Reproduction (2002) **124**, 713–719

Research

Effects of ions on the motility of fresh and demembranated paddlefish (*Polyodon spathula*) spermatozoa

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This study investigated the effects of different environmental conditions on the motility parameters of paddlefish (*Polyodon spathula*) spermatozoa. Paddlefish spermatozoa demonstrated the following characteristics: (i) all spermatozoa were motile 10 s after activation with a velocity of 130–160 μm s^{-1}; (ii) after 2 min, velocity decreased to 80–130 μm s^{-1}; and (iii) motility was maintained for up to 9 min. Concentrations of 0.5–5.0 mmol KCl l^{-1} prevented activation of spermatozoa. After transfer into a swimming medium (20 mmol Tris l^{-1}, pH 8.2 and 1 mg BSA ml^{-1}) containing 0.5 mmol KCl l^{-1} (combined with 5 mmol NaCl or $MgCl_2$ l^{-1}), 80–100% of cells were motile with a velocity of about 120–150 μm s^{-1}. $MgCl_2$ significantly improved the velocity of spermatozoa at 10, 40, 50 and 60 s after activation and the stable velocity of spermatozoa was about 140 μm s^{-1}. Very low concentrations of $CaCl_2$ (0.125 mmol l^{-1}) combined with 0.5 mmol KCl l^{-1} initiated motility in 20% of spermatozoa, whereas all spermatozoa were activated after 2 min with 0.25 mmol $CaCl_2$ l^{-1} in similar medium for the full period of swimming with velocity of about 120 μm s^{-1}. This study demonstrated that potassium (5–15 mmol l^{-1}) inhibits demembranated spermatozoa. Thus, initiation of movement in paddlefish spermatozoa is under the reciprocal control of potassium and calcium ion concentrations.

Introduction

Two species from the family Polyodontidae are extant: the American paddlefish (*Polyodon spathula*) which is confined to North America, and the Chinese paddlefish (*Psephurus gladius*) which is native to mainland China. Both species were commercially important, but numbers have declined markedly over the past 100 years (Carlson and Bonislawsky, 1981; Gengerke, 1986; Liu and Zeng, 1988; Mims *et al.*, 1993a). Artificial propagation and stocking have been successful only for the North American species. Wild stocks have been harvested for flesh and caviar production, and *P. spathula* is a popular sport fish in some areas in the USA (Gengerke, 1986; Liu and Zeng, 1988). *P. spathula* can reach a maximum of 83.5 kg and 216 cm in length (Adams, 1942; Folz and Mezers, 1985) and *P. gladius* grows much larger. *P. spathula* feeds on plankton, especially copepods, some insects, algae and plant fragments, whereas *P. gladius* is piscivorous (Mims *et al.*, 1993a). Adult *P. spathula* filter zooplankton (microscopic food) through their gill rakers while swimming with their mouth open (Mims *et al.*, 1993b; Kroll *et al.*, 1994). The spawning period of *P. spathula* is in early spring, when adults migrate upstream in the Mississippi River and its major tributaries. Spawning occurs from the middle of March to the end of May at 11–14°C in the Ohio River (Purkett, 1963; Ballard and Needham, 1964) or from April to June at 16°C in the Missouri River (Purkett, 1963; Ballard and Needham, 1964). Needham (1965) and Graham *et al.* (1986) developed artificial reproductive methods for paddlefish in the USA, and Mims and Shelton (1998) have modified this procedure.

Spermatozoa of sturgeons and paddlefish are essentially immotile in the seminal plasma (Linhart *et al.*, 1995; Cosson and Linhart, 1996; Cosson *et al.*, 2000). Spermatozoa are immediately activated when they are transferred into swimming medium, usually fresh water or low salt concentration solutions (Drabkina, 1961), in a similar way to spermatozoa of freshwater teleosts. Sperm motility is greatest immediately after activation. All sperm motility parameters (frequency, velocity and wave amplitude) decrease rapidly during the period after activation and the percentage of motile cells also gradually decreases (Cosson *et al.*, 2000). During the earliest period of motility, spermatozoa of sturgeons and paddlefish move at velocities of 175–250 μms^{-1}

