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Chemical and thermal effects on the viability and motility of spermatozoa from the turtle epididymis

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The viability and motility of spermatozoa harvested from the epididymides of turtles were estimated to elucidate properties that might enable them to be stored over long periods of time. Spermatozoa from the painted turtle, *Chrysemys picta*, were analysed and compared with spermatozoa from two other turtles, *Trachemys scripta* and *Sternotherus odoratus* using the Cellsoft analysis system for videotaped images. Spermatozoa from *C. picta* and *T. scripta*, suspended in F-10 medium, showed low motility (3–6% motile) and motion velocities, whereas the motility of spermatozoa from *S. odoratus* was higher (40% motile). Spermatozoa from *C. picta* and *S. odoratus*, but not *T. scripta*, had higher motilities and motion velocities when incubated at 2°C before analyses. *C. picta* spermatozoa were unresponsive to calcium concentrations ranging from 10^{-8} to 10^{-1} mol l^{-1}, potassium concentrations ranging from 0.1 to 10 mmol l^{-1}, and to pH values in the range 5.9–8.4. Spermatozoa from *C. picta* were sensitive to hypo-osmotic media, and showed reduced motility at 25% of normal osmolarity and no motility at 10% of normal osmolarity. Distorted cells and missing flagellae were noted at 50% of normal osmolarity. *C. picta* spermatozoa were viable up to 40 days after harvest when incubated at 4°C; during this time, both motility and motion velocity were increased in response to 0.5 mmol 3-isobutyl-1-methylxanthine l^{-1}. Spermatozoa from turtles have osmotic properties and resistance to changing chemical environments similar to spermatozoa from other vertebrates that have internal fertilization, and appear to be stable over long periods of time compared with spermatozoa from other vertebrate species.

Introduction

Turtles are among the most primitive of reptiles, diverging from other reptilian stock in the Paleozoic era. As such, turtles are the first extant group to show characteristics of amniote vertebrates. One of these characters is internal fertilization. Although external fertilization occurs in most fish and amphibians, all but a few amniote vertebrates deposit spermatozoa directly into the female reproductive tract in a copulatory act. Thus, a change from external to internal fertilization might be accompanied by changes in the structure or behaviour of the male gamete, but this issue has received little attention from gamete biologists.

Spermatozoa are among the most fragile of cells and do not survive for long outside the male reproductive tract (Restall, 1967). Fish spermatozoa live only minutes after spawning or artificial stripping (Billard and Cosson, 1992). The motility of spermatozoa within the ductus deferens of the quail declines markedly after 72 h (Clulow and Jones, 1982) and diluted rooster spermatozoa are viable for only 12–24 h after collection (Bakst, 1988). Despite the fragility of spermatozoa, fertility is preserved in those species in which spermatozoa are stored within the female tract. For example, bat spermatozoa are fertile after overwinter storage in the female uterus (Wimsatt, 1942, 1944). The life of spermatozoa stored within the sperm storage tubules of birds (SST) is prolonged and the storage time is correlated with the interval between egg clutches (Birkhead and Moller, 1992). Salamander spermatozoa stored within the spermathecae of females remain morphologically intact up to 60 days after cessation of sexual activity (Zalisko and Larsen, 1989). Spermatozoa are stored within the oviducts of many reptiles and fertile eggs may be oviposited up to several years after isolation from males (Gist and Jones, 1987).

Freshwater turtles are excellent models to test whether the extended fertility of spermatozoa is a property of the male gamete or of the storage structures in the female oviduct. These turtles possess an unusual reproductive cycle in which the production and maturation of male and female gametes is not synchronized (Licht, 1984). In males, spermatogenesis commences in spring and is completed by autumn, whereas in females vitellogenesis begins after oviposition in early

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summer but ovulation does not occur until the next spring. Thus, there is a 5–6 month difference in the production of male and female gametes. Male turtles retain spermatozoa in the epididymis over winter, presumably using them in spring matings. However, some freshwater turtles mate in the autumn (Gist *et al*., 1990) and spermatozoa transferred in those matings are stored over winter within oviductal glands of females to fertilize eggs ovulated the following spring (Gist and Jones, 1987). In either scenario, there is an extended interval during which the fertility of spermatozoa must be preserved, either in the male or the female.

