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Optimal physicochemical conditions for the manipulation and short-term preservation of koala (*Phascolarctos cinereus*) spermatozoa

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Protocols for the successful manipulation and preservation of semen in a given species depend upon a fundamental knowledge of how spermatozoa respond to the physicochemical conditions of the extension media; methods developed for the preservation of eutherian spermatozoa may not necessarily be suitable for marsupial semen. The aim of this study was to investigate the effects on koala sperm motility of serial dilution, changes in temperature, diluent pH and osmolality to establish the optimal physicochemical conditions for short-term semen storage. This study showed that electroejaculated koala semen diluted 1:1 (v/v) with PBS frequently coagulated after incubation at 35°C, but that further dilution and incubation resulted in a corresponding increase in the percentage of spermatozoa swimming in a non-linear trajectory. The effect of rapid temperature change on the motility of koala spermatozoa was investigated by exposing semen, initially diluted at 35°C, to temperatures of 45, 25, 15 and 5°C. Although sperm motility was reduced after incubation at 45°C, a rapid decrease in temperature of up to 20°C did not result in a significant reduction in sperm motility. However, contrary to evidence in other marsupials, there was a small but significant decrease in sperm motility after rapid cooling of diluted semen from 35 to 5°C. The effects of diluent pH and osmolality on the motility of koala spermatozoa were investigated. These experiments indicated that diluents for koala sperm manipulation should buffer in a pH range of 7–8 and have an osmolality of approximately 300 mmol kg^{-1}. The final experiment compared the relative effectiveness of Tris–citrate buffer (1% glucose) and PBS to maintain koala sperm motility over a range of incubation temperatures (5–35°C) for up to 8 days. Reduction in sperm motility was directly related to temperature, and motility was sustained for the longest duration when stored at 5°C. The Tris–citrate buffer solution was superior to PBS as a preservation diluent at all temperatures, and koala spermatozoa remained motile even after 42 days storage at 5°C. Spermatozoa diluted in PBS (with Ca^{2+} or Mg^{2+}) and cooled to 5°C showed evidence of an unusual motility pattern, similar to that of hyperactivated eutherian spermatozoa. This study showed that koala spermatozoa respond to different physicochemical conditions associated with short-term liquid storage in essentially the same way as the spermatozoa of eutherian mammals, although koala spermatozoa appear to be more tolerant of rapid temperature shock. The results of this study can be used to make informed selections with regard to appropriate diluent composition and improved short-term sperm preservation protocols and represent the first such database for any species of marsupial.

Introduction

Successful preservation of semen is an important part of the development and implementation of artificial breeding programmes for marsupials (Johnston, 1999). When combined with techniques such as artificial insemination, the ability to preserve semen offers significant advantages for improving the reproductive success and genetic management of captive and free-ranging populations (Rodger, 1990; Taggart *et al*., 1997; Johnston, 1999). While cryopreservation is the ultimate objective for the long-term storage of semen, short-term or liquid storage also has potential benefits, particularly in species in which cryopreservation of spermatozoa has proven difficult (for

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example, the tammar wallaby, *Macropus eugenii*; Molinia and Rodger, 1996) or only moderately successful (for example, the koala, *Phascolarctos cinereus*; Johnston *et al.*, 1993).

The metabolic activity of spermatozoa must be reduced by chemical inhibition or by lowering of temperature if they are to remain viable for manipulation or preservation; either approach requires dilution (Watson, 1990). Since diluent requirements for the preservation of spermatozoa may vary among species (Watson, 1979; Mann and Lutwak-Mann, 1981; Holt, 1997), studies that investigate the tolerance and behaviour of diluted spermatozoa to a range of physicochemical conditions are likely to be useful in the selection of media for semen manipulation, short-term liquid storage of spermatozoa at refrigerated temperatures (5°C) and cryopreservation. Such studies are particularly important for marsupial semen, as there is no guarantee that spermatozoa from this taxa will behave in a similar manner to eutherian spermatozoa. The most significant of the physical and chemical influences on diluted spermatozoa *in vitro* are variations in ionic strength (pH and osmolality), dilution rate and temperature (Mann, 1964).

Apart from a study on the collection, handling and properties of brushtail possum (*Trichosurus vulpecula*) semen by Rodger and White (1978), details of the manipulation of marsupial semen is mostly found in references to methodology in studies dealing with sperm motility patterns (Taggart and Temple-Smith, 1989; Taggart, 1990, 1994; Taggart *et al.*, 1994, 1995a,b), short-term sperm preservation (Rodger and White, 1978; Johnston *et al.*, 1992, 1997a; Taggart *et al.*, 1996), cryopreservation (Rodger *et al.*, 1991; Johnston *et al.*, 1993; Taggart *et al.*, 1994, 1996; Molinia and Rodger, 1996), *in vitro* fertilization (Moore and Taggart, 1993) and acrosomal function (Mate and Rodger, 1991; Sistena *et al.*, 1993a,b,c).