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(Cosson *et al.*, 2000) and then the forward motility gradually reduces to between 50 and 100 μm s^{-1} at 3–6 min after activation, even when considering only the fraction of the population of cells with forward motility. Occasionally, some spermatozoa are motile for up to 9 min (Cosson *et al.*, 2000). The concentration and ratio of ions in seminal fluid is important for maintaining energy. The motility of paddlefish spermatozoa is partly controlled by osmotic pressure (Linhart *et al.*, 1995), as described for freshwater cyprinids (Redondo-Müller *et al.*, 1991) and for marine fish (Morisawa and Suzuki, 1980). When the concentration or ratio of Na^+:K^+ or the osmotic pressure change, sperm motility in sturgeon and paddlefish is initiated (Linhart *et al.*, 1995; Cosson and Linhart, 1996; Toth *et al.*, 1997; Cosson *et al.*, 2000). A concentration of ⩾ 0.5 mmol K^+ l^{-1} prevents motility of white sturgeon (*Acipenser fulvescens*) spermatozoa (Toth *et al.*, 1997). The concentration of potassium in seminal fluid of paddlefish prevents sperm motility, but cells can be activated after dilution by about four (Linhart *et al.*, 2002) or eight times (Mims, 1991). Linhart *et al.* (2002) observed a high correlation between osmolality of seminal fluid and concentration of GnRHa, and between velocity of spermatozoa and osmolality of seminal fluid. As a consequence it appears that high osmotic concentration in seminal fluid increases the velocity of paddlefish spermatozoa.

The main aims of the present study were to examine the effects of potassium, calcium and magnesium on the motility of fresh and demembranated spermatozoa of the American paddlefish.

Materials and Methods

Induction of spermiation and collection of spermatozoa

The experiments were carried out in March and April at the Aquaculture Research Center, Kentucky State University (Frankfort, KT). Paddlefish of 7.0–12.0 kg were captured below McAlpinne Dam, Louisville, KT.

Five males were selected and held separately in a circular tank (3000 l) with a water flow rate of 12 l min^{-1}, 9.0 mg O_2 l^{-1}, at controlled water temperature of 17–19°C. Mature male paddlefish can be identified during the spawning period by the presence of tubercles on their head and opercular flaps.

Spermatogenesis was induced hormonally by i.p. injections of the GnRH analogue [des-Gly10 (D-Ala6) GnRH ethylamide] (Sigma, St Louis, MO) at 50 μg kg^{-1} body weight (Linhart *et al.*, 2000). Tygon tubing (5 cm in length) attached to a 10 ml plastic syringe was used to collect spermatozoa. The tubing was inserted into the urogenital pore and the syringe was filled with milt. Spermatozoa were collected and stored in aerobic conditions on wet ice until motility analysis.

Evaluation of sperm motility parameters

The velocity and percentage of motile spermatozoa were evaluated, and motility parameters were measured under dark field and phase-contrast microscopy (Fig. 1) (Cosson *et al.*, 2000). Spermatozoa were observed for spontaneous movement immediately after collection and before dilution. A drop of 10 μl undiluted spermatozoa was spread on a prepositioned glass slide and examined at × 200. The swimming ability of spermatozoa was measured by the addition of 0.5 μl spermatozoa to 49 μl swimming medium, which was composed of 20 mmol Tris–HCl l^{-1}, pH 8.2, with 1 mg bovine serum albumin ml^{-1} (Sigma A-7511; to prevent cells from adhering to the glass slide) and other component ions, such as K^+, Mg^+ and Ca^{2+}. The swimming medium was placed on a glass slide previously positioned on the microscope stage, and immediately after mixing with spermatozoa, examined at × 200. In this part of the study, the final dilution was 1:100. Motile spermatozoa were video-recorded beginning < 5 s after activation for measurement of velocity and percentage of motile spermatozoa. The movements of spermatozoa were observed through a × 20 lens using dark field microscopy and were recorded at 60 frames s^{-1} using a CCD video camera (Sony SSC-DC50AP) mounted on a dark field microscope (Nikon Optiphot 2). The focal plane was always positioned near the glass slide surface. Sperm movement was recorded using a tape recorder (Sony VHS, SVO 1520), visualized on a colour video monitor (Sony) and using stroboscopic illumination. The stroboscopic flash (Strobex; Chadwick-Helmut, El Monte, CA) illumination with adjustable frequency was set in automatic register with the video frames (60 Hz). The successive positions of the recorded sperm heads were measured from video frames using a video-recorder (Sony SVHS, SVO-9500 MDP). Velocity and percentage of motile spermatozoa were analysed from five successive frames, each by 'Micro Image Analysis' (Version 4.0 for Windows, special macro of three colours).