This study was undertaken to examine properties of turtle spermatozoa that might contribute to this longevity. The painted turtle (*Chrysemys picta*) is abundant in the ponds of North America and was selected for that abundance and also because much is known of its mating habits and sperm storage (Gist *et al*., 1990; Gist and Jones, 1987). As the study progressed, it became necessary to use spermatozoa from other less abundant turtles for comparison.

Materials and Methods

Animals

Spermatozoa from three species of pond turtle were used in this study. Painted turtles (*Chrysemys picta*) were collected from traps placed in ponds in the vicinity of Cincinnati, Ohio, or were purchased from commercial suppliers. Stinkpot (*Sternotherus odoratus*) and slider (*Trachemys scripta*) turtles were captured from pond traps placed in the vicinity of Aiken, South Carolina, and were subsequently shipped to Cincinnati. Turtles were housed in running water tanks, fed crickets once a day, and were generally killed within 1 week of capture or arrival.

Sperm collection

Turtles were killed by i.p. pentobarbital injection using a protocol approved by the institution. The epididymides were removed, placed in cold (4°C) Krebs–Ringer phosphate buffer (KRP), pH 7.4, and allowed to exsanguinate for 10–15 min. Except when noted, all chemicals were purchased from Sigma (St Louis, MO). After transfer to fresh cold KRP, connective tissues surrounding the epididymis were removed, the tubules were ruptured, and the spermatozoa were allowed to drain into the KRP for 10–15 min. Buffer containing spermatozoa was transferred to centrifuge tubes and concentrated by centrifugation at 700 ***g*** for 20 min in a refrigerated centrifuge. Preliminary experiments compared the motility of spermatozoa from two turtles suspended in various buffers (PBS, Krebs–Ringer bicarbonate, Tyrode's, Hams F-10) and at different osmolarities within the range measured in seminal fluid. The highest motility was obtained using F-10 medium (NaCl, 100 mmol l^{-1}; $NaHCO_3$, 14 mmol l^{-1}; Hepes, 10 mmol l^{-1}; glucose, 6 mmol l^{-1}; $MgSO_4$, 1 mmol l^{-1}; KH_2PO_4, 1.6 mmol l^{-1}; KCl, 0.4 mmol l^{-1}; $CaCl_2$, 0.3 mmol l^{-1}; NaH_2PO_4, 0.1 mmol l^{-1}; pH 7.4) at an osmolarity of 275 mosm kg^{-1}, and this was used in all experiments except when noted. After centrifugation, the KRP was replaced by F-10 medium, and the spermatozoa were resuspended. After two additional washes with F-10, spermatozoa were suspended in 10 ml F-10 medium containing various ionic concentrations or osmolarities as described below.

Incubations

Except when noted, spermatozoa were incubated for 1–3 h on ice (2°C) in F-10 medium at various ionic concentrations, pH or osmolarity. At the end of the incubation, an aliquot was taken and diluted for motion analysis videotaping as described below, and another aliquot was taken to determine sperm viability.

Viability was assessed by fluorescence microscopy using ethidium homodimer (0.15 µmol l^{-1}) and calcein-AM (2 µmol l^{-1}) (live/dead viability kit; Molecular Probes, Eugene, OR) and was based on counts of 200 cells. With this kit, live spermatozoa fluoresce green and dead spermatozoa fluoresce red when viewed with fluorescein isothiocyanate (FITC) optics. The efficacy of this method was verified using spermatozoa killed with formalin.

