Journal of Fish Biology (2000) **56,** 1348–1367
doi:10.1006/jfbi.2000.1256, available online at http://www.idealibrary.com on IDEAL®

Analysis of motility parameters from paddlefish and shovelnose sturgeon spermatozoa

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(*Received 3 September 1999, Accepted 28 January 2000*)

Ninety to 100% of paddlefish *Polyodon spathula* were motile just after transfer into distilled water, with a velocity of 175 μm s^{-1}, a flagellar beat frequency of 50 Hz and motility lasting 4–6 min. Similarly, 80–95% of shovelnose sturgeon *Scaphirhynchus platorynchus* spermatozoa were motile immediately when diluted in distilled water, with a velocity of 200 μm s^{-1}, a flagellar beat frequency of 48 Hz and a period of motility of 2–3 min. In both species, after sperm dilution in a swimming solution composed of 20 mM Tris–HCl (pH 8·2) and 20 mM NaCl, a majority of the samples showed 100% motility of spermatozoa with flagella beat-frequency of 50 Hz within the 5 s following activation and a higher velocity than in distilled water. In such a swimming medium, the time of motility was prolonged up to 9 min for paddlefish and 5 min for sturgeon and a lower proportion of sperm cells had damage such as blebs of the flagellar membrane or curling of the flagellar tip, compared with those in distilled water. The shape of the flagellar waves changed during the motility phase, mostly through a restriction at the part of the flagellum most proximal to the head. A rotational movement of whole cells was observed for spermatozoa of both species. There were significant differences in velocity of spermatozoa between swimming media and distilled water and between paddlefish and shovelnose sturgeon.

Key words: motility; velocity; sperm; flagellum; paddlefish; shovelnose sturgeon.

INTRODUCTION

Two species of the family Polyodontidae, the paddlefish *Polyodon spathula* Walbaum, confined to North America, and the Chinese swordfish *Psephurus gladius* (Martens) also called Chinese paddlefish, have declined dramatically in abundance in the wild over the past 100 years (Carlson & Bonislawsky, 1981; Gengerke, 1986; Mims *et al.*, 1993; Wei *et al.*, 1997). Both species have been commercially important in the past, but efforts to establish artificial propagation and stocking have been successful only for the North American paddlefish. Wild stocks of this species have been harvested for flesh and caviar, and used for angling (Gengerke, 1986; Wei *et al.*, 1997). The maximum weight of North American paddlefish is about 84 kg (Thuemler, 1997) and maximum recorded

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0022–1112/00/061348+20 $35.00/0

length is 216 cm (Adams, 1942). The shovelnose sturgeon *Scaphirhynchus platorynchus* (Rafinesque) is indigenous to the Mississippi River drainage including 21 states of the U.S.A. (Wallus, 1990). It is a small species (<3 kg) that can mature sexually in 5–6 years, more quickly than most other sturgeons. Spermatozoa of many fish species are immotile in the testes and in the genital tract. They are activated only after release into the external medium and have a short period of motility (Scott & Baynes, 1980). Most of the knowledge on flagellar movement comes from studies on sea urchin sperm motility, used as a model for such studies (Gibbons, 1981*a*). Nevertheless, some characteristics of sperm cells of fish show original features (Cosson *et al*., 1999): motility duration (Billard, 1978; Billard & Cosson, 1992), initiation of motility (Morisawa, 1985; Cosson *et al*., 1995) or motility pattern (Boitano & Omoto, 1992; Cosson *et al*., 1997). Rainbow trout *Oncorhynchus mykiss* (Walbaum) sperm has been used as a model for freshwater species. Environmental factors, such as ions, pH or osmolality, may hyperpolarize the cell membrane and stimulate motility of spermatozoa (Morisawa *et al*., 1999): this is probably the case for paddlefish, where several generations of spermatozoa are present in the testis at the same time (Cosson & Linhart, 1996). Scheuring (1925) was the first to report that in trout sperm, ions such as Na^+, Ca^{2+}, and Mg^{2+} reduce the inhibitory action of K^+ ions, the bivalent cations being more effective than Na^+. Synergistic effects between ions led to studies demonstrating a possible control of motility by the membrane potential which resulted from the combined effect of several ions (Blaber *et al*., 1988; Gatti *et al*., 1990; Boitano & Omoto, 1991). Osmotic pressure, concentration of K^+ and sucrose, and seminal plasma with a pH<7 are the main factors inhibiting sperm motility in salmonids (Morisawa *et al*., 1983*a*), and osmotic pressure seems to be the major controlling factor in cyprinids (Morisawa *et al*., 1983*b*; Linhart *et al*., 1991; Billard *et al*., 1995). The motility of paddlefish spermatozoa is controlled partly by osmotic pressure (Linhart *et al*., 1995) as described in cyprinids (Redondo-Müller *et al*., 1991) as well as in marine species (Morisawa & Suzuki, 1980). The motility of paddlefish spermatozoa is inhibited by an increase in K^+ concentration, even at low values, at pH 7·0 without any cumulative effect of Ca^{2+} as described in salmonids by Scheuring (1925), Cosson *et al*. (1986, 1989), and Christen *et al*. (1987). Baynes *et al*. (1981) demonstrated that solutions of K^+ (or K^+ and Na^+), which did not induce motility of trout sperm cells could be used as activating solutions when small amounts of Ca^{2+} were added. Trout spermatozoa describe circular trajectories, which become tighter with time elapsed after activation. The influx of Ca^{2+} ions occurring during the short motility period is responsible for this circularization of the tracks of trout spermatozoa (Cosson *et al*., 1989) and that Ca^{2+} ions can overcome the K^+ inhibitory effect on motility. No similar effect of Ca^{2+} ions was observed with membranated or demembranated spermatozoa of several teleost fishes, for example turbot *Scophthalmus maximus* (L.) sperm (Chauvaud *et al*., 1995), halibut *Hippoglossus hippoglossus* (L.) (Billard *et al*., 1993), silurids *Silurus glanis* L. (Billard *et al*., 1997), or eel *Anguilla* sp. (Gibbons *et al*., 1985).

In the present study, percentage motility, frequency, velocity, wave characteristics, rotational movements and eventual damage to paddlefish and shovelnose sturgeon spermatozoa were investigated in order to describe in detail the motility

phase of spermatozoa and its regulation in two closely related fish species of increasing aquacultural interest (Wei *et al.*, 1997).

MATERIALS AND METHODS

INDUCTION OF SPERMIATION AND SPERM COLLECTION

The experiments were carried out in April 1997–1998 at the Aquaculture Research Center, Kentucky State University, Frankfort, Kentucky, U.S.A. Shovelnose sturgeon (1–3 kg) were captured below Uniontown Dam, Uniontown, Kentucky. Paddlefish (7·0–12·0 kg) were captured below McAlpinne Dam, Louisville, Kentucky. Broodfish were transported to the ponds of the Aquaculture Research Center in Kentucky. Four different male sturgeons were used for the present experiments. Sturgeons were selected and kept together in 3000-l tanks with a water flow rate of 12 l min^{-1}, 9·0 mg O_2 l^{-1}, at controlled water temperature 17–19° C. Paddlefishes were selected and held separately in circular tanks (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. Four different male paddlefishes were used for these experiments. Maturity of male paddlefish can be determined by tubercle formation on their head and opercular flags, whereas male shovelnose sturgeon are identified by a concave abdomen.