Some studies have reported that marsupial spermatozoa are tolerant of sudden changes in temperature (Rodger and White, 1978; Taggart *et al.*, 1996, Molinia and Rodger, 1996) but, to date, no systematic experiments examining the effect of sudden temperature shock have been described. Similarly, although Rodger and White (1978) noted that brushtail possum spermatozoa were able to tolerate frequent washing and fivefold dilution in Krebs–Henseleit–Ringer solution, no quantitative data were given. However, a dilution-like effect (Watson, 1990) has been described by Rodger *et al.* (1991) and Taggart *et al.* (1996) in studies investigating the cryopreservation of brushtail possum and southern hairy-nosed wombat (*Lasiorhinus latifrons*) spermatozoa, respectively. Both of these studies found that spermatozoa did not cryopreserve well if the concentration of sperm cells was below 4×10^6 ml^{-1}.

The effect of diluent pH and osmolality on sperm motility has not been described for any marsupial, although Jones and Clulow (1994) noted that Tammar wallaby epididymidal spermatozoa were irreversibly immobilized by dilution in media of low pH and containing lactate. Jones and Clulow (1994) suggested that regulation of intracellular pH might provide a mechanism for manipulating sperm motility; such investigations would also be extremely useful in the development of marsupial sperm encapsulation technology (Nebal *et al.*, 1993; Holt, 1997). Most studies of marsupial semen have used buffer systems and additives that maintain the diluent pH and osmolality in the range of 6.8–7.4 and 280–320 mmol kg^{-1}, respectively.

There have been few studies of the short-term preservation of marsupial semen. Rodger and White (1978) showed that washed spermatozoa from brushtail possums could maintain motility for 6 h if incubated in Krebs–Ringer–phosphate solution at 37°C. After a 1:1 dilution in 'Kiev' diluent (Johnson and Aalbers, 1984) and storage at 16°C, Johnston *et al.* (1992) were able to maintain the motility of electroejaculated koala spermatozoa for up to 48 h. Spermatozoa obtained from eastern grey kangaroos (*Macropus giganteus*) by electroejaculation have also been stored at 5°C for 48 h in PBS containing combinations of egg yolk and D-glucose (Johnston *et al.*, 1997a). The limited storage time of spermatozoa in both these studies may be related to the lack of antibiotics in the semen diluent and the high osmolality of the 'Kiev' diluent. Taggart *et al.* (1996) found that fat-tailed dunnart (*Sminthopsis crassicaudata*) epididymidal spermatozoa left intact within the scrotum and refrigerated (5°C), could remain motile for up to 8 days. Such a finding has important implications in the potential recovery of spermatozoa from post-mortem specimens.

Given the limited information on the manipulation and preservation of marsupial spermatozoa, the present study examined the response of koala sperm to a range of physicochemical conditions to provide data for the selection of the most appropriate media and protocols for short-term semen storage in this species. The effect of serial dilution, rapid changes in temperature (temperature shock), diluent pH and diluent osmolality on the motility of koala spermatozoa extended in PBS with no Ca^{2+} or Mg^{2+} were investigated and compared with similar observations of eutherian spermatozoa (Watson, 1990). Two semen diluent buffers, PBS (with Ca^{2+} or Mg^{2+}) and Tris–citrate, were also examined for their ability to maintain the motility of koala spermatozoa over a range of incubation temperatures.

Materials and Methods

Animals and semen collection

Semen was collected from 30 sexually mature (3–10 years of age) captive male koalas located in four captive breeding colonies in south-east Queensland, either by electro-ejaculation ($n = 10$) (Johnston *et al.*, 1994) or using an artificial vagina ($n = 20$) (Johnston *et al.*, 1997b). Details of the husbandry of these animals has been described by Johnston *et al.* (1994) and Blanshard (1994). All koalas were clinically healthy at the time of semen collection.

Semen manipulation for Expts 1–5

After collection, each koala semen sample was immediately diluted into a 6 ml stoppered polypropylene test tube (Becton Dickinson Labware, New Jersey) containing a defined volume of warm (35°C) PBS (pH 7.2; osmolality 280–320 mmol kg^{-1}; Commonwealth Serum Laboratory, Brisbane). Details of dilution volumes are given in the

descriptions of individual experiments below and varied depending on whether the semen was collected by artificial vagina or electroejaculation. The sperm concentration of the original semen sample was determined using a calibrated sperm chamber (Makler, Sefi-Medical Instruments, Haifa, Israel). The motility of diluted koala spermatozoa was assessed using criteria developed for frozen–thawed bovine spermatozoa (Barth, 1995). A drop of semen was placed onto a pre-warmed microscope slide (35°C) with a coverglass, and motility was evaluated using a phase-contrast microscope at a magnification of × 400. Estimates of the percentage of forwardly motile spermatozoa, the total percentage of forwardly and circularly motile spermatozoa and a rate of sperm movement (0 = no sperm movement; 5 = very rapid sperm movement) were calculated. All assessments of sperm motility were made on a warm stage fitted to the microscope and set at 35°C. Spermatozoa were evaluated at 35°C as this is the core body temperature of koalas.