Temperature. Epididymal spermatozoa from all three species were incubated for 1 h in F-10 medium on ice (2°C) or at ambient (23°C) temperature. It was not possible to videotape sperm motility at the same temperature as the incubation with the available equipment configuration. For recordings of spermatozoa incubated at 2°C, the equipment (see below) was moved into a cold room and videotaping was performed at 12°C. For sperm incubated at ambient temperature, the cold room was opened and the cooling system was disengaged. Under these conditions, temperature in the cold room during videotaping was 16–19°C.

Osmotic variation. *C. picta* spermatozoa were incubated in F-10 medium diluted with Hepes-buffered water (10 mmol l^{-1}) to yield 100, 50, 25 and 10% solutions; the osmolarity of these solutions was measured using a Precision Systems Osmette osmometer and averaged 274, 154, 94 and 58 mosm l^{-1}, respectively. Videotaping was performed at 12°C.

Ionic variations. The effects of varying ionic concentrations were assessed using *C. picta* spermatozoa in F-10 medium. For potassium studies, spermatozoa were incubated in medium containing K^+ at 0, 0.4 or 4.0 mmol l^{-1}; compensatory adjustments were made in NaCl and NaH_2PO_4 concentrations to preserve osmotic equality at 275 mosm kg^{-1}. Other spermatozoa were incubated in medium containing Ca^{2+} at 0 (Ca^{2+}, 10^{-8} mmol l^{-1}, buffered with EGTA), 0.1, 0.3 or 1.0 mmol l^{-1}; compensatory adjustment was made in NaCl to preserve osmotic equality. Videotaping was performed at 12°C.

pH. *C. picta* spermatozoa were incubated in F-10 medium in which the acidity was adjusted to yield pH values of 5.9, 6.4, 6.9, 7.4, 7.9 or 8.4 and the osmolarity was maintained at 300 mosm kg^{-1}. Videotaping was performed at 12°C.

Longevity. The behaviour of *C. picta* spermatozoa was observed over an extended period after collection. Semen antibiotics (gentamicin, 50 mg ml^{-1}; tylosin, 10.8 mg ml^{-1}; linconycin, 30 mg ml^{-1}; spectinomycin, 60 mg ml^{-1}; Lorton *et al*., 1988) were added to spermatozoa suspended in F-10 medium. These were separated into aliquots, sealed and incubated in the refrigerator at 4°C. At various intervals up to 40 days after collection, an aliquot was removed, allowed to warm to ambient temperature, and videotaped at ambient temperature (23°C). Because of low motility estimates, the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) was used to verify the capacity for movement of isolated spermatozoa. At intervals of approximately 1 week, an additional subsample was taken and exposed to 0.5 mmol IBMX l^{-1} for 30 min before videotaping. IBMX is known to stimulate sperm motility in mammals (Sinha *et al*., 1995) and in pilot experiments was found to do so in *C. picta* (Dawes, 1993). The viability of spermatozoa in each of the aliquots was determined as described above.

Motion analysis

Spermatozoa maintained under the various conditions for 1–3 h were resuspended in F-10 medium containing 10% BSA, diluted when appropriate to reduce the number of cells, and an aliquot placed in a Cell-Vu 20 µl counting chamber (Spectrum Technologies, Healdsburg, CA). BSA was necessary to prevent sperm heads from adhering to the coverslip or slide. Spermatozoa were videotaped at × 30 total magnification using a Sony (XC-75) CCD camera attached to an Olympus BX40 microscope equipped with a × 10 negative phase objective, a time-date generator and a JVC HR-S69000U video recorder. A total of eight arbitrary fields were recorded for each sample. Subsequent analysis of the videotapes was performed using a computer assisted semen analysis system (Cellsoft, Cryo Resources, New York). The use of this system for turtle spermatozoa was verified by tracking sperm movement manually across video frames. Settings for the computer analysis system are presented (Table 1) and the data reported include percentage motility, curvilinear velocity (Vc), straight line velocity (Vs), and linearity of movement (Vs/Vc).