For detailed examination of other swimming parameters, such as the flagella beating and wave characteristics, 0.1–0.5 μl spermatozoa (by Eppendorf pipette from 0.1 to 2.5 μl) was directly mixed on a glass slide with 49 μl distilled water or swimming medium, and a cover slip was added for oil immersion application. Immediately after mixing, sperm motility was recorded under × 400 or × 1000. In this part of the study, the final dilution was between 1:100 and 1: 500. Motile spermatozoa were recorded within 6–8 s after activation for measurement of swimming parameters. The movements of the flagellum were recorded with the technique described above, but also using phase-contrast × 40 or × 100 oil immersion lenses. The stroboscopic flash illumination was set manually at an adjustable frequency of 150–800 Hz, depending on the time resolution required. During recording, the microscope stage was slowly moved by hand: this allowed the visualization of multiple well-defined successive images of a motile spermatozoon without overlap of flagella images within every video frame (Cosson *et al.*, 1997).

Demembranation and reactivation of spermatozoa

Two microlitres of undiluted spermatozoa were mixed with 50 μl demembranation medium, which was composed

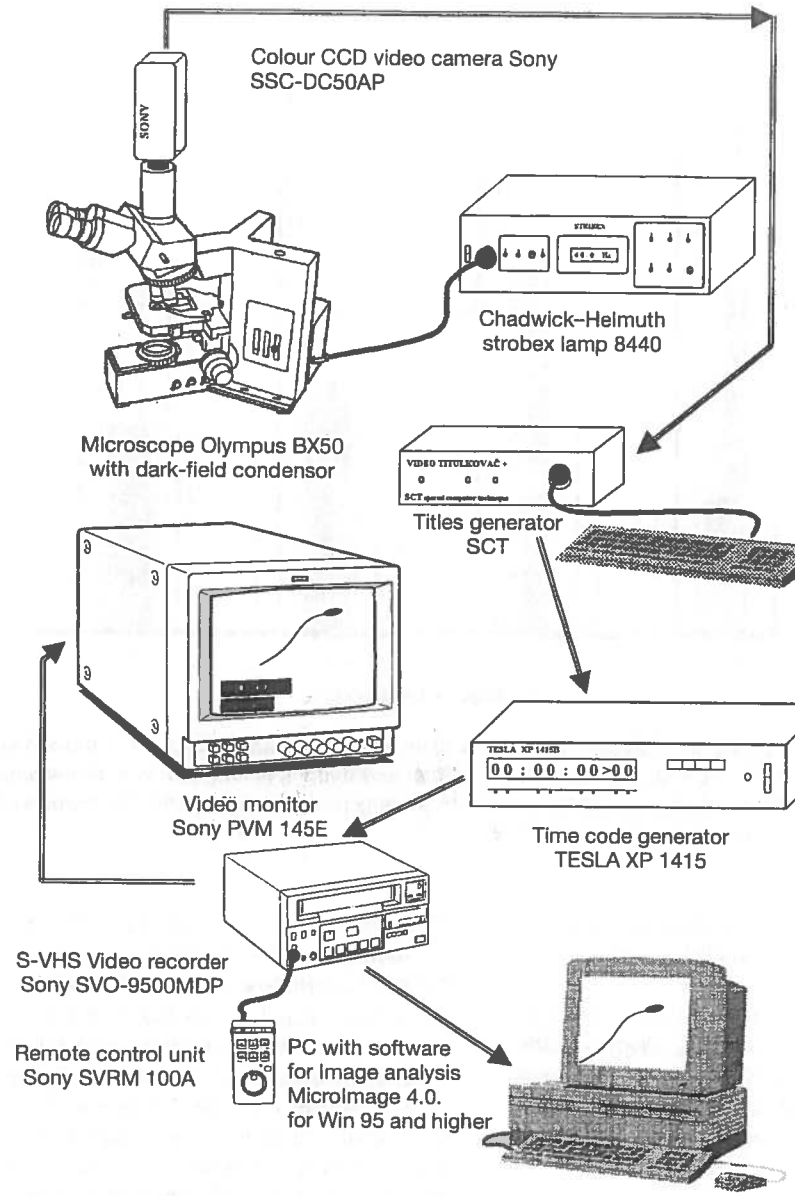

Fig. 1. Equipment for evaluation of sperm motility in paddlefish.