Experiment 1: effect of serial dilution

Semen samples were collected from six koalas by means of electroejaculation during March–April 1995. The initial concentration of the six semen samples before dilution ranged from 20×10^6 to 120×10^6 spermatozoa ml^{-1}. Immediately after collection, approximately 0.5 ml of the electroejaculate was serially diluted (v/v) 1:1, 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64 into 0.5 ml PBS medium. The diluted semen was then maintained for 2 h in an incubator (Thermoline Scientific Equipment Pty Ltd., Smithfield, NSW) set at 35°C. Sperm motility was assessed four times over a 2 h period, such that 400 spermatozoa per treatment were evaluated.

Experiment 2: effect of rapid temperature shock

Semen samples were collected from seven koalas by electroejaculation in June 1995. Semen was diluted 1:4 (v/v) in PBS so that the final concentration of spermatozoa ranged from 15×10^6 to 90×10^6 spermatozoa ml^{-1}. Aliquots of diluted semen (100 µl), originally at 35°C, were dispensed into stoppered test tubes and placed directly into a water bath set at 45, 25 or 15°C. A similar aliquot of diluted semen was also placed directly into a refrigerator set at 5°C. The approximate cooling or warming rates to each of the set temperatures described above were +6, –7, –13 and –16°C min^{-1}, respectively. Each test tube was held at the set temperature for 10 min and subsequently re-warmed or cooled as appropriate to 35°C for analysis of motility. Two hundred spermatozoa were evaluated per treatment. Each treatment sample was compared with control koala semen samples maintained at 35°C throughout the experiment (1.5 h).

Experiment 3: effect of diluent pH

Semen was collected from eight koalas by means of an artificial vagina from January to February 1997. Koala semen was immediately diluted 1:10 (v/v) in PBS media of different pH prepared from stock solutions of AR grade KH_2PO_4 and Na_2HPO_4 (Ajax Chemicals Pty Ltd, Auburn, NSW). A dilution rate of 1:10 (rather than the 1:4 dilution of earlier experiments) was used in Expts 3–5 because of the higher concentration of spermatozoa recovered in semen collected with the artificial vagina (Johnston *et al.*, 1997b) and to ensure that the final pH and osmolality of diluents in Expts 3 and 4 were not confounded by the buffering capacity and osmolality of koala seminal plasma. Diluents with a pH of 4.7, 5.9, 6.5, 7.0, 8.1 and 9.1 were used. All pH treatment diluents were adjusted to a final osmolality of 280–320 mmol kg^{-1} by the addition of NaCl. The final sperm concentrations of diluted semen ranged from 2×10^6 to 25×10^6 spermatozoa ml^{-1}. The final diluted volume of each treatment semen sample was 400 µl. Sperm motility was assessed three times over a 2 h period, such that 300 spermatozoa were evaluated per treatment. Throughout the experiment spermatozoa were maintained at 35°C.

Experiment 4: effect of diluent osmolality

Semen was collected from seven koalas by means of an artificial vagina in January 1997. The semen was immediately diluted 1:10 (v/v) in a series of PBS diluents ranging from 95 to 884 mmol kg^{-1}. Hyperosmotic diluents were prepared by the addition of sucrose (Ajax Chemicals Pty Ltd). Hypo-osmotic diluents were prepared by dilution of PBS media with sterile water. The osmolality of each diluent was determined by a vapour pressure osmometer (Wescor, UT). The pH of treatment diluents before the addition of semen ranged from 6.9 to 7.2. Sperm motility was assessed as in Expt 3. The final sperm concentration of the diluted semen ranged fron 2×10^6 to 25×10^6 spermatozoa ml^{-1}. The final diluted volume of each treatment semen sample was 400 µl. Throughout the experiment spermatozoa were maintained at 35°C.

Experiment 5: comparison of PBS–glucose and Tris–citrate media for the liquid preservation of koala semen stored at 35, 25, 15 and 5°C