Table 1. Settings for Cellsoft motility parameters

Number of frames to analyse	15
Number of frames per second	30
Minimum sampling for motility	4
Minimum sampling for velocity	4
Maximum velocity (µm s^{-1})	300
Threshold velocity (µm s^{-1})	10
Threshold grey level	105
Cell colour	White
Pixel scale (µm per pixel)	0.635
Dilution factor	1
Approximate cell size range (pixels low, high)	3, 45

Statistical analyses

Motion analysis data were analysed using a one way ANOVA with a nested design using the Systat statistical package. When appropriate, a post hoc test of significance (Tukey) or a *t* test was applied and significance was accepted at $P < 0.05$.

Results

Spermatozoa were collected from turtles in autumn at the end of the spermatogenic period and in the spring before the regression of the epididymis; spermatozoa generally were not collected during the summer (July, August) or winter (January, February). Analysis of variance of the data revealed no differences in any of the motility parameters attributable to the timing of sperm collection and thus all data were combined for statistical purposes.

There was substantial variation in the motility of *C. picta* spermatozoa. The highest motility recorded was 49% and the lowest was 0%; the average motility was 2.5%. Epididymal spermatozoa from *T. scripta* and *S. odoratus* were examined under identical conditions to verify that the low motility was not an artefact of the methods used. Average motility for *T. scripta* spermatozoa was 4.1%, whereas that for *S. odoratus* was 23.0%. The low motility in *T. scripta* and *C. picta* was not the result of fewer live cells, since viability estimates were above 90% in all three species. The low motility in these two species was accompanied by a reduced linear velocity of sperm movement, which was 23–25 µm s^{-1}. Higher motility in *S. odoratus* was accompanied by a higher average linear velocity of 40 µm s^{-1}.

Temperature

Motion parameters of spermatozoa from *S. odoratus* and *C. picta* were higher at the lower (2°C) incubation temperature (Table 2). Motility was significantly increased at the lower temperature in *S. odoratus* even though linearity was significantly lower. Motility was not significantly increased at the lower temperature in *C. picta*, but both curvilinear and linear velocity were. In contrast, the same parameters measured in *T. scripta* spermatozoa did not vary with temperature.

Osmolarity

Preliminary experiments indicated that the osmolarity of seminal fluid collected from *C. picta* epididymides was 260–320 mosm l^{-1}. Flagellae were lost from a few of the spermatozoa when F-10 was diluted to 50% of normal osmolarity. At 25% of normal osmolarity, motility was significantly ($P < 0.05$) lower, and forward movement was virtually undetectable in all spermatozoa (Table 3). The viability of spermatozoa remained above 90% at 50% osmolarity but dropped to 75% at 10% of normal osmolarity.

Table 2. Effects of incubation temperature on turtle sperm motion parameters

Turtle	Number of turtles	Motile spermatozoa (%)	Linear velocity (μm s^{-1})	Curvilinear velocity (μm s^{-1})	Linearity[a]
Sternotherus odoratus					
23°C	5	28.4 ± 11.7	48.8 ± 4.6	129.9 ± 15.4	37.1 ± 3.3
2°C	6	51.2 ± 9.4*	37.7 ± 2.2	143.7 ± 9.5	25.7 ± 1.8*
Trachemys scripta					
23°C	3	3.3 ± 1.5	26.1 ± 1.3	137.9 ± 8.7	17.5 ± 0.8
2°C	3	4.3 ± 1.8	25.3 ± 1.2	152.1 ± 9.9	18.8 ± 1.1
Chrysemys picta					
23°C	13	2.4 ± 0.7	16.5 ± 3.8	83.5 ± 22.1	15.3 ± 4.2
2°C	23	5.0 ± 0.9	23.6 ± 1.6*	141.5 ± 13.8*	16.3 ± 1.2

Spermatozoa were isolated from the epididymis and incubated in F-10 medium containing 10 mg BSA ml^{-1} either on ice (2°C) or at ambient temperature (23°C) for at least 1 h before videotaping.
Data are presented as the mean ± standard error.
[a] Curvilinear velocity/straight line velocity.
*Significantly different from higher temperature ($P < 0.05$; Student's *t* test).