In both species, spermatogenesis was induced hormonally (Needham, 1965) and males were injected intraperitoneally with the LHRH analogue des-Gly10(D-Ala6) LHRH ethylamide (Sigma, St Louis, Missouri, U.S.A.) at doses of 0·05 mg kg^{-1} of body weight, or with carp hypophysis (Linhart *et al.*, 1995). For milt collection, Tygon tubing (5 cm length) attached to a 10 ml plastic syringe was inserted into the urogenital pore and the syringe was filled with milt. Milt was collected and stored on wet ice for <3 h in 100-ml containers until motility analysis and counting of sperm concentrations with a Thoma hematometer. Milt of each male from either species was collected on the second and the third day after injection.

EVALUATION OF MOTILITY PARAMETERS

Motility was evaluated for percentage of motile spermatozoa, velocity, beat frequency and wave characteristics. Dark field and phase contrast microscopy images were used for measurements of spermatozoa motility parameters, including those of flagellar waves (Cosson, 1996).

Immediately after milt collection, spermatozoa were observed for spontaneous movement prior to dilution; a drop of 10 µl of undiluted milt was spread directly on a glass slide on the microscope stage and examined under 200 × magnification.

For testing the swimming ability of spermatozoa, 0·5 µl of milt was mixed directly within a 49 µl drop of distilled water or swimming medium (SM composed of 20 mM NaCl and 20 mM Tris–HCl, pH 8·2), placed on a glass slide positioned on the microscope stage and immediately after mixing examined under 200 × magnification. In this study, final dilution was 1 : 100. Motile spermatozoa were video recorded within 5 s after activation for measurement of velocity and percentage of actively swimming 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 3 CCD video camera (SONY DXC-970MD, Japan) mounted on a dark-field microscope (NIKON Optiphot 2, Japan). The focal plane was always positioned near the glass slide surface. Spermatozoa movement was recorded using a tape recorder (SONY VHS, SVO 1520, Japan), visualized on a colour video monitor (SONY) and using stroboscopic illumination. The stroboscopic flash (Strobex, Chadwick-Helmut, El Monte, California, U.S.A.) 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, Japan) and analysed from three successive frames each by Micro Image Analysis (version 3.0.1. for Windows, with special application from Czech Olympus) for determination of velocity and percentage of moving spermatozoa.

For detailed examination of other swimming parameters, such as the flagellar beat, wave characteristics etc., 0·1–0·5 µl of milt was mixed directly with a 49-µl drop of distilled water or SM, placed on a glass slide, covered with a cover-slip, positioned on the microscope stage and immediately after mixing, recorded under 400 × or 1000 × magnification. In this part of the study, the final dilution was thus 1 : 100 to 1 : 500. Motile spermatozoa were recorded within 6–8 s after activation for measurement of swimming parameters. The movements of sperm flagella were recorded with both techniques described above and 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 needed. During the process of recording, the microscope stage was slowly hand-translated: this allowed the visualization of multiple well-defined successive images of a moving spermatozoon without overlap of flagellar images within every video frame (Cosson *et al*., 1997). Parameters such as track curvature were analysed from video tapes on a recorder (AG 7330 S-VHS, Panasonic, Japan) together with a video processor image enhancer using the trace function (DVS 3000, Hamamatsu-Photonics, Japan). The number of waves per flagellum was counted on video frames as the number of maxima and minima along the flagellum. Wave propagation and wave amplitude were measured on enlarged images from video tape records. The wave amplitude was measured as the distance between two segments tangential to the wave envelope, with one segment joining two successive principal (*P*) waves and the opposite segment joining two successive reverse (*R*) waves. The measurements of bend curvature characteristics of axonemes were according to Brokaw (1991), with some simplifications. The local bend angle was defined as the angle between tangents to the two straight segments adjacent to the bend. The wavelength was defined as twice the length of the segment between the two inflexion points adjacent to a bend. The dampening factor of waves was evaluated by measuring the ratio (in percentage) of the amplitude of two waves appearing successively along the flagellum from head to tail.

For the measurement of the beat frequency of the flagellum, 0·5 µl of milt was mixed directly with 49·5 µl distilled water or of SM, placed on a glass slide previously settled on the microscope stage; immediately after mixing (at least 4–5 s) with the pipette tip, beating of each spermatozoon flagellum was measured using a stroboscopic flash, as described above, by adjusting its flash light frequency to stabilize the flagellar waves in synchrony.

DATA ANALYSIS

The data acquired from several replications (four fish for each species with two sperm collections each) were evaluated and statistical significance was assessed using analysis of variance (ANOVA, Statgraphics version 5), followed by multiple comparison tests (Figs 3 to 5). Probability values $<0{\cdot}05$ were considered significant.

RESULTS

SWIMMING PARAMETERS AT INITIATION OF SPERM MOTILITY

Direct observation of undiluted sperm by dark field microscopy showed that most spermatozoa in the seminal plasma of paddlefish and sturgeon were immotile, but with a straight shivering flagellum, and only a few spermatozoa were motile but for <10 s. Sperm cells did not move even though flagella showed transiently propagated curvatures (up to 0·15 radians) along flagella (low beat frequency=0·1 Hz), so-called shivering (Fig. 1). Such shivering can be used as a good indicator of the potentiality for spermatozoa to be activated. Spermatozoa density was measured by counting sperm head number after dilution in a non-swimming medium containing formaldehyde and counting with a Thoma hematometer. The sperm density varied from 0·1 to 4 billion spermatozoa ml^{-1} and a total volume of 30–90 ml per collection.

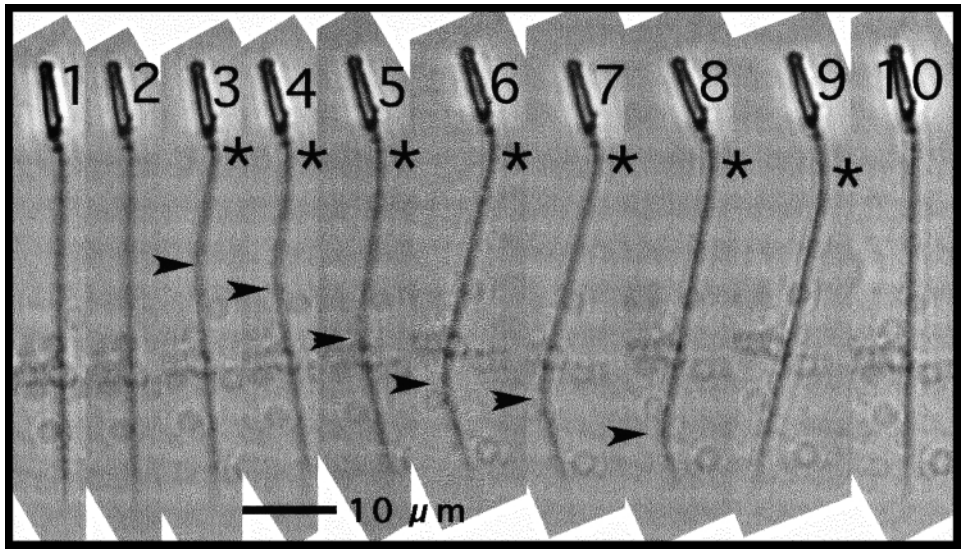

FIG. 1. Shivering of a paddlefish spermatozoon. A resting spermatozoon in seminal fluid showing no progressive motility but with flagellum exhibiting transiently local bending of low amplitude. Positions 1–10 are successive video frames obtained every 17 ms. Arrowhead in frames 3–8 shows progression of a local bend from head to tip. Star in frames 3–9 shows an opposite bend progressing slowly. Bar scale next to position 1.