Semen was collected from nine koalas by means of an artificial vagina from December 1997 to January 1998. Immediately after collection, the ejaculate was divided into two samples and diluted 1:10 in either PBS containing Ca^{2+} (200 µg ml^{-1}) , Mg^{2+} (98 µg ml^{-1}) and 1% D-glucose (ICN Biomedical, Seven Hills, NSW) or Tris–citrate–glucose (TCG) extension medium. TCG media was prepared using 3.63 g of Tris (hydroxymethyl) aminomethane, 1.99 g of citric acid (monohydrate) and 1.00 g of D-glucose (Ajax Chemicals Pty Ltd) made up to 100 ml with sterile deionized water. The pH of the TCG diluent was adjusted to 7.4 by the addition of a 0.1 mol sodium hydroxide l^{-1}. Both diluents also contained 1000 iu penicillin G ml^{-1} and 100 µg gentamicin ml^{-1} (Commonwealth Serum Laboratory, Brisbane, Queensland; Johnston *et al.*, 1998). Approximately 0.5 ml semen from each diluent was incubated at 35, 25, 15 and 5°C and sperm

motility was assessed as described in Expt 3 at 1, 2, 3, 4, 5, 6, 7 and 8 days after dilution. Sperm concentration of diluted semen samples ranged from 5×10^6 to 25×10^6 ml^{-2}. During this experiment, it was found that koala spermatozoa diluted in TCG diluent and held at 5°C could maintain their motility for periods significantly longer than 8 days. Subsequently, diluted semen from six of the eight koalas was stored at 5°C for a further 5 weeks and sperm motility was assessed after 22, 35 and 42 days of storage.

Statistical analysis

The effect of serial dilution, diluent pH and osmolality on the numbers of motile spermatozoa and on the rate of sperm movement were analysed using a single factor analysis of variance (Feldman and Gagnon, 1991); multiple comparison tests of significant analyses were made using Fisher's protected least significant difference (PLSD). The motility of 'temperature shocked' spermatozoa in Expt 2 was compared with that of control samples using unpaired two-group *t* tests (Feldman and Gagnon, 1991). The effect of diluent type, temperature and time on the sperm motility of liquid-preserved koala semen in Expt 5 was analysed using a three-factor analysis of variance (Feldman and Gagnon, 1991). The percentage of forwardly motile and the total percentage of forwardly and circularly motile spermatozoa were transformed to arcsines for calculation of analysis of variance and *t* tests.

Results

Experiment 1

The effect of serial dilution on the motility of spermatozoa when the koala electroejaculate was extended with PBS is shown (Fig. 1). Both the percentage of forwardly motile spermatozoa ($F = 3.02$, $P = 0.03$) and the rate of sperm movement ($F = 10.40$, $P < 0.01$) were significantly reduced with increasing dilution. However, the loss of total motility was not significant ($F = 2.20$, $P = 0.08$), indicating an increase in the percentage of non-linear motility with increased dilution. Four of the six electroejaculates diluted 1:1 with PBS coagulated within 1 h of dilution and therefore could not be included in the statistical analysis. For the purposes of this study, coagulation refers to the formation and precipitation of seminal particulate matter formed from aggregations of degenerating prostatic bodies, entrapped or dead spermatozoa and seminal plasma debris, rather than the formation of a solidified rubbery mass typified by ejaculated macropod semen (Rodger and White, 1975).

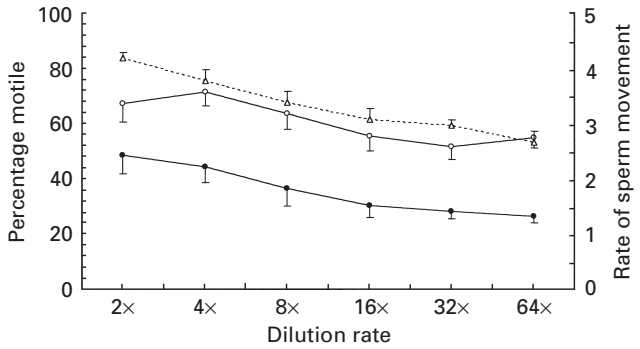

Fig. 1. Effect of dilution rate on the mean (± SEM) motility of koala spermatozoa extended in PBS. ●, Percentage of forwardly motile spermatozoa; ○, total percentage of forwardly and circularly motile spermatozoa; △, rate of sperm movement (subjective assessment based on criteria of Barth (1995)).

Experiment 2

The motility of diluted koala spermatozoa when exposed to environmental temperatures of 5, 15, 25 and 45°C for 10 min and when compared with the motility of control samples of koala spermatozoa maintained at 35°C over the same duration is shown (Table 1). There was no significant difference between the motility (percentage of forwardly motile spermatozoa, the total percentage of forwardly and circularly motile spermatozoa and rate of sperm movement) of spermatozoa held at 35°C and those exposed to 15 or 25°C. However, exposure of koala spermatozoa to a temperature of 5°C for 10 min resulted in a small but significant loss of motility (forwardly motile, $P = 0.01$; total percentage motile, $P < 0.01$; rate of sperm movement, $P = 0.01$) when these spermatozoa were compared with control spermatozoa maintained at 35°C. Similarly, the total percentage motile ($P = 0.02$) and the rate of sperm movement ($P = 0.03$) of koala spermatozoa stored for 10 min at 45°C were also significantly lower than those of control spermatozoa.