Table 3. Effects of varying medium osmolarity on motion parameters of *Chrysemys picta* spermatozoa

F-10 (%)	Motile cells (%)	Curvilinear velocity (μm s^{-1})	Straight line velocity (μm s^{-1})	Linearity[a]	Viability[b]
100	3.83 ± 1.3	126.20 ± 25.8	22.25 ± 4.6	15.32 ± 3.2	98
50	2.67 ± 1.6	88.08 ± 39.7	15.32 ± 7.0	10.63 ± 5.3	98
25	0.5 ± 0.2	0.0	0.0	0.0	80
10	0.0	0.0	0.0	0.0	75

Spermatozoa were isolated from the epididymis and incubated in F-10 medium at 22°C.
Data are presented as the mean ± standard error of six turtles.
[a] Curvilinear velocity/straight line velocity.
[b] Percentage of live cells based on count of 200 cells.

The few intact cells remaining at the lower osmolarities showed little movement and possessed flagellae that were bent over at the midpiece (Fig. 1).

Ionic and pH sensitivity

Motion parameters of *C. picta* spermatozoa remained unchanged after incubation in K$^+$-free medium or 0.1 or 1.0 mmol K$^+$ l^{-1}. They were also unaffected by Ca^{2+} concentrations ranging from 10^{-8} to 1.0 mmol l^{-1} as well as variations in pH ranging from 5.9 to 8.4. Motility data for these experiments are presented (Fig. 2).

Longevity

Despite the presence of antibiotics, contamination eventually developed in the sperm samples, and for two of the four turtles the experiment was terminated early. The viability of spermatozoa from all four turtles remained high for the duration of the experiment; at 40 days, viability had only declined to 70%. Spermatozoa showed some motility during the first 5 days of the experiment (Fig. 3a), but this decreased to < 1% thereafter. IBMX consistently increased the activity of spermatozoa. Despite the small number of motile spermatozoa in these samples, the velocity of motile spermatozoa (Fig. 3b) remained constant throughout the 40 days of the experiment, and likewise was increased by IBMX treatment.

Discussion

This study was initiated to obtain data on the motility and viability of spermatozoa from the painted turtle and is the first to examine such parameters systematically in any turtle species. It is impossible to predict from motility parameters the fertility or infertility of a given gamete, but studies on mammalian spermatozoa have indicated that the number of motile spermatozoa in a sample and the velocity of movement of those spermatozoa are positively correlated with fertilizing success. Thus the observation of low sperm motility in both painted and slider turtles was unexpected. In the painted turtles, reduced motility and swimming velocities were consistently observed in preliminary experiments using different buffers and suspending media. The low motility was not the result of fewer live cells, since viability in all experiments exceeded 90%; in both species, it was accompanied by a reduced swimming velocity, both linear and curvilinear. In contrast, spermatozoa from the stinkpot turtles had considerably higher motility and motion velocities under identical experimental conditions. Unlike mammals, and some other reptiles, turtles lack accessory glands that contribute to seminal fluid, and the epididymis is located only a short distance from the penile groove. Thus, the probability of post-epididymal activation of spermatozoa is low. Therefore, it is concluded that the low motility of *C. picta* spermatozoa is not an artefact of the method of collection or