of 20 mmol NaCl l^{-1}, 0.5 mmol EDTA l^{-1} (Sigma), 1 mmol dithiothreitol l^{-1} (DTT, Sigma D-0632), 20 mmol Tris–HCl l^{-1}, pH 8.2 and 0.04% Triton-X100 at 0°C. After 30 s at 0°C, a 2 µl aliquot was pipetted and mixed at room temperature (18–20°C) on the glass slide with 50 µl of reactivation medium (20 mmol NaCl l^{-1}, 1 mmol DTT l^{-1}, 20 mmol Tris–HCl l^{-1}, pH 8.2, 1 mmol $MgCl_2$ l^{-1}, 2 mg BSA ml^{-1} and 1 mmol ATP l^{-1}, vanadate free from Boehringer). BSA was required to prevent the sperm cells from adhering to glass or particles. The addition of 50–200 µmol cAMP l^{-1}, either in demembranation or reactivation media, was necessary to initiate motility. The swimming ability of spermatozoa was assessed as described above.

Statistical analysis

The data acquired from several replications were evaluated and statistical significance was assessed using ANOVA (Statgraphics version 5), followed by multiple comparison tests. Probability values of $P < 0.05$ were considered significant.

Results

Motility of undiluted and diluted spermatozoa in swimming medium

Direct observation of undiluted spermatozoa by dark field microscopy showed that most spermatozoa in the

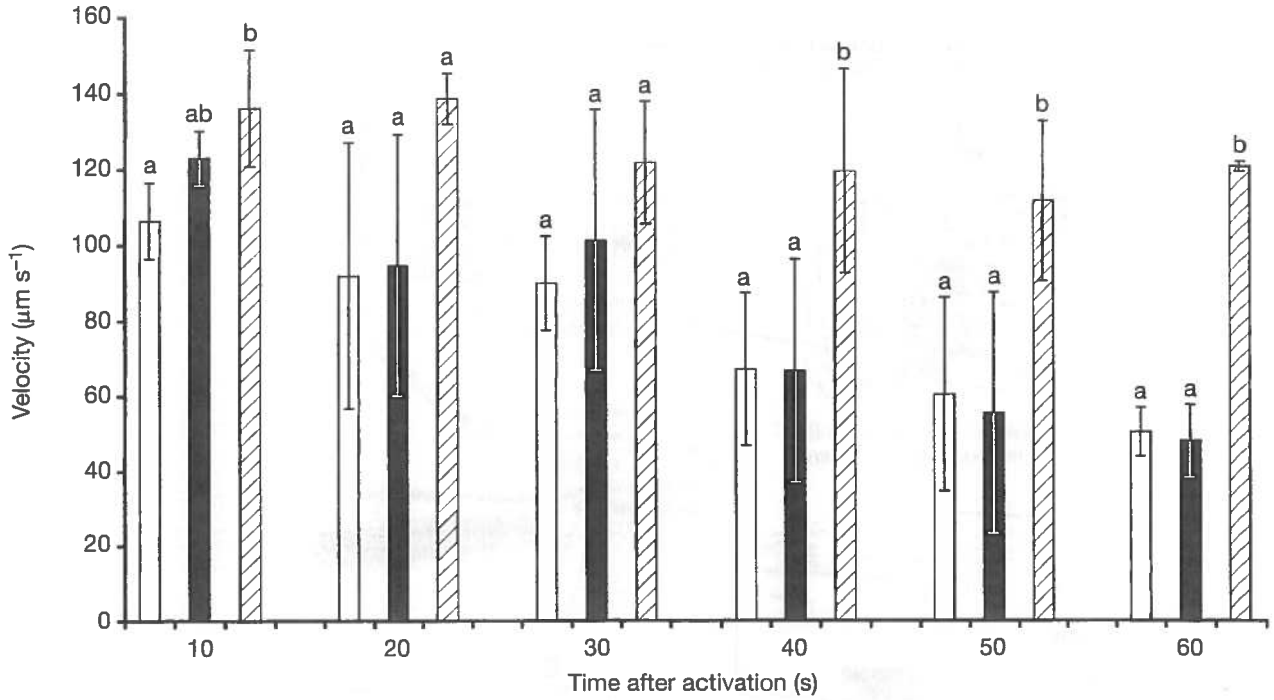

Fig. 2. Velocity of paddlefish (*Polyodon spathula*) spermatozoa activated with 5 mmol $CaCl_2$ l^{-1} (□) and swimming medium (20 mmol Tris–HCl l^{-1} at pH 8.2 and BSA), with 5 mmol $MgCl_2$ l^{-1} (■) and swimming medium, or with swimming medium only (control, ⊡). Values are mean ± SD. ^{ab}Columns within the same swimming periods (10, 20, 30, 40, 50 and 60 s after activation) with a common superscript are not significantly different ($P < 0.05$).

