation in combination could improve the efficiency of sturgeon males exploitation in aquaculture. It should be underlined that even previously conducted experiments show the possibility for sequential sperm collection in paddlefish (Linhart *et al.* 2001) and sturgeons (Alavi *et al.* 2006), in our study we for the first time prooved effectiveness of combination of sequential sperm collection with cryopreservation. These findings are also important as they demonstrate that prolonged natural storage in hypotonic conditions in the testes (see chapter 2.2) does not influence sturgeon spermatozoa fertilizing ability and cryoresistance.

As intra-species sperm cryoresistance heterogeneity is one of the factors that decreases applicability of sperm cryobanking in aquaculture (Kopeika and Kopeika 2008), elaboration of methods for increasing sperm cryoresistance is a practical task.

It was demonstrated (attachement 11) that higher post-thaw motility rate, spermatozoa velocity, and fertilization rate were observed in sperm pretreated with hypotonic solutions compared to non-treated sperm or sperm treated with the isotonic solution. Cryoresistance can be improved by specific hypotonic treatment of spermatozoa before cryopreservation in carp. These results represent the first report of the potential for improvement of post-thaw spermatozoa motility parameters through hypotonic treatment of sperm prior to freezing. We have found no previously published information regarding the influence of hypotonic treatment before freezing on fish sperm cryoresistance. Hypotonic treatment before freezing is known to have a negative effect on human red blood cells cryoresistance, as was shown by Pegg and Diaper (Pegg and Diaper 1991). Discrepancies between our data and results of Pegg and Diaper (1991) could arise from possible intra-specific variation in membrane response to hypotonicity, as, in contrast to mammals, evolution of the physiological activity of fresh water fish sperm took place in a hypotonic environment. Apart from motility activation survival, little is known about the consequences of fish spermatozoa transfer from the isotonic seminal fluid to a hypotonic environment. The rapid increase in carp spermatozoa membrane fluidity in response to hypotonicity (Krasznai *et al.* 2003) could facilitate sperm of water/cryoprotectant fluxes occurring during the freeze–thaw and following fertilization under hypotonic conditions. This speculation is also supported by observations of rainbow trout, in which increased membrane fluidity was correlated with higher sperm post-thaw fertilizing ability (for review, see Ciereszko et al. (2011)). We suggest that the phenomenon observed in the present study may arise from

spermatozoa volume changes, which lead to spermatozoa membrane modification, enhancing the ability of spermatozoa to survive the freeze–thaw processes. We consider these our results to be preliminary and needing further study for application of this methodology to become practical for fish sperm cryopreservation.

2.5. Conclusions

Ionic composition and osmolality of the aquatic environment are natural factors that regulate fish spermatozoa motility. While, naturally, they act synergistically, there are taxa-specific hierarchies of these signals that determine either “osmotic” or “ionic” mode of sperm motility activation. An ionic mode is considered essential for reproduction of euryhaline fish such as some species of tilapia and for specific parental care during embryogenesis in pipefish, which are adapted to reproduction in a wide range of environment salinities. Ionic mode also plays an important role during sperm production in sturgeon. An osmotic mode of sperm motility activation can be considered as an evolutionary advanced trait, as it is found in teleost fish only. Spermatozoa from species with an osmotic mode of motility activation are characterized by a low ability to resist non-isotonic conditions, which makes them more susceptible to damage during cryopreservation. However, manipulation by environment osmolality before sperm freezing can improve outcomes of cryopreservation.

Description of sperm maturation process in sturgeon, determination of basic mode of sperm motility activation in euryhaline fish species, improvement of male use in fisheries practice by combination of sequential stripping and spermatozoa cryopreservation are novel aspect of fish spermatology presented in the study.

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4. Abstract in English-

The work presented in this dissertation is written as a set of 11 selected scientific articles published by the author (as a first author or coauthor) and complemented by his comments. The selection of articles is based on the three following assumptions: 1) spermatozoa motility is a prerequisite for natural fertilization in fish, 2) specific environment signals such as osmolality and ionic composition regulate this motility, and 3) there are species-specific hierarchies of environment signals that determine either “osmotic” or “ionic” mode of sperm motility activation. Compilation of selected articles in this thesis was performed with the aim to illustrate the species-specific interrelations between spermatozoa motility activation mode, features of reproductive organ anatomy, spawning environment and spermatozoa cryopreservation outcome.

Articles 1-4 are based on studies of fish sperm motility in relation to spawning environment and specific mode of spawning. It was demonstrated that some representatives of both groups of fish, euryhaline (blackchin tilapia *Sarotherodon melanotheron heudelotii*, blackstripe pipefish *Syngnathus abaster*) and freshwater (sterlet *Acipenser ruthenus*), can possess ionic mode of motility activation. Thus, sperm can be activated in media that are hypotonic, isotonic, or hypertonic relative to the seminal plasma (cases of blackchin tilapia and sterlet), and in media that are hypotonic or isotonic (case of blackstripe pipefish). In case of blackchin tilapia and sterlet, it was demonstrated that motility activation mechanism involves external Ca^{2+} ions. Such feature is important in species which spawn in an extremely wide range of environment salinity, and allows sperm motility to be manipulated for purposes of basic research. Further, it was proposed that specific spermatozoa activation mechanism in pipefish is mandatory for realization of specific paternal care, which involves the male participation in sperm production, external sperm release and embryo incubation within a male brood pouch.

Articles 5-7 summarize recent findings on mechanism specific to sturgeon sperm maturation, which occurs by natural mixing of testicular sperm with hypotonic urine. This mechanism is supported by spermatozoa respiration as energy source and is dependent on external Ca^{2+} and K^{+} ions.

Sperm cryopreservation is considered as an important tool for aquaculture and conservation measures, however drastic changes of environment osmotic pressure during the freezing/thawing process result in damaging factors, which limit the success of freezing. Articles

8-9 focused on understanding of the basic aspects of fish sperm cryopreservation. It was demonstrated that fish sperm cryopreservation outcomes depend on spermatozoa osmoresistance. Spermatozoa of euryhaline haarder *Mugil soiuy* possess high resistance to both environment osmolality and damaging factors of cryopreservation, while spermatozoa of silver carp *Hypophthalmichthys molitrix* have comparatively lower cryoresistance, a feature associated with low resistance to environment osmolality. Resistance to environmental osmolality was evaluated as the capability to keep spermatozoa cellular volume unchanged under non-isotonic conditions. Based on differential relationship between spermatozoon cellular volume changes and motility activation, the existence of species-specific motility activation phenomenon (cryoactivation) induced by the freezing/thawing process itself was found (Article 9).

For sperm cryopreservation, we found that osmotic cryoresistance in sterlet spermatozoa is not determined by osmolality of seminal fluid. This finding makes it possible to freeze sturgeon sperm collected by sequential strippings, where different osmolality of seminal fluid in samples from different strippings was found (Article 10). For sperm that are sensitive to osmotic changes, such as carp spermatozoa, it was demonstrated that spermatozoa cryoresistance can be improved by specific hypotonic treatment before cryopreservation (Article 11).

5. Abstract in Czech (abstrakt česky)

Práce prezentovaná v této dizertaci je sepsána jako soubor 11 vybraných vědeckých článků publikovaných jejím autorem (jakožto prvním autorem nebo spoluautorem) a je doplněná o jeho komentáře. Výběr publikací je založen na třech následujících předpokladech: 1) motilita spermatu je je nezbytnou podmínkou přirozeného oplodnění u ryb, 2) specifické environmentální signály jako je osmolalita a iontové složení regulují motilitu spermií, a 3) existuje druhově specifická hierarchie enviromentálních signálů určující buď „osmotický" nebo „iontový" způsob aktivace motility spermatu. Sestavení vybraných publikací v této práci bylo provedeno s cílem ilustrovat druhově specifické vzájemné vztahy mezi způsobem aktivace motility spermatu, znaky anatomie reprodukčních orgánů, prostředím výtěru a výsledkem kryokonzervace spermatu.

Publikace 1-4 pojednávají o studiích motility rybích spermií ve vztahu k prostředí a specifickému způsobu výtěru, ve kterém se dané ryby vytírají. Bylo zjištěno, že obě skupiny ryb, euryhalinní (tilápie *Sarotherodon melanotheron heudelotii*, jehla černopruhá *Syngnathus abaster*) a sladkovodní (jeseter malý *Acipenser ruthenus*) mohou disponovat iontovým způsobem aktivace spermatu. Z toho důvodu mohou být spermie aktivovány v mediích, která jsou hypotonická, isotonická nebo hypertonická vzhledem k seminální tekutině (platí pro tilápii a jesetera malého), a v mediích, která jsou hypotonická nebo isotonická (platí projehlu černopruhou). U tilápie a jesetera malého bylo zjištěno, že mechanismus aktivace motility zahrnuje externí ionty Ca^{2+}. Tato vlastnost je významná u druhů, které se třou v extrémně širokém rozsahu salinity prostředí, a umožňuje tak manipulovat s motilitou spermií pro účely základního výzkumu. Dále se předpokládá, že zvláštní mechanismus aktivace spermií u jehly je nezbytný pro realizaci specifické otcovské péče, která zahrnuje samčí účast na produkci spermatu, vypuštění spermatu do prostředí a inkubaci embryí uvnitř samčího břišního vaku.

Články 5-7 shrnují nejnovější poznatky o specifickém dozrávání spermatu u jeseterů, které nastává přirozeným mísením testikulárního spermatu s hypotonickou močí. Tento mechanismus je podporován respirací spermatu k získání energie a je závislý na externích Ca^{2+} a K^{+} iontech.

Kryokonzervace spermatu je považována za významný nástroj pro akvakulturu a ochranářská opatření, nicméně drastické změny v osmotickém tlaku prostředí během procesu zmrazování/rozmrazování jsou škodlivým faktorem, který limituje úspěšnost zmrazování. Studie

8-9 jsou zaměřené na základní aspekty kryokonzervace rybího spermatu. Bylo zjištěno, že výsledky kryokonzervace rybích spermií jsou závislé na osmoresistenci spermatu. Sperma euryhalinního cípala východního (*Liza haematocheila,* v původní práci uváděného jako *Mugil soiuy)* vykazuje vysokou odolnost jak vůči osmolalitě prostředí, tak vůči škodlivým faktorům kryokonzervace, zatímco sperma tolstolobika bílého (*Hypophthalmichthys molitrix*) má srovnatelně nižší kryoresistenci, což je rys spojený s nízkou odolností vůči osmolalitě prostředí. Odolnost vůči osmolalitě prostředí byla vyhodnocována jako schopnost spermií udržet nezměněný buněčný objem v neizotonických podmínách.

Na základě rozdíleného vztahu mezi buněčným objemem spermie a aktivací motility byl objasněn fenomén existence druhově specifické aktivace motility spermií indukovaný samotným procesem zmrazování/rozmrazování, tedy jde o tzv. kryoaktivaci (článek 9).

Pro kryokonzervaci jeseteřího spermatu je důležité zjištění, že osmotická kryoresistence u spermatu jesetera malého není určena osmolalitou seminální tekutiny. Toto zjištění umožňuje zmrazovat sperma jesterů z několikanásobných výtěrů (opakovaných odběrů spermatu), při kterých byla u jednotlivých odběrů zjištěna rozdílná osmolalita semenné plazmy (článek 10). U spermatu, které je senzitivní na osmotické změny, jako je sperma kapra, bylo zjištěno, že kryoresistence spermatu může být zlepšena specifickým hypotonickým ošetřením před kryokonzervací (článek 11).

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7. Attachments

Attachment 1

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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 $[Ca^{2+}]$ 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 $[Ca^{2+}]/[Na^+]$ 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 lifelong 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, Roebling, 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^+, Mg^{2+}, and Ca^{2+}) 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 Ca^{2+} 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, $CaCl_2$, and $MgCl_2$) 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 Roebling micro-osmometer. Some media (see Section 3) were supplemented with ethylene glycol tetraacetic acid (EGTA) or $CaCl_2$ at various concentrations for evaluating the Ca^{2+} requirements for the activation of spermatozoa. The free Ca^{2+} concentration ($[Ca^{2+}]$) of AM was calculated by using a specific software [25] using total concentration values of the three components involved in the Ca^{2+} equilibrium (Ca^{2+}, EGTA, and H^+). The effects of replacing Ca^{2+} by Mg^{2+} 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 × 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 × 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 ± 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 $P < 0.05$.

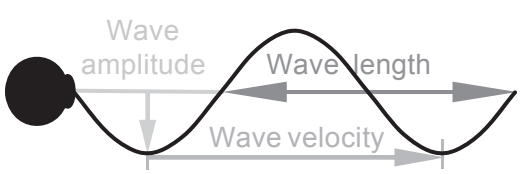

Fig. 1. Schematic representation showing measurements carried out on active sperm flagellum (see text for details).

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 μL and 220 μL, and mean values were very low (<60 μL) at all salinities. Similarly, the mean GSI never exceeded 0.3% whatever the water salinity. The osmolality of the seminal plasma tented to increase with the fish maintenance salinity and was significantly higher for HW males (349 ± 5 mOsm kg^{-1}) than for FW or SW males (318 ± 6 and 330 ± 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), Mg^{2+} (1.2–1.9 mM), or Ca^{2+} (0.2–0.9 mM) (Table 2). Nevertheless, the Ca^{2+} content in HW sperm (0.2 ± 0.2 mM) tended to be lower than that in FW sperm (0.9 ± 0.2 mM), with an intermediate situation being observed for SW sperm (0.5 ± 0.2 mM).

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

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 ± 11^a (40)	0.23 ± 0.02^a (40)	37 ± 8^a (40)	318 ± 6^a (14)	5.1 ± 1.5^a (5)
35 (SW)	227 ± 10^a (50)	0.26 ± 0.02^a (50)	59 ± 7^a (50)	330 ± 4^a (30)	4.6 ± 0.8^a (18)
70 (HW)	205 ± 10^a (47)	0.22 ± 0.02^a (47)	48 ± 7^a (47)	349 ± 5^b (20)	5.6 ± 1.0^a (11)

Mean ± 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 ± 24.6^a	127.1 ± 14.9^a	19.8 ± 4.0^a	1.1 ± 0.4^a	0.9 ± 0.2^a
35 (SW)	5	149.0 ± 18.0^a	112.2 ± 11.0^a	23.1 ± 3.0^a	1.9 ± 0.3^a	0.5 ± 0.2^a
70 (HW)	3	110.9 ± 23.3^a	130.4 ± 14.1^a	16.6 ± 3.8^a	1.2 ± 0.3^a	0.2 ± 0.2^a

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

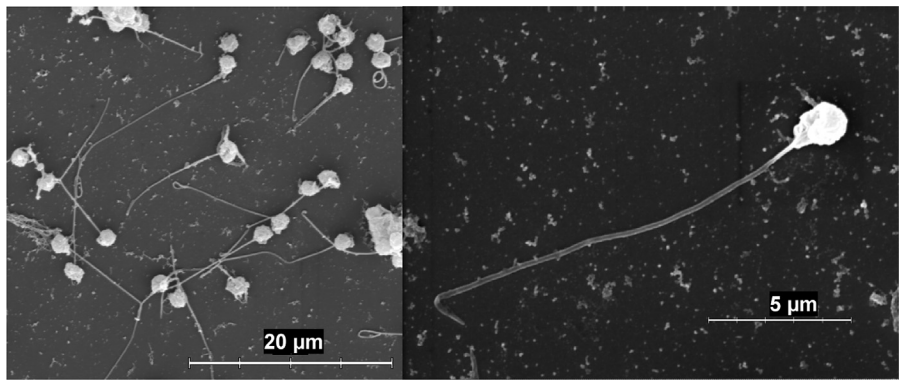

Fig. 2. General morphology of *Sarotherodon melanotheron heudelotii* sperm using scanning electron microscopy after sperm fixation in PBS buffer with 2.5% glutaraldehyde (example from fish reared in sea water).

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

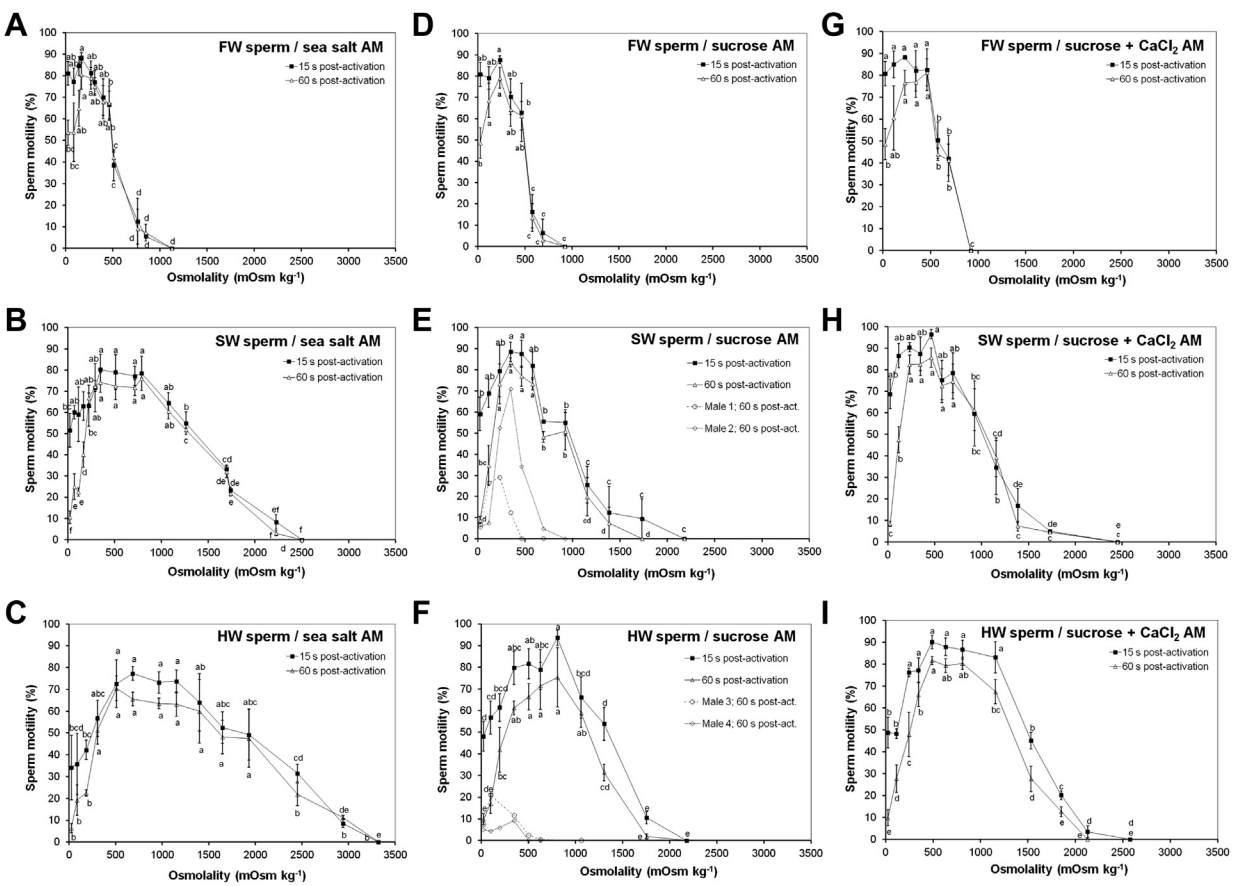

Fig. 3. Percentage of motile spermatozoa (mean ± SE) 15 seconds and 60 seconds after activation in different media (AM) at various osmolalities (left column: synthetic sea salt; central column: sucrose; and right column: sucrose added with 1 mM Ca^{2+}) for *Sarotherodon melanotheron heudelotii* reared 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.

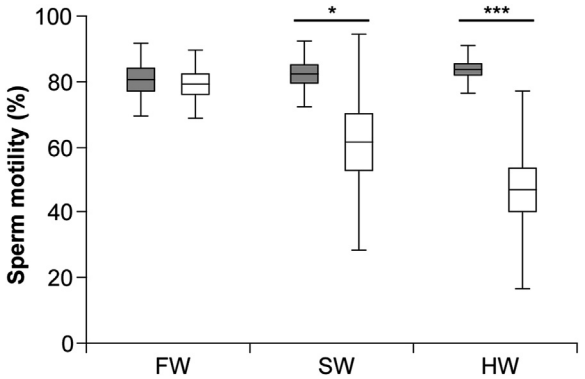

Fig. 4. Motility of sperm activated in diluted sea water (gray) or in sucrose (white) solutions for males of *Sarotherodon melanotheron heudelotii* reared in fresh water (FW, n = 12), sea water (SW, n = 14), or hypersaline water (HW, n = 20). The osmolality of swimming medium was 300 mOsm kg^{-1} for FW fish and 600 mOsm kg^{-1} for SW and HW fish. Only fish with sperm motility ≥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 ± SE and whiskers are ±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 (60–75 μm $second^{-1}$) than in those from SW or HW males (40–45 μm $second^{-1}$). 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 (40–50 μm $second^{-1}$) than those in diluted sea water (60–75 μm $second^{-1}$).

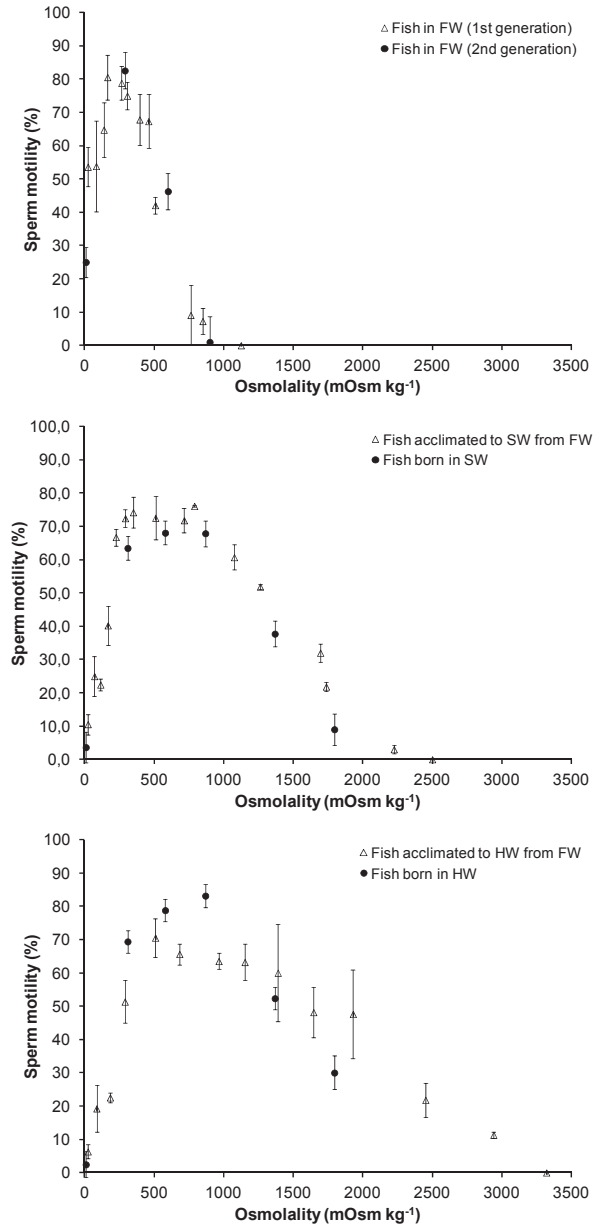

Fig. 5. Percentage of motile spermatozoa (mean ± SE) as a function of osmolality, salinity of the fish rearing environment (FW: 0; SW: 35; and HW: 70), and fish generation (i.e., fish acclimated from FW to SW or HW vs. fish hatched and maintained all lifelong at the same salinity). Data correspond to 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

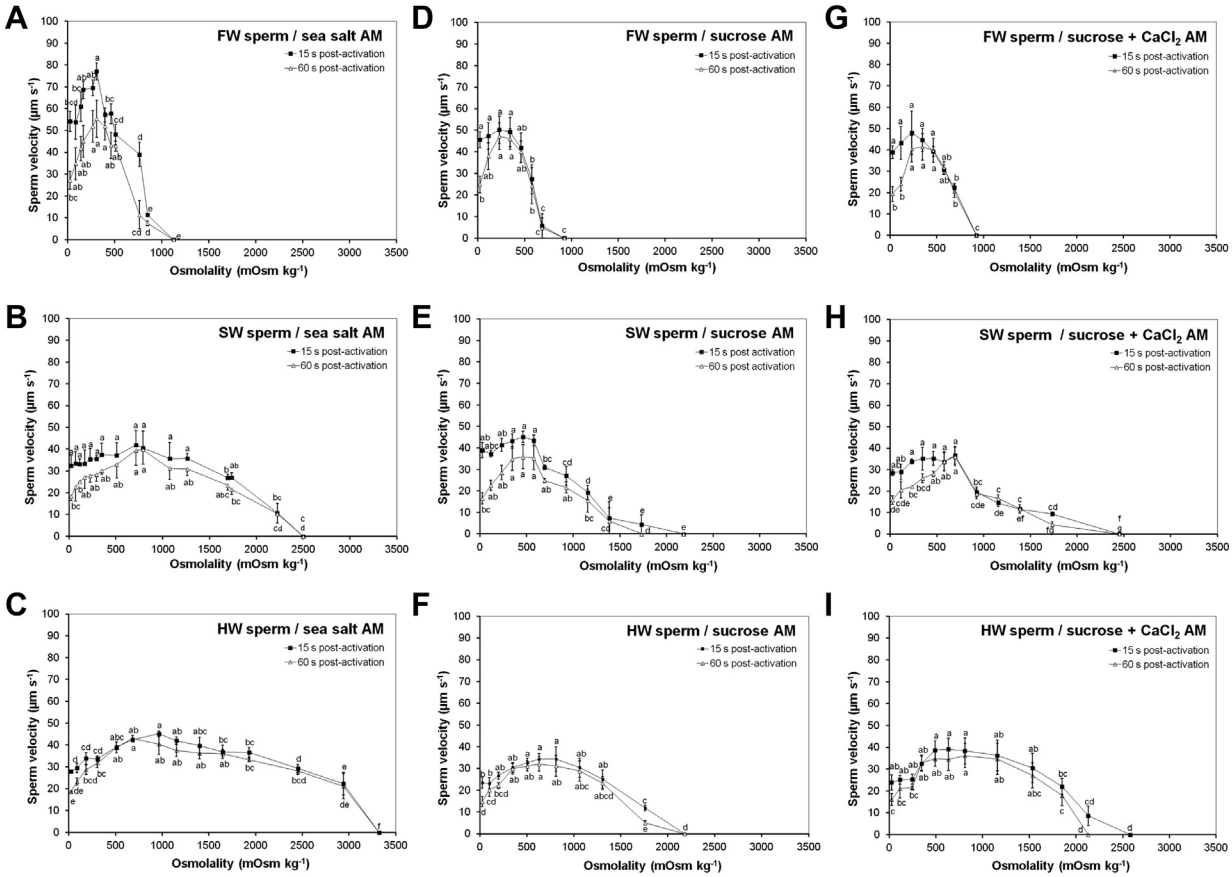

Fig. 6. Average velocity of motile sperm (mean ± SE) 15 seconds and 60 seconds after activation in different media (AM) of various osmolalities (left column: synthetic sea salt; central column: sucrose; and right column: sucrose added with 1 mM $Cacl_2$) for *Sarotherodon melanotheron heudelotii* reared 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 ± 13.8 µm second^{-1}, $n = 60$) than that in FW or SW sperm (419.6 ± 20.6 µm second^{-1}, $n = 27$ and 456.6 ± 15.8 µm second^{-1}, $n = 46$, respectively; ANOVA, $F_{2,130} = 16.90$, $P < 0.0001$). The initial beating frequency of flagella (15 seconds after sperm activation) was much higher in FW sperm (40.0 ± 1.5 Hz) than that in SW or HW sperm (31.2 ± 0.8 Hz and 30.9 ± 0.8 Hz, respectively; Fig. 8B). As a result, the sperm velocities differed significantly between FW (53.5 ± 1.7 µm second^{-1}), SW (36.9 ± 1.8 µm second^{-1}), and HW fish (31.0 ± 1.5 µm second^{-1}; ANOVA, $F_{2,129} = 35.35$, $P < 0.0001$; Fig. 8B).