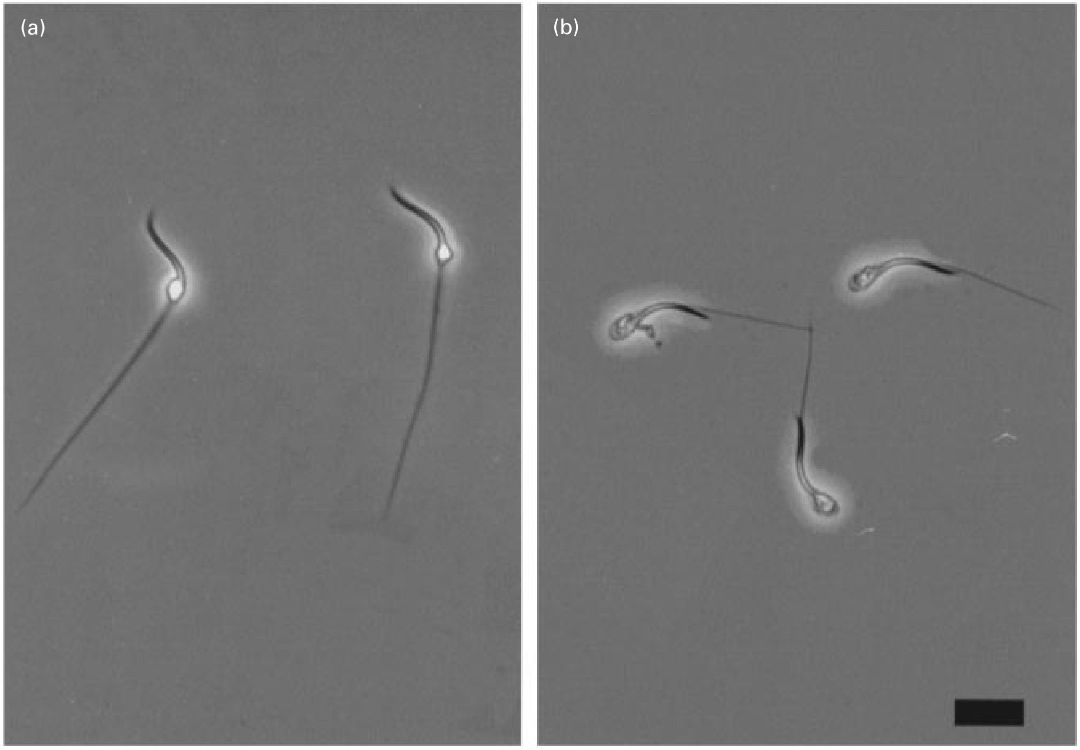

Fig. 1. Phase-contrast micrographs of epididymal spermatozoa from *Chrysemys picta*. (a) Cells maintained in normal F-10 buffer (274 mosm l^{-1}). (b) Cells maintained in 25% F-10 buffer (94 mosm l^{-1}). Scale bar represents 10 μm.

processing. Motility of spermatozoa from *T. scripta* was also low, averaging 4%, which is lower than the values (11–17%) reported for this species under similar conditions by Garstka and Gross (1990); reasons for the discrepancy are not clear. The reduced motility might have a taxonomic base: painted turtles and slider turtles both belong to the family Emydidae, whereas the stinkpot turtle is taxonomically more distant. Otherwise, there is little basis for comparison. Epididymal spermatozoa from the lizard *Lacerta vivipara* displayed a much higher motility than the spermatozoa of turtles (Depeiges and Dacheux, 1985). In these lizards, maturation of male and female gametes is synchronized, and fertilization occurs shortly after copulation, which is quite different from the reproductive cycle of chelonians. What advantage low motility might have in these two species of turtles is uncertain, but it may contribute to the longevity of spermatozoa within the oviduct.