Experiment 3

The effect of diluent pH on the motility of koala spermatozoa collected by artificial vagina is shown (Fig. 2). The total percentage of motile spermatozoa ($F = 4.11$, $P < 0.01$) and the rate of movement ($F = 8.03$, $P < 0.01$) changed significantly over the range of diluent pH tested. The total percentage of motile spermatozoa and the rate of sperm movement were significantly reduced ($P < 0.05$) in media of pH 4.7 and 5.9 compared with media of pH 7.0. The percentage of forwardly motile spermatozoa was not significantly affected ($F = 1.62$, $P = 0.15$).

Experiment 4

The effect of diluent osmolality on the motility of koala spermatozoa is shown (Fig. 3). The percentage of forwardly motile spermatozoa ($F = 40.2$, $P < 0.01$), the total percentage of motile spermatozoa ($F = 36.9$, $P < 0.01$) and the rate of sperm movement ($F = 21.7$, $P < 0.01$) changed significantly across the range of diluent osmolality tested. Koala sperm motility was highest when diluted in media of approximately 300 mmol kg^{-1}. Koala spermatozoa appeared

Table 1. The effect of temperature shock on the mean (± SEM) motility of koala spermatozoa diluted 1:4 in PBS and stored for 10 min at 5, 15, 25 or 45°C and compared with a control sample of spermatozoa maintained at 35°C

Temperature (°C)	Motility	Control spermatozoa	Treatment spermatozoa	Significance
5	FM	77.6 ± 2.3	65.7 ± 2.8	$P < 0.01$
5	TM	86.7 ± 1.3	79.9 ± 1.4	$P < 0.01$
5	R	4.1 ± 0.1	3.4 ± 0.2	$P = 0.01$
15	FM	70.6 ± 3.7	69.0 ± 3.5	$P = 0.75$
15	TM	82.9 ± 3.0	82.3 ± 2.6	$P = 0.85$
15	R	4.1 ± 0.1	4.0 ± 0.2	$P = 0.59$
25	FM	71.0 ± 4.9	63.0 ± 5.4	$P = 0.31$
25	TM	81.7 ± 2.5	75.7 ± 4.6	$P = 0.31$
25	R	3.9 ± 0.1	3.4 ± 0.2	$P = 0.11$
45	FM	68.6 ± 5.3	51.6 ± 7.2	$P = 0.07$
45	TM	83.3 ± 3.6	69.1 ± 3.9	$P = 0.02$
45	R	3.9 ± 0.1	3.1 ± 0.3	$P = 0.03$

FM: percentage of forwardly motile spermatozoa; TM: total percentage of forwardly and circularly motile spermatozoa; R: rate of sperm movement.

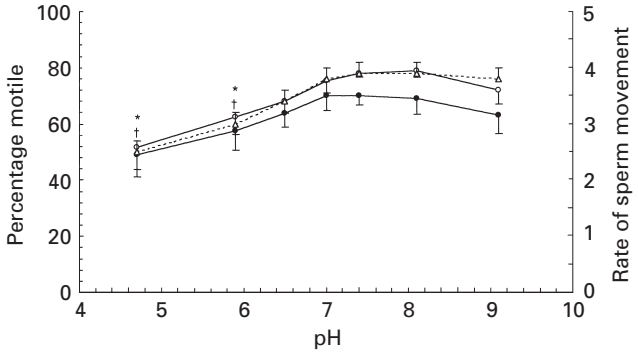

Fig. 2. Effect of diluent pH on the mean (± SEM) motility of koala spermatozoa extended in PBS. ●, Percentage of forwardly motile spermatozoa; ○, total percentage of forwardly and circularly motile spermatozoa; △, rate of sperm movement (subjective assessment based on criteria of Barth (1995)). *Total motility significantly different ($P < 0.05$) from that of spermatozoa diluted in pH 7 PBS; †rate of sperm movement significantly different ($P < 0.05$) from that of spermatozoa diluted in pH 7 PBS.

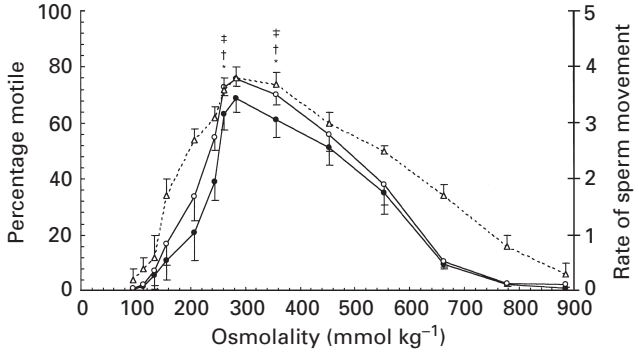

Fig. 3. Effect of diluent osmolality (mOsm kg^{-1}) on the mean (± SEM) motility of koala spermatozoa extended in PBS. ●, Percentage of forwardly motile spermatozoa; ○, total percentage of forwardly and circularly motile spermatozoa; △, rate of sperm movement (subjective assessment based on criteria of Barth (1995)). ‡Forward motility not significantly different from that of spermatozoa diluted in 300 mOsm PBS; *total motility not significantly different from that of spermatozoa diluted in 300 mOsm PBS; †rate of sperm movement not significantly different from that of spermatozoa diluted in 300 mOsm PBS.

to tolerate hyperosmotic conditions considerably better than hypo-osmotic conditions.