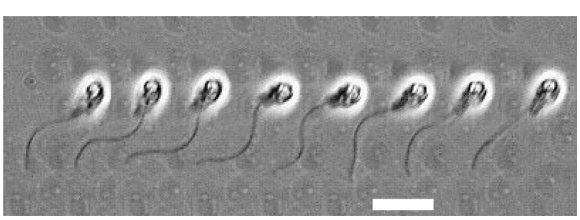

Fig. 7. Illustration of flagellar movements in sperm of *Sarotherodon melanotheron heudelotii*, as observed using high-speed camera images (white bar = 10 µm).

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 (100–150 mOsm kg^{-1} for FW fish, 500–600 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

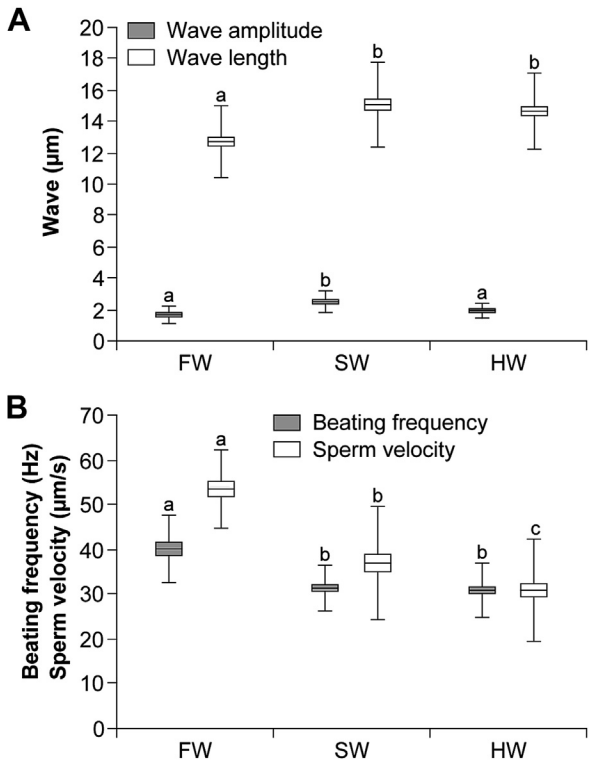

Fig. 8. Average wavelength and amplitude of flagellar movement (A) and flagellar beating frequency and sperm velocity (B) 15 seconds after activation in diluted sea water at 300, 600, and 900 mOsm kg^{-1} for *Sarotherodon melanotheron heudelotii* reared in FW (n = 27), SW (n = 46), or HW (n = 60), respectively. Boxes represent mean ± SE and whiskers are ±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 Ca^{2+} 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 Ca^{2+} necessary to fully restore sperm motility was higher for HW fish (10 mM Ca^{2+}) than that for FW or SW fish (1 mM Ca^{2+}; 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 Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} 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

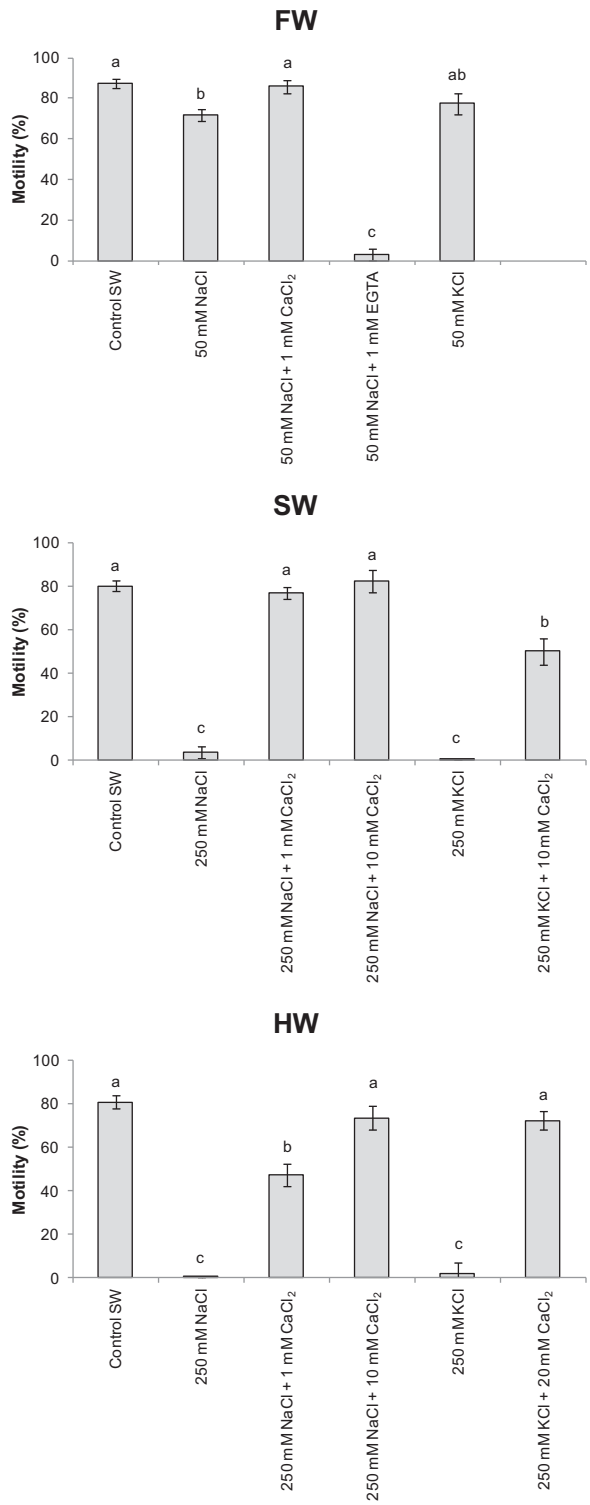

Fig. 9. Effects of electrolytic activation media containing Na^+, Ca^{2+}, or K^+ on the percentage of motile sperm cells (mean ± SE) from *Sarotherodon melanotheron heudelotii* males reared in fresh water (FW), sea water (SW), or hypersaline water (HW). Control (sea salt solution) and other media tested 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 ($[Ca^{2+}]$) 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 $[Ca^{2+}]$ 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 $[Ca^{2+}]$ 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.

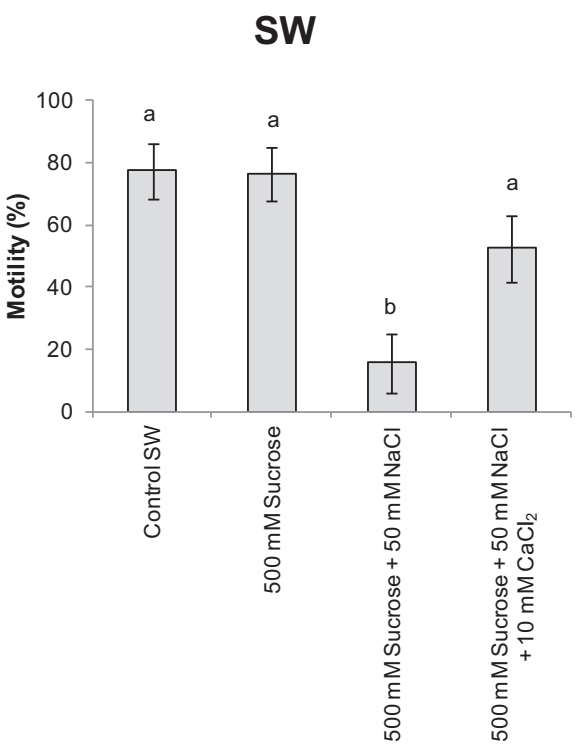

Fig. 10. Effects of Na^{+} and Ca^{2+} in sucrose activation medium on the percentage of motile sperm cells (mean ± SE) from *Sarotherodon melanotheron heudelotii* males reared in sea water (SW). Control (sea salt solution) and other media tested are at osmolalities of 500 to 600 mOsm kg^{-1}. Data sharing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

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

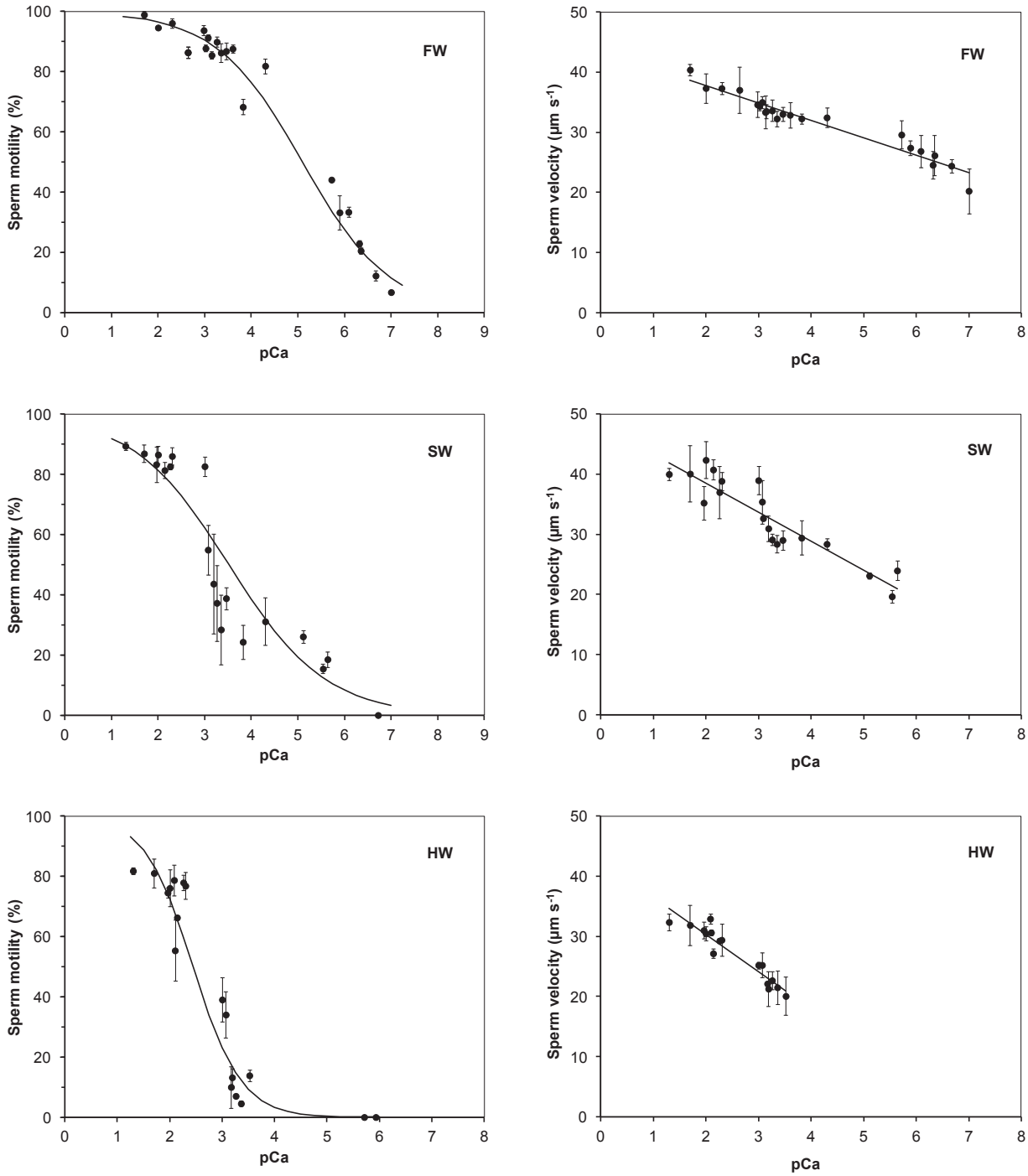

Fig. 11. Effects of free Ca^{2+} concentration (pCa = lg (1/[Ca^{2+}])) on sperm motility (left column) and sperm velocity (right column) at 15 seconds after activation for *Sarotherodon melanotheron heudelotii* acclimated in fresh water (FW), sea water (SW), or hypersaline water (HW). Activation media 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: $M\% = 100\ (e^{(a + b\ pCa)}/1 + e^{(a + b\ pCa)})$ (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 "pCa_{10-80}" are given by the models for sperm motility of 10%, 50%, and 80%, respectively (maximal motility observed in HW series).

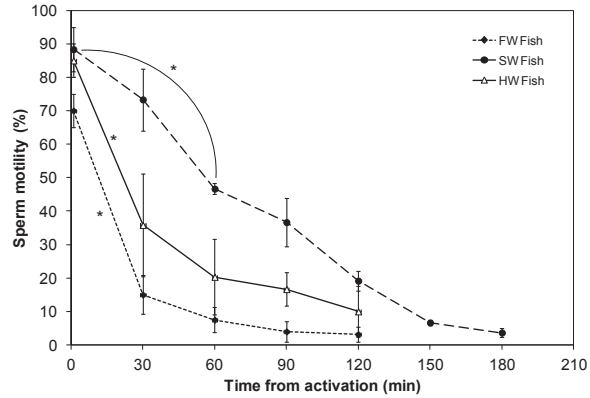

Fig. 12. Sperm motility (mean ± SE) as a function of time since activation in diluted sea water for male *Sarotherodon melanotheron heudelotii* reared in fresh water (FW, n = 3), sea water (SW, n = 3), or hypersaline water (HW, n = 3). Sperms were maintained at constant room temperature (21 °C–22 °C) 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.

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, 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 tilapias 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

Table 4
Coefficients (a, b), R^2, and probability 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 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

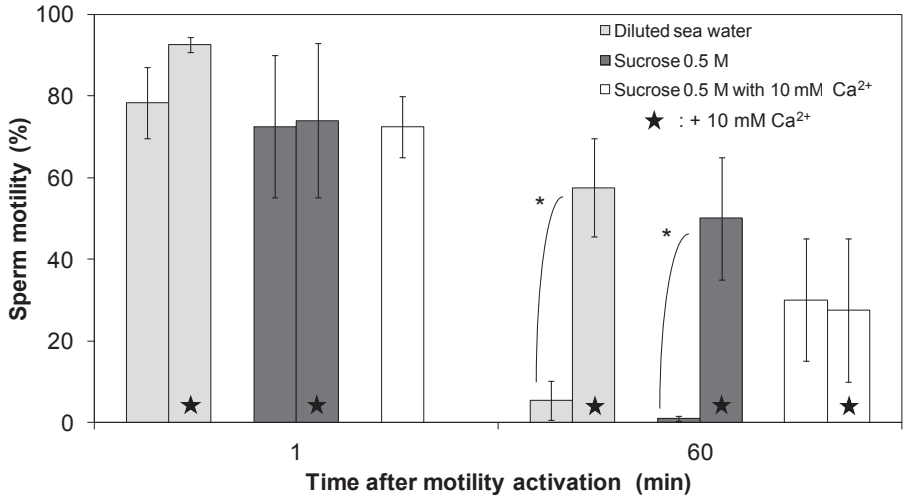

Fig. 13. Effect of calcium on sperm motility (mean ± SE) in different swimming media (diluted sea water, sucrose 0.5 M, or sucrose 0.5 M containing 10 mM $CaCl_2$; osmolality close to 600 mOsm kg^{-1} in all situations) for *Sarotherodon melanotheron heudelotii* reared in hypersaline water. Sperm is 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. heudelotii* 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 μm $second^{-1}$) than that in SW or HW sperm (about 40 μm $second^{-1}$). These velocities are close to those reported for the sperm of *O. mossambicus* acclimated to FW or SW (40–70 and 35–50 μm $second^{-1}$, 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 μm second^{-1} and 135 μm 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 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. Avarre 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. Avarre 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 $[Ca^{2+}]/[Na^+]$ 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 $(Ca^{2+})/(Na^+)$ 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 $[Ca^{2+}]/[Na^+]$ 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 $[Ca^{2+}]/[Na^+]$ ratio for the sperm of SW fish (0.03) is very close to that in synthetic sea salt (0.022). Similar effects of the $[Ca^{2+}]/[Na^+]$ 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 ($[Ca^{2+}]_i$). In sperm of FW fish activated in hypotonic conditions, the increase of $[Ca^{2+}]_i$ is independent of extracellular $[Ca^{2+}]$ 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, $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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-μM 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. Tiersh 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*, demembranated 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 [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 [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 [Ca^{2+}]/[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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Attachment 2

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Variable sperm size and motility activation in the pipefish, *Syngnathus abaster*; adaptations to paternal care or environmental plasticity?

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Abstract. Like seahorses, some of the closely-related pipefish species (Family *Syngnathidae*) incubate their eggs within a male brood pouch. This has contributed to considerable confusion about sperm transfer mechanisms to the eggs; some authors have reported that ejaculates are released directly into water before they reach the eggs, while others have suggested that eggs are fertilised using spermatozoa deposited directly into the brood pouch via an internal sperm duct. Here we present anatomical evidence from the freshwater pipefish, *Syngnathus abaster*, showing not only that direct sperm deposition into the pouch is impossible, but that spermatozoa must somehow travel a significant distance (>4 mm) outside the body of the male, to reach and fertilise eggs in the pouch. We have also used several putative sperm-activating solutions to identify the type of environment most conducive to sperm activation. Spermatozoa released from the testis were active for a brief period (<5 min) in water or 150 mM saline, but showed prolonged (>25 min) motility in ovarian fluid. This suggests that spermatozoa are released into a mixture of ovarian fluid and eggs while the male and female are in close contact. Our data also suggest that the fertilisation mechanism is highly efficient (sperm : egg ratio <200 : 1) even though this pipefish species produces dimorphic spermatozoa (with long and short flagellae). The shorter (<40 μm) morphotypes were not capable of motility activation, and are therefore probably incapable of fertilisation. If so, the sperm : egg ratio reported here would represent an overestimate.

Additional keywords: dimorphism, pouch, spermatozoa, sperm : egg ratio, sperm morphology, sperm transport.

Introduction

The extensive variability of reproductive modes and systems among fish species is thought to reflect the equally extreme variety of behavioural adaptations they have developed, as well as the range of environments in which they live (Reid and Hall 2003). Evolutionary biologists and taxonomists have taken considerable advantage of this diversity to study relationships between the fish species, exploiting sperm structural characteristics in particular as indicators of relationships and adaptations (Jamieson and Leung 1991; Gwo *et al*. 2004). In this context, representatives of the fish family *Syngnathidae*, the seahorses and pipefishes, might be expected to differ from other major groups because of their unique mode of paternal care, which involves the male in sperm production, external sperm release and internalised embryogenesis (Carcupino *et al*. 2002; Foster and Vincent 2004; Van Look *et al*. 2007). While seahorses possess a well-developed brood pouch, there is considerable variation in brood pouch sophistication among the pipefish genera. In some species the female simply attaches her eggs to the skin of the caring male, while in others the females deposit their eggs into a brood pouch resembling that of the seahorses (Wilson *et al*. 2003).

The mechanism of gamete transfer in these species is still unclear, and it is not easy to classify the mode of fertilisation as either 'internal' or 'external' as with other species (Jamieson and Leung 1991). The spermatozoa emerge from a testicular excurrent duct some distance from the pouch opening and to date there is no satisfactory explanation of how they reach the eggs, although several hypotheses have been proposed (Van Look *et al*. 2007). Although it is known that female pipefish deliver their eggs into the male's brood pouch, it is not certain whether the spermatozoa (*1*) swim through water towards the eggs, which are then fertilised within the pouch environment, (*2*) are somehow collected by the female's ovipositor during egg transfer and moved actively towards the pouch, or (*3*) engulfed in ovarian fluid during egg transfer, then swim towards the egg surface within this medium. The first option requires sperm motility activation within water itself or pouch fluid, while the second and third options require sperm activation to take place in pouch fluid or ovarian fluid.

Given that gamete interactions are normally very sensitive to the conditions in their immediate environment, one logical approach to gaining a better understanding of sperm transfer

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and fertilisation mechanisms in pipefishes and seahorses is to examine sperm motility activation responses to the potential environments that they might encounter. In general, spermatozoa from freshwater fishes are activated by the sudden decrease in ionic strength that they experience upon exposure to water (Alavi and Cosson 2006). This activation is usually short-lived (a few minutes), and fertilisation takes place within that period. Conversely, spermatozoa from externally-fertilising marine fishes are activated when they encounter the elevated osmolarity of the marine environment, while the spermatozoa of species that fertilise internally remain within a protected environment throughout sperm transfer and maintain viability for long periods within the female reproductive tract (Billard 1986; Pavlov and Moksness 1994). Here we have exposed spermatozoa to solutions of different osmolarities in an effort to identify the appropriate activation conditions.

The results of these experiments are relevant to the life history of pipefishes, which are euryhaline species capable of living and breeding in environments that differ in salinity and tonicity (Cakic *et al*. 2002). Such flexibility in breeding environment would not normally be expected if the spermatozoa have to suffer exposure to a hostile environment, whether high or low tonicity, and we therefore predict that the spermatozoa are maintained in a protective environment before fertilisation. Here we investigated this prediction, starting from the premise that knowledge of the environment needed for sperm motility activation might provide clues about the likely course of events. We combined this approach with estimates of the likely number of deposited spermatozoa and anatomical studies of the male genital area. Such studies addressing the possibility that spermatozoa are freely released into the water may offer a sustainable explanation for the mode of sperm transfer. In addition, we measured sperm swimming speeds in fluids likely to interact with spermatozoa around the time of sperm release, to examine the possibility that the spermatozoa could reach the pouch opening through their own intrinsic motility. We additionally examined the structural features of pipefish spermatozoa to see how much morphological diversity exists within single ejaculates. As high levels of sperm competition are thought to result in reduced structural variation among spermatozoa (Calhim *et al*. 2007), it seems reasonable to propose that the existence of significant sperm diversity would indicate the opposite, i.e. lack of sperm competition. Sperm competition should only be possible in pipefish if males actually do release their spermatozoa into the surrounding water. In general, reduced sperm production is a feature of reproductive systems that operate in the absence of sperm competition (Stockley *et al*. 1997). As members of the family *Syngnathidae* are believed to exhibit this mode of reproduction, we also asked whether there are any specific features of sperm motility which maximise the effectiveness of the small amount of sperm produced. Finally, we have also examined the structure of the pipefish egg to see whether its micropyle (the opening in the egg surface that permits access of motile spermatozoa to the oolemma) possesses any kind of specific structure which could have evolved as a result of evolution towards fertilisation by a small number of spermatozoa produced in the absence of sperm competition; e.g. unusually large or small micropyle size or specific oolemmal organisation in the vicinity of the micropyle (Riehl and Kokoscha 1993).

Materials and methods

All procedures involved in the sampling of fish and gonads, as well as methods of fish maintenance, were approved by the Institute for Problems in Cryobiology and Cryomedicine Ethics Committee.

Fish

The species used for the present study was from a freshwater population of *Syngnathus abaster*, obtained from Lakes Liman and Krasnyi Oskol (Kharkov region, Ukraine). The fish were sampled during their spawning season (May–July); the samples were fish that had been accidentally caught by trawls during commercial fishing activities. The samples were delivered to the laboratory within 3 h. Fish were housed in aquarium systems with controlled illumination (16 h day–8 h night), aeration and water quality parameters (pH 7.8, 23°C, salinity 0 g L^{-1}) for less than one week before being used in experiments. The fish were fed daily with fresh *Artemia nauplii*. While in the aquarium systems fish exhibited mating behaviour and bore pouch embryos.

Collection of gonads

For the collection of gonads, fish were anesthetised by placing them into 1% 3-aminobenzoic acid ethyl ether (MS222; Aldrich, Milwaukee, WI, USA) solution in tank water for 1 min and decapitated. Photographs of the external reproductive organ were prepared using an Olympus SP-350 camera.

Ovaries and testes were carefully removed from the body cavity and placed on a glass plate. Eggs were removed from the ovary onto the glass plate surface and fluid surrounding the eggs (ovarian fluid) was collected into a pipette (20–40 μL) for subsequent use in sperm motility activation experiments.

Eggs were transferred into fixative containing 2% (v/v) glutaraldehyde and 2% (w/v) paraformaldehyde dissolved in 100 mM cacodylate buffer pH 7.4. After 24 h of fixation, eggs were transferred into 100 mM cacodylate buffer pH 7.4, dehydrated in a graded ethanol series from 50% to 100% (v/v), transferred into 25% sucrose in 100 mM cacodylate buffer, freeze-dried, coated with gold and examined by scanning electron microscopy. Testes were used for sperm collection and motility experiments as described below.

Sperm motility experiments

Testes were cut with a blade on the glass plate and mixed with 20 μL of (*1*) fresh water (water from an aquarium tank), (*2*) isotonic physiological saline solution (150 mM NaCl), (*3*) ovarian fluid, (*4*) a mixture of fresh water and ovarian fluid (1 : 1 v/v) or (*5*) seawater (salinity 17 g L^{-1}). Within 30 s, 10 μL of the mixture of testis and testing solution were transferred on to a glass slide precoated with 1% bovine serum albumen and mounted under a coverslip. Samples were transferred to a microscope stage (Carl Zeiss CS 25 series; Carl Zeiss, Welwyn Garden City, Hertfordshire, UK) and viewed using ×10, ×20 or ×40 positive-phase contrast objectives (and a green filter). Images were captured using a CCD monochrome video camera (Panasonic WV- BP 330; Opta, Kharkov, Ukraine) and recorded for 5–30 min using a TV-tuner (AVerMedia Technologies UK,

Ltd Newport Pagnell, UK) and stored on the hard drive of a personal computer. Records of sperm motility were viewed using Light Alloy 4.0 (http://www.softella.com) software. Sperm shapes (head and flagellum morphology), sperm trajectories and sperm flagellar patterns were traced on to acetate films using 'screenshots' and the 'frame by frame' function. Acetates were then scanned and sperm flagellum lengths, sperm head lengths and widths, and sperm trajectory lengths were measured using Image-Pro software (version 4, Media Cybernetics, Marlow, UK). Velocity of motile spermatozoa (V) was calculated as $V = L \times t^{-1}$ $\mu m\,s^{-1}$ where L = length (in micrometres) of the head trajectory recorded at 12 frames per second and t = duration of the measured trajectory (in seconds). The duration of motility, from the initiation of movement to the complete cessation of sperm movement in the sample, where appropriate, was also recorded.

Statistical analysis

Sperm head width and length measurements and corresponding flagellar lengths, with and without being transformed by standardisation (i.e. subtracting each value from the mean and dividing it by the standard deviation (s.d.) to produce a new set of values for which mean = 0 and s.d. = 1), were examined and classified using hierarchical (minimum spanning tree) and non-hierarchical (K-means) clustering techniques (Statistica V6.1; Statsoft UK, Herts, UK). The use of standardisation prevents variables with numerically large data values from dominating the cluster analysis. Sperm dimensions of the resultant clusters were subsequently compared by analyses of variance (ANOVA) to test their validity as distinct groups (Sharma 1996).

Sperm activation experiments were analysed by calculating the number of samples (individual pipefish testes) for which sperm motility was observed, and expressing this value as a percentage of the total number tested. The maximum duration of motility (t_{max}) was observed and recorded as the maximum time between motility initiation and the complete cessation of motility. The reported values represent the minimum and maximum duration of motility observed within any given experimental treatment.

Results

Male and female external reproductive organs and structures

The morphological characteristics of male and female pipefish were approximately equal in terms of body length and body mass (two-tailed *t*-test; $P > 0.1$). The mean (± s.e.m.) body mass and lengths of fish used in the study were: male body mass 1.21 ± 0.36 g ($n = 19$) and body length 13.54 ± 1.28 cm ($n = 42$): female body mass 1.48 ± 0.33 g ($n = 27$) and body length 14.27 ± 1.1 cm ($n = 33$).

The female possesses an ovipositor that is located in an approximately equal position to the sperm duct and pouch area of the male (Figs 1*a–d*). Detailed examination of the male genital area indicated that the sperm duct opens to the exterior environment ~0.5 cm anterior to the skin folds that mark the pouch opening (Fig. 1*c*). An anal fin is positioned approximately halfway between the sperm duct and the pouch opening.