When activated by transfer into distilled water, the motility of paddlefish and sturgeon was activated immediately [Fig. 2(a)–(c), (f)–(i)]. Beating waves propagated along the full length of flagella, while waves appeared dampened later during the motility phase [Fig. 2(c)], then completely absent at the end of the motility phase [Fig. 2(d) and (e)]. A series of images [Fig. 2(a) or (c)] allowed measurement of several flagellar parameters (Figs 3 to 5, Table I). Paddlefish spermatozoa demonstrated the following characteristics: the percentage of motile cells was 90–100% (Fig. 3), their velocity was 175 μm s^{-1} (Fig. 4), beat frequency was 50 Hz (Fig. 5) and period of motility was 4–6 min (Fig. 3). Initial motility of shovelnose sturgeon spermatozoa, also observed in distilled water, was 80–95% (Fig. 3), with a velocity of 200 μm s^{-1} (Fig. 4), a beat frequency of 48 Hz (Fig. 5) and a period of motility lasting 2–3 min (Fig. 3).

The effects of a swimming solution composed of 20 mM Tris–HCl and 20 mM NaCl, pH 8·2 were tested (Figs 3 to 5). After sperm dilution in such swimming solution, a majority of the samples showed 100% motility with a flagellar beat frequency of 50 Hz within 5 s after activation in both species. Analysis of variance indicated no significant difference between SM and distilled water in the two species, on the percent motility and on the flagellar beat frequency of spermatozoa. In such a swimming medium, the spermatozoa of paddlefish or shovelnose sturgeon showed a higher velocity within 10 s (175 and 250 μ s^{-1}, respectively), compared with values in distilled water (see above). Other flagellar parameters such as number of wave curvatures, wave amplitude, wave length, rotation frequency, beat frequency and dampening factor were measured (Table I). For simplification, data from only two time periods are grouped: short periods after initiation versus longer periods.

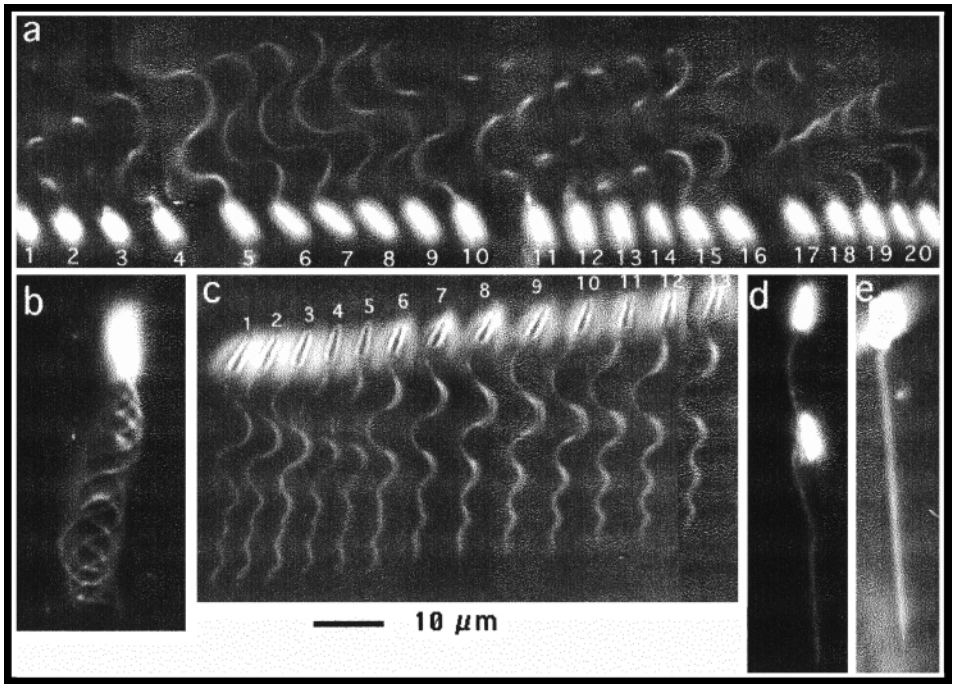

FIG. 2 (a)–(e).

FIG. 2. Actively swimming paddlefish and sturgeon spermatozoa. Motility was triggered by transfer of spermatozoa from seminal fluid to swimming solution. Dilution rate of sperm to SM was 1 : 50. Sturgeon sperm in panels (a)–(e), bar scale in (c): (a) swimming activation after 12 s; successive images of the same sperm cell every 0·003 s; (b) as (a) but with overlapping images showing the flagellar envelope; (c) as (a) but 55 s after activation showing dampening of waves in the distal part of the flagellum; (d), (e) 3 min after activation swimming has ceased, (d) shows slightly damaged flagellum and (e) an intact flagellum. (f)–(i) Paddlefish spermatozoa immediately after activation [bar scale in (f)]; (f), (g) phase contrast optics; (f) 12 s after activation, each video-frame is illuminated by one flash and shows the homogeneous behaviour of the sperm flagella; (g) as (f) but two flashes separated by 20 ms applied within the time of a video-frame showing two successive positions of each spermatozoon; (h), (i) dark field microscopy with double flash separated by 17 ms; (h) the two head positions illustrate the lateral deviation of the head track; (i) the two head positions illustrate the angular deviation of the head track.

SWIMMING PARAMETERS AFTER LONGER PERIODS OF MOTILITY

During the progress of the motility period, the percentage of actively swimming cells decreased with time (Fig. 3); for paddlefish spermatozoa, it reached 50% at 3–4 min in distilled water and at 7–8 min in the swimming medium. In sturgeon the percentage of swimming cells dropped below 50% after 80 s in distilled water and after 3 min in SM.

After activation of paddlefish and shovelnose sturgeon spermatozoa in the SM, the motility period was prolonged up to 9 and 5 min, respectively. Analysis of variance indicated significant influence of the swimming media and distilled water for both species ($P<0{\cdot}001$) on velocity of spermatozoa. In these conditions, values of mean velocity were still in the range of 100 µm s^{-1} for the spermatozoa of sturgeon or paddlefish still active at this time. Multiple range analysis (LSD) of the velocity of spermatozoa indicated significant differences