seminal plasma of paddlefish were immotile, but that the flagella were straight and shivered slightly. Only a few spermatozoa were motile for < 10 s.

When transferred into swimming medium, paddlefish spermatozoa were immediately activated and displayed the following characteristics: (i) 100% of spermatozoa were motile at 10 s after activation with a velocity of 130–160 µm s^{-1}; (ii) after 2 min, the velocity decreased to 80–130 µm s^{-1}; and (iii) motility was maintained for up to 9 min.

Inhibitory effect of low ionic concentrations

The motility of paddlefish spermatozoa in low concentrations of KCl, $MgCl_2$, NaCl or $CaCl_2$ solution in swimming medium (20 mmol Tris–HCl l^{-1} at pH 8.2 and BSA) was investigated. The spermatozoa were highly motile in swimming medium with low concentrations (0.5–5.0 mmol l^{-1}) of NaCl, $MgCl_2$ or $CaCl_2$. Concentrations of 0.5–5.0 mmol KCl l^{-1} did not initiate sperm motility. When swimming medium containing 0.5 mmol KCl l^{-1} was combined with 5.0 mmol NaCl l^{-1} or $MgCl_2$, 80–100% of spermatozoa became motile with a velocity of about 120–150 µm s^{-1}. $MgCl_2$ significantly improved velocity of sperm motility at 10, 40, 50 and 60 s after activation (Fig. 2) and stable velocity of spermatozoa was 140 µm s^{-1} at 2 min after activation. A low concentration of KCl (1 mmol l^{-1}) fully inhibited sperm movement, but motility was re-initiated by the addition of a low concentration of $CaCl_2$. A solution of 0.125 mmol $CaCl_2$ l^{-1} and 0.5 mmol KCl l^{-1} resulted in movement of 20% of the spermatozoa, whereas 100% of the spermatozoa were activated by the addition of 0.25 mmol $CaCl_2$ l^{-1} plus 0.5 mmol KCl l^{-1}; spermatozoa swim for 2 min at a velocity of about 120 µm s^{-1}. In addition, spermatozoa were activated in higher concentrations of KCl (5 mmol l^{-1}) and 3 mmol $CaCl_2$ l^{-1} final concentration, resulting in 50–60% motile sperm cells. All spermatozoa were fully motile after combining 5 mmol KCl l^{-1} plus 5 mmol of $CaCl_2$ l^{-1}; velocity was about 120 µm s^{-1} at 2 min after activation. Paddlefish spermatozoa inhibited with 0.5 mmol KCl l^{-1} could be activated in a solution composed of 40 mmol sucrose l^{-1}, 2 mg BSA ml^{-1} (to prevent spermatozoa adhering to the glass slide) and at 125 µmol $CaCl_2$ l^{-1}. Low concentrations of Ca^{2+} appeared to reverse the inhibitory effects of 0.5 mmol KCl l^{-1}. In addition, the EGTA used to complex Ca^{2+} could abolish the protective calcium ion effect. In the latter case Ca^{2+} combined with the calcium ionophore A23187 completely re-established the inhibitory effect of K^+ probably by increasing of Ca^{2+} permeability of the sperm membrane.

Demembranation and reactivation of spermatozoa

In this experiment, the inhibitory effect of K^+ on the axonemes of demembranated spermatozoa (Fig. 3) in the reactivating solution containing ATP was examined. The presence of KCl or K^+ at 5–15 mmol l^{-1} was used to inhibit