Sperm subpopulations

The total number of spermatozoa recovered from pipefish testes (right and left) varied widely from 700 to 37 000 (mean ± s.e.m. $14\,200 \pm 3121$; $n = 14$ fish). Morphological measurements revealed that there was considerable heterogeneity of sperm shape and size within each of the sperm samples. It was not possible to obtain many clear, freeze-framed, images of spermatozoa from each sample and therefore a pooled group of 93 spermatozoa from the 14 sperm samples was assessed for their overall dimensions: mean sperm tail length (minimum and maximum) was 53.05 μm (11–92.1 μm), sperm head length was 4.23 μm (3.1–5.9 μm), and sperm head width was 1.33 μm (0.72–1.86 μm).

The application of hierarchical and non-hierarchical (K-means) cluster analysis to this set of (standardised) sperm data identified 2–5 morphotype categories that were significantly different from each other in all parameters when compared by *post-hoc* one-way ANOVA ($P < 0.0001$). Although classification into five morphotypes was mathematically valid, it seemed unrealistically high and difficult to explain biologically. We therefore used the 'root mean squared standard deviation' technique (Sharma 1996) to judge that the data could be reasonably clustered into three clusters. The three biologically valid morphotypes could mainly be distinguished by differences in their relative tail length and sperm head width. The group average data are presented in Table 1 and a profile plot of the standardised data is shown in Fig. 2*a*.

The functional meaning of this sperm dimorphism became clearer when motility characteristics were examined as a function of flagellar length (Fig. 2*b*). When suspended in ovarian fluid, only spermatozoa whose flagellae exceeded 40 μm in length were motile and most of the motile spermatozoa were longer than 60 μm in length. One interpretation of these data is that spermatozoa with shorter flagellae were actually immature and had not completed the process of spermiogenesis. Velocity measurements varied widely (Fig. 2*c*), between 10 and 140 $\mu m\,s^{-1}$, but there was no correlation between velocity of motile spermatozoa and flagellar length (Pearson product moment $R^2 = 0.016$; $n = 82$). Qualitative assessment of sperm trajectories showed that they were mostly linear (Fig. 2*d*).

Sperm activation experiments

The results of the sperm motility activation study are shown in Table 2. These results were treated qualitatively and not subjected to statistical analysis. Sperm motility was more effectively activated in isotonic saline and ovarian fluid than in any of the other media tested. Tank water was capable of inducing relatively short-lived motility in some samples (2/9 samples tested); the proportion of samples activated was markedly improved by the addition of ovarian fluid to the tank water (3/5 samples), but the duration of motility was not extended above that of tank water alone. Mixing the ovarian fluid with 150 mM NaCl did not increase the proportion of samples in which sperm activation took place; unexpectedly, this mixture was less effective at sustaining prolonged sperm motility than either ovarian fluid or 150 mM NaCl alone. Natural seawater failed to induce any motility.

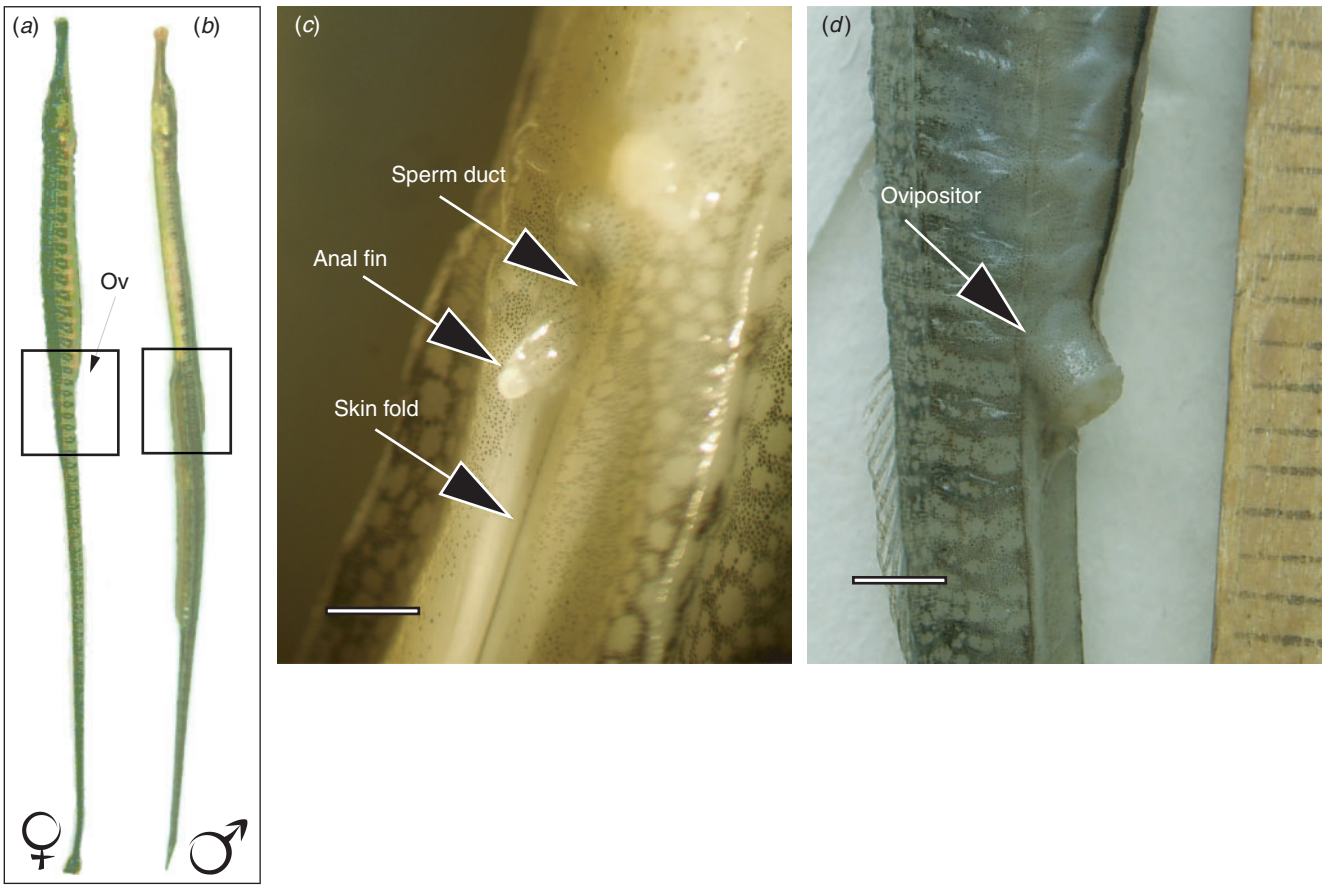

Fig. 1. External reproductive organ structures in: (*a*) female and (*b*) male *Syngnathus abaster*. Panel (*c*) illustrates an enlarged view of the boxed region shown in (*b*) and Panel (*d*) shows an enlarged view of the boxed region in (*a*). 'Ov' in panel (*a*) indicates the position of the ovipositor. Bar in (*c*) = 5 mm; bar in (*d*) = 2 mm.

Table 1. Summary data for the three groups of spermatozoa identified by non-hierarchical cluster analysis

Group ID	Number of sperm	Tail length ± s.d. (μm)	Head length ± s.d. (μm)	Head width ± s.d. (μm)
1	26	45.55 ± 16.23	4.68 ± 0.404	1.52 ± 0.196
2	42	62.78 ± 10.93	3.99 ± 0.344	1.37 ± 0.196
3	25	28.76 ± 15.45	4.13 ± 0.362	1.05 ± 0.177

Ovarian fluid on its own was capable of activating sperm motility in nearly 70% of the samples tested; however, the duration of motility sustained by ovarian fluid was exceptional (up to 25 min).

The behaviour of motile pipefish spermatozoa in ovarian fluid and isotonic media was unusual in that the spermatozoa exhibited a marked tendency to start or stop very suddenly. This sperm behaviour is illustrated by the series of tracings presented in Fig. 2*e*; in this example, the spermatozoon is static for the first four plotted frames except for lateral movement of the sperm head. Motility is then suddenly switched on, as represented by the tracings numbered 5–7. Interestingly, when the spermatozoa were immotile the flagellum was extended and straight. The onset of motility activation was indicated by proximal bending of the flagellar region and lateral sperm head motion for ~0.5 s before forward motion occurred, but within one second the forward motion reached maximum velocity. The process of stopping was very sudden; actively-motile spermatozoa become immotile almost instantly, and once again the flagellum assumed a straight structure.

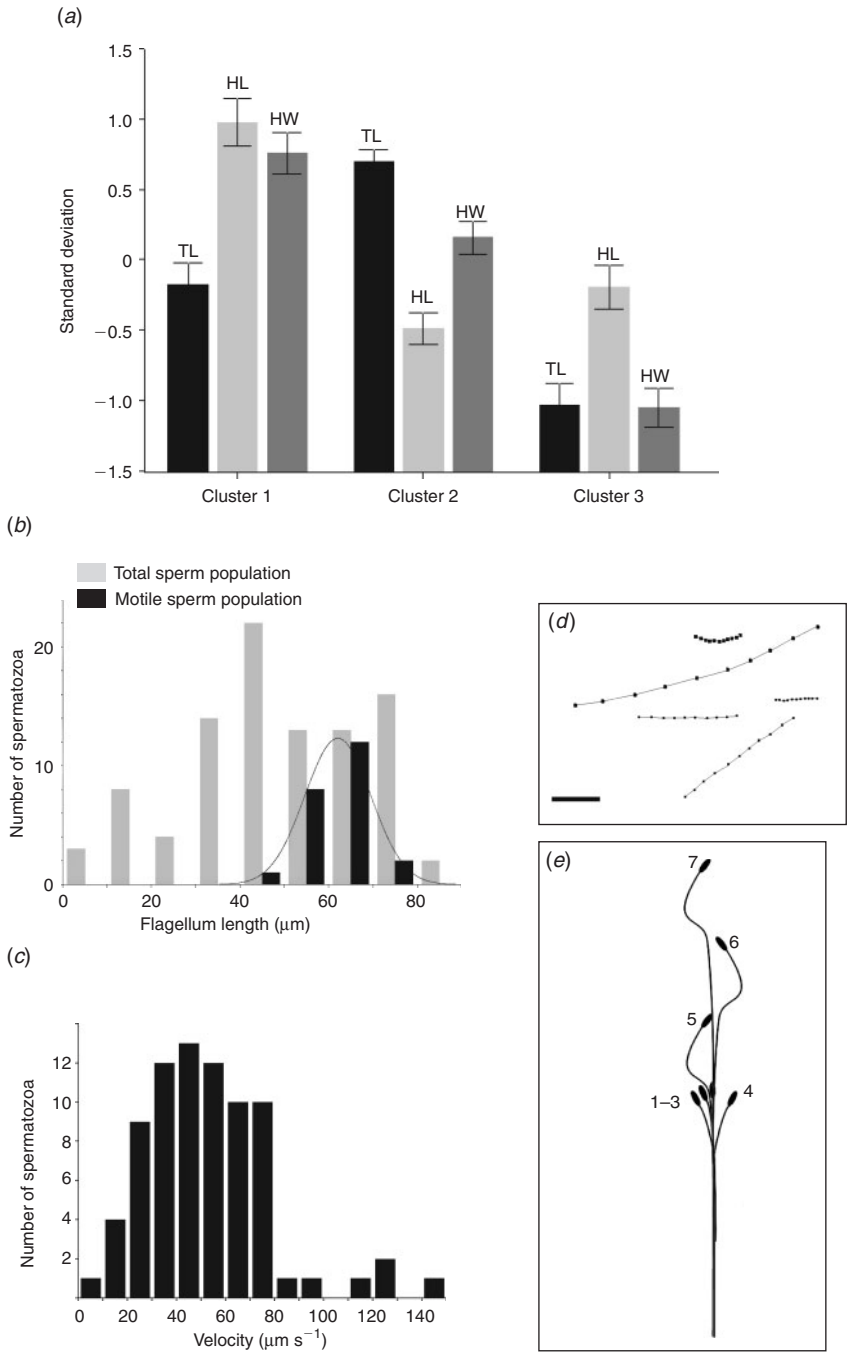

Fig. 2. (*a*) Profile plot showing the standardised (overall mean = 0; standard deviation = 1) mean values for sperm tail length, head length and head width for the three sperm clusters. TL, HL and HW = tail length, head length and head width, respectively. (*b*) Histogram showing the distribution of flagellar lengths in all measured spermatozoa ($n = 95$; grey bars) compared with the distribution of flagellar lengths in the motile population ($n = 23$; black bars). (*c*) Histogram showing the average curvilinear velocity of pipefish spermatozoa added to ovarian fluid. (*d*) Representative tracings of 2-dimensional sperm movements observed within ovarian fluid. Individual dots represent sperm head positions every 0.08 s. Bar = 20 μm. (*e*) Schematic representation of a sequence in which a spermatozoon undergoes motility activation involving bending of the proximal, but not the distal, region of the flagellum. Sequential sperm tracings were made at 0.08 s intervals; tracings labelled 1–4 represent bending of the proximal region of the flagellum without forward progression, while tracings 5–7 show that sperm progression was suddenly initiated.

Table 2. Tests of the sperm activation responses of pipefish spermatozoa in different media

Solution tested	Males tested (n)[A]	Males showing sperm motility[B]	% males showing sperm motility	Duration of motility (min–max)
NaCl, 150 mM	12	6	50	47 s–7 min 45 s
Tank water	9	2	22	32 s–2 min
Ovarian fluid	26	18	69	1 min 24 s–25 min
Tank water + ovarian fluid (1 : 1 v/v)	5	3	60	42 s–1 min 30 s
NaCl, 150 mM + ovarian fluid (1 : 1 v/v)	7	4	57	2 min–4 min 50 s
Seawater	20	0	0	Not applicable

[A]Number of samples (from separate males) containing mature spermatozoa.
[B]Number of samples in which motile spermatozoa were observed.

Size and localisation of the micropyle

In view of the possibility that there might be different size classes of pipefish spermatozoa, we hypothesised that the micropyle of the oocyte might be able to select the fertilising class on the basis of sperm head width. Examination of 20 pipefish eggs (from 3 females) by SEM revealed that there was a single micropyle and that it was ~10 μm in diameter (Fig. 3*a–c*). Based on our morphological observations, we estimate that five or six spermatozoa could theoretically enter the exterior region of the micropyle at the same time.

Estimation of sperm : egg ratio

Sperm : egg ratio was estimated using data on the total number of spermatozoa from the testes of 14 males and comparing this with the number of embryos observed in the pouches of 11 other gravid males. The resultant mean was 191 sperm per egg (range 5.2–771). The wide range resulted from variations in both observed total sperm numbers (mean and range were 14 200 and 700–37 000, respectively) and embryo numbers (mean and range were 74 and 48–135, respectively).

Discussion

The mode of mating in pipefishes, and more especially the mechanism involved in sperm transfer, is still surprisingly unclear despite several investigations aimed at studying the significance of sperm competition. Kvarnemo and Simmons (2004) discussed the various possibilities in some detail and concluded that the evidence for external fertilisation, after sperm release into the environment, was weak and insubstantial. However, they were also uncertain about the likelihood that eggs are first deposited into the male brood pouch by the female, after which the male then 'fertilises them internally', as suggested in some previous publications (Lourie *et al*. 1999). Such a mechanism implies the existence of a sperm duct that opens directly into the pouch, but, as we show here, this is anatomically inaccurate and the sperm duct opens directly to the external environment. The same anatomical observation was recently reported for the Yellow seahorse (*Hippocampus kuda*; Van Look *et al*. 2007) emphasising that spermatozoa cannot be shed directly into the pouch in either the seahorses or pipefishes.

Knowing that the spermatozoa must be released some distance from the pouch opening does not explain how the spermatozoa actually meet the eggs. In *S. abaster* it is likely that less than 50 000 spermatozoa are required to travel about half a centimetre between the excurrent duct and the pouch opening without being dispersed into the surrounding fluid. Moreover, our data on sperm activation further demonstrates that this is unlikely to involve exposure to the external environment because the spermatozoa were optimally activated in ovarian fluid and not activated in seawater.

S. abaster occurs widely in marine environments, including the Eastern Atlantic Ocean, the Mediterranean Sea and the Black Sea (Cakic *et al*. 2002), and our present data supports the view that they would be unable to breed if the fertilisation mechanism involved sperm transport through seawater. Although the marine and freshwater forms of *S. abaster* appear morphologically identical, there is still no unequivocal genetic proof that they are the same species. With that caveat in mind we have to qualify our speculation about the putative survival of marine-type spermatozoa in seawater. If genetic studies do indeed demonstrate that the marine and freshwater forms are the same species, then we could assert more confidently that sperm transport from the testes to the eggs cannot involve exposure to seawater. Our present observations suggest that spermatozoa are likely to be protected during transfer from the excurrent sperm duct to the vicinity of the eggs, possibly by being engulfed in ovarian fluid as the eggs are passed from the female to the male. This would explain how a small number of spermatozoa could be used to fertilise oocytes with great efficiency. Our observation that spermatozoa remained motile for up to 25 min within ovarian fluid suggests that under natural conditions there would be sufficient time for spermatozoa to interact with the oocytes once both sets of gametes reach the pouch environment. Similar sperm longevity in ovarian fluid has previously been observed in the three-spined stickleback (*Gasterosteus aculeatus*) (Elofsson *et al*. 2006); moreover Le Comber *et al*. (2004) demonstrated the ability of ovarian fluid to restore stickleback sperm motility after an initial period of motility activation had finished.

S. abaster lives in both brackish water and freshwater as well as seawater, and like the stickleback it has been characterised as a euryhaline species. Our observations on sperm activation are consistent with this degree of flexibility, and explain why it is

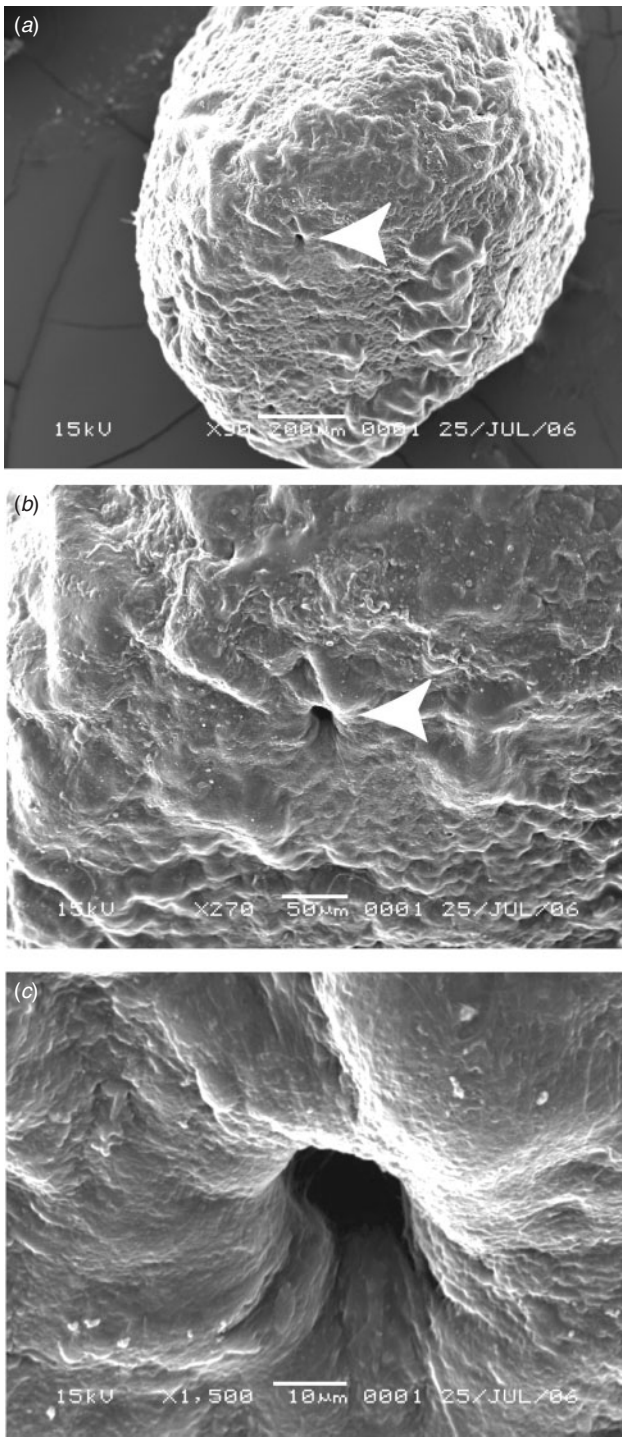

Fig. 3. Scanning electron micrographs showing the micropyle of a pipefish oocyte at three magnifications; arrows indicate the micropyle opening in panels (*a*) and (*b*). (Bar in (*a*), (*b*), (*c*) = 200, 50 and 10 μm, respectively.)

possible for the species to breed in such a variety of environments without the induction of irreversible sperm damage.

Although the total number of spermatozoa in the testes was always very low it may, in fact, still represent an overestimate of the true number available for fertilisation. The observation that pipefish spermatozoa are polymorphic, with only the longer ones (~50% of the total) exhibiting motility, suggests that the functional sperm population is much smaller than initially apparent. The occurrence of sperm dimorphism in pipefishes and seahorses has been reported previously (Watanabe *et al*. 2000; Van Look *et al*. 2007), but the functional importance of this observation is still speculative. One possibility is that the shorter spermatozoa are immature but, for *S. abaster* at least, this is not supported by the observation that sperm head dimensions were not as highly variable as might have been expected if the short spermatozoa were actually developing spermatids. These would probably have larger sperm heads, owing to the presence of uncondensed DNA and residual cytoplasm (Billard 1986). The alternative possibility that small spermatozoa are the result of a degenerative process (Pavlov and Moksness 1994) cannot be ruled out, but seems unlikely. Recent studies by Thunken *et al*. (2007) of a cichlid species, *Pelvicachromis taeniatus*, demonstrated considerable within-male variation in sperm length. In their interpretation of the data, the authors drew attention to theoretical studies (Parker and Begon 1993) implying that while large spermatozoa may evolve in response to inter-male sperm competition, they may also possess a within-male fertilisation advantage. As there is no likelihood of sperm competition in *S. abaster*, it may be possible to validate this hypothesis by examining spermatozoa that are in the early stages of fertilisation.

One unusual feature of pipefish sperm movement was the tendency to start and stop motility very abruptly without any changes in the surrounding environment. This may be a cellular strategy for prolonging sperm survival. We speculate that although pipefish produce small quantities of spermatozoa because sperm competition is non-existent, the functional limitation of small numbers might be offset by their ability to continue fertilising eggs over a prolonged period, perhaps even allowing them to fertilise eggs received in subsequent matings.

Our observations of pipefish eggs showed that they are characterised by the possession of a single micropyle that was not surrounded by any specific or unusual structures. We were only able to examine the exterior opening of the micropyle, so although it would be feasible for approximately five or six spermatozoa to enter this region simultaneously, it is likely that only one spermatozoon would be able to make further progress (Ginzburg 1972). This is tentatively consistent with our conclusion that the functional sperm : egg ratio in *S. abaster* is very low; our mean estimate of 191 spermatozoa per egg, although higher than previously reported values for the yellow seahorse (5 : 1) (Van Look *et al*. 2007), is several orders of magnitude lower than estimates for other fish species. The zebrafish (*Danio rerio*), which has one of the lower sperm concentrations in fish, 480 000 per ejaculate, has a sperm : egg ratio of 48 000 : 1, while that in sea trout (*Salmo trutta*) is 1.79×10^9 : 1 (Stockley *et al*. 1996).

External features of the reproductive organs in *S. abaster*, in combination with existing descriptions of pipefish mating behaviour (Jones and Avise 1997; Watanabe *et al*. 2000) suggest that sperm competition is almost certainly impossible in this species. This supposition is supported by recent conclusions about the absence of sperm competition in pipefish species with a simpler mode of male parental care, namely bearing embryos on

the male's ventral surface (Ah-King *et al*. 2006). Unfortunately Ah-King *et al*. (2006) did not indicate whether there were many or few spermatozoa in the testes of these species or calculate sperm : egg ratios. However, decreased sperm competition intensity can lead to reduced sperm production (Stockley *et al*. 1997); therefore on this basis we could predict '*a priori*' that *S. abaster* should exhibit very low sperm production. Here we found that the sperm concentration per testis and the sperm : egg ratio were among the smallest ever observed for fish species, being matched only by the seahorses, which are close relatives of pipefish (Van Look *et al*. 2007). Low sperm production is additionally related to the occurrence of fertilisation in the small volume of the pouch. While other close relatives such as the three-spine stickleback, *Gasterosteus aculeatus*, also characterised by an absence of sperm competition, produce large numbers of spermatozoa, up to 88×10^7 sperm per male (Zbinden *et al*. 2001), that is likely to be associated with the need for fertilisation in the large volume of their nest. Given the similarity in the functional anatomy of seahorse and pipefish genitalia, i.e. the substantial separation of the sperm duct and the pouch opening in both species, we attribute the difference in sperm : egg ratio between these species to the possibility that pipefish may need to fertilise several clutches of eggs from different females within a short period of time; that is not the case for the seahorse. Evidence for multiple maternity and therefore possible 'egg competition' has previously been confirmed for other pipefish species (Jones and Avise 2001).

Studies in other species have demonstrated that the micropyle actively participates in fertilisation by releasing signalling molecules that stimulate motility in their immediate vicinity (Creech *et al*. 1998). Indeed, spermatozoa from some marine species are immotile in seawater but become activated by proximity to the micropyle (Pillai *et al*. 1993). We cannot rule out this mechanism of sperm activation for *S. abaster*, but believe the occurrence of a convincing sperm activation response in ovarian fluid militates against it. The oocytes of some fish species, such as the pikehead (*Luciocephalus pulcher*), possess elaborate surface morphologies that are believed to guide spermatozoa towards the micropyle (Riehl and Kokoscha 1993), although the validity of this explanation is still uncertain. No special morphology was observed in the present study, suggesting that there is perhaps less need for a guidance system if the spermatozoa are maintained in close contact with the eggs by being contained within a small volume of ovarian fluid.

Acknowledgements

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Attachment 3

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Motility of sturgeon spermatozoa can sustain successive activations episodes

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ABSTRACT

Here we report for the first time the possibility of sequential sperm motility activation in sturgeon (sterlet, *Acipenser ruthenus*), a fish with external fertilization, through changes either in osmolality (global solute concentration) or in the Ca^{2+} concentration of the medium surrounding the spermatozoa. Sperm motility was initiated in any of three solutions containing buffer and sucrose at 80, or 40 or 10 mM (called S80, S40, S10, respectively); S80 is hypertonic relative to sterlet seminal fluid, while S40 is isotonic and S10 is hypotonic. After cessation of sperm movement at the end of this first motility period, a second and then a third, subsequent motile phase were observed. The second motility period was induced at cessation of motility in S80 by imposing a two-fold decrease in osmolality. After arrest of motility in this half-diluted S80, a third motility period could be initiated by addition of $CaCl_2$ to 1 mM final concentration. At the end of a first motility period in either S40 or S10, subsequent motility re-activation episodes were achieved only by addition of 1 mM $CaCl_2$. Depending on conditions in which sperm samples were activated, significant differences in curvilinear velocity, percent motile spermatozoa, motility duration time, and specific external features of spermatozoa flagella were observed. Altogether, these observations on the ability of sturgeon spermatozoa to sustain sequential activation episodes by experimental adjustment of their environmental conditions represent a potent model for deeper investigations on the sperm motility activation mechanisms.

1. Introduction

Activation of sperm flagellar motility occurs differently according to animal species, either progressively during a "maturation" process or as a response to a specific signal perceived by the spermatozoa (Darszon et al., 2001). In the case of animals with external fertilization such as fish, several sperm activating factors have been described in fish of differing taxa, with changes in osmolality and/or ion composition of the environment being considered the most common (Cosson, 2004; Alavi and Cosson, 2006).