Experiment 5

The results of the effect of diluent type, temperature and period of preservation on the motility of extended koala spermatozoa are shown (Table 2). On initial dilution of koala semen with the TCG, sperm motility (percentage of forwardly motile spermatozoa, $t = 5.5$, $P = 0.0001$; total percentage of motile spermatozoa, $t = 4.9$, $P = 0.0001$; rate of sperm movement, $t = -13.62$, $P = 0.001$) decreased significantly compared with spermatozoa diluted in PBS. However, with continued preservation of diluted koala semen, the TCG diluent maintained significantly higher sperm motility across all storage temperatures (percentage of forwardly motile spermatozoa: $F = 830.3$, $P < 0.01$; total percentage of motile spermatozoa: $F = 421.8$, $P < 0.01$; rate of sperm movement: $F = 297.8$, $P < 0.01$).

The lower motility of koala spermatozoa diluted in PBS was confounded somewhat by a consistent pattern of seminal coagulation (in eight of nine samples) at all

Table 2. Mean (± SEM) motility of koala spermatozoa diluted 1:10 in either PBS or TCG and stored at 35, 25, 15 or 5°C for up to 8 days

			Days of preservation			
Diluent	Temperature (°C)	Motility	0.04	1	4	8
PBS	35	FM	–	6.1 ± 4.1	0 ± 0	0 ± 0
		TM	–	11.9 ± 7.9	0 ± 0	0 ± 0
		R	–	0.7 ± 0.5	0 ± 0	0 ± 0
PBS	25	FM	66.2 ± 5.5	26.7 ± 7.3	0.9 ± 0.8	0 ± 0
		TM	74.3 ± 4.8	29 ± 7.2	1.3 ± 1.2	0 ± 0
		R	3.9 ± 0.2	2.0 ± 0.5	0.4 ± 0.4	0 ± 0
PBS	15	FM	–	32.2 ± 7.2	9.3 ± 3.1	0 ± 0
		TM	–	38 ± 7.1	12.0 ± 3.5	0 ± 0
		R	–	3.6 ± 0.2	2 ± 0.3	0 ± 0
PBS	5	FM	–	6.9 ± 3.0	0.9 ± 0.7	0 ± 0
		TM	–	39.1 ± 5.2	25.3 ± 6.1	0 ± 0
		R	–	3.2 ± 0.3	2.5 ± 0.2	0 ± 0
TCG	35	FM	–	13.1 ± 5.5	0.4 ± 0.4	0 ± 0
		TM	–	17.6 ± 6.3	0.4 ± 0.4	0 ± 0
		R	–	2.1 ± 0.3	0.2 ± 0.2	0 ± 0
TCG	25	FM	52.6 ± 7.1	52.0 ± 4.1	22.8 ± 7.5	8.0 ± 3.9
		TM	70.3 ± 3.6	59.6 ± 4.5	26.5 ± 7.6	8.6 ± 4.0
		R	3.9 ± 0.2	3.3 ± 0.3	2.2 ± 0.4	1.4 ± 0.3
TCG	15	FM	–	56.0 ± 4.7	50.8 ± 3.1	33.1 ± 6.6
		TM	–	66.3 ± 4.3	52.0 ± 3.2	33.4 ± 6.5
		R	–	3.6 ± 0.2	3.1 ± 0.2	2.3 ± 0.4
	5	FM	–	51.0 ± 4.7	49.7 ± 3.9	45.8 ± 4.4
		TM	–	63.8 ± 4.3	56.0 ± 4.1	46.7 ± 7.0
		R	–	3.8 ± 0.3	3.7 ± 0.2	3.2 ± 0.2

TCG: Tris–citrate–glucose; FM: percentage of forwardly motile spermatozoa; TM: total percentage of forwardly and circularly motile spermatozoa; R: rate of sperm movement.

temperatures. Although koala seminal plasma diluted in PBS failed to solidify completely, a significant proportion of seminal 'particulate matter' precipitated out of solution, resulting in spermatozoa becoming trapped in aggregations of degenerating prostatic bodies and seminal plasma debris. However, despite the apparent decomposition of prostatic bodies in semen samples diluted in PBS, there was no evidence of bacterial contamination. This form of seminal plasma coagulation was generally first evident in semen incubated at higher temperatures approximately 24–48 h after initial dilution. While prostatic bodies remained intact, as was the case in the TCG diluent, sperm motility was maintained. Even after 42 days of storage at 5°C, there was no microscopic evidence of prostatic body degeneration or seminal coagulation in TCG-extended semen samples.