Variations in ionic content or the pH of the suspending medium are considered important factors influencing sperm behaviour, but had little effect on spermatozoa from *C. picta.* Fish seminal fluid is high in potassium ion (Kruger et al l984), and dilution results in an increase in motility of trout but not cyprinid spermatozoa (Billard and Cosson, 1992) even under iso-osmostic conditions. Likewise, acidic conditions (< 7.5) are inhibitory to sperm motility in trout and salamander (Hardy and Dent, 1986) but not carp (Billard and Cosson, 1992). Fish sperm are immobile at calcium concentrations lower than 10^{-9} mol l^{-1}, and Ca^{2+} channel blockers are known to inhibit sperm motility in salmonids (Cosson, 1986; Tanimoto and Morisawa, 1988). Both avian and mammalian spermatozoa are activated via a Ca^{2+}-induced increase in cAMP. The low endogenous motility observed in *C. picta* spermatozoa could preclude responses to ionic variations, particularly those in which motility may have been reduced. However, despite the fact that viability remained above 90%, *C. picta* spermatozoa remained unresponsive to ionic concentrations that activate or otherwise increase sperm motility in other species.

C. picta spermatozoa showed little motility after removal from the epididymis, but remained over 70% viable for 40 days. They remained capable of activation as evidenced by their response to the phosphodiesterase inhibitor IBMX. This behaviour is unusual, since fish and amphibian spermatozoa become immotile within minutes after isolation (Billard and Cosson, 1992) and gametes from mammals and birds lose motility within 24–48 h (Ashizawa *et al*., 1976; Cupps, 1987) unless steps are taken to extend motility. The low motility of turtle spermatozoa and their ability remain viable under conditions as diverse as the epididymis, the oviduct, or refrigerated buffer indicates that longevity of turtle spermatozoa is primarily a property of the male gamete rather than structures or conditions in which it is stored and that chelonian spermatozoa may be more resilient than those of other vertebrates. The refractoriness and longevity of turtle spermatozoa could be of value in efforts to preserve the fertility of gametes of endangered chelonians.

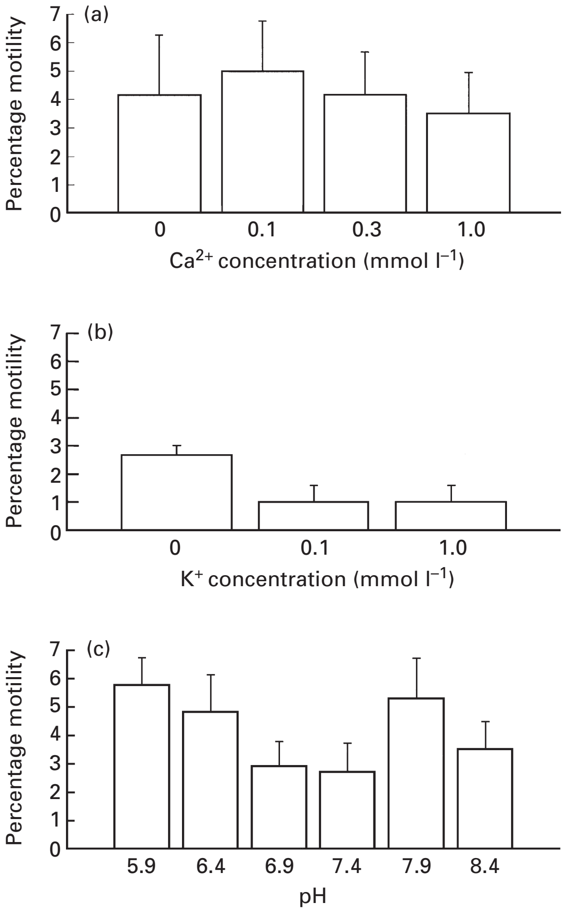

Fig. 2. Motility of spermatozoa isolated from the epididymis of *Chrysemys picta* and incubated for 1 h at 2°C in medium adjusted to contain the appropriate ion concentration or pH. Motion recordings were performed at 12°C. Data are presented as the mean percentage motility ± standard error. (a) Calcium ion, *n* = 6; (b) potassium ion, *n* = 8; (c) pH, *n* = 5.