The study of sperm motility by varying the activation media has been applied to several species of the order Acipenseriformes (sturgeon and paddlefish) because of its potential applications for artificial propagation. Almost all representatives of these taxa have a high market demand but an endangered status (Billard and Lecointre, 2001). Sturgeon sperm immotility in seminal plasma, along with immediate activation at transfer into fresh water, are considered common features of most freshwater spawning fish species. The ability of sturgeon and paddlefish spermatozoa to be activated in both hypo- or

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hyper-tonic (relative to seminal fluid) activating media (Alavi et al., 2006) can be considered a specific property of the activation mode of sperm motility in this group of species. The well-known suppression of sperm motility in the presence of a high potassium ion (K^+) concentration in activating medium, which can be abolished by the presence of calcium (Ca^{2+}) ions (Linhart et al., 2002, 2003), has been summarized as "antagonism between K^+ and Ca^{2+} ions" (Cosson, 2004, 2010). This suggests that sturgeon and paddlefish possess an ionic mode of sperm motility activation. Moreover, the participation of Ca^{2+} in the sequence of cellular events leading to sperm motility in fish possessing an ionic mode of activation has also been extensively documented in salmonid fishes (Cosson et al., 1989; Boitano and Omoto, 1992). Furthermore, changes in internal Ca^{2+} concentrations are also thought to be an essential step of motility activation in tilapia (Morita et al., 2003). However, the involvement of environmental osmotic changes in sterlet sperm motility activation has also been suggested (Alavi et al., 2011).

In the spermatozoa of several fish species with an osmotic mode of motility activation, motility can be stopped and immediately reactivated by applying successive alterations in osmotic pressure (Boryshpolets et al., 2009; Hu et al., 2009; Takai and Morisawa, 1995). The same result can be achieved by, for example, changes in K^+ (in salmonids (Benau and Terner, 1980)) or CO_2 (in flatfish species (Inaba et al., 2003)) concentrations, which alter sperm metabolic conditions and are considered the main activating signals for motility control. So, investigation of the ability of fish sperm to sustain successive activation episodes could be considered as a tool for a more precise study of sperm motility activation mechanisms.

In sturgeon, the changes in motility parameters under various conditions of osmolality and ionic composition of the activating media have been described (Alavi et al., 2004, 2008), but the extent of sperm reactivation abilities, whether only once or multiple times, is still unclear. A more complete description of environmental conditions for motility activation and reactivation is required in order to understand the basis of sperm motility signaling in fish of Acipenseriformes order.

In the present study we hypothesized that factors influencing motility activation of sturgeon sperm such as environment osmolality and Ca^{2+} concentration could operate separately and that relationships between them can be elucidated by studying the ability of spermatozoa to exhibit successive motility periods. The sterlet, *Acipenser ruthenus*, was used as a model sturgeon species.

2. Materials and methods

2.1. Fish rearing conditions and sperm collection

The study was carried out at the hatchery of the Research Institute of Fish Culture and Hydrobiology, Vodnany, Czech Republic. Broodstock of sterlet (3–5 years old, 0.67–1.18 kg body weight) were kept during the natural spawning season in 4 m^3 outdoor plastic tanks with a constant pond water flow rate of 20 L min^{-1} and temperature of 8–12 °C. Prior to hormone treatment, fish were moved to a closed water recirculation system, with water temperature gradually elevated to 15 °C over the course of the subsequent 24 h. Males were injected intramuscularly with carp pituitary extract (CPE, product of Rybníkářství Pohořelice a.s., Czech Republic) at 4 mg kg^{-1}. Thirty-six hours post-injection, the urogenital tract was emptied by aspiration using a plastic catheter (4 mm diameter). Thirty-nine hours post-injection, semen was collected from the urogenital papilla by catheterization into 50 mL plastic vials and stored on ice for experimentation within 3 h (Podushka, 2003).

2.2. Chemicals

Sucrose, $CaCl_2$, Tris, ethylene glycol tetra-acetic acid (EGTA) were purchased from Sigma-Aldrich Co. Prague, Czech Republic.

The pH of 10 mM EGTA stock solution was adjusted to 8.0 by addition of NaOH.

The free Ca^{2+} concentration in media used in the study was calculated using shareware Macintosh software, taking into account concentrations of sucrose, Tris, EGTA, and $CaCl_2$ (Saudrais et al., 1998). Concentration of free Ca^{2+} in S80, S40, and S10 was calculated as 8, 4, and 1 μM respectively and in S80, S40, and S10 containing 0.1 mM EGTA was calculated as 5×10^{-5}, 2×10^{-5}, and 6×10^{-6} μM respectively.

2.3. Estimation of seminal fluid and activating media osmolalities

Seminal fluids (SF) were obtained after sperm sample centrifugation at 16,000 × *g* for 10 min. Osmolalities of SF and media used in these experiments were evaluated using a Vapor Pressure Osmometer 5520 (Wescor, USA), and expressed as mOsm kg^{-1}.

2.4. Evaluation of sperm motility parameters

Immediately following dilution, sperm motility was recorded in each mode of treatment until motility arrest, using a CCD video camera (Sony, SSCDC50AP) mounted on a dark-field microscope (Olympus BX50, ×200) and illuminated with a stroboscopic lamp (Chadwick-Helmut, 9630, USA) set to a flash frequency of 50 Hz. Video recordings were made using a DVD recorder (Sony, DVO-1000 MD). Video recordings were analyzed to estimate spermatozoa curvilinear velocity (VCL, length of sperm head track divided by the time of the track, μm s^{-1}), percent motile spermatozoa (motility, %), and motility duration (s). To compute the VCL and percent motile spermatozoa at 10 sec post-activation, five successive frames were analyzed by image analyzer (Olympus Micro Image 4.0.1 for Windows, Hamburg, Germany). Ten to 50 spermatozoa were evaluated in each frame. Spermatozoa with velocity lower than 3 μm s^{-1} were considered as immotile and excluded from further analysis. Only motile spermatozoa

were considered in the VCL calculation. Motility duration was considered as the period of time required to reach motility cessation in 95% of cells at each step of treatment. Total sperm motility duration was calculated for each activating medium and used as a sum of motility duration of each of the three steps of the treatment mode.

2.5. Immotile spermatozoa image analysis

Images of quiescent spermatozoa were captured from video recorded sequences by VirtualDub 1.5.10 software; these images were contrast-enhanced by image analyzer (Olympus Micro Image 4.0.1 for Windows, Hamburg, Germany) by applying negative contrasting mode.

2.6. Successive activations of sperm motility by changing osmolality and Ca^{2+} concentrations

Sperm suspensions were subjected to three stepwise and sequential treatments in order to modify the ionic or osmotic conditions of their surrounding media. Step 1 involved treating the sperm suspensions with one of three solutions of varying osmolality consisting of sucrose 80, 40, or 10 mM in 10 mM Tris–HCl buffer pH 8.5 (designated S80, S40, S10, respectively). To evaluate motility parameters of spermatozoa at this step of treatment, 50 μL of sucrose solution (S80, S40, S10) was placed on a glass microscope slide, and 0.5 μL of sperm suspension was added and thoroughly mixed, followed immediately by observation and video recording. Immediately after sperm motion arrest, one of the following two treatments ("Mode of treatment") was applied.

2.6.1. Mode of treatment 1

Samples were supplemented with 1 μL solution of 50 mM $CaCl_2$, to achieve a final Ca^{2+} concentration of 1 mM (Step 2) and after motility arrest the osmolality of the swimming medium was halved by the addition of 50 μL distilled water (Step 3).

2.6.2. Mode of treatment 2

The osmolality of the swimming medium was halved by the addition of 50 μL of distilled water (Step 2) and, after motility arrest, the $CaCl_2$ concentration was increased to 1 mM by adding 2 μL solution of 50 mM $CaCl_2$ (Step 3).

2.7. Effect of EGTA on sperm motility

To investigate the effect of Ca^{2+} ions on sperm motility activation and termination, EGTA was used as a specific Ca^{2+} chelating agent. The influence of EGTA on sperm motility was assessed in 2 experiments:

2.7.1. To determine the inhibitory effect of EGTA on motility activation and to check whether this effect is reversible by Ca^{2+} ions

A volume of EGTA stock solution required to achieve final concentrations of 0.01, 0.05, and 0.1 mM was added to volume calibrated drops (50 μL) of S80, S40, and S10 on glass slides and thoroughly mixed. A small volume of sperm suspension (0.5 μL) was then added and sperm behavior was immediately observed and recorded. The percentage of motile spermatozoa in EGTA-containing solutions was considered as an estimator of the Ca^{2+} effect on sperm motility activation. Subsequently, after motility cessation, $CaCl_2$ was added (1 mM final concentration) and motility was recorded to estimate percent motile spermatozoa. Percent motile spermatozoa was determined as described in paradraph 2.4.

2.7.2. To determine the role of external Ca^{2+} concentration during the sperm motility period

Sperm motility was initiated in S40 and S10 (S80 was not used because of short motility duration) containing 0.1 mM Ca^{2+}; after 20 s of motility initiation, EGTA was added to 0.5 mM final concentration and motility was recorded. In this experiment, the concentration of $CaCl_2$ was selected from previous observations showing that 0.1 mM $CaCl_2$ increased motility duration (unpublished data), and also, taking into account the results of the first experiment.

2.8. Data presentation and statistical analysis

All experiments were conducted with sperm from 5 different males (replicates). Each replicate involved triplicate measurements of osmolality of SF, percent motile spermatozoa, and motility duration and the triplicate means were used for statistical analysis.

The values of parameters used were checked for distribution characteristics and homogeneity of dispersion using Shapiro–Wilk's and Levene's tests, respectively. The values of spermatozoa velocity were normally distributed with similar dispersion values. For this parameter, the mean values with 95% CI are presented in figures. To compare these parameters, parametric ANOVAs were applied and Tukey's honest significant difference test (HSD test) was used for differences between subgroups. When values were not normally distributed (percent motile spermatozoa and motility duration), they were presented as median and interquartile range in figures, and min-max values are given in the text. Nonparametric statistics by Kruskal–Wallis test with following Mann–Whitney *U*-test were performed for comparison of percent motile spermatozoa and motility duration data.

Statistical significance was considered at $P < 0.05$. All analyses and plots were conducted using STATISTICA V 9.1 computer program (Statsoft Inc., USA).

3. Results

3.1. Osmolality of seminal fluid and media used in experiments

Mean osmolality of SF was 52 ± 4 mOsm kg^{-1}. Osmolality values of S10, S40, S80 solutions were 28 ± 3, 52 ± 1, and 87 ± 3 mOsm kg^{-1} respectively. Therefore, S10, S40, and S80 were identified as hypo-, iso-, and hyper-tonic activating media, respectively, relative to SF.

Table 1
Qualitative parameters of successive sperm motility activation episodes in sterlet.[a]

Activating medium	STEP 1		STEP 2			STEP 3		
	Results of treatment			Results of treatment			Results of treatment	
	Motility	Sperm flagella shape at motility cessation	Mode of treatment	Motility	Sperm flagella shape at motility cessation	Mode of treatment	Motility	Sperm flagella shape at motility cessation
S80	+		1 (Ca^{2+})	-		1 (W)	+	
			2 (W)	+		2 (Ca^{2+})	+	
S40	+		1 (Ca^{2+})	+		1 (W)	-	
			2 (W)	-		2 (Ca^{2+})	+	
S10	+		1 (Ca^{2+})	+		1(W)	-	
			2 (W)	-		2 (Ca^{2+})	+	

[a] Symbols: "+" and "–" – motility is observed or not respectively; (Ca^{2+}) – calcium ions are added after motility cessation at previous step of treatment (1 mM final concentration); (W) – water is added after motility cessation at previous step of treatment (dilution 1:1). For a more detailed description, see Section 2.

3.2. Successive sperm motility activation by osmolality and Ca^{2+} concentration

3.2.1. Qualitative description of spermatozoa during successive activation steps and flagellar shape after motility cessation

The results of qualitative observations of sterlet spermatozoa after successive activations are presented in Table 1, in the form of a comprehensive summary. From this table the following derivations are extracted:

(1) Sperm motility was observed in hyper (S80)-, iso (S40)-, and hypo (S10)-tonic conditions.
(2) Three episodes of motility activation were observed when hypertonic (S80) medium was applied at the treatment Step 1 followed by mode of treatment 2. This mode of treatment includes subsequent decrease of environmental osmolality at Step 2 and Ca^{2+} concentration increase at Step 3.
(3) Two episodes of motility were achieved under the following conditions:
   a. First activation by S80 (Step 1), followed by mode of treatment 1. This mode of treatment includes Ca^{2+} concentration increase at Step 2 (no motility) and osmolality decrease at Step 3 (second motility episode).
   b. First activation by S40 or S10 (Step 1), followed by mode of treatment 1. This mode of treatment includes Ca^{2+} concentration increase at Step 2 (second motility episode), no motility was induced by the osmolality decrease at Step 3.
(4) Visualization of spermatozoa during the initial phases of motility in hyper (S80)-, iso (S40)-, and hypo (S10)-tonic solutions showed similar flagellar shape at any step of treatment (images not presented). The positions of flagella after motility cessation depended on

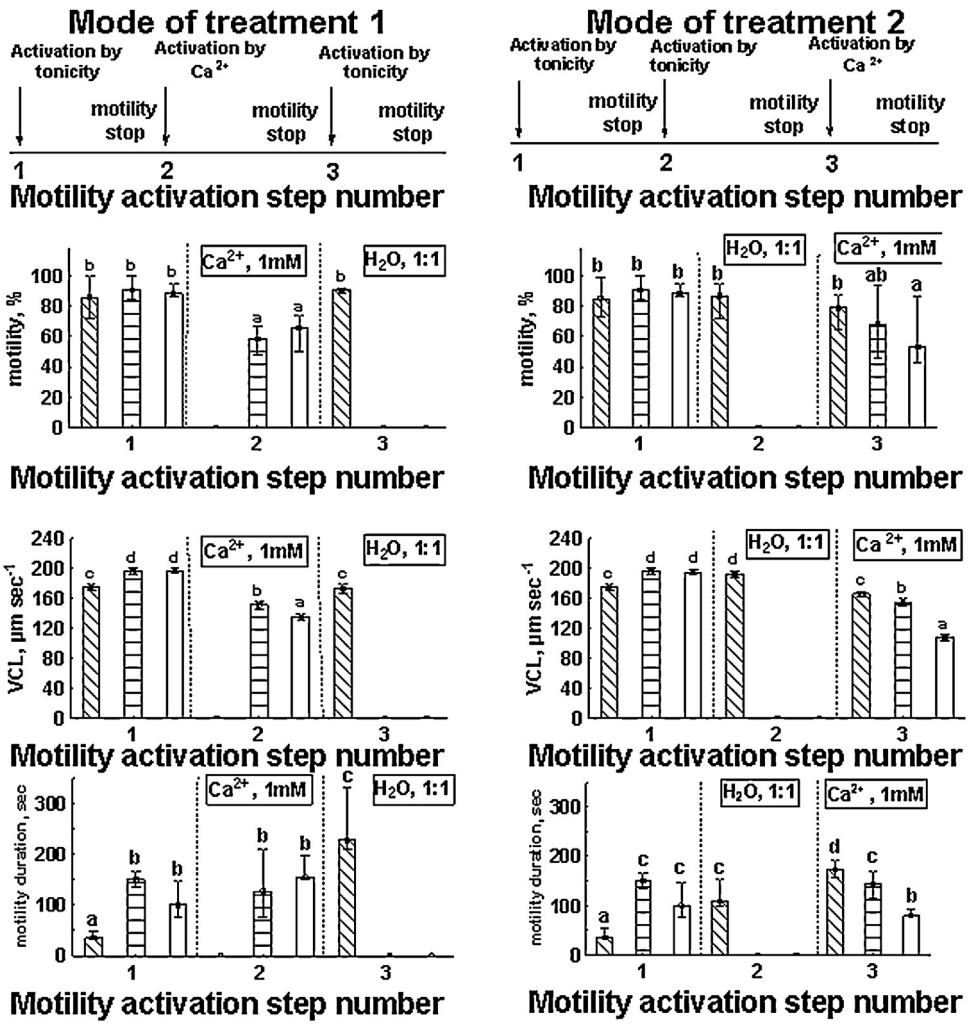

Fig. 1. Sperm motility parameters during successive activation episodes resulting from variations in osmolality and Ca^{2+} concentration. Values for percent motile spermatozoa (motility, median value with 25% and 75% percentiles), curvilinear velocity (VCL, mean and 95% CI), and motility duration (median value with 25% and 75% percentiles). Motility and VCL were measured at 10 s after motility initiation. Schematic descriptions of successive mode 1 and 2 of sperm motility activation are given at the top of each column. Parameters obtained in S80, S40, and S10 are shown by columns marked ▨, □, ▤, respectively. Values with different letters are significantly different ($P < 0.05$, Mann–Whitney U-test for motility and motility duration, Tukey's test for VCL).

the osmolality of activating media and on the treatment modes applied. In hypertonic conditions, the flagella of arrested spermatozoa remained in a bent configuration.

The first activation by isotonic or hypotonic conditions resulted in sperm motility cessation with flagella in a curved configuration. Spermatozoa which ceased movement with their flagella in a bent or curved position had the potential to be activated a second time. It was observed that if spermatozoa ceased motility with flagella in a distorted position successive motility episode could not be achieved.

3.2.2. Quantitative assessment of sperm motility parameters during successive steps of activation

Median values of percent motile spermatozoa activated in all three (S80, S40, and S10) solutions at Step 1 ranged from 86 to 91% with no significant differences observed among these values. VCL and motility duration at Step 1 in S80 were significantly lower compared to values in S40 and S10 (Fig. 1).

Sperm motility activation at Steps 2 and 3 of treatment was dependent on osmolality of the medium and on mode of treatment as well. The second and third successive activations were observed immediately after application of appropriate treatment steps.

3.2.2.1. Treatment mode 1 (Step 2 – activation by Ca^{2+}, Step 3 – activation by tonicity decrease). A second motility activation episode was observed for each of the three media applied (Fig. 1). In the case of S80, a second activation period was observed at Step 3 of treatment. Percent motile spermatozoa (62–100% min–max values) and VCL ($174 \pm 32\ \mu m\ s^{-1}$) values at Step 1 were not significantly different from those observed at Step 3 (81–100% and

$174 \pm 32\ \mu m\ s^{-1}$ respectively (Fig. 1, treatment mode 1, S80). Motility duration was significantly increased from 30–65 sec during Step 1 to 220–330 s (min–max values) during Step 3 (Fig. 1, treatment mode 1).

In the cases of S40 and S10, a second motility activation period was observed at Step 2. Comparison between Step 1 and Step 2 treatments shows that the percentage of motile spermatozoa was significantly decreased from 83–100% (S40) and 88–90% (S10) during Step 1 to 51–71% (S40) and to 47–75% (S10) during Step 2 (Fig. 1, treatment mode 1, S40 and S10). Similarly, VCL was significantly decreased from $195 \pm 30\ \mu m\ s^{-1}$ (S40) and $197 \pm 34\ \mu m\ s^{-1}$ (S10) during early Step 1 to $150 \pm 25\ \mu m\ s^{-1}$ (S40) and to $135 \pm 25\ \mu m\ s^{-1}$ (S1) during early Step 2. Motility duration values at Step 1 for S10 and S40 were not significantly different from those at Step 2, as shown in Fig. 1 (treatment mode 1, S40 and S10), and no significant differences were seen in results between S10 and S40 for percent motile spermatozoa and motility duration within Step 1 and 2.

3.2.2.2. Treatment mode 2 (Step 2 – activation by tonicity decrease, Step 3 – activation by Ca^{2+}). Three sequential sperm motility episodes were observed after activation by hypertonic medium (Fig. 1, treatment mode 2, S80). In this case percent motile spermatozoa median values were 79–87%, and no significant differences among steps were found for these values. The mean value of VCL for samples activated in S80 was significantly higher ($192 \pm 30\ \mu m\ s^{-1}$) at Step 2 of activation, while no differences were found between VCL at first ($174 \pm 32\ \mu m\ s^{-1}$) and third ($165 \pm 23\ \mu m\ s^{-1}$) episodes (Fig. 1, treatment mode 2, S80, Steps 1–3). Motility duration (median value) was significantly increased to 107 sec at Step 2 and 173 s at Step 3 compared to Step 1 (35 s) (Fig. 1, treatment mode 2, S80, Steps 1–3).

Two successive activation episodes were observed with S40 and S10. A second activation could occur only in the case of treatment Step 3 (Fig. 1, treatment mode 2). Application of S40 resulted in a significant decrease in VCL, from $195 \pm 30\ \mu m\ s^{-1}$ at treatment Step 1 to $154 \pm 20\ \mu m\ s^{-1}$ at treatment Step 3. Percent motile spermatozoa and motility duration during treatments Steps 1 and 3 were not significantly different (Fig. 1, treatment mode 2, S40). Percent motile spermatozoa, VCL, and motility duration were significantly decreased during Step 3 as compared to Step 1 with S10 application (Fig. 1, treatment mode 2, S10).

In comparison with other treatment modes, a significant decrease in total motility duration ranging from 162 to 220 s (min–max values) was observed by use of treatment mode 2 when applying S10 (Fig. 2).

3.3. Effect of EGTA on sperm motility

3.3.1. Is there an inhibitory effect of EGTA and can it be reversed by adding Ca^{2+} ions?

At Step 1 of treatment, percent motile spermatozoa progressively decreased with increasing concentrations of EGTA (0.01, 0.05, and 0.1 mM), reaching 0% when concentration of EGTA was 0.1 mM in all three activating media used (S80, S40, and S10) (Fig. 3A). Motility recovered fully when adding 1 mM Ca^{2+} to sperm exposed to S80, S40, and S10 supplemented with 0.1 mM EGTA (Fig. 3A). In S80, S40, and S10 containing 0.1 mM EGTA, sperm flagella exhibited almost perfectly straight tails (Fig. 3B).

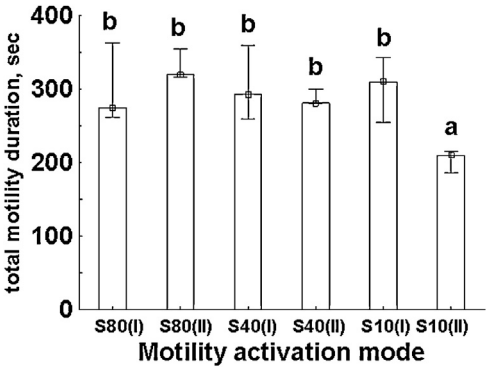

Fig. 2. Total sperm motility duration. Sum of motility duration (median with 25% and 75% percentiles) at each step of treatment mode 1 using S80, S40, and S10 (S80(I), S40(I) and S10(I)) and treatment mode 2 (S80(II), S40(II) and S10(II)) are shown. Values with different letters are significantly different, $P < 0.05$, Mann–Whitney *U*-test.

3.3.2. Determination of role of external Ca^{2+} concentration during sperm motility period in sterlet

Addition of EGTA to motile spermatozoa activated by S10 and S40 (containing 0.1 mM Ca^{2+}) did not significantly change percent motile spermatozoa (Fig. 4A) and velocity (Fig. 4B) at 30 s after addition.

4. Discussion

The present study of sterlet sperm motility observed in hyper-, iso-, and hypo-tonic conditions describes for the first time their ability to sustain successive sperm motility activations solely through changes of osmolality and Ca^{2+} concentration in the surrounding environment. The first step of sperm activation, which was not dependent on osmotic pressure, has been previously described in the case of sterlet (Alavi et al., 2011) and the Persian sturgeon (Alavi et al., 2006). Reduction of sperm VCL and motility duration observed in hypertonic conditions with treatment Step 1 of our study were similar to previous results (Alavi et al., 2011). In the present study, it was possible to trigger a second motile phase by decreasing environmental osmolality only when a hypertonic medium was used for activation at the initial step. Therefore, we suggest that environmental hypertonicity is the factor reducing motility at the first step of activation and its elimination leads to the second phase of motility. The necessity for the presence of Ca^{2+} in the activating medium observed in our study strongly suggests the existence of an ionic mode of sperm activation in sterlet. No activation in the presence of 0.1 mM EGTA was found in the present study, in contrast to a previously published report (Alavi et al., 2011). This discrepancy could arise from differences in sperm sampling procedures. Since sturgeon testes possess no externally opening sperm ducts, and the spermatozoa pass through the urinary collecting ducts (Wrobel and Jouma, 2004), we suggest that, in the present study, spermatozoa could have been mixed with

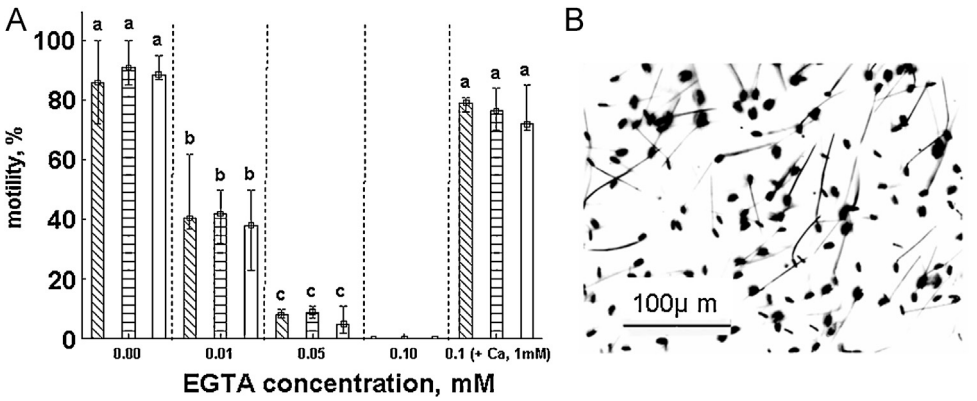

Fig. 3. Percent motile spermatozoa in samples activated with EGTA containing solutions (A) and representative flagellar shapes for spermatozoa diluted with 0.1 mM EGTA containing solutions (B). A. Motility was initiated in S80, S40, S10 (shown by columns marked ▨, ▭ and ▭, respectively) containing 0 (control), 0.01, 0.05, and 0.1 mM EGTA. In group of 0.1(+Ca, 1 mM) Ca^{2+} was added subsequently, after sperm dilution with S80, S40, S10 containing 0.1 mM EGTA. Percent motile cells was estimated at 10 s post-activation. The values (median values with 25% and 75% percentiles are presented) with different superscripts are significantly different (Mann–Whitney *U*-test, $P < 0.05$). B. Shapes of immotile sterlet spermatozoa flagella in S40, containing 0.1 mM EGTA.

urine for a shorter period (see M&M) than samples used in the previous study. According to results of Krayushkina and Semenova (2006) the Ca^{2+} ion content in urine is 0.70 ± 0.19 mM, while in seminal plasma it was determined as 0.16 ± 0.11 mM (Psenicka et al., 2008) or 0.15 ± 0.09 mM (Li et al., 2011). Therefore, during this prolonged period of exposure to urine, the internal Ca^{2+} concentration of spermatozoa may increase to a level sufficient for activation, and spermatozoa would not require additional external Ca^{2+} for motility activation. However, the high external K^+ concentration in seminal fluid is believed to constitute an inhibitory factor, which prevents activation of sturgeon sperm motility (Gallis et al., 1991; Toth et al., 1997; Linhart et al., 2002). This suggests that sperm dilution by activating medium results in a decrease in external K^+, which itself facilitates motility activation of sturgeon spermatozoa. During hypotonic treatment by the activation medium, the decrease in external osmolality could also be converted into a decrease of the intracellular K^+ concentration and this drop down of K^+ constitutes one major step in sperm motility signaling. Such a pathway has already been proposed by Takai and Morisawa (1995) for teleost spermatozoa activation, but it appears from our results that the same mechanism would apply to sturgeon species as well. As sperm motility was inhibited by 0.01–0.1 mM EGTA containing solutions, it should be emphasized that free Ca^{2+} external concentration is probably required for motility activation but in a low (1 μM – no inhibition, 5×10^{-5} μM – full inhibition) concentration range. Therefore, we suggest that motility activation in sterlet spermatozoa is highly dependent on the ionic environment while decreased osmolality is required for prolongation of the motility period. In sturgeon, the mode of sperm motility activation, which involves the participation of external concentration of both K^+ and Ca^{2+} ions, is similar to that proposed for trout sperm motility activation (Morisawa and Morisawa, 1990). By using such an analogy, a model of sperm motility activation mechanism in sturgeons was proposed recently by Alavi et al. (2011). In this model, K^+ efflux out of spermatozoa (due to either drop of external K^+ concentration or osmolality decrease) is considered as a source of sequential membrane hyperpolarization/depolarization events, which leads to Ca^{2+} influx. This membrane signal would initiate a Ca^{2+} dependent cascade of intracellular regulatory events involving Ca^{2+}-calmodulin-activated phosphodiesterase, a sequence of steps, which needs experimental

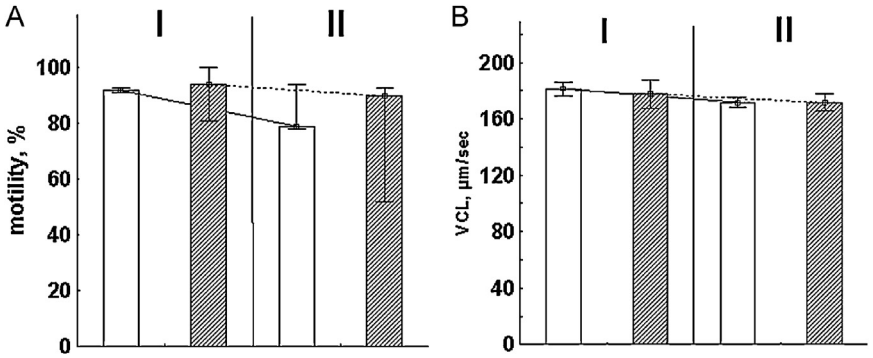

Fig. 4. Percent motile spermatozoa (A) and VCL (B) before and after EGTA treatment. Motility was initiated in S40 plus 0.1 mM Ca^{2+} or S10 plus 0.1 mM Ca^{2+} (I). At 20 s of motility EGTA was added (0.5 mM final concentration); after 30 s, motility parameters were measured (II). Evolution of parameters obtained in S40 and S10 are shown by columns marked ▨ and ▭, respectively. For A, median values with 25% and 75% percentiles are presented, $P < 0.05$, Mann–Whitney *U*-test. For B, mean and 95% CI are presented. No significant differences were found ($P > 0.05$, Mann–Whitney *U*-test and Tukey's test respectively).

confirmation in sturgeon spermatozoa. In addition, a direct influence of K^+ and Ca^{2+} ions concentration on the motility of demembranated sturgeon spermatozoa has also been shown previously (Linhart et al., 2002) and suggests that the intracellular concentration of those two ions could be directly responsible for motility control. Therefore, it looks as though sturgeon sperm motility regulation by K^+ and Ca^{2+} could also occur via an alternative ion sensitive signaling pathway, involving more straightforward regulation at the axonemal level. The description of a precise sequence of events responsible for sturgeon sperm motility activation is still awaiting better experimental elucidation in the future.