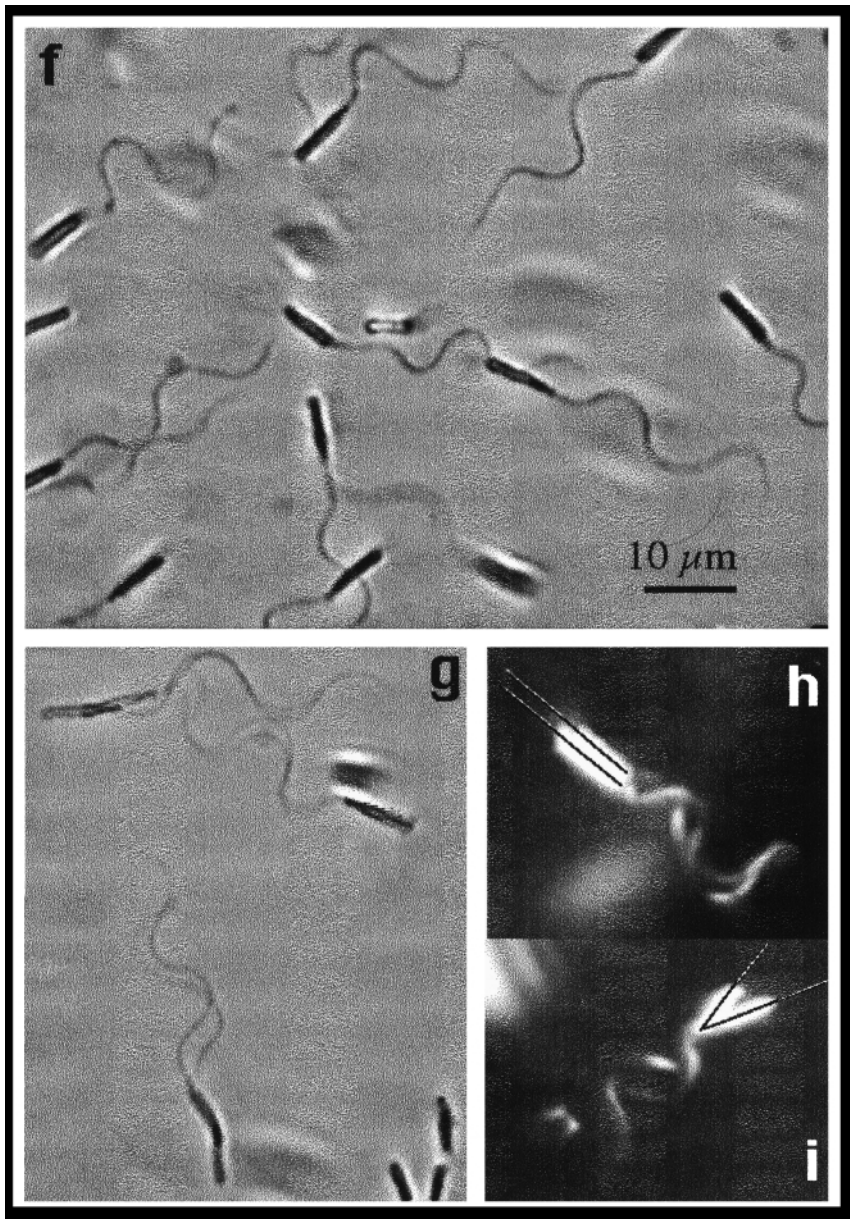

FIG. 2 (f)–(i).

($P<0{\cdot}01$) between swimming medium and distilled water, and between paddlefish and shovelnose sturgeon.

During the motility phase, many parameters which characterize the swimming behaviour were changing (Fig. 6). The wave shape develops homogeneously for the whole population of sperm cells from situation 1 before initiation, to 2 characterized by a large amplitude wave pattern and waves occupying the whole length, then later 4 with smaller amplitude waves and 5 where a dampening process appears in the distal portion, ending with 6 where very slow waves

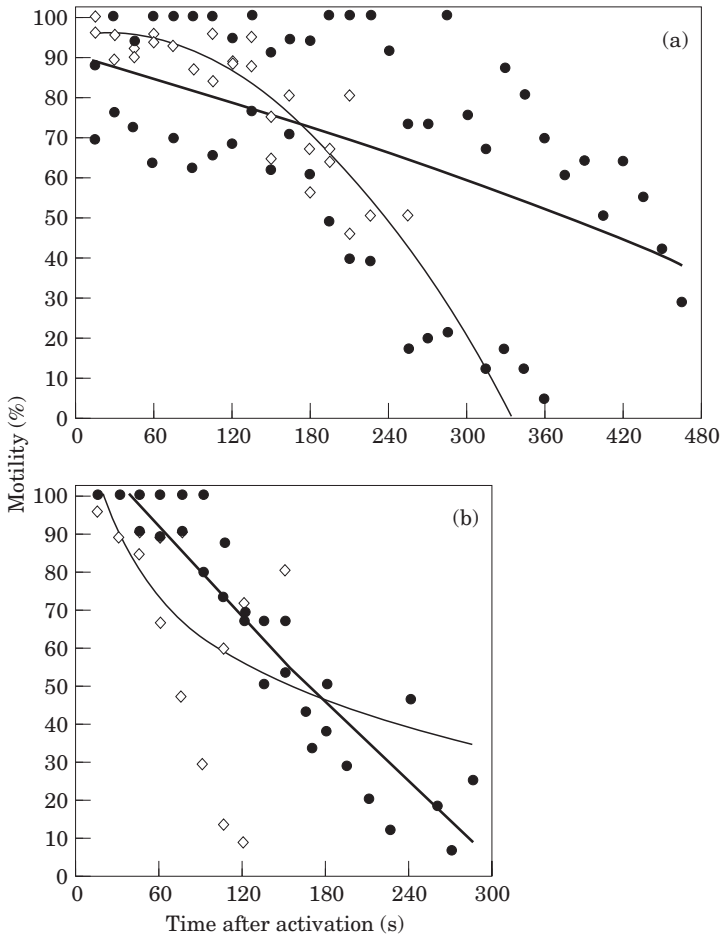

FIG. 3. Percentage motility of paddlefish (a) and shovelnose sturgeon (b) spermatozoa activated in distilled water (◇) and in SM (●). Analysis of variance showed no significant difference between SM and distilled water or between species, on the percentage motility (four different fishes and two samples per fish). The curve fits were drawn as mean lines for clarity of illustration.

provide only some shaking of the sperm cells. In order to quantify comparatively the evolution of this wave pattern (Fig. 6), a computer assisted method was used (Cosson *et al.*, 1997), allowing measurement of the local curvature; thus, several parameters were used to quantify these changes (Table I). It appears that the number of curvatures increases as a function of time elapsed since activation, while the wave amplitude decreases. The beat frequency as well as the rotation frequency both decrease during the same period. Also, the change of the dampening factor illustrates the restriction of waves to the proximal portion of the flagellum. Thus, the combined modifications in parameters such as decrease of wave amplitude, of beat frequency, or increase of dampening, leads to a rapid decrease in overall swimming performance of the spermatozoa in both species. This handicap is emphasized by damage occurring in parallel to the flagella during the motility period.