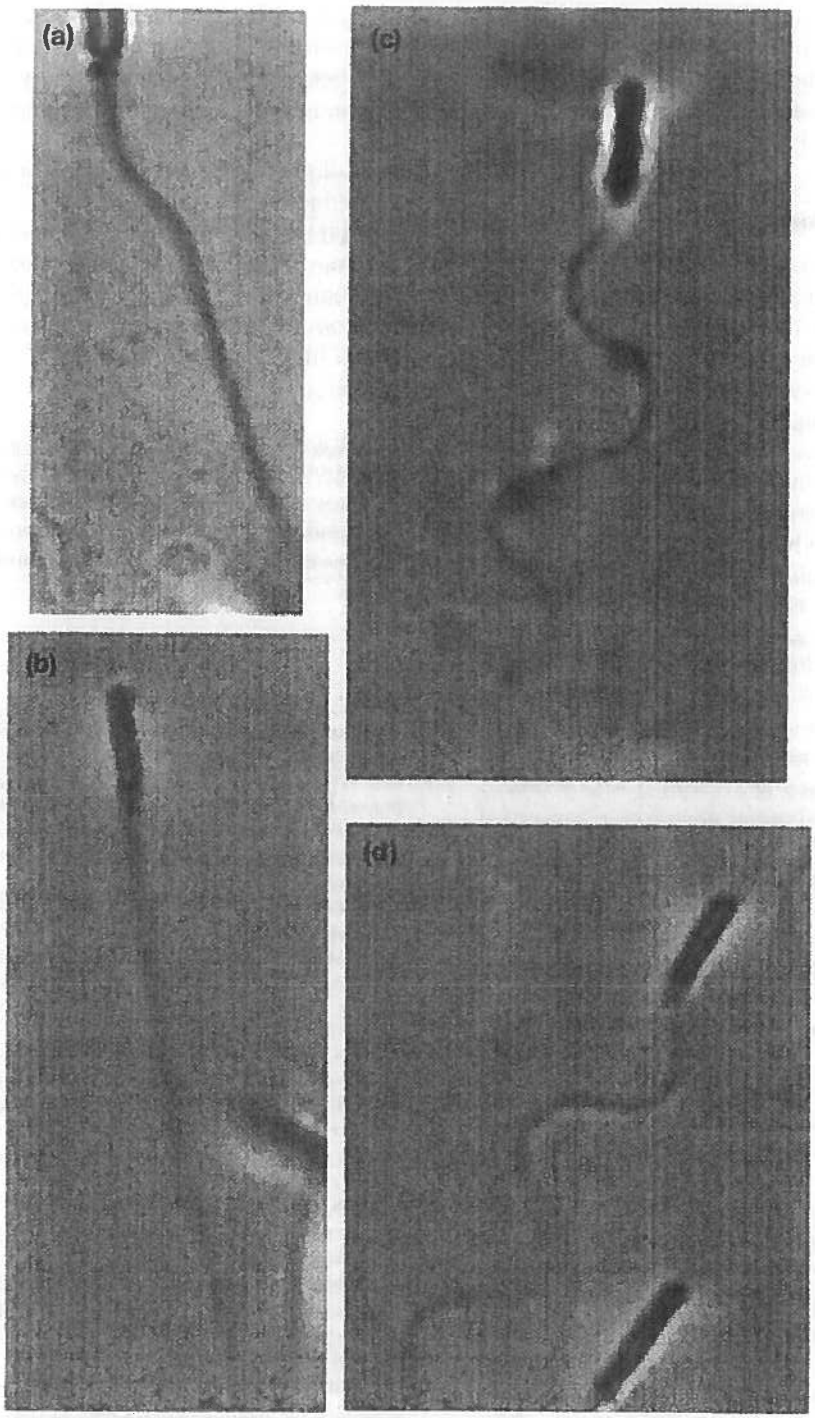

Fig. 3. (a) Shivering, not activated spermatozoa with membrane, (b) resting demembranated spermatozoa, (c) fully-activated spermatozoa with membrane damaged by low osmotic shock (distilled water) and (d) fully activated demembranated spermatozoa of the paddlefish (*Polyodon spathula*).

demembranated flagella. The addition of 20 mmol NaCl l^{-1} allowed reactivation of fully demembranated sperm flagella after the addition of 50–200 μmol cAMP l^{-1} to the reactivated medium. The effect on motility was greater when the spermatozoa were demembranated and reactivated in solution demembranated medium and reactivated medium, respectively, both containing 0.5 mmol $CaCl_2$ l^{-1}. The functional sensitivity of sturgeon axonemes to Ca^+ was confirmed. Even when using demembranated medium and reactivated medium without K^+ but with $\leq$ 100 μmol $CaCl_2$

l^{-1} in reactivated medium, the flagella of demembranated ATP-reactivated spermatozoa had only twitching motion with a very small wave amplitude. In contrast, waves with large amplitude developed when the reactivated medium contained $\geqslant 250$ µmol $CaCl_2$ l^{-1}.