For both diluents used, sperm survival as estimated by motility was inversely related to incubation temperature (percentage of forwardly motile spermatozoa: $F = 182.2$, $P < 0.01$; total percentage of motile spermatozoa: $F = 231.1$, $P < 0.01$; rate of sperm movement: $F = 365.6$, $P < 0.01$). There was a characteristic change in the motility pattern of spermatozoa diluted in PBS and stored at 5°C. Without exception, spermatozoa from all nine koalas swam in a continuous loop, similar to the swimming pattern of hyperactivated spermatozoa described in various domestic species (for example, see Gordon, 1994). Prolonged warming of these spermatozoa on a warm stage set at 35°C for 5–10 min did not affect the trajectory of their motility. A similar motility pattern was not observed at 35, 25 or 15°C for spermatozoa diluted in PBS or at any temperature for spermatozoa diluted in the TCG diluent. Although the main part of this experiment was terminated after 8 days, TCG-diluted semen from six koalas was stored for a further 42 days at 5°C. The mean (± SEM) percentages of forwardly motile spermatozoa and rate of sperm movement of the six diluted semen samples after 22, 35 and 42 days storage were 46.5 ± 2.4, 3.1 ± 0.2; 30.8 ± 5.6, 2.7 ± 0.3; and 15.6 ± 4.3, 1.9 ± 0.2, respectively.

Discussion

Excessive dilution of semen is thought to lead to the removal of sperm surface components and changes in the permeability of sperm membranes, thereby hastening sperm senescence (Watson, 1990). While the results of this study show that the trajectory and rate of koala sperm motility was affected by increased dilution, the percentage of total motile spermatozoa was not affected. The loss of forward motility and rate of sperm movement of koala spermatozoa with increased dilution in PBS may have been evidence of the early stages of sperm membrane damage, and further incubation or dilution may have resulted in a more substantial loss of motility. Four of the six electroejaculated semen samples diluted 1:1 (v/v) with PBS coagulated. This tendency for koala semen obtained by electroejaculation to flocculate (Mann, 1964), whereby spermatozoa become entrapped in clumps of degenerating prostatic bodies and seminal particulate matter, has been described by Johnston *et al.* (1994) and highlights the importance of adequate dilution of electroejaculated koala semen samples for short-term storage or cryopreservation.

It has been generally accepted that marsupial spermatozoa are not susceptible to 'cold shock' (Rodger and White, 1978; Molinia and Rodger, 1996; Taggart *et al*., 1996), yet this claim has not been systematically tested in any species of marsupial. Findings from the present study revealed that koala spermatozoa cooled rapidly to 5°C (–16°C min^{-1}) in PBS showed a small but significant decrease in motility when re-warmed to 35°C. These findings represent the first preliminary evidence of 'cold shock' to be reported in marsupial semen, but it should be noted that the loss of forward motility was limited to 12%, substantially less than that which occurs when bull or ram semen is 'cold shocked' (White, 1993). These results indicate that the phospholipids that compose the sperm membrane in marsupials may have a relatively low proportion of unsaturated to saturated fatty acids and a relatively high cholesterol content (White, 1993). However, there are currently no data on the composition of marsupial sperm membranes. Approximately 85% of koala spermatozoa retained forward motility after 10 min storage at 45°C. A similar tolerance of exposure to high environmental temperatures has also been observed in the spermatozoa of various macropod species (W. V. Holt, L. Penfold, S. D. Johnston, P. Temple-Smith, C. McCallum, J. Shaw, W. Lindemans and D. Blyde, unpublished).

Hydrogen ion concentration has been identified as an important factor influencing the motility, viability and metabolism of extended spermatozoa in a wide range of species (for review, see Mann, 1964). The results of the effect of diluent pH on koala sperm motility reported in the present study indicate that koala sperm motility was highest when the diluent pH was in the range of 6.5–8.1. These results are consistent with general trends of the effect of pH on sperm motility across a range of mammals (Watson, 1990), including work on marsupials by Jones and Clulow (1994) who showed that diluents with low pH and containing lactate can reversibly immobilize *M. eugenii* spermatozoa dissected free from the tail of the epididymis.

Buffer systems that have been used for semen manipulation in marsupials typically buffer in a pH range of 6.8 to 7.4, and include minimum essential medium (MEM) (Taggart *et al*., 1996), 'Kiev' (Johnston *et al.*, 1992), Tris–citrate (Johnston *et al.*, 1993; Taggart *et al*., 1996), PBS (Sistena *et al.*, 1993a; Molinia and Rodger, 1996; Johnston *et al.*, 1997a) Krebs–Henseleit–Ringer (KHR) (Rodger and White, 1978; Rodger *et al.*, 1991), Eagle's minimum essential media (EMEM) (Sistena *et al*., 1993b) and Dulbecco's modified Eagle's medium (DMEM) (Sistena *et al*., 1993a). Future studies on koala semen manipulation should also investigate other organic buffer alternatives (for example, TES (*N*-Tris[hydroxymethyl]methyl-2-aminoethane-sulphonic acid; pKa 7.4). However, buffer systems that promote sperm motility, and hence increase sperm metabolism, may actually be detrimental to their long-term storage, particularly for chilled (16°C) semen or semen maintained at ambient (20–30°C) temperatures (Mann and Lutwak-Mann, 1981).