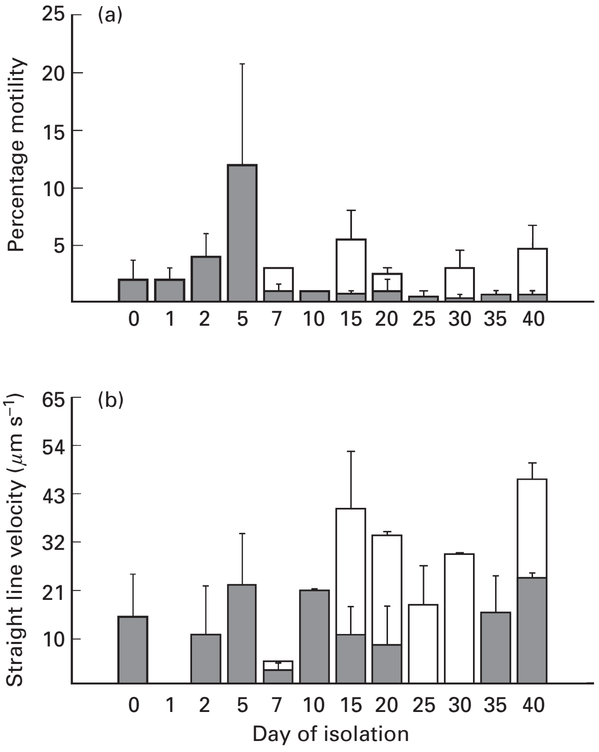

Fig. 3. Motility parameters of spermatozoa isolated from the epididymis of *Chrysemys picta*, maintained at 4°C, and recorded at ambient temperature (23°C). Data are presented as the mean ± standard error of one to four samples per day. (■) Controls; (□) spermatozoa incubated for 30 min in 0.5 mmol 3-isobutyl-1-methylxanthine (IBMX) l^{-1} on the days indicated. (a) Percentage of motile spermatozoa; (b) linear velocity of motile spermatozoa.

Vertebrate spermatozoa vary widely in their tolerance to altered osmotic environments. Spermatozoa from species that have external fertilization tolerate altered osmotic environments well in the short term. Dilution of seminal fluids will initiate motility in freshwater fish and amphibian spermatozoa (Hardy and Dent, 1986; Inoda and Morisawa, 1987; Billard and Cosson, 1992). Spermatozoa from marine fish are activated upon exposure to hyperosmotic solutions (Stoss, 1983). In contrast, spermatozoa from species that have internal fertilization such as birds or mammals undergo rapid distortion in hypo-osmotic medium (Bakst, 1980; Schrader *et al.*, 1986; Willoughby *et al.*, 1996). Turtle spermatozoa clearly fall into the latter category, since the dilution of the suspending medium severely affected the motility of spermatozoa, and at higher dilutions caused damage and deformation of the cells. Despite the fact that freshwater turtles mate in the aquatic environment, fertilization is internal and one adaptation of germ cells to internal fertilization may be the loss of osmolarity as a stimulus for activation.

It is well known that temperature has marked effects on the motility and fertility of spermatozoa isolated from endothermic males, and that efforts must be taken to ensure that semen samples are maintained as close as possible to body temperature for maximum fertility. It was anticipated that the motility of turtle spermatozoa might be greatest at ambient temperatures and thus the observation that motility parameters were actually higher at reduced temperatures in two of the three species examined was unexpected. Other studies on these species indicate that they begin copulations at a time of reduced or decreasing temperatures (Gist *et al.*, 1990; Gist and Congdon, 1998). The higher activity of spermatozoa at depressed temperatures indicates that autumnal and winter matings might result in more active gametes, possibly facilitating the movement of inseminated spermatozoa into oviductal sperm storage glands. To the extent that increased sperm motility reflects favourably on sperm fertility, stored gametes, selected on the basis of their higher activity at reduced temperatures, could be the ones participating in fertilizations using stored spermatozoa.

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