Discussion

Spermatozoa of sturgeons and paddlefish are essentially immotile in the seminal plasma (Cosson and Linhart, 1996), but are fully activated when transferred into swimming medium. Fish spermatozoa are generally immotile in the testes and seminal plasma. Environmental factors, such as ions, pH or osmolality, may lead to the depolarization of the cell membrane and, therefore, stimulate motility of spermatozoa (Cosson *et al.*, 1999). Several generations of paddlefish spermatozoa are present in the testis at the same time. The motility of paddlefish spermatozoa is inhibited by an increase in K^+ concentration even at pH values of 7.0 (Cosson and Linhart, 1996). No cumulative effect of Ca^{2+} was observed in paddlefish as described for salmonids by Scheuring (1925), Billard (1978), Cosson *et al.* (1986, 1989) and Christen *et al.* (1987). In the case of paddlefish spermatozoa, motility is sensitive to very low concentrations of K^+ (0.5 mmol l^{-1}), which is lower than that for salmonids (Cosson *et al.*, 1986; Morisawa *et al.*, 1983). The potential for movement in paddlefish spermatozoa was preserved after dilution in a 5 mmol KCl l^{-1} plus 20 mmol Tris–HCl l^{-1} at pH 8. This solution also regenerated the sperm potential for movement, especially when the pH of fresh spermatozoa was < 7, which might be due to urine contamination (Cosson and Linhart, 1996). A similar regenerative effect of K^+ was reported by Redondo *et al.* (1991) for carp spermatozoa, but using a medium of high osmotic pressure (380 mOsmol), in contrast to paddlefish spermatozoa, for which the effect of regeneration was observed at a lower osmotic pressure (40 mOsmol). The spermatozoa of paddlefish were motile in a range of osmotic pressures from 0 to 120 mOsmol kg^{-1}. There was a trend of a rapid reduction from 80 to 0% sperm motility with increases in osmotic pressure from 70 to 120 mOsmol kg^{-1} (Linhart *et al.*, 1995). The effects of the KCl, $CaCl_2$, glucose and Li_2CO_3 were also tested by Cosson and Linhart (1996). Sperm movement was inhibited in solutions of 120 mmol glucose l^{-1} or 10 mmol $CaCl_2$ l^{-1} in combination with 20 mmol Tris–HCl l^{-1} at pH 8. The spermatozoa were motile in solutions of 20 mmol Li_2CO_3 l^{-1}, which indicates a specific effect for K^+. No cumulative effect on storage time of spermatozoa was observed between 1 mmol KCl l^{-1} and 100 mmol glucose l^{-1} (Cosson and Linhart, 1996).

A demembranated flagellum is directly in contact with chemicals and the potential of the motility 'effectors' can be tested directly. Demembranation is obtained by application of a mild non-ionic detergent (for example Triton-X100) and reactivation is initiated by addition of ATP–Mg^{2+}, the substrate of flagella dynein-ATPases (Gibbons and Gibbons, 1972). In this respect, the present study demonstrated that inhibition of fresh or demembranated spermatozoa was effected with low concentration of K^+ (0.5 mmol l^{-1}) and that this effect was eliminated usually with low Ca^{2+} concentration or replacement of K^+ by Na^+ in demembranated flagella.

The results from the present study indicate that movement of paddlefish spermatozoa is not significantly influenced by osmotic pressure (Linhart *et al.*, 1995; Cosson and Linhart, 1996), but is under reciprocal control of the concentration of K^+ and Ca^{2+}. In this respect, paddlefish spermatozoa are more similar to those of salmonids than cyprinids (Billard, 1978; Billard *et al.*, 1995 and Morisawa *et al.*, 1983).

This work was supported by USDA 1890 Capacity Building Grant Program (KYX-01-11469) Kentucky State University, Frankfort, KY. This project was funded by the CNRS-France (J. Cosson), Czech Grand Foundation and Ministry of Education no. J06/98:126100001, Czech Republic (O. Linhart and M. Rodina) and Embassy of France in Prague.

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Received 4 March 2002.
First decision 18 April 2002.
Revised manuscript received 23 July 2002.
Accepted 1 August 2002.