To elucidate the source of discrepancy between our results (full inhibition of sterlet sperm motility by EGTA) and the previous results mentioned above (Alavi et al., 2011), we conducted screening experiments oriented towards the checking the responses of different sturgeon sperm samples to the presence of EGTA in activating media. Although the results are not directly related to phenomenon of successive spermatozoa motility, they are presented in Table A1 from Appendix A. These observations show that the ability of sperm motility activation to be suppressed by the presence of EGTA in the activating medium is sample dependent. Additionally, no relationships between EGTA inhibiting effect and spawning season, in vitro storage time, collection number after injection were observed, making the cause of this phenomenon rather unclear. However, to our knowledge, individual differences in sperm sensitivity to Ca^{2+} in sturgeon have not previously been described in the literature. Therefore, we conclude, that this phenomenon requires a future more precise study. We suppose in this respect that the study of sperm internal Ca^{2+} concentrations could be useful for better understanding of the roles of external Ca^{2+} ions in sturgeon sperm motility activation.

It has been shown previously that changes in sperm motility occurring in response to environmental conditions, namely osmolality and/or Ca^{2+} concentration, are species-specific. For example the presence of Ca^{2+} ions makes the range of activating media osmolality wider in Marble goby *Oxyeleotris marmorata* (Morita et al., 2006) while the presence of Ca^{2+} in activating media significantly decreases percent motile spermatozoa and motility duration in Java carp *Puntius javanicus*. Moreover, sperm motility in catfish *Clarias batrachus* seems to be independent of environmental Ca^{2+} concentrations (Morita et al., 2006).

Fish spermatozoa possess a typical eukaryotic flagellum structure and are rendered motile by mechanical activity of the axonemal structures, which consume chemical energy liberated from ATP hydrolysis to generate movement. This mechano-chemical process involves a complicated network of signaling and structural changes in any flagellum and is generally considered as not completely understood (Cosson, 2008; Inaba, 2003). However, some simple parameters such as flagellar shape and characteristics of flagellar wave propagation and head trajectories already provide information that reveals how complicated is such a biological scaffold (Gibbons, 1981). Our quantitative descriptions of percent motile spermatozoa and VCL suggest that motility activation in media of varying osmolalities probably initiates and supports the activity of flagellar structures regardless of the activation media used. However, either the lowering of external Ca^{2+} (e.g. by EGTA addition) or the increase in external osmolality of activating media can inhibit motility, both conditions leading flagella to cease their movement in a wide range of positions.

Following activation in hypertonic conditions, sperm motion is arrested with the flagellar position differing from that of spermatozoa activated under iso- and hypotonic conditions. In those spermatozoa demonstrating the ability for a second and a third motility phase, flagella remain in positions that differ from those that had not been activated (because of the presence of EGTA) or cannot be activated for an additional round (fully 'exhausted' spermatozoa). Interestingly enough, at very low external Ca^{2+} concentration (in presence of EGTA), flagella of sterlet spermatozoa present fully straight shapes, suggesting that Ca^{2+} ions are also involved in the regulation of flagellar bending in non-motile spermatozoa. A similar dependency of flagellar shape of arrested spermatozoa on the presence of Ca^{2+} ion concentration has been observed in sea urchin spermatozoa (Gibbons, 1980). A short-duration of the second activation (resulting from Ca^{2+} concentration increase or from osmolality decrease) suggests that the first motility period arrest occurred due to environmental conditions unable to sustain motility rather than due to a lack of energy stores. The similarity of accumulated motility duration observed with successive activations under most experimental conditions (except in one case, all activation steps were performed under hypotonic conditions) supports this hypothesis. Finally, it appears that the presence of low external Ca^{2+} concentration during the period following the sturgeon sperm motility activation step could be unimportant for the motility signaling process.

We consider that the phenomenon of successive sterlet sperm activation described here supports our hypothesis about possible independent regulation of sturgeon sperm motility by osmotic pressure decrease or by Ca^{2+}concentration increase in experimental conditions. Approaching successive activations should help to better understand the basis of signaling processes in the sturgeon sperm motility.

Next steps in elucidating the activation cascade following perception of the signals (osmotic and/or ionic) at the membrane level as well as transduction from membrane to axoneme need deeper investigations. It is clear in this respect that sturgeon spermatozoa offer a unique model on which to address these questions.

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Appendix A.

Table A1.

Table A1
Sturgeon sperm motility in EGTA containing activating medium.[a]

Species	Condition of sperm sample collection, sperm storage time	Motility in EGTA containing media	Number of observation
Acipenser ruthenus	First collection after hormonal treatment, 2 h	+	5
		–	16
	Second collection after hormonal treatment, 2 h	+	4
		–	15
Acipenser gueldenstaedtii	First collection after hormonal treatment, 48 h	+	3
		–	2
Huso huso	First collection after hormonal treatment, 48 h	+	2
		–	2
Acipenser baerii	First collection after hormonal treatment, 48 h	+	2
		–	3

[a] Activating medium: 40 mM sucrose, 10 mM Tris, pH 8.5, 0.1 mM EGTA. Symbols: "+" – motility (no less 80%) is observed; "–" – no motility is observed.

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Attachment 4

Prokopchuk, G., **Dzyuba, B**., Rodina, M., Cosson, J., 2016. Control of sturgeon sperm motility: Antagonism between $K(^{+})$ ions concentration and osmolality. Animal Reproduction Science 164: 82-89. (IF 2015 = 1.377)

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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.

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 Aquaculture and Biodiversity of Hydrocenoses, Research Institute of Fish Culture and Hydrobiology, Vodnany, Czech Republic. Authorization for use of experimental animals No.: 53100/2013-MZE-17214 valid from 08/30/2013 to 08/30/2016.

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 μL of sperm suspension was transferred to a glass slide into a 50 μL 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 μM 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× 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, μm/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 ± 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

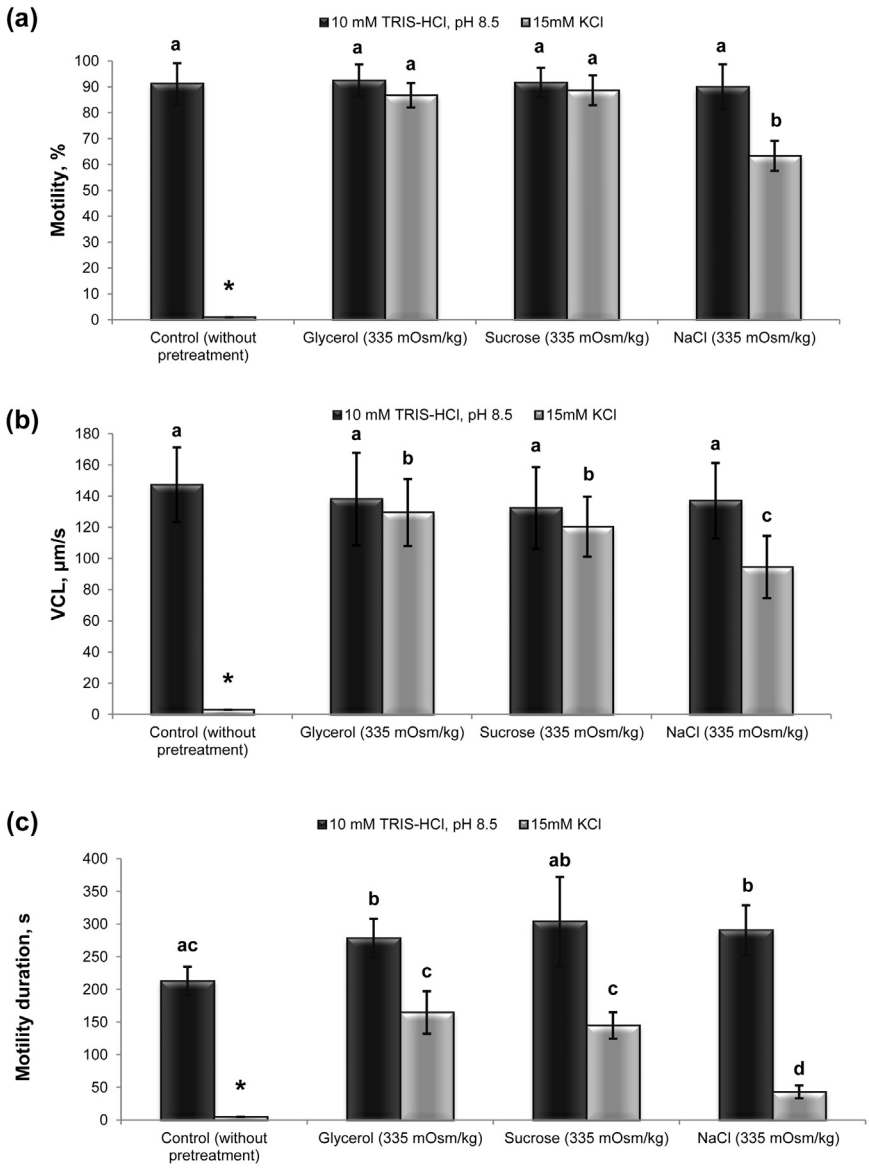

Fig. 1. Beluga sperm motility (a), VCL (b), and duration of motion (c) after 5 s pre-incubation in hypertonic (335 mOsm/kg glycerol, 335 mOsm/kg sucrose, or 335 mOsm/kg NaCl) media followed by activation in either 10 mM TRIS-HCl (pH 8.5) alone or supplemented with 15 mM KCl. Data are presented as mean ± 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 hyperosmolarity 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).

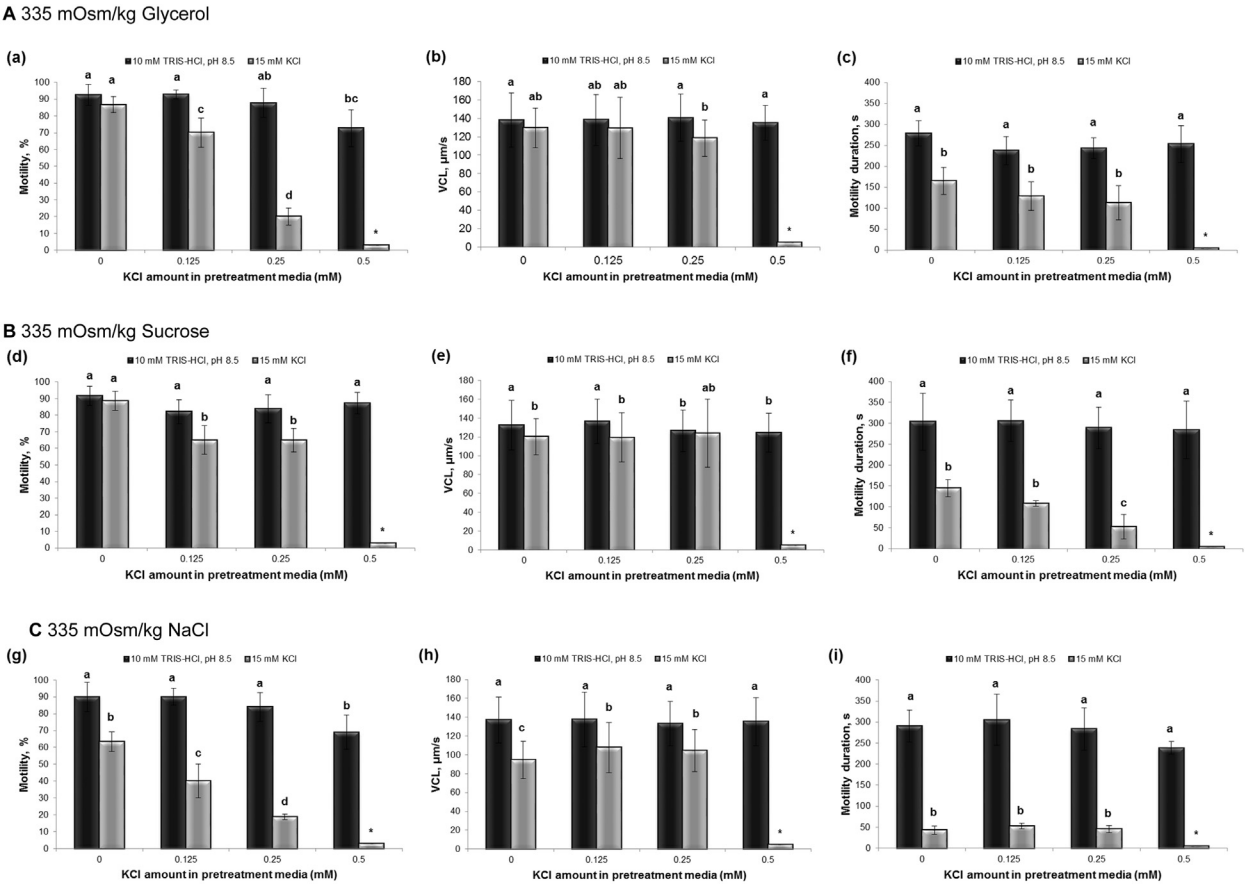

Fig. 2. Effects of hyperosmotic treatment by 335 mOsm/kg glycerol (A), 335 mOsm/kg sucrose (B), or 335 mOsm/kg NaCl (C) supplemented with different K^+ concentrations (0.125, 0.25, or 0.5 mM) on motility percentage (a, d and g), velocity (b, e, and h) and motility duration (c, f, and i) of beluga sperm in either 10 mM TRIS-HCl (pH 8.5) alone or supplemented with 15 mM KCl. Data are presented as mean ± 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,

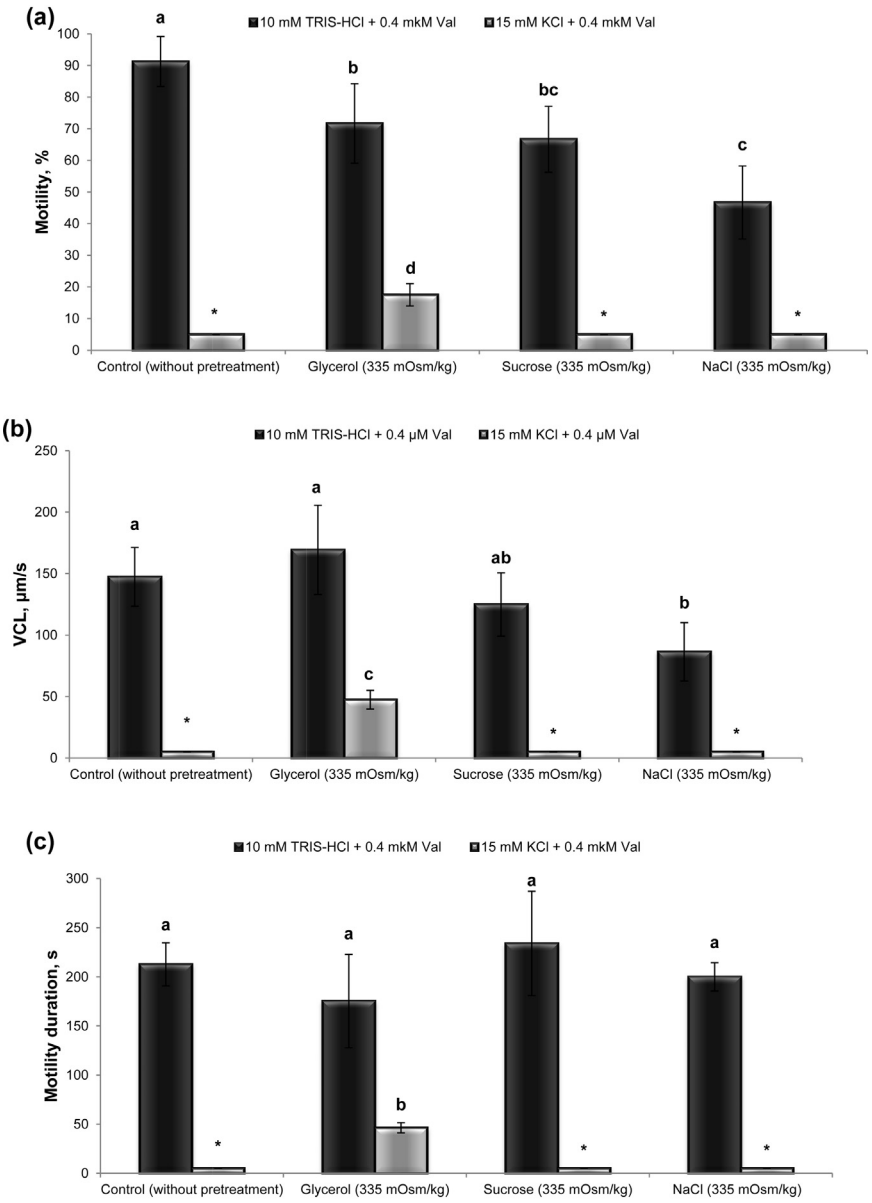

Fig. 3. Effects of hyperosmotic treatment by 335 mOsm/kg glycerol, 335 mOsm/kg sucrose, or 335 mOsm/kg NaCl on motility percentage (a), velocity (b), and motility duration (c) of beluga sperm activation. 0.4 μM valinomycin was added into the activating media composed of either 10 mM TRIS-HCl (pH 8.5) alone or supplemented with 15 mM KCl. Data are presented as mean ± 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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Attachment 5

Dzyuba, B., Cosson, J., Boryshpolets, S., Bondarenko O., Dzyuba V., Prokopchuk, G., Gazo, I., Rodina, M., Linhart, O., 2014. In vitro sperm maturation in sterlet, *Acipenser ruthenus.* Reproductive Biology 14:160-163. (IF 2014 = 1.524)

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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.

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 m^3 closed water recirculation system, located at the hatchery of the South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Vodnany, Czech Republic. Thereafter, the water temperature was increased to 15 °C within 24 h, and before the beginning of the experiment, fish were held four days without feeding. Urine was collected from the urogenital sinus by aspiration using a plastic catheter (4 mm diameter) connected to a 10 mL syringe, and was stored at 4 °C for 24–30 h. Spermiation was stimulated by one intramuscular injection of carp pituitary powder dissolved in 0.9% (w/v) NaCl solution (4 mg/kg bw). Spermatozoa were collected from: (1) urogenital (Wolffian) ducts by the procedure described for urine collection, 24 h after stimulation of spermiation (Wolffian duct sperm, used in fisheries for artificial sturgeon propagation), and (2) testis (testicular sperm). Immediately after Wolffian duct sperm collection, the fish were euthanized by striking the cranium followed by exsanguination. After euthanasia, the digestive tract was removed, and testicular sperm was collected after incision of the efferent ducts [6].

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

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

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

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

3. Results and discussion

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

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

Fluid	Examined parameters					
	$[Na^+]$ (mM)	$[K^+]$ (mM)	$[Ca^{2+}]$ (mM)	Total protein (mg/mL)	Osmolality (mOsm/kg)	pH
Urine	15 ± 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 Ca^{2+} [12]. Thus, high K^+ and low Ca^{2+} concentrations in seminal fluids from Wolffian duct sperm, conditions which may result from testicular sperm dilution with hypotonic urine [8,12], seem to be the factors that prevent sperm motility activation. We have observed significant differences in testicular sperm and Wolffian duct sperm responses to dilution with activating medium. The activation medium activated the Wolffian duct sperm motility, but was not able to initiate testicular sperm motility immediately after collection and at the end of the experiment (2 h later). The Wolffian duct sperm was characterized by high motility rate and VCL values (Table 2) typical for sterlet [13]. In our opinion, the difference in the ability to trigger motility may be an indicator of sperm maturity.

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

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

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

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

Sperm type	Pre-incubation time	Treatment during pre-incubation	Motility (%)	VCL (μm/s)	Motility duration (s)
WS	–	Control, no treatment	90 ± 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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Attachment 6

Fedorov, P., **Dzyuba, B.**, Fedorova, G., Grabic, R., Cosson, J., Rodina, M., 2015. Quantification of adenosine triphosphate, adenosine diphosphate, and creatine phosphate in sterlet *Acipenser ruthenus* spermatozoa during maturation. Journal of Animal Science 93: 5214-5221. (IF 2015 = 2.014)

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

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

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

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INTRODUCTION

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

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

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

MATERIALS AND METHODS

Fish and Sperm Sampling

Sterlet (*A. ruthenus*) was selected as a model sturgeon species. All experiments were performed according to the principles of the Ethical Committee for the Protection of Animals in Research of the University of South Bohemia, Research Institute of Fish Culture and Hydrobiology, Vodnany, Czech Republic. Experiments were conducted in the experimental facilities of Faculty of Fisheries and Protection of Waters, University of South Bohemia in Ceske Budejovice, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Vodnany, Czech Republic. During the natural spawning season (April–May), 5 sterlet males (3–4 yr and 0.6–1.0 kg BW) were transferred from aquaculture ponds (water temperature 8–10°C) into a 0.8-m^3 closed water recirculation system. The system water temperature was increased to 15°C within 24 h, and before experimentation, fish were held 4 d without feeding.

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

Sample Preparation

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

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

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

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

Activation of Spermatozoa

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

Motility Analysis

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

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

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

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

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

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

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

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

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

Statistical Analysis

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

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

RESULTS

Motility Parameters

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

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

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

Macroergic Phosphates Content during In Vitro Maturation of Sterlet Spermatozoa

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

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

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

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

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

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

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

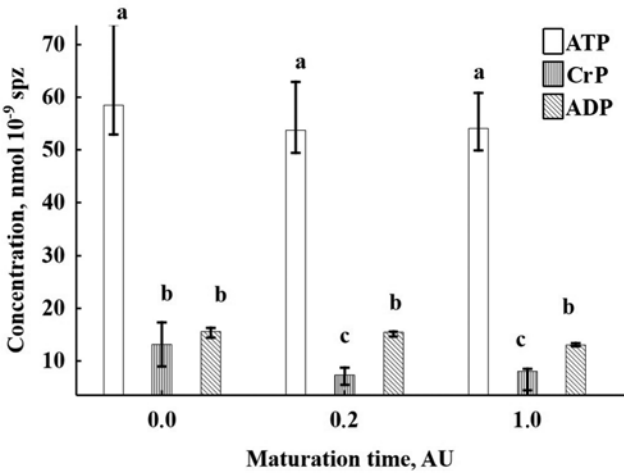

Figure 1. Adenosine triphosphate, creatine phosphate (CrP), and ADP content during the maturation of testicular sperm after dilution in seminal fluid ($n = 5$). [a–c]Columns with different letters differ significantly as determined by the nonparametric Kruskal–Wallis ANOVA test ($P < 0.05$). spz = spermatozoa;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

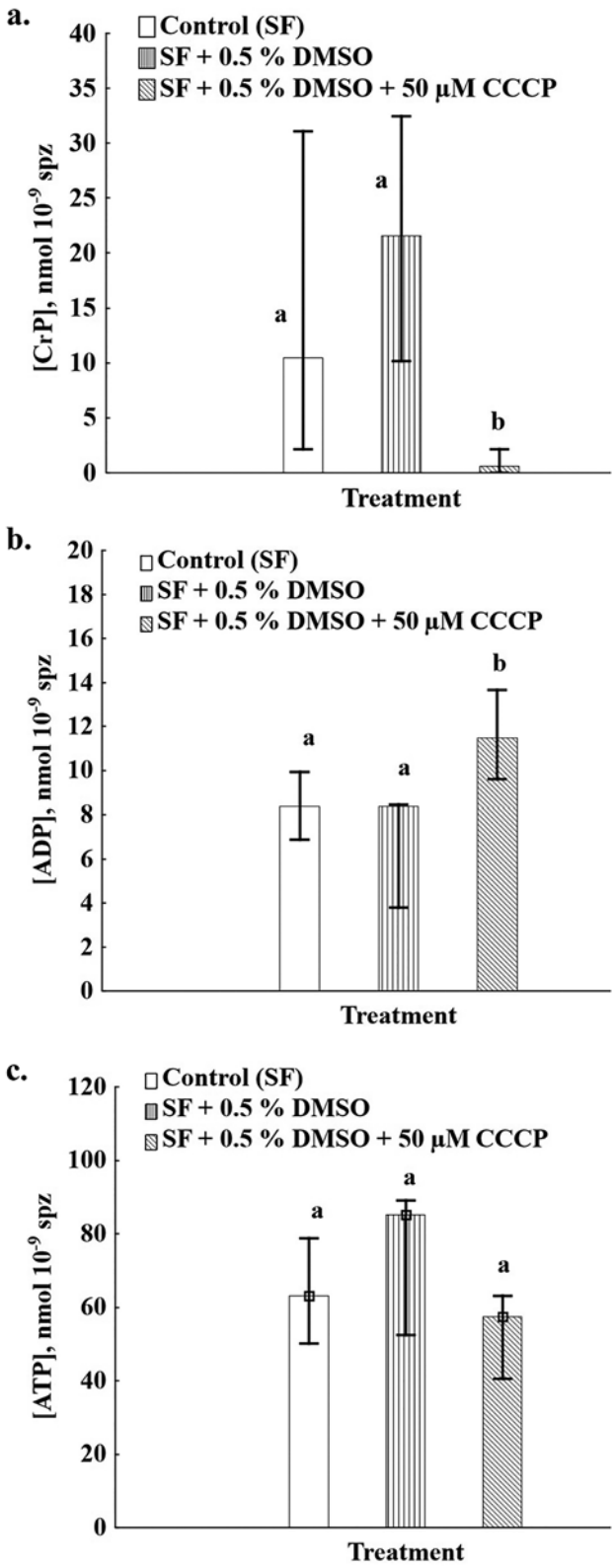

Figure 2. Creatine phosphate (CrP; a), ADP (b), and ATP (c) content during the maturation of testicular sperm after dilution in seminal fluid (SF) and treatment with dimethyl sulfoxide (DMSO) and carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP; $n = 5$). [a,b]Columns with different letters differ significantly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

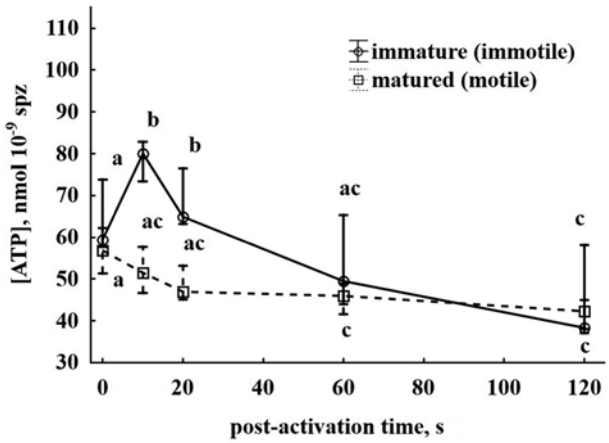

Figure 3. Adenosine triphosphate content of immature sperm (0 arbitrary units [AU] incubation time with seminal fluid [SF]) and matured sperm (1 AU incubation time with SF) after dilution with activation medium ($n = 5$). [a–c]Values with different letters differ significantly as determined by the nonparametric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

DISCUSSION

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

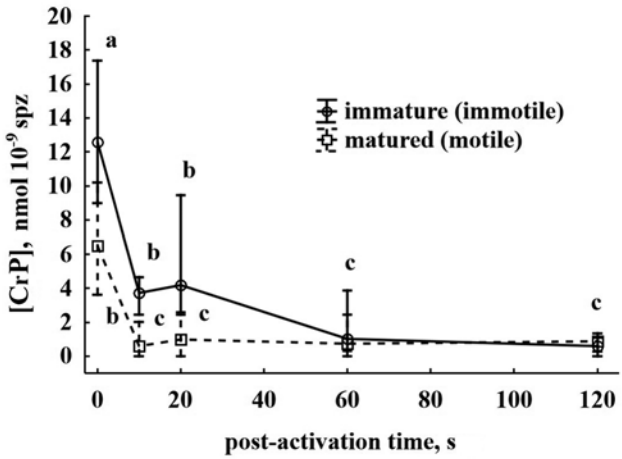

Figure 4. Creatine phosphate (CrP) content of immature sperm (0 arbitrary units [AU] incubation time with seminal fluid [SF]) and matured sperm (1 AU incubation time with SF) after dilution with activation medium ($n = 5$). [a–c]Values with different letters differ significantly as determined by the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

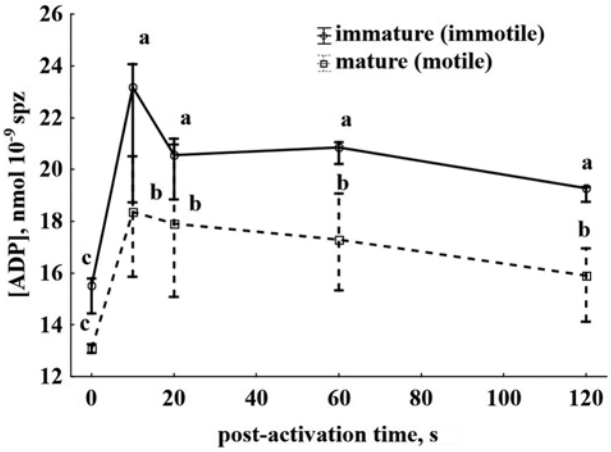

Figure 5. Adenosine diphosphate content of immature sperm (0 arbitrary units [AU] incubation time with seminal fluid [SF]) and matured sperm (1 AU incubation time with SF) after dilution with activation medium ($n = 5$). [a–c]Values with different letters differ significantly as determined by the nonparametric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

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

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

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

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

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

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

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

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

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Attachment 7

Dzyuba, B., Boryshpolets, S., Cosson, J., Dzyuba, V., Fedorov, P., Saito, T., Psenicka, M., Linhart, O., Rodina, M., 2014. Motility and fertilization ability of sterlet *Acipenser ruthenus* testicular sperm after cryopreservation. Cryobiology 69: 339-341 (IF 2014 = 1.587).