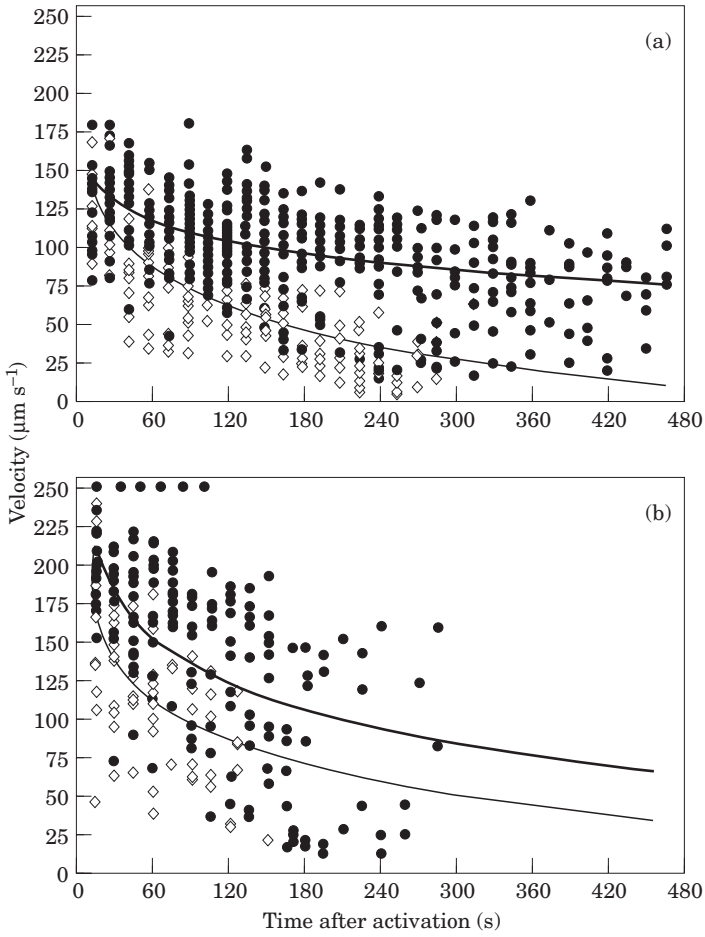

FIG. 4. Velocity of paddlefish (a) and shovelnose sturgeon (b) spermatozoa activated in distilled water (◇) and in SM (●) respectively. Only sperm cells with velocity >5 μm s^{-1} are taken into acount. Analysis of variance showed significant influence of SM and distilled water and species (P<0·001) on velocity of spermatozoa. Multiple range analysis (LSD) showed significant difference between SM and distilled water and between species in velocity of spermatozoa (P<0·01, four different fishes and two samples per fish). Curve fitting is drawn as exponential decrease for clarity of illustration.

DAMAGE TO SPERMATOZOA RESULTING FROM EXPOSURE TO LOW OSMOLALITY ACTIVATING SOLUTIONS

From a comparison of the swimming ability of sturgeon and paddlefish spermatozoa in various media, it appears that at activation, sperm cells are exposed to a hostile environment, i.e. low osmolality compared with that of the seminal fluid. In this respect, the activation in distilled water is certainly the most harmful because of its lower osmolality (Fig. 6). One minute after transfer to distilled water, blebs appeared along the flagellum [Fig. 7(a)–(c)], which prevent correct and efficient wave propagation. At later periods in the motility phase, the tip of flagellum became curled into a loop which shortened the flagellum and restricted waves to the third or the fourth proximal part of its

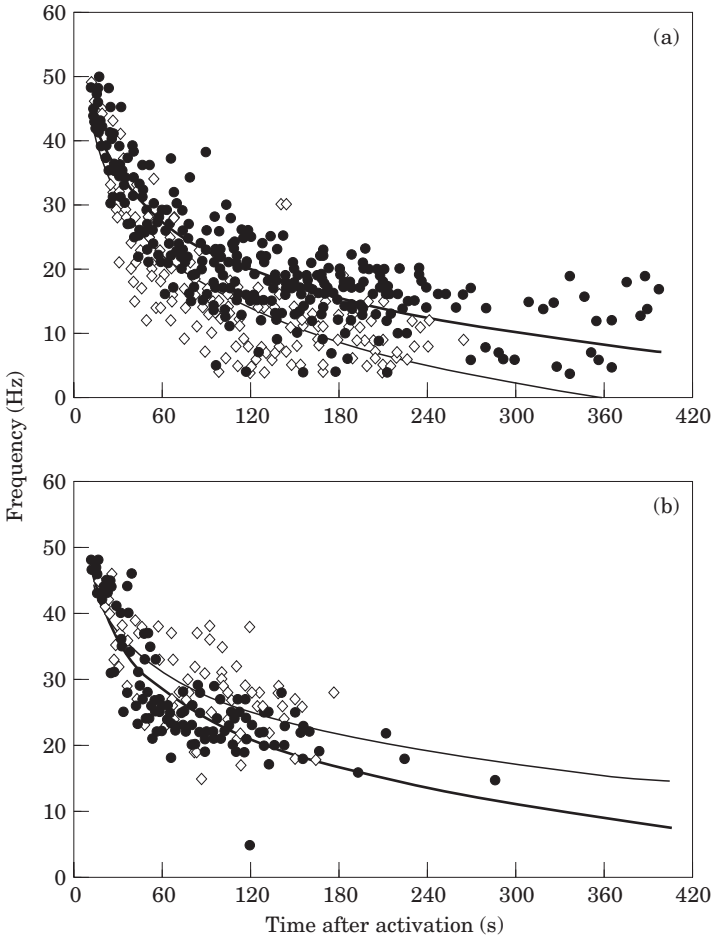

FIG. 5. Flagella beat-frequency of paddlefish (a) and shovelnose sturgeon (b) spermatozoa activated in distilled water (◇) and in SM (●) respectively. Only the motile cells were used for beat frequency measurements. Analysis of variance showed no significant difference between SM and distilled water or between species, on flagellar beat-frequency of spermatozoa (four different fishes and two samples per fish). Curve fitting is drawn as exponential decrease for clarity of illustration.

length [Fig. 7(d)–(q)]. These defects were not induced in flagella when activation of spermatozoa was triggered in a solution comprising NaCl/Tris (SM).

ROTATION OF SPERM CELLS DURING TRANSLATIONAL MOTION

When successive images of a single spermatozoon were observed for a very short period of time, flagellar waves could be visualized alternatively (Fig. 8) from side or from top view, i.e. waves were coplanar with the microscope focal plane or orthogonal to it. Thus, it is possible to measure the rotation frequency of these spermatozoa [Fig. 8(g), Table I]. The rotation of sperm cells contributes to the high degree of linearity observed for the sperm tracks (Table I), as a local circular portion of the track is compensated by the opposite circular portion occurring after a half turn of the same sperm cell. Figure 8(b)–(d) are successive positions of a same spermatozoon and illustrate the alternating view of waves

TABLE I. Detailed characterization of flagellar parameters in paddlefish and shovelnose sturgeon sperm flagella

Species	Time after activation	Number of curvatures	Wave amplitude (μm)	Wave length (μm)	Rotation frequency (Hz)	Beat frequency (Hz)	Dampening factor (%)
Sturgeon	10–30 s	$3{\cdot}85 \pm 0{\cdot}36^{a}$	$9{\cdot}50 \pm 0{\cdot}76^{c}$	$17{\cdot}70 \pm 0{\cdot}90^{b}$	$9{\cdot}58 \pm 2{\cdot}16^{b}$	$38{\cdot}25 \pm 6{\cdot}95$	$-29{\cdot}63 \pm 5{\cdot}68^{a}$
	>1·5 min	$7{\cdot}09 \pm 0{\cdot}82^{c}$	$5{\cdot}34 \pm 0{\cdot}94^{a}$	$12{\cdot}80 \pm 1{\cdot}16^{a}$	$2{\cdot}98 \pm 0{\cdot}41^{a}$	$11{\cdot}48 \pm 5.39$	$+30{\cdot}67 \pm 6{\cdot}54^{b}$
Paddlefish	10–30 s	$3{\cdot}82 \pm 0{\cdot}39^{a}$	$9{\cdot}69 \pm 0{\cdot}75^{c}$	$18{\cdot}60 \pm 0{\cdot}83^{c}$	$10{\cdot}58 \pm 2{\cdot}38^{c}$	$40{\cdot}85 \pm 5{\cdot}20$	$-29{\cdot}43 \pm 7{\cdot}17^{a}$
	>1·5 min	$6{\cdot}69 \pm 0{\cdot}75^{b}$	$6{\cdot}13 \pm 0{\cdot}67^{a}$	$12{\cdot}80 \pm 0{\cdot}91^{a}$	$3{\cdot}22 \pm 0{\cdot}42^{a}$	$23{\cdot}31 \pm 5{\cdot}06$	$+29{\cdot}54 \pm 7{\cdot}14^{b}$

Wave number, wave amplitude, wave length, rotation frequency and dampening factor were measured as described in the Materials and methods. Groups with a common superscript do not differ significantly ($P<0{\cdot}05$; four different fishes of each species and two samples per fish).