The present study reports the first description of the effect of diluent osmolality on the motility of ejaculated marsupial spermatozoa and clearly indicates that koala sperm motility is more adversely affected by hypo-osmotic media than by hyperosmotic conditions. In this regard, koala spermatozoa behave similarly to those of eutherian species (Mann, 1964; Watson, 1990). The motilities of koala spermatozoa over the range of diluent osmolality were similar to those reported for mice (Willoughby *et al*., 1996) and men (Gao *et al.*, 1995). The behaviour of koala spermatozoa to anisosmotic media provides insight into how the spermatozoa may respond osmotically to changing water flux during cryopreservation (Watson, 1979). Future studies should examine not only motility but also the integrity of the koala sperm membranes to repeated changes in osmotic pressure (Willoughby *et al.*, 1996).

The TCG diluent was clearly the diluent of choice for liquid preservation. If stored at 5°C, the motility of koala spermatozoa can be maintained for up to 35 days at approximately 59% of the original dilution motility. Studies on the fertilizing potential of liquid-stored semen have shown that extended spermatozoa from many species lose fertilizing capacity with the passage of time, despite remaining highly motile (Watson, 1990; Vishwanath and Shannon, 1997). While insemination studies are required to ascertain the fertility of liquid-preserved koala spermatozoa, 35 days (the approximate length of the koala anovulatory oestrous cycle; Johnston *et al.*, 1997c) is an extremely long period for the spermatozoa of any species to remain motile. Murdoch and Jones (1994) have concluded that *M. eugenii* spermatozoa have a greater store of endogenous substrate for energy metabolism than eutherian spermatozoa and are able to oxidize this substrate for up to 12 h *in vitro*. Large stores of endogenous energy may also occur in koala spermatozoa. Clulow *et al.* (1992) have suggested that *M. eugenii* spermatozoa may be able to utilize lipid from the membraneous region around the neck of the sperm, but the ultrastructure of the koala spermatozoon is far less elaborate (Harding and Aplin, 1990).

After 35 days, there was also no microscopic evidence of bacterial contamination or prostatic body degeneration. These results confirm the efficacy of 1000 iu penicillin G ml^{-1} and 100 μg gentamicin ml^{-1} for use in extended koala semen

(Johnston *et al.*, 1998). However, the effect of antibiotics on the fertilizing capacity of extended koala spermatozoa has yet to be determined.

The results from Expts 1 and 4 indicate that a high dilution at 35°C and storage of koala spermatozoa at 5°C for 24 h in PBS resulted in an increased percentage of spermatozoa swimming in a non-linear trajectory. This change in the swimming pattern of koala spermatozoa may be related to the PBS medium in which they were diluted, as spermatozoa similarly held and stored at 5°C in the TCG diluent showed no such increases in the percentage of non-linear motile spermatozoa, even after 42 days of storage. It is difficult to explain why such marked changes in motility patterns were not observed in spermatozoa extended in TCG at a similar dilution and temperature. As the Tris–citrate buffer has the ability to chelate Ca^{2+} in solution, semen diluted with TCG is likely to have a reduced extracellular concentration of Ca^{2+} compared with semen diluted with PBS which, in the present study, had a Ca^{2+} concentration of 200 μg ml^{-1}. It is possible that there is an influx of extracellular Ca^{2+} into the sperm cell during the cooling process as a result of structural damage to the sperm membrane (White, 1993). Furthermore, an increase of Ca^{2+} concentration into the koala spermatozoon may inadvertently trigger capacitation-like events leading to hyperactivation. On the basis of these observations it is recommended that diluents for the short-term preservation of koala spermatozoa exclude Ca^{2+} and contain agents, such as EDTA, that chelate Ca^{2+}. Preliminary evidence (S. D. Johnston, unpublished) using electro-ejaculates from two koalas has revealed that spermatozoa refrigerated at 5°C in PBS media with no Ca^{2+} or Mg^{2+} showed significantly less 'hyperactive' motility than spermatozoa extended in PBS with Ca^{2+} or Mg^{2+}.

The results of the present study indicate that koala spermatozoa exposed to a range of physicochemical conditions associated with short-term liquid semen storage respond in an essentially similar way to the spermatozoa of eutherian mammals. This conclusion contrasts with the findings from studies of marsupial sperm cryopreservation, the success of which requires fundamentally different protocols to those used for eutherian spermatozoa (Johnston, 1999).

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