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Brief Communication

Motility and fertilization ability of sterlet *Acipenser ruthenus* testicular sperm after cryopreservation ☆

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ABSTRACT

Sturgeon spermatozoa are immotile in the testis and acquire the potential for motility after contact with urine in Wolffian duct. The present study tested if *in vitro* incubation of testicular sperm in seminal fluid from Wolffian duct sperm leads to the acquisition of sperm fertilization ability. Sterlet sperm was taken from the testes, matured *in vitro* and cryopreserved. The fertility and motility of cryopreserved semen were tested. Matured testicular sperm showed freeze–thaw survival rates similar to Wolffian duct sperm, which is commonly used in sturgeon artificial propagation. Matured testicular sperm and Wolffian duct sperm post-thaw motility rate and curvilinear velocity were not significantly different, while duration of matured testicular sperm motility was significantly shorter than that of Wolffian duct sperm. Development rates of embryos obtained with post-thaw matured testicular sperm and Wolffian duct sperm were not significantly different. *In vitro* maturation of sterlet testicular sperm can potentially be useful in sperm cryobanking.

Use of testicular sperm is required in artificial reproduction when collection of ejaculated sperm leads to obtaining of sperm of low quantity or in case of the death of the donor. Testicular sperm is commonly used in culture of some fish species, such as catfish [7]. Postmortem collected sperm is a source of viable genetic material for fish sperm cryobanking [9]. However, two problems are linked with the use of testicular sperm: (1) The collection of non-fully mature sperm because of the maturation process along the genital ducts, (2) The aging of sperm in the case of the death of the breeder. Sturgeons represent a group of highly exploited fish species that are considered threatened or endangered [8]. Expansion of cryobiological methods for preservation of sturgeon testicular sperm is needed and potentially useful for application in aquaculture and species conservation.

In sturgeon, testicular spermatozoa do not become motile upon transfer to, and dispersion in, fresh water. That is associated with specific anatomy of excretory pathways – the efferent ducts coming from the testes directly contact the kidneys, and testicular sperm is diluted by urine in Wolffian ducts. Recently we have demonstrated that a maturation step is necessary for sturgeon spermatozoa to acquire the potential for activation, and this step takes place exactly in Wolffian duct (from which mature sperm is collected by catheterization for practical purposes) [4]. Testicular sturgeon spermatozoa maturation can be simulated *in vitro* by pre-incubation in a urine-containing solution such as seminal fluid obtained from Wolffian duct sperm or urine itself. However, it is not clear whether spermatozoa matured *in vitro* possess fertilizing ability and could retain the ability after cryopreservation. Motility itself is not sufficient for sturgeon spermatozoa fertilizing ability because of the presence of an acrosome and dependence on acrosome reaction [10]. Investigation of these questions is important for understanding the processes involved in sturgeon sperm maturation and could be applied to expand existing methods of sturgeon artificial propagation.

The goal of this study was to investigate the cryoresistance and post-thaw fertilizing ability of sturgeon testicular spermatozoa subjected to a maturation phase before and after cryopreservation.

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0011-2240/

Sterlet *Acipenser ruthenus* was selected as a model sturgeon species as procedures of *in vitro* gamete manipulation and fertilization are widely applied in artificial propagation of this species.

All experiments were performed according to the principles of the Ethics Committee for the Protection of Animals in Research of the University of South Bohemia in Ceske Budejovice. Experiments on sperm were conducted in experimental facilities of Faculty of Fisheries and Protection of Waters, University of South Bohemia in Ceske Budejovice, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Vodnany, Czech Republic.

During the natural spawning season (April – May), 6 sterlet males (3–4 years old, 0.6–1.0 kg body weight, BW) were transferred from fish-farming ponds (water temperature 8–10 °C) to a 0.8 m^3 closed water recirculation system. The water temperature in closed water recirculation system was increased to 15 °C over the course of 24 h. Prior to initiation of experiments, fish were held 4 days without feeding.

Spermiation was stimulated by a single intramuscular injection of carp pituitary powder dissolved in 0.9% (w/v) NaCl solution (4 mg/kg BW) [3]. Twenty four hours post-stimulation, spermatozoa were collected from the urogenital ducts by aspiration using a 4 mm plastic catheter connected to a 10 ml syringe (Wolffian duct sperm, commonly used in fisheries for artificial sturgeon propagation). Immediately after Wolffian duct sperm collection, the fish was euthanized by a blow to the skull and exsanguination. The digestive tract was removed, and testicular sperm was collected via incision of the efferent ducts [1].

Seminal fluid was obtained from Wolffian duct sperm centrifugation at 4 °C 300*g* for 10 min. The supernatant was collected and centrifuged at 5000*g* for 15 min. Supernatants obtained from the second centrifugation were used in the experiments.

For maturation, testicular sperm was incubated for 25 min in the seminal fluid at a dilution rate of 1 vol of testicular sperm to 50 vol of seminal fluid [4]. Wolffian duct sperm and matured testicular sperm were frozen using conventional freezing procedures: prior to freezing the samples were diluted 1:1 in an extender composed of 23.4 mM sucrose, 0.25 mM KCl, and 30 mM Tris–HCl, pH 8.0 [5] containing 10% methanol (methanol concentration after sperm dilution is 5%). Diluted sperm was placed in 0.5 ml straws (CRYO-VET, France) and suspended 3 cm above liquid nitrogen in a Styrofoam box for 10 min and then plunged into the liquid nitrogen. Sperm solution was thawed for 6 s in a 40 °C water bath. Thawed sperm was used immediately for motility or fertilization assays.

To investigate whether premature spermatozoa survive cryopreservation, testicular sperm was frozen after 1 min incubation in seminal fluid from Wolffian duct sperm, a period insufficient for sperm maturation. Samples were frozen and thawed as described above. Motility of these samples was evaluated immediately after thawing and after 25 min post-thaw storage. Fertilizing ability of spermatozoa immediately after thawing was also evaluated.

For triggering motility, sperm samples were diluted at 1:100 in an activation medium consisting of 10 mM Tris–HCl buffer, pH 8.0, containing 0.25% Pluronic® F-127 (SIGMA-ALDRICH, catalogue number P2443) to prevent spermatozoa from adhering to microscope slides. Sperm suspensions were thoroughly mixed for 2 s. Motility was recorded for 1–2 min post-activation using video microscopy combined with stroboscopic illumination (ExposureScope®, Czech Republic). Video records were analyzed to estimate spermatozoa curvilinear velocity (VCL) and percent of motile cells (motility rate) by micro-image analyzer (Olympus Micro Image 4.0.1. for Windows, Japan) on five successive overlapping video frames. After overlapping of frames, tracks of spermatozoa heads became visible, permitting calculation of VCL (defined as total point-to-point distance traveled by the spermatozoon in 0.16 s, the time period between the first and fifth frames) and motility rate [3]. Sperm motility duration was measured as the time from activation to cessation of motility in approximately 95% of spermatozoa. Spermatozoa concentration was estimated using a Burker cell hemocytometer (Meopta, Czech Republic) at 200× magnification on Olympus BX 50 phase contrast microscope (Olympus, Japan).

For evaluation of fertilization and embryo development, sterlet females were injected with carp pituitary powder dissolved in 0.9% (w/v) NaCl solution 36 h (0.5 mg kg^{-1}) and 24 h (4.5 mg kg^{-1}) before stripping [2]. Eggs from three females pooled in equal parts (2 g, approx 140 eggs) were inseminated in a Petri dish with sperm immediately after thawing. Based on the spermatozoa concentration of the sample, the volume of sperm was adjusted to obtain a 10^5 spermatozoa/egg ratio. Gametes were activated with 8 ml hatchery water, and, after 1 min, ova were transferred to an incubator with aerated, dechlorinated, and UV-sterilized tap water at 16 °C. Living (transparent, with visible embryo inside) and dead (opaque, whitish) eggs were counted in each Petri dish during incubation and dead eggs were removed. Live embryos were counted after the second cleavage division at 4 h post-fertilization to calculate fertilization rate, and at the eyed stage at 72 h post-fertilization to calculate development rate. Fertilization and development rates were expressed as the proportion of live embryos at corresponding post-fertilization times of the initial number of eggs incubated according to recommendations for sturgeon fishery practices according to Dettlaff et al. [2].

Table 1
Motility parameters of Wolffian duct and testicular spermatozoa after maturation before and after freeze/thaw.

Sperm sample	Motility (%)	VCL (μm s^{-1})	Motility duration (s)
Wolffian duct sperm			
Fresh	92 ± 8[a]	187 ± 25	125 ± 38[a]
Frozen/thawed	57 ± 11[b]	185 ± 37	117 ± 15[a]
Matured testicular sperm			
Fresh	80 ± 9[a]	187 ± 21	54 ± 13[b]
Frozen/thawed	48 ± 16[b]	195 ± 31	48 ± 8[b]
No matured testicular sperm			
Fresh	0*	0	0
Frozen/thawed	0	0	0
Frozen/thawed after 25 min post-thaw storage	0	0	0

Values with different letters are significantly different ($P < 0.05$, Mann–Whitney *U*-test with Bonferroni correction). No significant differences were found in VCL (ANOVA, $P = 0.37$).

*Samples of no matured testicular sperm, showing no motility, were not included into statistical analysis to avoid artificial decrease of sensitivity of statistical method applied.

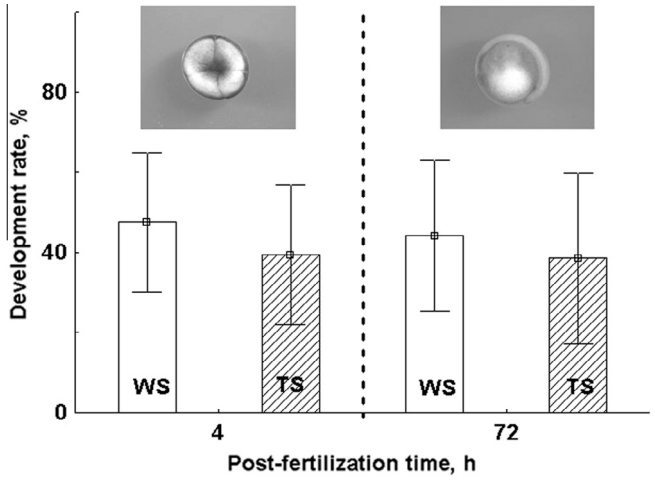

Fig. 1. Development rate of sterlet embryos obtained by frozen–thawed Wolffian duct sperm (WS) or testicular sperm after *in vitro* maturation (TS). The photos of developing embryo at 4 and 72 h post-fertilization are shown above the columns. No significant difference among groups was found ($P = 0.48$,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

Data distribution and homogeneity of dispersion were tested by the Shapiro–Wilks test and Levene's test, respectively. Normally distributed data were analyzed by ANOVA followed by Fisher's LSD test. Due to a low number of observations ($n = 6$), a nonparametric Kruskal–Wallis test followed by the Mann–Whitney *U*-test with Bonferroni correction was used for comparison of motility rate, motility duration, and fertilization and development rates. Data are presented as mean ± SD. Statistical significance was accepted at $P < 0.05$. All analyses and plotting were conducted using Statistica v 9.1 (Statsoft Inc, Tulsa, OK, USA).

We confirmed that testicular sturgeon spermatozoa acquire the potential for activation only after pre-incubation in seminal fluid from Wolffian duct sperm. In fresh sperm, the motility rate and VCL of matured testicular sperm in the initial period of motility were not significantly different from that in Wolffian duct sperm (Table 1). These results are in accordance with those of our previous study [3]. However, motility duration of matured testicular spermatozoa was significantly shorter than observed for Wolffian duct spermatozoa. This finding could not be explained from the limited existing data. As sperm motility is an ATP consuming process [6], we propose that future studies include investigation of bioenergetic pathways affected by sturgeon sperm maturation.

Testicular sperm frozen after 1 min pre-incubation in Wolffian duct seminal fluid did not become motile either immediately after thawing or after a post-thaw incubation period. A fertilization test conducted with those sperm samples resulted in no normally developing embryos.

Matured sterlet testicular spermatozoa showed freeze–thaw survival similar to Wolffian duct sperm. Their motility rate and VCL were not significantly different while motility duration was significantly lower (Table 1). However, the shorter motile period did not appear to be a limiting factor for post-thaw fertilizing ability, as development rates of embryos obtained with testicular and Wolffian duct sperm were not significantly different ($P = 0.48$, Kruskal–Wallis test, Fig. 1). The absence of significant differences between embryo development rates at 4 and 72 h post-fertilization together with previously published results of similarity of hatching rate and development rate at 72 h post-fertilization [3] indicate high fertilizing ability. Thus, we observed that with *in vitro* maturation, sterlet testicular sperm acquires fertilizing ability, which, after the freeze/thaw process, is not different from that of Wolffian duct sperm. These results constitute a solid base for development of methods allowing the use of testicular sperm in cases such as accidental death of valuable broodstock being at spermiating stage or failure to obtain Wolffian duct sperm required for artificial sturgeon propagation in manner similar to practice with other fish species [7].

Acknowledgments

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Attachment 8

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CryoLetters **23**, 353-360 (2002)

RELATIONSHIP BETWEEN THE CHANGES IN CELLULAR VOLUME OF FISH SPERMATOZOA AND THEIR CRYORESISTANCE

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Abstract

We have investigated the hypothesis that the spermatozoa of marine fish are more resistant than freshwater species to the dynamic changes in osmotic pressure that occur during the process of cryopreservation. We show that while the spermatozoa of marine fish can be successfully activated across a wide range of osmotic pressures (0-2000 mOsmol/l), those of the freshwater species only survive activation within a more restricted range (0-300 mOsm/l). After freeze-thawing, up to 30% of motile cells were found in silver carp samples, while up to 90% of motile cells were observed in samples from the haarder (*Mugil soiuy* B). Haarder spermatozoa showed no change of cell volume after dilution in activating or cryoprotective media, while the silver carp spermatozoa responded by swelling and eventual cell disruption. We propose that the differences in cryoresistance of silver carp (*Hypophthalmichthys molitrix* V.) and haarder spermatozoa may be determined by the ability to preserve cellular volume under non-isotonic conditions.

Keywords: Sperm, freeze-thawing, cellular volume, haarder, silver carp

INTRODUCTION

The factors which determine species differences in the stability of fish spermatozoa during cryopreservation are unclear (2). As a result the systematic development and use of cryopreservation methods for fish breeding and conservation is difficult. Attempting to identify logical and biologically-based reasons which define the general relationship between peculiarities of sperm activation and cryoresistance between these species may offer useful guidance when designing suitable cryopreservation procedures.

Spermatozoa of fish with external fertilisation begin to move actively only after release into the external environment and consequently the mechanisms of motility activation in various fish groups differ considerably (3,9,18). Initiation of motility of carp *Cyprinus carpio* L. spermatozoa occurs under hypotonic conditions, resulting in the activation of ionic K^+ channels accompanied by cellular swelling (12,19). For spermatozoa from marine species of haarder the distinct mechanisms of movement activation have not been described, but it was found that this process was not related to cell volume changes (8).

It is known, that in the course of freeze-thawing, cells are subjected to considerable changes in osmotic pressure of the external medium; these are likely result in changes of cellular volume (14,15). We propose a hypothesis that marine fish spermatozoa would be more resistant to cryopreservation because of their resistance to cell volume changes during activation. The aim of this work was to determine the relationship between the ability of fish spermatozoa to survive freeze-thawing and their ability to preserve cellular volume following exposure to solutions of different osmotic pressure.

MATERIALS AND METHODS

Source of spermatozoa

Spermatozoa of the silver carp, *Hypophthalmichthys molitrix* V., were obtained from mature males of 3-5kg body weight, 24 hr after injection with 0.2 ml suspension, containing 150 mM NaCl and carp lyophilised pituitary powder (dose rate, 5 $mg \cdot kg^{-1}$ body weight).

Haarder *Mugil soiuy* B. spermatozoa were obtained from mature males without preliminary hormonal injections, during the natural spawning period in Azov Sea region.

The milt was stripped by abdominal massage into dry 20 ml vials and stored during the experiment (up to 2 hrs) in a water bath at a temperature of $5\text{-}7^0$C.

For this work we used milt which exhibited a minimum sperm motility not lower than 80%.

Activating media

To study activation of sperm movement we used various concentration of NaCl in 10 mM Tris-HCl buffer, pH 8.0. NaCl (50 mM) and tris-HCl (10 mM), pH 8,0 was used (10) for activation of movement of silver carp sperm after freezing-thawing. For activation of the movement of haarder spermatozoa after freeze-thawing we used a solution, containing 500 mM NaCl and 10 mM Tris-HCl, pH 8.0 (8).

Sperm microscopic investigations

Spermatozoa motility was visually determined at the start of activation using a microscope at 1x200 magnification, and expressed as the percentage of forwardly moving cells in relation to the total number of cells in the field of view of the microscope. For this purpose, the spermatozoa were diluted (1:400) in the media under study. In addition, the time-period within which about 95% of cells terminate their movement (t_m) was recorded. Independent observers made observations and counted the percentage of motile spermatozoa.

Optical density measurement

The measurements of optical density were accomplished using a recording photoelectrocolorimeter KF-77 (Poland), equipped with thermostatically controlled cell chamber and magnetic stirrer, in a 1cm cell at the wavelength of 610 nm. To measure optical density the spermatozoa were diluted 1:200 v/v into the medium under study, and in this case the cell concentration in the suspension reached 1-2 x 10^7 cells/ml. Cell concentration was determined with Goryaev's chamber. The measurements were carried out in the following way: 10 µl of native sperm was added into the cuvette with 2 ml of the solution studied, then the cell was intensively shaken for 2 seconds to obtain a homogeneous suspension and placed into the cell chamber of the photoelectrocolorimeter.

For numeric characteristics of cellular volume change in carp spermatozoa activated before and after freeze-thawing the value of relative change in optical density (D_v) was calculated (7) using the following equation:

$D_v = (D_1-D_2) / D_1$,

where D_1 - value of optical density just after activation

D_2 - value of optical density at the termination of the process of change in optical density.

Sperm cryopreservation procedure

For sperm cryopreservation we used the method developed earlier for carp sperm. Native spermatozoa (milt) were diluted into saline cryoprotective medium, containing 16% DMSO, without adding egg yolk and was frozen in 1.5 ml plastic ampoules according to the following programme (10):

Cooling to -12ºC at a rate of 2-3ºC /min;

from -12^{0}C to -70ºC at a rate of 30ºC /min;

once the temperature fell below -70ºC the samples were immersed in liquid nitrogen.

Sperm ampoules were thawed in a water bath at 40ºC for 1 min (10).

Within 1 min after thawing the spermatozoa were diluted in activating solution (see *Activating media*) and the percentage of motile cells was determined.

Statistical analysis

During statistical processing mean values of the parameters under study and their standard deviations were calculated. The results are presented as mean values (Dv, T_m, percentage of motile cells) plus/minus standard deviations and number of measurements. Student's *t*- test was used to confirm the statistical significance of the differences at $P \leq 0.05$.

RESULTS

Determination of the ranges of NaCl concentration in activating solutions

The ranges of NaCl concentration were determined for activating spermatozoa motility solutions. For spermatozoa of silver carp the motility range was observed in 0-150 mM NaCl. A significant reduction ($P \leq 0.05$) in the percentage of motile cells was observed when using activating solution, containing 125 mM NaCl (Fig.1, curve 1). When using 150 mM NaCl solution there was no motility activation observed. Spermatozoa of the haarder became motile in the solutions with NaCl concentrations in the range of 0 - 1000 mM; in this case the maximum cell motility for haarder was observed in the solutions with 500 mM NaCl concentration (Fig 1, curve 2).

Spermatozoa started to move immediately after being transferred into activating media. After the movement period, which differed for every activating medium studied, most cells stopped and only a few individuals continued to move. We therefore defined t_m (see Materials and Methods) as the value characterising the duration (in minutes) of sperm movement. The results for spermatozoa of the silver carp and haarder in activating solutions with NaCl various concentrations are shown in Table 1.

Table 1. Duration of motility for freshly collected spermatozoa of silver carp and haarder in various activating media (t_m , min)

Species	Concentration NaCl, (m?)							
	0	50	100	150	250	500	750	1000
Haarder	1,6±1	-	-	-	3±1*	7±0,5*	3±1*	1±0,5*
Silver carp	0.3±0,1	2,5±0,5*	1,5±0,1*	0	0	0	0	0

Legends: * - statistically true differences when comparing them with each previous column (n=10, $P \leq 0.05$)

The movement time for haarder increased significantly with the rise in NaCl concentration up to 500 mM and then reduced as the concentration was increased further. By comparison, silver carp spermatozoa were activated in narrower range of concentrations, 0-125 mM NaCl. Maximum of movement time was observed under NaCl concentration of 50 mM.

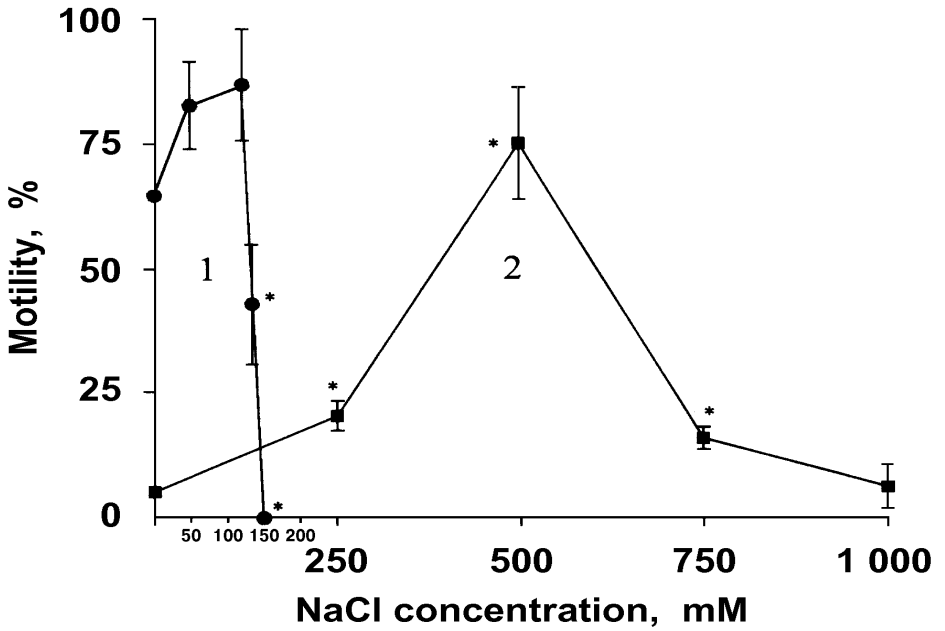

Figure 1. Sperm motility for silver carp and haarder in the solutions of NaCl under various concentrations. Legends: 1- silver carp; 2- haarder.

Investigations of optical density of sperm suspension in motility period

The optical density of haarder sperm suspension only slightly changed its value in the activation period for both samples native and survived after cryopreservation. Optical density of silver carp sperm suspension changed considerably. The extent of these changes depended on the osmotic pressure of the activating solution. Typical behaviour of optical density of spermatozoa suspension in various experimental variants is presented in Figure 2.

Results of freezing-thawing

After freezing-thawing samples of spermatozoa from harder, not less than 70% of motile cells (80+10%, n =12) was observed. In the samples of silver carp sperm not more than 30% of motile cells was observed (15±10%, n=15). After freezing-thawing of samples of spermatozoa from 15 silver carp males it was determined that in six samples the cell motility was not higher than 10% (5±3%, n =6) – designated group A, and in seven not less than 20% (24±4%, n=7) – designated group B, the remaining two males could not be assigned to either of these groups because the motility values (approximately 15%) were between these two ranges of motility.

The value of D_v for silver carp sperm increased significantly after freezing-thawing ($P \leq 0{,}05$). However no significant differences in the D_v values were observed between the groups A and B, described in the item 3) (Fig.3).