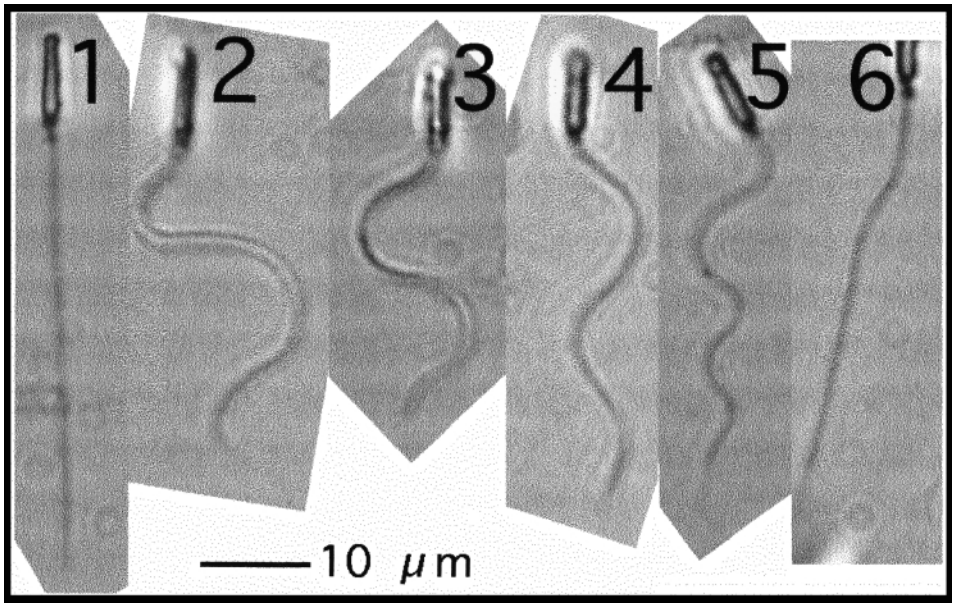

FIG. 6. The succession of wave pattern observed during the swimming period. (1) Resting sperm in seminal fluid is not swimming progessively; (2) fully activated sperm 3–15 s after activation in a swimming solution showing wave amplitude increasing from head to tip (see first wave on left side smaller than second wave on right side); (3) at 15–30 s, sperm flagella show first and second waves of similar amplitude; (4) at 30–60 s, wave amplitude decreases slightly; (5) after 1 min most sperm flagella still show progressive motility but with dampening of waves from base to tip; (6) at much longer periods after activation, non-progressive sperm cells show transient slight bends, similar to those in Fig. 1. The representative images of paddlefish spermatozoa were observed by phase contrast microscopy, but the principle was the same in sturgeon spermatozoa. Flash illumination = 60 Hz. Bar scale in (6).

from the top and from the side. In Fig. 8(e), (f) the proximal segment of the flagellum shows waves from the side while the distal portion shows waves from the top (flat view). Figure 8(h) illustrates the transient situation of Fig. 8(c) or (e) where a proximo-distal distortion occurs along a single flagellum. Such distortion, leading to compensation in lateral deviations during swimming, also contributes to the observed linearity of tracks.

DISCUSSION

The spermatozoa of sturgeons and paddlefish are essentially immotile in seminal plasma (Fig. 1; Cosson & Linhart, 1996) and are activated when transferred to the swimming medium (Fig. 2), usually freshwater or low salt solutions (Drabkina, 1961), with a behaviour similar to other teleost fish species. Immediately after activation, they have rapid forward motility. All motility parameters such as beat frequency, velocity, and wave amplitude, decrease abruptly during the motility period (Figs 3 to 5) including the percentage of motile cells which also decreases gradually (Fig. 3). During the earliest period, spermatozoa of sturgeon and paddlefish move at 175–250 μm s^{-1} (Fig. 4). The forward motility of the spermatozoa then slows down gradually to 50–100 μm s^{-1} at 3–6 min after activation (Fig. 4); these values take into account only the

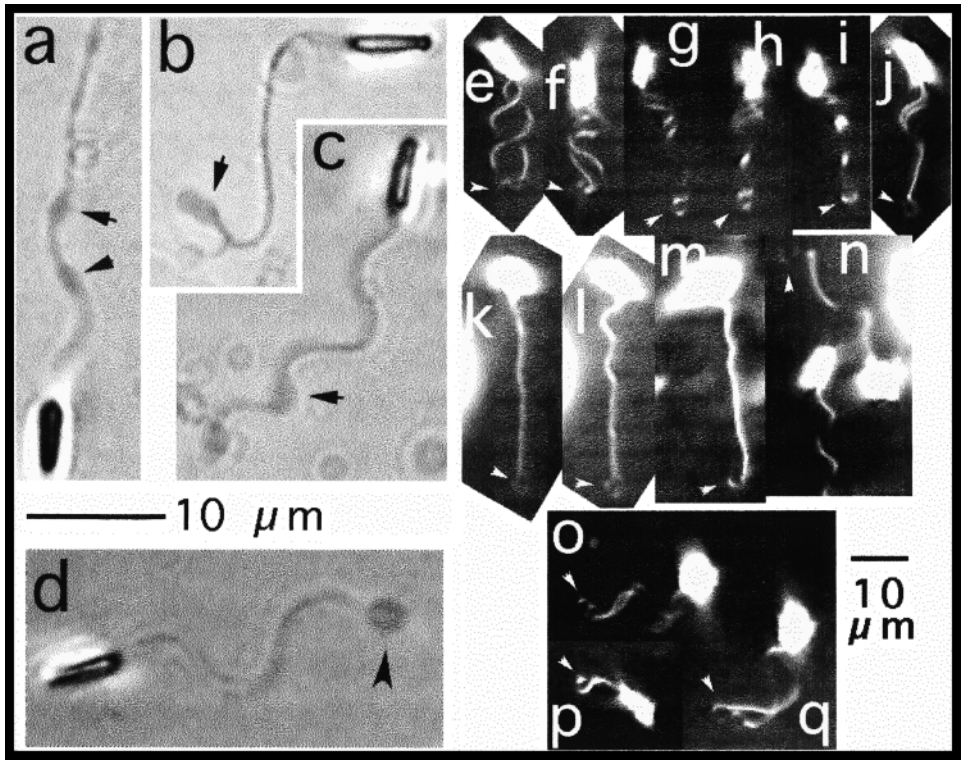

FIG. 7. Damage observed on flagella of shovelnose sturgeon and paddlefish spermatozoa. (a)–(d) Shovelnose sturgeon; phase contrast optics [bar scale in (d)], (e)–(q) paddlefish; dark-field optics, bar scale in (q). In (a)–(d), sturgeon sperm after several minutes of activation in pond water shows blebs (arrow heads) along [(a)–(c)] or at the tip of their flagella (d); loops on other flagella are seen out of focus in the same field. In (e)–(q) different paddlefish spermatozoa activated in pond water; (e), (f) are early after activation showing wave propagaiton limited by the presence of a curl appearing at the flagellar tip (arrowhead). In (g)–(n), later stages in the motility period with waves propagating proximally to the head and with a curl (arrowhead) at the flagellar tip. In (o), (p), (q), the main length of flagellum is curled (arrowhead) which impairs drastically the wave propagation. Illumination rate = 50 Hz in (a)–(d) and 100 Hz in (e)–(q).