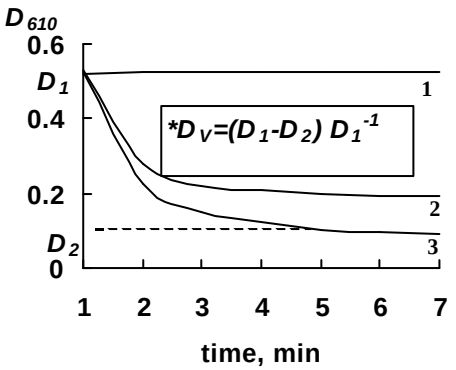

Figure 2. Typical dynamics of optical density in fish sperm suspension Legends: 1- for haarder in any considered solutions, 2- for silver carp in 50 mM NaCl in control, 3- for silver carp in 50 mM NaCl after freezing-thawing. *- the formula for Dv calculation

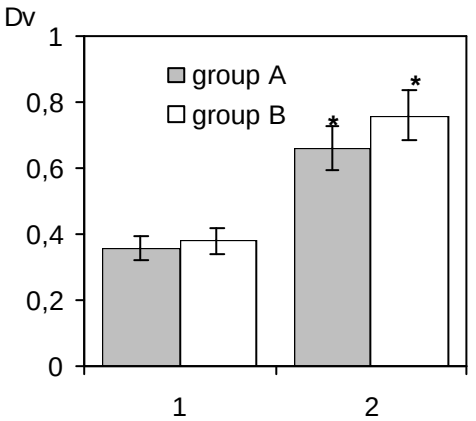

Figure 3. Dv values for silver carp samples with different percentage of motile cells after freeze-thawing. Legends: 1-control, 2- after freeze-thawing; * - statistically significant differences in comparison with the control (*P* =0,05); group A - frozen-thawed samples with cell motility not higher than 10% (5±3%, n=6), group B - frozen-thawed samples with cell motility not less than 20% (24±4%, n=7).

DISCUSSION

Data presented in the work testify to a significant difference in osmolality of activating solutions for spermatozoa between the haarder and the silver carp. It should be noted that the osmotic pressure of seminal fluid of marine and fresh water fishes is within the range of 290-400 mOsmol/l (21). Thus a distinguishing feature of haarder spermatozoa is that activation of these cells is possible not only within a wide range of hypertonic external conditions, typical for marine species (9,18), but also under hypotonic conditions (Fig.1). Low percentages of motile spermatozoa of haarder and the low value of movement time (Table 1) under hypotonic conditions point to the fact that these conditions are not optimal for motility activation in this species. However it should be noted that such a wide range of osmolality of activating media for haarder demonstrated activation under both hyper- and hypotonic

conditions. Such a "combined" activation is abnormal for fish sperm motility. This is probably due to the fact that haarder is eurohaline species. This is testified by the history of its introduction into Azov Sea from the Pacific Ocean (water salinity for the Pacific Ocean is twice higher). In addition, it is possible to breed the species in fresh water ponds (20).

The determined limits of osmolality for activating media (Fig. 1, curve 1) point to the fact that silver carp spermatozoa are capable of being activated under conditions identical to those for spermatozoa of carp (12,19). Hypotonic conditions of activation of the movement of silver carp spermatozoa are characteristic for fresh water fish (6,9,17).

These peculiarities of activation of marine and fresh water fishes are likely related to considerable differences in the biochemical composition of the plasma membrane of these cells. A previous paper (4) showed that in membranes of marine fish spermatozoa there is a higher ratio of cholesterol to lipids in comparison with fresh water fish. These differences may confer a high degree of cryoresistance to marine fish spermatozoa. Thus the observed differences between cryoresistance of spermatozoa of the haarder and the silver carp are the most vivid example of the described above differences between cryoresistance for marine and fresh water species. However the inverse relationship between the ratio of cholesterol to membrane total lipids and cryoresistance is observed during attempting to explain the differences within one species in rainbow trout sperm cryoresistance (13). So additional studies of this question are needed.

We attempted to reveal the causes of the relationship of differences in the activation conditions, swelling degree within motility period and cryoresistance of spermatozoa of studied species. The paper (7) describes in details the method we selected for measurement of cell swelling degree. For carp sperm we have shown that a reduction of optical density of the suspension in motile period was related to cell swelling and the usage of D_v value was possible as the index of the degree of swelling. Thus the behaviour of optical density of spermatozoa suspension is the index of cellular volume changes.

Therefore the results (Fig. 2) of measurements of sperm suspension optical density allow to determine, that in activating media before and after freezing the spermatozoa of haarder and silver carp considerably differ in the dynamics of cellular volume change. We suppose that the absence of changes in optical density for haarder in all considered activating media before and after freeze-thawing is stipulated by the stability of cellular volume (Fig.2, curve 1). In contrast, optical density of silver carp sperm suspension in activating media decreased due to cell swelling (Fig. 2, curve 2).

Measurements of movement time (Table 1) and optical density (Fig. 2, curve 2) for silver carp spermatozoa showed that main changes in cellular volume take place exactly within the period of active movement.

It has previously been established, that during freeze-thawing the cells are subjected to large variations of osmotic pressure, which result from both dilution by hypertonic cryoprotective media and the processes of freezing-out of intra- and extracellular water (14,15,16). In this connection a high cryoresistance of haarder spermatozoa is explained by the ability of these cells to survive the differentials of osmotic pressure without changes in cellular volume. Freezing-thawing increases the swelling extent of silver carp sperm, that manifests in an enhanced difference between optical density in the start and the end of motile period.

Taking into account the data (4) we propose, that the higher ratio of cholesterol to lipids in membranes of marine fish spermatozoa, compared to those from fresh water ones, confers a high degree of their ability to survive differentials of osmotic pressure both during motility activation and freezing-thawing. So sperm of marine fish species is characterised by higher cryoresistance.

It is known that adding of egg yolk to cryoprotective medium results in a rise in carp spermatozoa motility after freezing approximately twice. Therefore, for maximum manifestation of the differences in cryoresistance between the samples of silver carp sperm, we carried-out their freezing without these additives (11).

The individual differences of sperm cryoresistance observed in the work are typical for fish in general (1,2) and for silver carp in particular (5). It was an attempt to establish a relationship between individual characteristics of physiologically essential swelling in activation period and silver carp spermatozoa cryoresistance.

Conducting of the studies on the relationship between the degree of cell swelling in motile period (characterised by D_v value) and cryoresistance of silver carp spermatozoa did not allow us to establish a correlation between these parameters (Fig.3). We found that degree of swelling in this species during sperm activation period after freezing-thawing statistically and significantly increased (see evident D_v differences between the control samples and those after freeze-thawing in Fig.3). This permitted us to suppose that this phenomenon was related to cryodamage in these cells and resulted in a reduction of cells' ability to control cellular volume under hypotonic conditions of activating media.

Thus we revealed that a high degree of cryoresistance of haarder spermatozoa was related to the ability to survive considerable changes of osmotic pressure without altering their cellular volume. The question on relationship between silver carp spermatozoa swelling and their related cryoresistance is still open. So we can conclude that such a relationship exists between two species and there is no one within one species.

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Attachment 9

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

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Summary

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

Introduction

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

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

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

Materials and methods

Fish

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

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

Gametes collection

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

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

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

Sperm cryopreservation

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

Evaluation of motility percentage and velocity

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

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

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

Data presentation and statistical analysis

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

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

Results

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

Sperm velocities and motility percentage in species possessing spontaneous motility activation

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

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

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

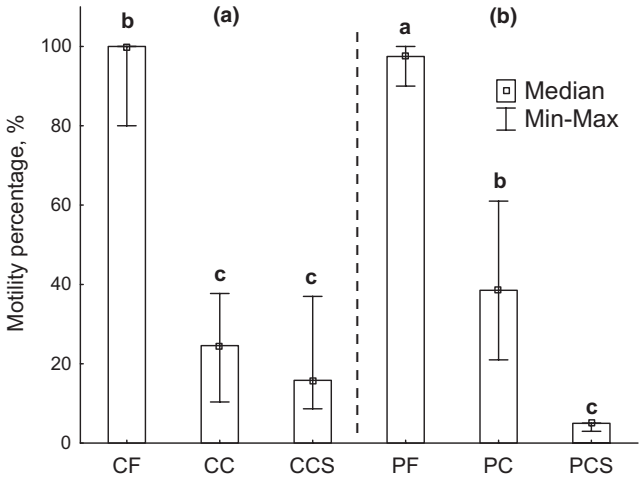

Fig. 1. Motility percentage before and after freeze-thawing in carp (a) and perch (b). CF, carp fresh sperm, hypotonic activation before freezing; CC, carp sperm hypotonic activation after cryopreservation; CCS, carp sperm spontaneous activation; PF, perch fresh sperm hypotonic activation before freezing;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

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

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

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

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

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

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

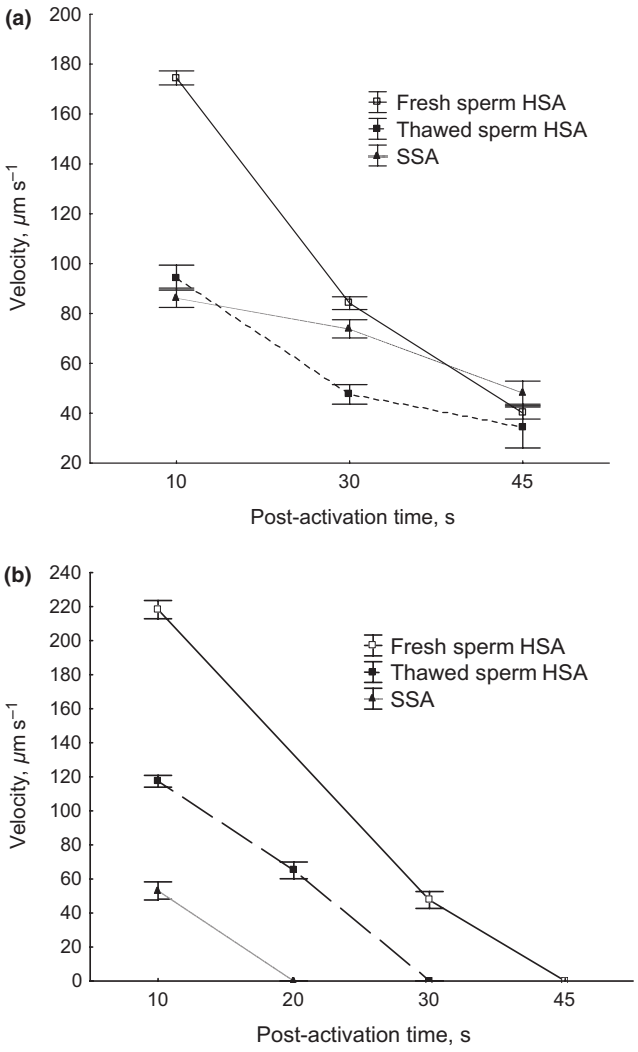

Fig. 2. Spermatozoa velocities during spontaneous and hypotonic activations in carp (a) and perch (b) before and after freeze-thawing. SSA, spontaneous sperm activation; fresh sperm HSA, hypotonic activation of sperm before freezing; thawed sperm HSA, hypotonic activation (dilution with AM) of thawed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

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

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

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

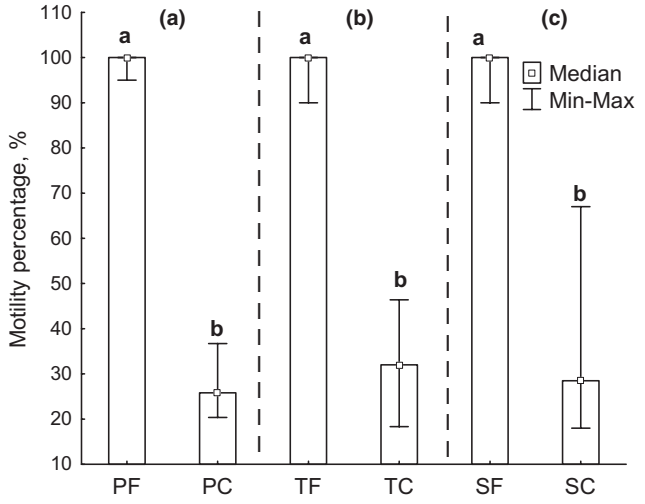

Fig. 3. Motility percentage in pike (a), rainbow trout (b) and sterlet (c) sperm before and after cryopreservation. PF,PC, pike fresh and thawed sperm, correspondingly; TF,TC, rainbow trout fresh and thawed sperm, correspondingly; SF,SC, sterlet fresh and thawed sperm, correspondingly. The values with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

Discussion

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

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

We studied the changes in sperm velocity after thawing in relation to the presence of spontaneous sperm activation. The absence of differences between motility percentage (Fig. 1a) and velocity (Fig. 2a) in SSA and HSA for carp thawed sperm

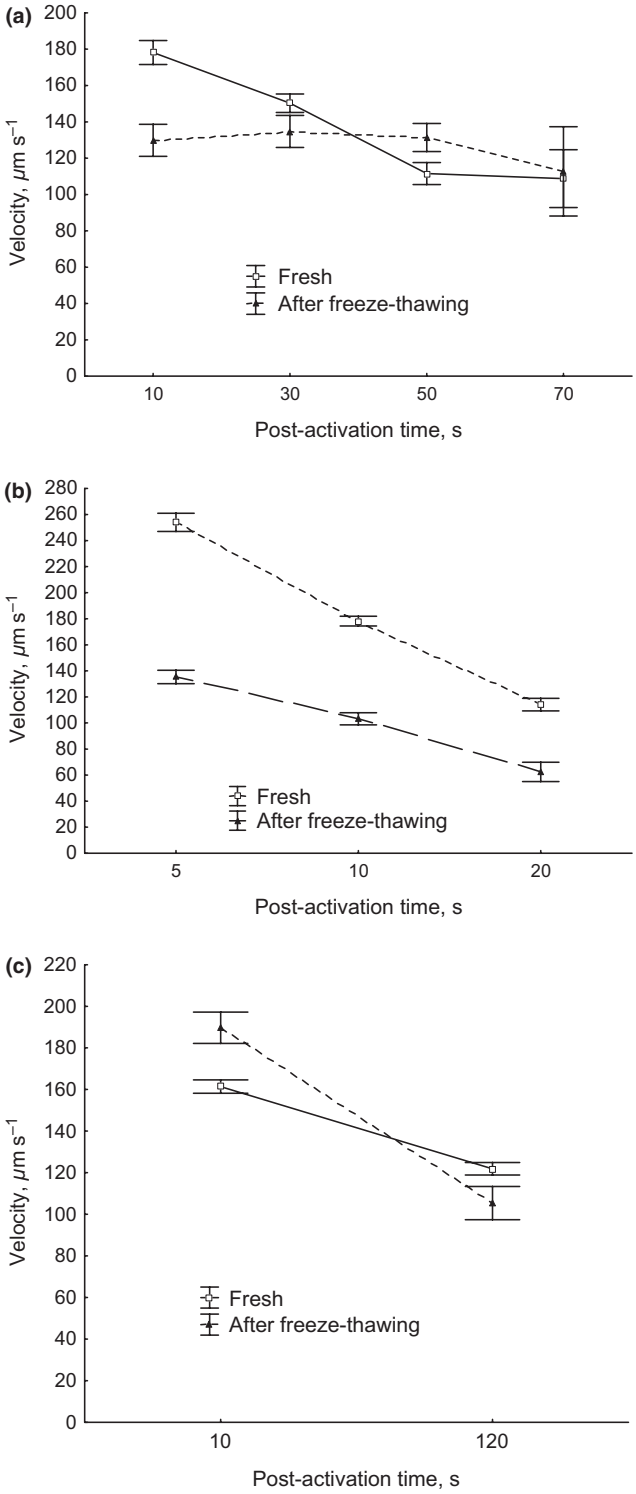

Fig. 4. Spermatozoa velocities during hypotonic activations in pike (a), rainbow trout (b) and sterlet (c) before and after freeze-thawing. Results are presented as mean ± 0.95 CI (Values with not overleaping whiskers are significantly different, HSD-test, $P < 0.05$)

suggested that in carp all the spermatozoa that possesss the ability for motility acquisition after cryopreservation are subjected to SSA. In perch freeze-thawing led to SSA just in part of sperm, since we were able to activate more cells under hypotonic conditions (Fig. 1b). In addition, this small part of activated sperm started motility with lower velocity (Fig. 2b).

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

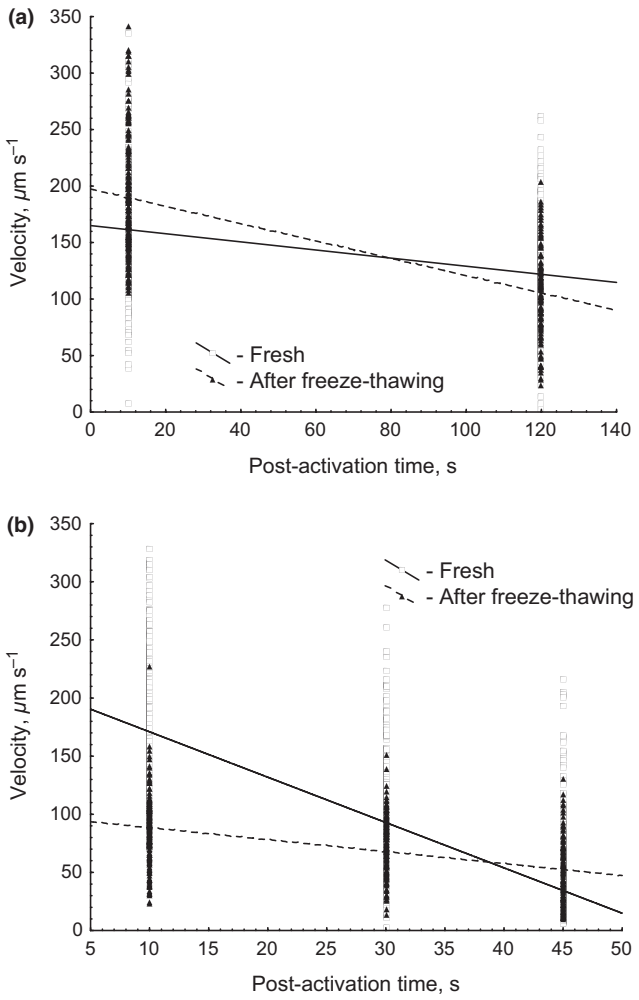

Fig. 5. Scatter plots of sperm velocities during post-activation time in sterlet (a) and carp (b) before and after cryopreservation

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

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

Conclusion

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

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Attachment 10

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Spermatozoa motility, cryoresistance, and fertilizing ability in sterlet *Acipenser ruthenus* during sequential stripping

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ABSTRACT

We describe spermatozoa characteristics from sequential collections in sterlet, *Acipenser ruthenus*, following a single dose of carp pituitary extract (CPE). Sperm production and spermatozoa fertilizing ability, percent motility, and curvilinear velocity (VCL) were investigated in fresh and frozen/thawed sperm. Sperm was collected by two procedures: (A) stripping 3 times per 24 h at 3 h intervals on 3 consecutive days beginning 12 h after CPE treatment, and (B) stripping 3 times over 6 h beginning 36 h after CPE treatment. Spermatozoa motility and VCL were evaluated by video microscopy, and sperm production was measured as volume and concentration. Sperm samples were frozen by a conventional freezing procedure in a cryoprotective medium containing 10% methanol. Fertilization was conducted using a ratio of 10^5 spermatozoa/egg. Both sequential stripping procedures yielded larger volumes of viable spermatozoa than did a single collection. Sperm parameters such as density and volume varied widely depending on collection time. The highest numbers of spermatozoa per individual were collected 15–42 h post-CPE treatment in (A) and at 42 h post-CPE treatment in (B) ($85 \pm 4\%$ and $64 \pm 5\%$ of total spermatozoa count, respectively). Median percent motility in spermatozoa before cryopreservation was 26–100% and 5–67% post-thaw. Fertilization rates obtained with frozen/thawed spermatozoa were 13–76% (median value). A significant increase in spermatozoa motility parameters and fertilizing ability at the second collection on each day was observed. Sequential stripping and spermatozoa cryopreservation in combination could improve the efficiency of sturgeon aquaculture.

1. Introduction

Sturgeon farming is considered a promising area of aquaculture with a growing world market for black caviar and sturgeon meat. Modern intensive sturgeon farming in Europe was predicted by Williot et al. (2001). Now worldwide production of cultured sturgeon has increased from 2500 metric tons in 1999 to 25,600 metric tons in 2008 (FAO, 2008), and aquaculture caviar production increased from 1.69 metric tons in 2003 to 27.32 metric tons in 2007 (Wuertz et al., 2009). Twenty-five of 27 sturgeon species are listed as extinct, critically endangered, endangered, or vulnerable (Billard and Lecointre, 2001). Thus sturgeon farming involves rare natural species and has potential for considerable development.

Although sturgeon farming has a history of more than one hundred years, the basis for intensive artificial reproduction and methods of *in vitro* gamete manipulation were developed only in the second half of the 20th century (Dettlaff et al., 1993). The importance of sperm storage to efficient husbandry was emphasized by Billard (2001). Several methods of hypothermic storage and cryopreservation have been developed for sturgeon sperm (Billard et al., 2004), allowing the use of sperm some time after collection. The effectiveness of male exploitation depends on collecting the maximum amount of spermatozoa with high fertilization capability that can be used immediately for direct egg fertilization or be cryopreserved for delayed fertilization. In sturgeon the possibility of repeat sperm collections after a single hormone treatment (sequential collections) has been extensively studied (Podushka, 2003). Sperm volume obtained through multiple collections at intervals of 2–3 h has been reported to be approximately fivefold that of a single collection, but no information about its quality or characteristics has been reported. Time intervals of 12 h (Alavi et al., 2006) and 24 h (Linhart et al., 2000) between collections have been applied in other studies, with precise measurement of sperm production indices and spermatozoa motility parameters. A cryoresistance assessment of spermatozoa obtained from 14 sequential strippings at 6 h intervals showed low motility in thawed spermatozoa, including in those exhibiting high motility prior to freezing (Kopeika and Kopeika,

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2008). However, no data on the fertilizing ability of spermatozoa obtained through sequential stripping have been published.

Testes of sturgeon possess no externally opening sperm ducts. Spermatozoa pass through urinary collecting ducts (Wrobel and Jouma, 2004), where they are believed to mix with urine. Research is needed to clarify the influence of this mixing process on spermatozoa motility, fertilizing ability, and cryoresistance.

The aim of the present study was to describe spermatozoa characteristics of sturgeon sperm samples obtained through multiple collections. Two procedures, differing in time post-hormone treatment of the initial stripping, were used. This time span was considered a possible determinant of spermatozoa quality influenced by the duration of residence in the urinary duct.

The sterlet, *Acipenser ruthenus*, was selected as a model species because of ease of handling due to its small size. The spermatozoa cryopreservation procedure was based on original studies (Glogowski et al., 2002; Urba´nyi et al., 2000) that reported high post-thaw fertilizing ability of sturgeon spermatozoa.

2. Materials and methods

2.1. Fish and rearing conditions

Sterlet broodstock (3–5 years old, weight 0.67–1.18 kg) were kept during the natural spawning season in outdoor 4 m^3 plastic tanks with a constant flow rate of 20 l min^{-1} pond water and temperature of 8–12 °C, located at the hatchery of the Research Institute of Fish Culture and Hydrobiology. Prior to hormone treatment, fish were moved to a closed water recirculation system with water temperature elevated to 15 °C within 24 h and held 3–4 days without feeding.

2.2. Procedures for sperm and urine collection

Ten male sterlet were injected intramuscularly with carp pituitary extract (CPE) at 4 mg kg^{-1} before being stripped. Females were injected with the same extract at 36 (0.5 mg kg^{-1}) and 24 h (4.5 mg kg^{-1}) before stripping (Dettlaff et al., 1993).

Sperm was collected at the urogenital papilla by aspiration using a plastic catheter (4 mm diameter) connected to a 10 ml syringe. Two procedures were used:

(A) sperm was collected from 5 of the males (first group of fish) 3 times per day at 3 h intervals on 3 consecutive days beginning 12 h after CPE treatment. As males were injected at 21.00 h, daily collection took place at 09.00, 12.00, and 15.00 h (12, 15, 18 h post-CPE treatment on day 1; 36, 39, 42 h post-CPE treatment on day 2; and 60, 63, 66 h post-CPE treatment on day 3).

(B) sperm was collected from the second group of 5 males (second group of fish) 3 times on a single day beginning 36 h after CPE treatment (36, 39, 42 h).

Sperm from (B) collected at 36 h was considered the control group, as this is the time routinely used for stripping in commercial practice (Gela et al., 2008). During each collection, the available sperm was thoroughly removed from the sperm–urinary tract.

Urine was collected by catheterization of the uro-genital papilla 5 days after hormone injection, when no spermatozoa were found by microscopic observation of the undiluted sample.

2.3. Evaluation of sperm velocity and percent motility

Sperm was diluted at a ratio of 1:50 with water from tanks in which the fish were kept, and spermatozoa motility was immediately measured until it ceased, using a CCD video camera (Sony, SSCDC50AP) mounted on a dark-field microscope (Olympus BX50, 200) and illuminated with a stroboscopic lamp (Chadwick- Helmut, 9630, USA) set to a flash frequency of 50 Hz. Video recordings were obtained (Sony SVHS, SVO-9500 MDP) at 25 frames s^{-1} and analyzed to estimate spermatozoa curvilinear velocity (VCL, μm s^{-1}) and percent motile using Olympus micro-image software (Olympus Micro Image 4.0.1. for Windows, Hamburg Germany). To compute VCL and percent motile spermatozoa at 10 s post-activation, head tracks were generated from five successive video frames, and VCL was calculated as length of spermatozoa head track divided by the time elapsed between the first and fifth video frame. Ten to 50 spermatozoa were evaluated in each frame. Spermatozoa with velocity lower than 3 μm s^{-1} were considered immotile and excluded from further analysis (Rodina et al., 2008).

Spermatozoa velocity and percent motility were also determined in urine as described above, with urine replacing water. In these trials, spermatozoa samples obtained from three males were assayed for motility in each of the three urine samples.

2.4. Evaluation of spermatozoa production indices, seminal fluid, and urine osmolality

Spermatozoa concentration of each sperm sample was estimated using a Burker cell hemocytometer (Meopta, Czech Republic) at 200× magnification on an Olympus BX 50 phase contrast microscope (Olympus, Japan).

Spermatozoa number (SN) was computed as spermatozoa concentration multiplied by the volume of each sperm sample. Collected SN per kg body weight was calculated as SN divided by body weight.

Total spermatozoa production (TSP) was computed as the sum of SN for each male. Percent spermatozoa collected per stripping (PS) was calculated by:

$$PS = SN * TSP^{-1} * 100.$$

Seminal fluids (SF) were obtained after centrifugation at 10 000 *g* for 10 min.

Osmolalities of SF and urine were evaluated using a Vapor Pressure Osmometer 5520 (Wescor, USA) and expressed in mOsm kg^{-1}.

2.5. Cryopreservation of sperm

Sperm from each stripping of each male was individually frozen. Before freezing the samples were diluted 1:1 in an extender consisting of 23.4 mM sucrose, 0.25 mM KCl, and 30 mM Tris–HCl, pH 8.0 (Glogowski et al., 2002) containing 10% methanol. The diluted sperm was placed into 0.5 ml straws (CRYO-VET, France), transferred to a styrofoam box containing liquid nitrogen, placed 3 cm above liquid nitrogen level on a styrofoam raft for 20 min, and then plunged into liquid nitrogen. Thawing was conducted in a water bath at 40 °C for 6 s. Thawed sperm was used immediately for motility or fertilization assays of spermatozoa.

2.6. Fertilization assay

Eggs from three females pooled in equal parts were used. To 2 g eggs (approximately 160 eggs), 8 ml of tank water was added along with sperm. Based on spermatozoa concentration in the sample, the volume of sperm was adjusted to obtain a 10^5 spermatozoa/egg ratio. After 2 min mixing, the fertilized eggs were incubated at 16 °C. As spermatozoa for experiments were collected over an extended period, it was not possible to control the fertilizing ability of fresh spermatozoa using the same batch of eggs; thus we recorded fertilizing ability of frozen–thawed sperm only. The use of eggs from three females in our experimental design had the potential to reduce variations in fertilization rates related to egg quality. The quality of eggs was evaluated by insemination with fresh spermatozoa from other males applying the same spermatozoa/egg ratio. Live embryos were counted at the eyed stage after 4 days incubation (stage 4), and dead eggs were removed. Yolk

sac larvae were counted at day 8 of incubation. Fertilization and hatching rates were expressed as the percent of live embryos and yolk sac larvae at days 4 and 8 of incubation, respectively.

2.7. Data presentation and statistical analysis

Five males were used for each stripping procedure. Measurements of SF osmolality, VCL, percent motility, fertilization, and hatching rates were conducted in triplicate for each male, while spermatozoa concentration and sperm volume were estimated from a single measurement.