fraction of the population of cells forwardly motile. Occasionally some spermatozoa swim for up to 9 min. This sequence of behaviours has been described for spermatozoa of several species (Cosson *et al.*, 1999): in the trout (Cosson *et al.*, 1989, 1991), in the European catfish (Billard *et al.*, 1997), in the carp (Perchec *et al.*, 1993), in *Cottus gobio* L. (Lahnsteiner *et al.*, 1997), in the perch *Perca fluviatilis* L. (Lahnsteiner *et al.*, 1995), in several sturgeon species (Cosson *et al.*, 1995; Tsvetkova *et al.*, 1996), in the paddlefish (Linhart *et al.*, 1995; Cosson & Linhart, 1996), in the sea bass, *Dicentrachus labrax* L. (Fauvel *et al.*, 1998; Dreanno *et al.*, 1999*a*), in the halibut (Billard *et al.*, 1993) and in turbot (Chauvaud *et al.*, 1995). These results contrast with those observed for eel spermatozoa: the latter swim for >20 min without change in their characteristics (Gibbons *et al.*, 1985). A decrease of the swimming performances as observed in most other species could be due primarily to the parallel decrease of the energy stores during the motility period (in trout: Christen *et al.*, 1987, in carp: Perchec

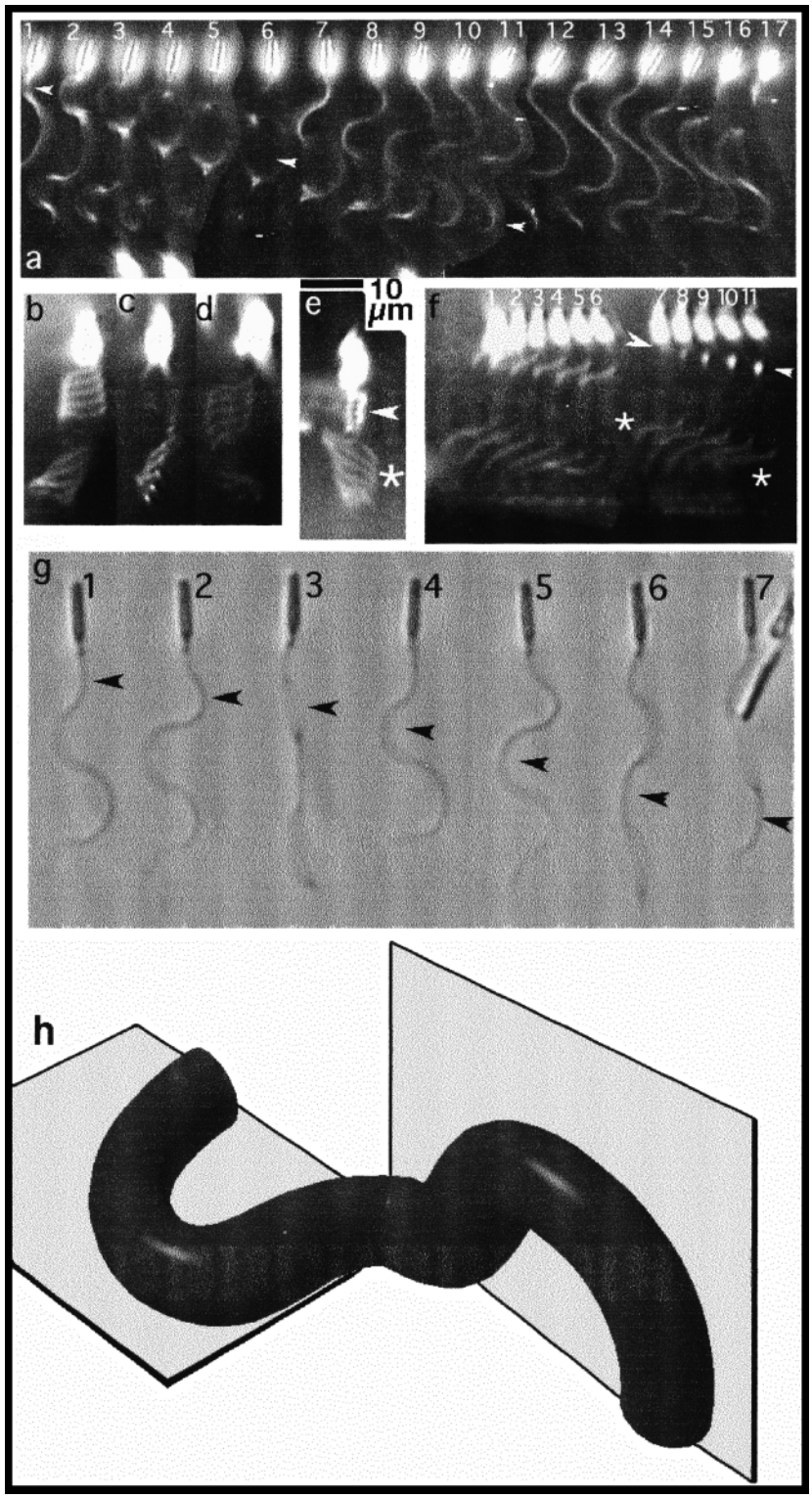

FIG. 8. For legend see page 1362.

et al., 1995, in turbot: Chauvaud *et al.*, 1995). A general discussion about the swimming characteristics of fish sperm motility can be found in Ishijima *et al.* (1998).

In sturgeon and paddlefish, only limited and contradictory information is available on the motility duration of native spermatozoa and data reported on the latter species as well as on the characteristics of motility are highly variable. Duration of 13 min in *Huso huso* L. was mentioned by Ginsburg (1968) and of 4·4 and 6 min in *Polyodon spathula* by Mims (1991) and by Linhart *et al.* (1995), while Toth *et al.* (1997) found motility of 30 min at 12° C in spermatozoa of the lake sturgeon *Acipenser fulvescens* Rafinesque. In the latter species, this may be because all spermatozoa were not activated synchronously initially, possibly due to the osmolality of the swimming solution; thus, the motility is initiated successively for individual cells, which could lead to an apparent extension of the period of motility. In the present experiments, only sperm samples that demonstrated at least 90% of the spermatozoa activated synchronously immediately after mixing were retained for experiments.

In *A. baeri* Brandt, Gallis *et al.* (1991) showed that motility, initiated after dilution in distilled water or in NaCl solution (<50 mOsmols kg^{-1}), declined regularly and stopped after about 5 min. Such decline of motility over 5 min results probably from the combination of the decreased percentage of motile spermatozoa and the change of the flagellar beating efficiency as shown by the progressive reduction of the wave amplitude (Fig. 6). A calculation of the ratio between the initial velocity (170 μm s^{-1}, Fig. 4) and the initial beat frequency (60 Hz, when extrapolated at zero time in Fig. 5) leads in the case of paddlefish spermatozoa to a value of 3 μm $beat^{-1}$ (velocity/frequency) for the so-called propulsive efficiency. In sturgeon, similar calculations accounting for velocity (200–250 μm s^{-1}, Fig. 4) and beat-frequency (60 Hz, Fig. 5) lead to a high propulsive efficiency (4 μm $beat^{-1}$). In both species, the latter will decrease as a function of time elapsed since activation, falling to values >1 μm $beat^{-1}$ at 1–2 min or later after activation. This decrease contributes to lower the swimming performance when combined with the decrease of other parameters like the wave amplitude or the proportion of the flagellar length occupied by