The values of parameters were checked for distribution characteristics and homogeneity of dispersion using the Shapiro–Wilk's and Levene's tests, respectively. Spermatozoa VCL before and after cryopreservation and SN showed normal distribution with similar dispersion values. For these values the mean values with a 95% CI were presented in figures and mean ± standard deviation are described in text. To compare spermatozoa velocity before and after cryopreservation, parametric ANOVA was applied, and Tukey's honest significant difference (HSD) test was used to identify differences among subgroups. When values (motility, sperm volume, spermatozoa concentration, and sperm production indices) were not normally distributed, they are presented as median and interquartile range in figures and as min–max ranges when described in the text. Nonparametric statistics using the Kruskal–Wallis test followed by the Mann–Whitney U-test were conducted for comparison among groups. Correlations among spermatozoa parameters were evaluated using Spearman rank analysis.

Statistical significance was considered at $P<0.05$. All analyses and plotting were conducted using Statistica V9.1 computer program (Statsoft Inc, USA).

3. Results

3.1. Sperm production indices with sequential stripping

Parameters such as spermatozoa concentration and volume of sperm sample varied widely among collections. Spermatozoa concentration ranged from 0.1 to 48.4 10^8 spz*ml^{-1} and volume of sperm sample from 1.8 to 24.0 ml. These variations precluded statistical analysis using parametric methods (Levene's test, $P<0.05$, Shapiro–Wilk's test $P<0.05$). With nonparametric Kruskal–Wallis ANOVA, significant differences were found in volume of sperm sample, spermatozoa concentration, percent spermatozoa collected per stripping, and seminal fluid osmolality.

Volume of sperm samples collected on the first day (12, 15, and 18 h post CPE treatment) for (A) was significantly higher than the volume of sperm samples collected on the second day (36, 39, and 42 h post CPE treatment). Volume of sperm samples collected on the third day (66 h post CPE treatment) was significantly lower than the volume of sperm samples collected on the second day (Fig. 1A, $P<0.05$, Mann–Whitney U-test, n = 5). In (B), a significant decrease in the volume of sperm samples collected was observed at the third stripping (Fig.1B, $P<0.05$, Mann–Whitney U-test, n = 5). Significant increases in spermatozoa concentration were found after the first stripping with both procedures (Fig. 2A and B). A similar increase over day 1 values was seen on the second day of stripping (36 and 39 h post-CPE treatment, Fig. 2A), while no significant changes compared to day 2 were observed on day 3 (60, 63, and 66 h post-CPE treatment, Fig. 2A).

Analysis of spermatozoa production showed a significant increase in PS at the second stripping for both procedures (Fig. 3). In (A), PS was not significantly altered with stripping at 15–42 h post-CPE treatment; however a decrease was observed 60–66 h post-CPE treatment (Fig. 3A). In (B), PS was significantly greater at 39 h post-CPE treatment compared with 36 and 42 h (Fig. 3B).

Collected spermatozoa number (SN) for (A) and (B) were $3.73*10^{11}$ and $1.49*10^{11}$, respectively, and were 12.4 and 5.0 times higher than for spermatozoa collected 36 h post-CPE treatment in (B) ($0.30*10^{11}$ spermatozoa), the control group. The highest SN was found 15–42 h post-CPE treatment in (A) and 42 h post-CPE treatment in (B) (85 ± 4% and 64 ± 5% of total spermatozoa production, respectively).

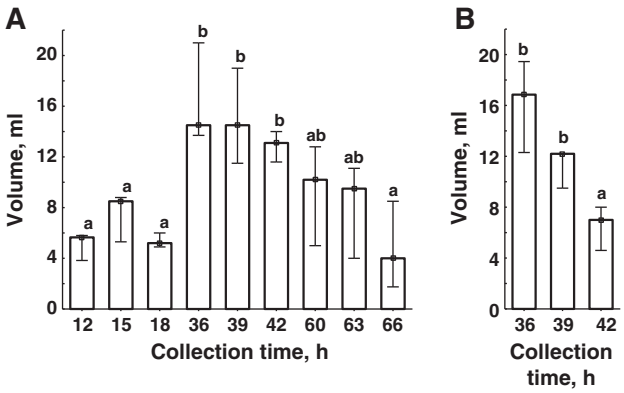

Fig. 1. Volume of sperm samples collected during sequential stripping. A: Procedure (A) sperm was collected 3 times per day at 3 h intervals on 3 consecutive days starting 12 h post-CPE treatment (12, 15, 18, 36, 39, 42, 60, 63, and 66 h). B: Procedure (B) sperm was collected 3 times in 1 day starting 36 h post-CPE treatment (36, 39, 42 h). Median value with 25% and 75% percentiles is presented, values with different letters are significantly different ($P<0.05$, Mann–Whitney U-test).

3.2. Spermatozoa motility

Spermatozoa motility was observed in both tank water and urine, while no motility was observed in seminal fluid. Significant decreases in percent motile spermatozoa were observed after freeze-thawing for all sperm samples (Fig. 4A–B). Motility in spermatozoa samples before cryopreservation ranged from 26 to 100%, while post-thaw motility was from 5 to 67%. The lowest percent motility was observed at 12 and 60 h post-CPE treatment in both fresh and frozen–thawed samples. Frozen–thawed samples collected at 12 h post-CPE treatment shown significantly lower motility ($P<0.05$, Mann–Whitney U-test, n = 5) from all other samples except those collected at 60 h post-CPE treatment.

Estimation of spermatozoa curvilinear velocity (VCL) showed normal distribution, and parametrical methods of ANOVA were used for comparison of mean values obtained for the groups. In sperm samples before freezing, VCL was significantly increased from 12 h post-CPE treatment (99 ± 19 μm s^{-1}) to 18 h post-CPE treatment (178 ± 37 μm s^{-1}), while, in samples collected after 36 h post-CPE treatment, significant reduction in VCL was observed only at 60 h post-CPE treatment (163 ± 29 μm s^{-1}) in (A) (Fig. 5A). There was no significant difference in VCL in (B). Freezing and thawing led to significant decrease of VCL in

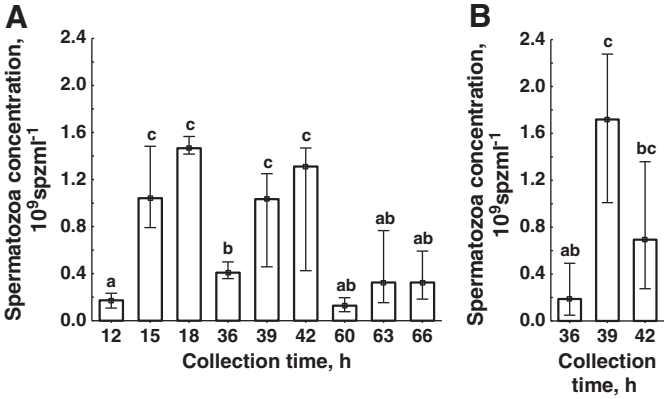

Fig. 2. Spermatozoa concentration in samples collected during sequential stripping. A: Procedure (A). B: Procedure (B). Median value with 25% and 75% percentiles is presented; values with different letters are significantly different ($P<0.05$, Mann–Whitney U-test).

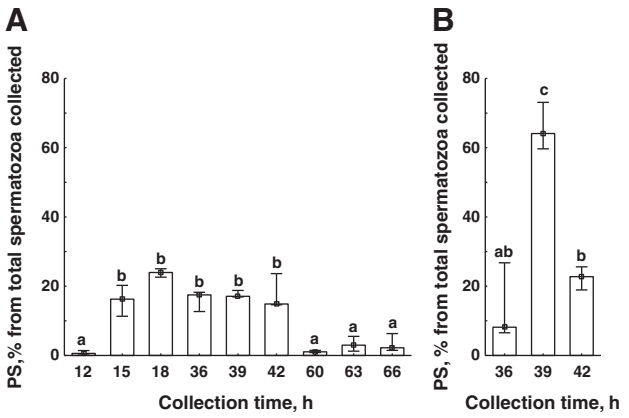

Fig. 3. Percent of spermatozoa collected per stripping calculated for samples obtained during sequential stripping. A: Procedure (A). B: Procedure (B). Median value with 25% and 75% percentiles is presented; values with different letters are significantly different ($P<0.05$, Mann–Whitney U-test).

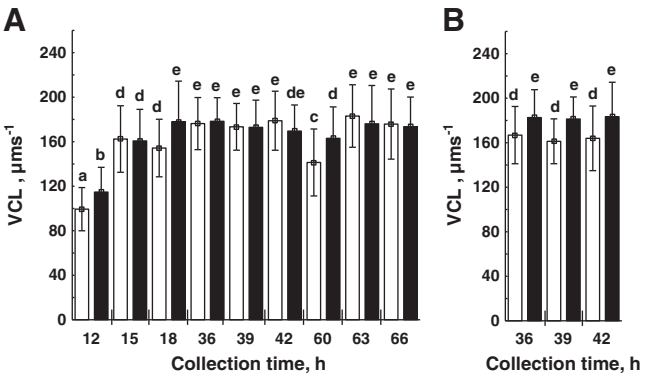

Fig. 5. Curvilinear velocity before and after freeze-thawing in sperm samples collected during sequential stripping. A: Procedure (A). B: Procedure (B). Mean with S.D. are presented; values with different letters are significantly different ($P<0.05$, Tukey's test). □ — post-thaw velocity, ■ — velocity before freezing.

spermatozoa samples collected by (A) at 12, 18 and 60 h post-CPE treatment. The same effect was observed in (B) (Fig. 5B).

Spermatozoa motility was observed in sperm collected 36 h post-CPE treatment diluted in urine. Percent motile spermatozoa ranged from 80 to 100% whether diluted with pond water or urine. VCL of spermatozoa activated by urine was 183 ± 31 µm s^{-1} and did not differ significantly from those activated by water (190 ± 22 µm s^{-1}).

3.3. Seminal fluids and urine osmolality

Gradual increase of seminal fluid osmolality was observed on the first day of stripping with subsequent decrease on days 2 and 3 in (A) (Fig. 6A, $P<0.05$, Mann–Whitney U-test, $n=5$). No significant change in seminal fluid osmolality was observed in sperm from (B) (Fig. 6B).

Osmolality of urine was 25 ± 7 mOsm kg^{-1} and was significantly lower than osmolality of seminal fluid obtained at any time of stripping (Mann–Whitney U-test, $n=5$).

3.4. Fertilization assays with sperm samples after freeze-thawing

After insemination by fresh spermatozoa, fertilization rate ranged from 90 to100% ($n=3$) showing a high viability of eggs used in the experiments. Fertilization and hatching rates using frozen–thawed spermatozoa are shown in Figs. 7 and 8, respectively. The parameters showed similar trends. While, in all cases, hatching rates were lower than corresponding values of fertilization rates, no significant differences were found between median values of these two parameters in sperm samples collected at the same time post-hormone treatment. Significant increases in both parameters were observed in the samples from the second collection on day 1 and day 3 in (A) (Figs. 7A and 8A) and from the second collection in (B) (Figs. 7B and 8B) compared with the first daily collection.

Results of analysis of the correlation among all spermatozoa parameters studied are presented in Table 1 with only significant ($P<0.05$) values shown. Fertilization and hatching rates correlated significantly with spermatozoa concentration, volume, count per stripping, post-thaw motility, and post-thaw VCL, although correlation coefficients were low (0.292–0.538). Spermatozoa count per stripping correlated with sperm volume, seminal fluid osmolality, and spermatozoa concentration with higher correlation coefficients (0.617–0.874). Spermatozoa concentration correlated with sperm osmolality with coefficient 0.722.

4. Discussion

It is clearly established that appropriate hormone treatment stimulates spermiation in sturgeon for a relatively long period (Alavi et al., 2006; Kopeika and Kopeika, 2008; Linhart et al., 2000; Podushka, 2003). In the present study, we demonstrated that, by using sequential stripping, it is possible to collect an increased amount of spermatozoa possessing both high fertilization ability and cryo-resistance.

As our experimental fish were kept in appropriate spawning conditions, hormone treatment with pituitary extract probably resulted in accelerated sperm production by release of fully developed spermatozoa from spermatocysts into the sperm ducts (spermiation) followed by

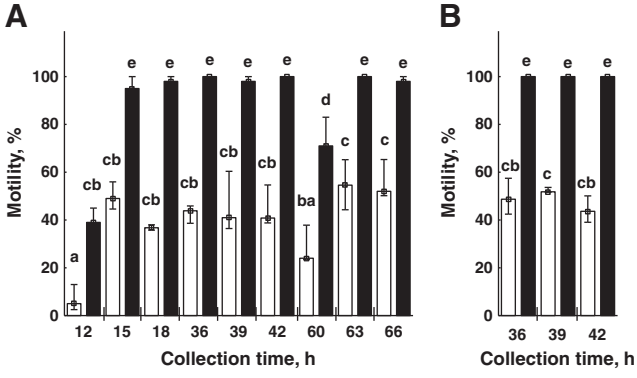

Fig. 4. Spermatozoa motility percent in samples collected during sequential stripping. A: Procedure (A). B: Procedure (B). Median values with 25% and 75% percentiles are presented; values with different letters are significantly different ($P<0.05$, Mann–Whitney U-test). □ — post-thaw motility, ■ — motility before freezing.

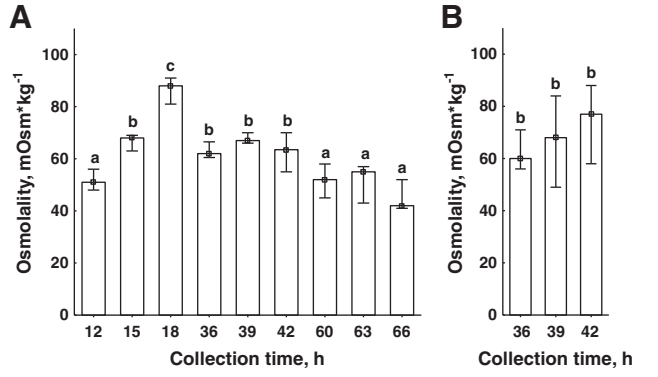

Fig. 6. Seminal fluid osmolality in sperm samples collected during sequential stripping. A: Procedure (A). B: Procedure (B). Median value with 25% and 75% percentiles is presented; values with different letters are significantly different ($P<0.05$, Mann–Whitney U-test).

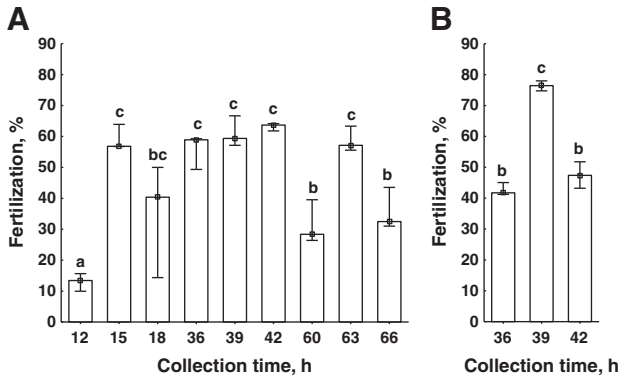

Fig. 7. Fertilization rate of eggs, fertilized by frozen–thawed sperm samples collected during sequential stripping. A: Sperm samples collected in procedure (A), B: sperm samples collected in procedure (B) (median value with 25% and 75% percentiles is presented; values with different letters are significantly different ($P<0.05$, Mann–Whitney U-test)).

production of seminal fluid (Mylonas et al., 2010; Schulz et al., 2010). As the precise sequence of sperm formation events has not been described in sturgeon, we assume that the wide variation in spermatozoa concentration during sequential stripping could be related to temporal asynchrony of spermatozoa release from spermatocysts relative to seminal fluid production. This mode of milt production could be the source of variation in the amount of spermatozoa obtained per stripping observed in our study. Similar results have been shown in paddlefish (Linhart et al., 2000).

In the present study more detailed information about milt production was obtained from measurements of spermatozoa volume and spermatozoa concentration. Increase in milt volume appeared 36 h after hormone treatment, while increased spermatozoa concentration was observed as early as 15 h post-CPE treatment, and a significant increase in seminal fluid osmolality was observed 15 and 18 h post-CPE treatment. Thus, we suggest that spermiation, as a response to hormone treatment, could be initiated before the increase of seminal fluid production associated with the phenomenon known as milt hydration. While it is certain that milt hydration is a hormone regulated process, it is not clear how the involved hormones exert their effects (Schulz and Miura, 2002). Increased spermatozoa concentration in sperm stripped 15 and 39 h after CPE treatment in (A) and 39 h post-CPE treatment in (B) (second strippings per day) suggests that sperm collection itself could stimulate the process of spermiation. While this suggestion is in accordance with results described previously

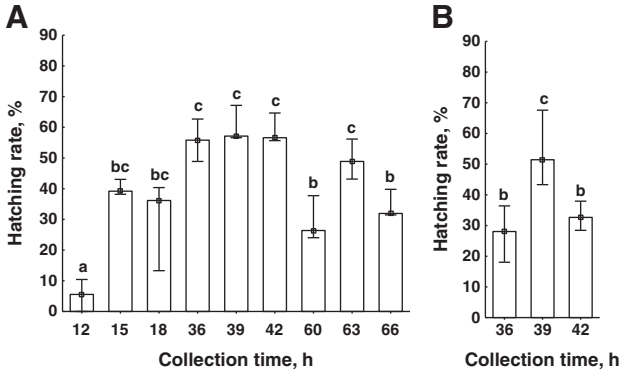

Fig. 8. Hatching rate of embryos obtained with frozen–thawed sperm samples collected during sequential stripping. A: Sperm samples collected in procedure (A), B: sperm samples collected in procedure (B). Median value with 25% and 75% percentiles is presented; values with different letters are significantly different ($P<0.05$, Mann–Whitney U-test).

(Podushka, 2003), more detailed study is required to understand the physiological basis of this phenomenon.

The specific structure of the sturgeon sperm-urinary excretory system, with a urogenital junction created by testes seminal ducts and opisthonephros (Hoar, 1969; Wrobel and Jouma, 2004), makes unambiguous determination of the source of milt volume increase almost impossible. Most likely, collected sturgeon milt consisted of a mixture of seminal fluid and urine. As we found no description of urine composition during spermiation in sturgeon, some related information should be mentioned. Both urine and seminal fluid of various sturgeon species are hypotonic compared to blood plasma (Krayushkina and Semenova, 2006; Potts and Rudy, 1972), and osmolality of urine measured in our study was significantly lower than osmolality of seminal fluid. Also, both fluids contain K^+ and Ca^{2+} ions, which have been shown to reciprocally control spermatozoa motility in sturgeon (Gallis et al., 1991; Linhart et al., 2002). Urine possesses similar concentrations of K^+ and Ca^{2+} ions (Krayushkina and Semenova, 2006) while, in seminal fluid, the concentration of K^+ ions ranges from two (Linhart et al., 2003) to 30 times (Toth et al., 1997) that of Ca^{2+}. Motility of sturgeon spermatozoa can be initiated in a wide range of environmental osmolalities [0–120 mOsm l^{-1}], but osmolalities of 25 and 50 mOsm kg^{-1} were found to produce only 60 and 10% motility, respectively, in sterlet (Lahnsteiner et al., 2004). The most likely reason is that both low osmolality and low K^+/Ca^{2+} ratio in urine determine its ability to activate spermatozoa motility, as observed in our study, as well as by Park and Chapman (2005). To investigate whether urine dilution of sperm occurs and has any effect on spermatozoa parameters, urine-specific compounds should be quantified, since variations in SF osmolality may not necessarily be due to urine contamination. To detect the underlying processes, additional studies are necessary.

As spermatozoa motility in fish can be activated several times by a gradual osmolality decrease (Boryshpolets et al., 2009; Dzyuba et al., 2011), it is likely that the first activation step may occur immediately upon spermatozoa release from testes into the urogenital tract. We suggest that low osmolality of seminal fluid observed in our study and previously described (Alavi et al., 2006; Linhart et al., 2003) is related to dilution of sperm with urine, and changes in osmolality of seminal fluid, in turn, could result from the extent of this dilution. High regression coefficients between spermatozoa concentration, spermatozoa number per stripping, and osmolality support this supposition, while more investigations are needed to confirm it.

While the process of sperm production in sturgeon is unclear, our study demonstrated that multiple collections provide the possibility of increasing the amount of spermatozoa with high motility parameters, cryoresistance, and fertilizing ability. Although sperm samples obtained at different stages of multiple collection differed in the parameters studied, only samples collected in the first stripping at 12 h after hormone treatment were of significantly lower quality compared to the control with respect to motility, VCL, and fertilizing ability. These results were similar to previously described observations of low percent motility in spermatozoa of Siberian sturgeon collected 12 h post-hormone treatment (Kopeika and Kopeika, 2008). In contrast to our observation, no decreased motility was observed in the early stages of hormonal stimulation of spermiation in paddlefish (Linhart et al., 2000). The increase in percent motile spermatozoa following post-CPE treatment is not fully understood, but spermatozoa maturation as a process of "development from non-functional gametes to mature spermatozoa fully capable of vigorous motility and fertilization" (Schulz and Miura, 2002) could be involved. Spermatozoa maturation as a sequence of events is described in a few teleosts (rainbow trout, chum salmon, and Japanese eel) but not in sturgeon. From the studies of these species, it is obvious that spermatozoa maturation is under the control of the endocrine system. This process includes the increase of seminal plasma pH in the sperm duct, which results in elevation of intra-spermatozoa cAMP levels (Schulz et al., 2010). We have not found in literature any description of physiological processes in sturgeon underlying spermatozoa maturation.

Table 1
Regression analysis of indexes of sperm samples collected in both procedures of stripping*.

	Sperm osmolality	Spermatozoa amount per stripping	Post-thaw motility	Post-thaw VCL	Fertilization rate	Hatching rate	Spermatozoa concentration	Volume of sperm
Sperm osmolality		0.652			0.292		0.722	
Spermatozoa amount per stripping	0.652		0.388	0.324	0.538	0.452	0.874	0.617
Post-thaw motility		0.388		0.385	0.347	0.292	0.312	0.333
Post-thaw VCL		0.324	0.385			0.330		0.318
Fertilization rate	0.292	0.538	0.347			0.838	0.497	0.356
Hatching rate		0.452	0.292	0.330	0.838		0.391	0.415
Motility percentage before freezing		0.278		0.362	0.391	0.366		0.412

* Spearman rank order coefficients, for which $P<0.05$, and $n=60$, are presented.

Our results describing changes of spermatozoa motility parameters during sequential stripping could be considered a promising model for future, more detailed, studies of the spermatozoa maturation process in sturgeon. Significant positive correlations between spermatozoa count per stripping and post-thaw motility, post-thaw VCL, fertilization, and hatching rates suggest that mature spermatozoa are produced during the increase in sperm production associated with sequential stripping.

Spermatozoa motility and VCL values of fresh and frozen–thawed samples, osmolality of seminal fluid, and rates of fertilization and hatching for embryos obtained with frozen–thawed sperm collected at different times of sequential stripping were in the same range as previously described in sturgeon (Alavi et al., 2006; Billard et al., 2004; Dzyuba et al., 2010; Lahnsteiner et al., 2004; Piros et al., 2002). However, in our study we found significant changes in spermatozoa fertilizing ability associated with sequential stripping. Increased fertilizing ability of spermatozoa collected at the second stripping of day 1 of both procedures and at the second stripping on day 3 of (A) was observed. The low correlation coefficients among spermatozoa motility, VCL, and fertilization and hatching rates indicate that spermatozoa motility parameters are not unambiguous determinants of fertilizing ability. This was shown in (B), when spermatozoa collected 39 h after hormone treatment demonstrated motility and VCL similar to that collected 36 h and 42 h after hormone treatment, but resulted in a significantly higher fertilization rate. Spermatozoa of sturgeon differ from those of freshwater teleost fish by the presence of an acrosome, and correct timing of the acrosome reaction is considered to be a crucial for fertilization success (Ginzburg, 1972; Psenicka, et al., 2008). Observed differences in fertilizing ability of spermatozoa may be associated with the acrosome maturation stage, and more investigation is needed to determine the functional state of the acrosome in spermatozoa collected at different stages of sequential stripping procedures.

5. Conclusion

Long-term sperm production after a single hormonal treatment in sturgeons is the basis of effective sequential stripping of males. Sequential stripping could be an appropriate model for future study of the spermatozoa maturation processes in sturgeon. While sperm samples collected at different stages of sequential stripping varied in motility parameters and fertilizing ability, these variations are not limiting for the most effective exploitation of the male fish. The initial stripping may stimulate production of spermatozoa possessing the highest motility and fertilizing ability. For artificial sturgeon breeding the identification of periods of the most intensive spermatozoa production is important. When sperm sampling begins within a short (12 h) period after hormonal stimulation, sperm from the first collection consists of spermatozoa of inadequate quality, but this collection stimulates a quick production of high quality spermatozoa.

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Attachment 11

Dzyuba, B., Cosson, J., Yamaner, G., Bondarenko, O., Rodina, M., Gela, D., Bondarenko, V., Shaliutina, A., Linhart, O., 2013. Hypotonic treatment prior to freezing improves cryoresistance of common carp (*Cyprinus carpio* L.) spermatozoa. Cryobiology 66: 192-194. (IF 2013 = 1.643)

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Brief Communication

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

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ABSTRACT

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

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

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

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

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

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

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

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

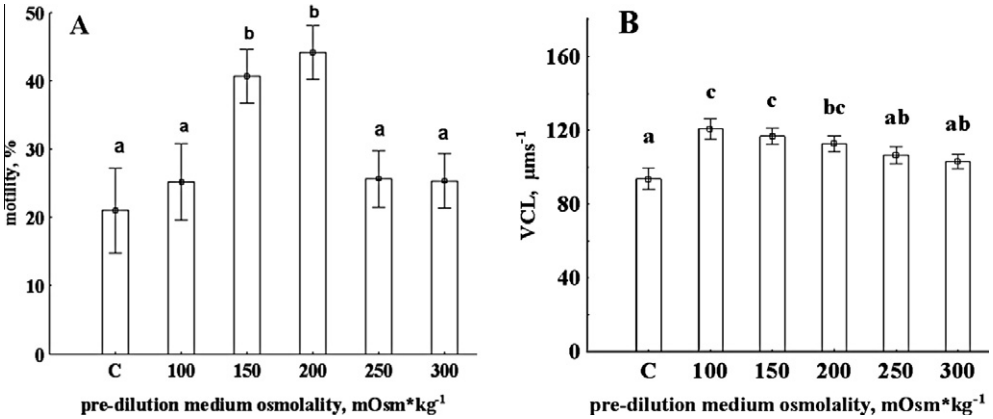

Fig. 1. Post-thaw motility rate (A) and curvilinear velocity (B) of *Cyprinus carpio* spermatozoa pretreated with NaCl solutions of different osmolalities before cryopreservation. Sperm sample osmolality after 10 s in pre-dilution medium was adjusted to 300 mOsm kg^{-1} by addition of 2 M KCl solution.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

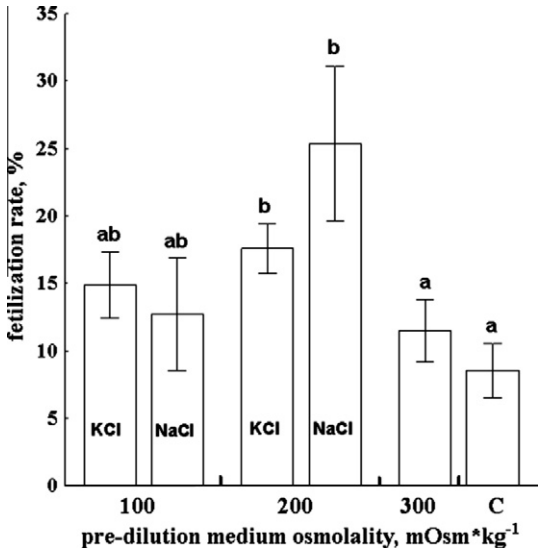

Fig. 2. Post-thaw fertilization rate for *Cyprinus carpio* sperm pretreated with NaCl solutions of different osmolalities before cryopreservation. Values with different letters are significantly different ($P < 0.05$). Osmolality was readjusted to isotonic values by addition of concentrated KCl or NaCl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

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

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

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

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

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

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