FIG. 8. Rotation of the actively swimming spermatozoa of sturgeon (a) and paddlefish [(b)–(g)]. Bar scale, above the (e) panel, applies to images in (a)–(f). (a) Dark-field microscopy, illumination at 300 Hz (3 ms between two flashes); in position 1, the newly growing wave (arrowhead) posterior to the head is coplanar with the observation plane (top view) and develops its bend left of the sperm track in positions 1, 2 and 3, then this wave is observed orthogonal to the observation plane (side view) in positions 4–8. The same wave is then observed again in top view but the bending direction is opposite, i.e. to the right of the sperm track in positions 10–13. (b), (c), (d) Flash rate 300 Hz, the same paddlefish spermatozoon observed as in (a) but with overlapping positions and showing successively top view of waves in (b), then mainly side view in (c) then top view in (d); (e), (f) dark field microscopy, flash rate 300 Hz, illustration of the 3D distortion of the flagellum: bending waves closest from the head are in top view (arrow head) while waves distal from head on the same flagellum are seen in side view (*). (g) Phase contrast optics, flash rate 60 Hz, a paddlefish spermatozoon with a flagellum developing a bend (arrow head) on the right of the sperm track seen in side view in positions 1 and 2 then in top view in 3, then in side view in 4 and 5, but with the bend left side to the sperm track. Such a series of sperm images in rotation allows the determination of the rotation frequency; it takes 0·05 s (three images at 60 frames s^{-1}) between positions 2 and 5 to accomplish a half turn rotation: this corresponds to a 10 Hz rotation frequency in this particular example. (h) Illustrates the 3D contortion as observed in (e) and (f): two successive bends are subscribed in planes orthogonal to each other.

waves, as well as an increase of the number of waves along the flagellar length (Table I). In contrast, the length of the waves still present in the proximal portion is affected little during the motility period.

Among fish with external fertilization, the traits of the motility behaviour of fish sperm flagella are quite similar in several respects (Cosson *et al.*, 1997; Ishijima *et al.*, 1998; Cosson *et al.*, 1999). An exception is the eel spermatozoon, which can swim for very long periods with a beat frequency reaching 95 Hz, and with an original wave pattern, having a rolling motion at 19 Hz, with flagella developing a helicoidal three dimensional (3D) bending (Wooley, 1998). In the case of paddlefish and sturgeon, due to the rotation of the whole sperm cell, each spermatozoon image appears alternatively with flagellar top view (waves in the plane of observation) or side view (waves orthogonal to the observation plane); in addition, waves were also observed as 3D transiently during rotation of sperm cells (rotation frequency of 9–10 Hz, Table I). Nevertheless waves are not arranged according to a helicoidal shape, but rather as successive waves subscribed in different planes [Fig. 8(h)]. The rolling motion was observed also for turbot spermatozoa but with a frequency of $5{\cdot}2 \pm 3$ Hz (Dreanno *et al.*, 1996*b*). The rotation of the sperm head has been observed also on native spermatozoa of sturgeon during swimming, as a consequence of the flagellar beating (Cosson *et al.*, 1997; Billard *et al.*, 2000). In all cases, rotation of the whole sperm cell adds complications to the measurement of the instrinsic flagellar beat frequency, as wave beating is not obvious to observe during periods where flagellar waves are transiently orthogonal to the observation plane.

In paddlefish or sturgeon sperm, the head trajectories remain close to linearity during the entire period after activation (results not shown). Furthermore, Ca^{2+} has no major effect on the motility, parameters such as the linearity of trajectories, reflecting the absence of asymmetric beating, the principal and reverse bends (as defined by Gibbons, 1981*b*) having similar amplitude. The effect of K^+ ions at low concentration was noted in both species, but this did not affect the track shape, but only induced arrest of wave propagation (not detailed in this paper).

In most fish species, the development of the wave pattern can be presented as illustrated in Fig. 6. During the motility phase, the wave pattern of paddlefish or sturgeon spermatozoa develops rapidly from a full beating situation where waves proceed through the whole length of the flagellum, to a partially beating situation where waves occupy only the part of the flagellum most proximal to the head; finally, absence of a wave leads to cessation of movement. When cells are devoid of progressive translation, their flagella are in a rigid state, mostly linear; this situation is similar to that of sperm flagella prior to their activation (Fig. 1). Nevertheless, in both cases, flagella are not completely immotile: transient shivering and local slight and slow bending are observed (Fig. 1): this can be used as an indicator of the potentiality to swim. A blocking process occurring in the distal part of the flagella is observed after activation (Fig. 6). In partially beating situations, the distal part of the flagellum appears passive, and devoid of any wave propagated in this segment, while a fully developed wave is initiated and propagated in the proximal portion of the flagellum over the third closest to the head. Due to the decrease of the ATP store of sperm cells during progress of the motility phase (Billard & Cosson, 1990), the proportion of motile sperm cells

also decreases as a function of time after activation, which also probably contributes to a decrease in fertilizing ability. The beat frequency and the wave amplitude decrease during the motility period, as well as a change in the dampening factor (restriction of waves to the proximal portion of the flagellum): all these modifications combine to cause a rapid drop in the swimming efficiency of spermatozoa of both sturgeon and paddlefish.

The decrease in swimming performance occurs very homogeneously for the whole sperm population, because the morphological changes during the motility phase affect simultaneously all flagella of paddlefish or of sturgeon spermatozoa. During the very earliest (but short) period, triggering of motility occurs simultaneously and swimming characteristics are then expressed at an optimal level. The progressive decrease of the wave amplitude in the distal part of the flagella was already reported on native spermatozoa of sturgeon *A. baerii* (Billard *et al*., 1999, 2000). It is similar to that observed in teleosts such as the turbot (Chauvaud *et al*., 1995), the sea bass (Dreanno *et al*., 1999*a*) or the carp (Perchec *et al*., 1995). This has been interpreted in terms of an energy transfer deficiency from the mid-piece (ATP production in mitochondria) to the distal part of the flagellum (Perchec *et al*., 1995) where ATP is consumed; this was documented in trout spermatozoa (Saudrais *et al*., 1998) in terms of insufficiency of the ATP/creatine-phosphate shuttle: similar problems of energy availability may exist also in sturgeon and paddlefish spermatozoa. This could also result from exposure to drastic environmental osmotic conditions encountered when fish sperm is delivered out of the seminal fluid (Cosson *et al*., 1999).

An additional feature, probably related also to the osmolality environment and contributing to the decrease of swimming capacity, is the appearance of loops (coiled axoneme) at the distal tip of the flagella [Fig. 7(a)–(d)] when exposed to low osmolality conditions, as mentioned in *A. baerii* by Tsvetkova *et al*. (1996) and also in carp spermatozoa after dilution in fresh water; in the latter, it probably reflects a response to hypo-osmotic exposure (Perchec *et al*., 1996) and is reversible. Nevertheless, such looped flagella still have the capacity to propel cells, even though more slowly, and such disabled spermatozoa probably have the capacity to fertilize eggs. In some instances, there were examples where waves were present and propagated only in the distal part of the flagellum. In all cases observed, this behaviour was transient and a full wave pattern reappeared within 1–2 s.

This work was supported by USDA 1890 Capacity Building Grant Program 96-388142582 to Kentucky State University, Frankfort, KY, and in collaboration with University of Oklahoma, Norman, U.S.A. Funding from CNRS-France (J.C.) and Czech Agriculture Foundation no. 6051, Czech Republic (O.L. and M.R.) also supported this work.

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