Use of computer-assisted sperm motility assessment and multivariate pattern analysis to characterize ejaculate quality in Mohor gazelles (*Gazella dama mhorr*): effects of body weight, electroejaculation technique and short-term semen storage

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Subjective and objective semen assessments were performed on 18 male Mohor gazelles (*Gazella dama mhorr*). Sperm motility assessments combined with sperm plasma membrane and acrosomal integrity evaluations were undertaken as part of a captive breeding programme. The primary objective was to test methodology for short-term preservation of gazelle semen for artificial insemination (storage in N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulphonic acid–Tris diluent (TEST) for up to 96 h at 17°C). However, the secondary objective was to investigate phenotypic and genotypic influences on semen quality within this small population, which was established in 1971 with only 12 genetic founders. Sperm motility was measured by computer-assisted semen assessment and the data were analysed using a pattern analysis technique to detect and quantify naturally occurring sperm subpopulations within the semen samples. Four sperm subpopulations distinguishable by their motion charac-

teristics were detected. The relative frequencies of two subpopulations (population 2: highly motile, non-linear; and population 4: poorly motile, non-linear) in fresh semen were correlated with the maximum voltage used during electroejaculation. The frequency of subpopulation 2 was negatively correlated with maximum voltage ($r = -0.875$, $P < 0.0001$) and the frequency of subpopulation 4 was positively correlated ($r = 0.727$, $P < 0.005$). The frequencies of all subpopulations varied significantly among the animals sampled (chi-squared = 2577.6, degrees of freedom = 54, $P < 0.0001$) and subpopulation 4 was also correlated with body weight ($r = -0.59$, $P < 0.005$). Semen stored at 17°C retained motility, plasma membrane and acrosomal integrity for 48 h, but these measures decreased thereafter. The frequency of a sperm subpopulation showing uncoordinated but active motility increased significantly over the first 48 h and then decreased.

Introduction

The potential benefits of using reproductive techniques to support breeding programmes for threatened species have been emphasized on many occasions (Holt and Moore, 1988; Mace, 1989; Ballou and Cooper, 1992; Wildt *et al.*, 1992; Johnston and Lacy, 1995; Holt *et al.*, 1996a; Holt and Pickard, 1999). Over the last 15 years there has been a considerable increase in the number of organized captive breeding programmes for threatened species. Many of these programmes aim to provide animals for reintroduction to their natural ranges and some have already shown a degree of success. One of the most successful captive breeding and reintroduction programmes has concentrated on the Mohor gazelle (*Gazella dama mhorr*). The captive population was founded in 1971 when four male and 13 female gazelles were taken to the Estación Experimental de

Zonas Áridas (EEZA) in southern Spain; the history of this population has been described by Alados *et al.* (1988). Animals in other captive collections all originated from this founder group and recently animals were reintroduced into protected areas in Tunisia, Morocco and Senegal. To date, this conservation programme has been managed carefully to minimize inbreeding effects as much as possible. All breeding within the programme has been achieved by natural matings, but the value of assisting genetic management by the use of artificial insemination with fresh or frozen semen is recognized (Wildt, 1997; Holt and Pickard, 1999), particularly as the entire gazelle population becomes more dispersed geographically. At present the captive population consists of about 250 individuals in several zoos around the world.

The development of successful artificial insemination techniques for this species commenced about 5 years ago with the establishment of a semen collection procedure and some preliminary research on semen cryopreservation and artificial insemination (Holt *et al.*, 1996b). Semen frozen in N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulphonic

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acid–Tris (TEST)–egg yolk (5% v/v) diluent with 6% (v/v) glycerol was used and 30% of females conceived when inseminated by a laparoscopic technique. Subsequently, non-invasive methods have been developed for monitoring and synchronizing the oestrous cycle and, moreover, pregnancy can be detected using faecal hormone analysis (Pickard *et al.*, 2000). Ideally, in addition to the long-term storage of semen for the establishment of a genetic resource bank, effective methods for storage of spermatozoa over short periods would be useful. Transport of liquid semen between breeding centres is generally possible within 24–48 h by road or air. The present study was undertaken to address the development of methods for such short-term storage and the design of the experiment was set up accordingly.

This study was made most effective by using a range of semen assessment techniques including computer-assisted semen assessment. Abaigar *et al.* (1999) showed that the large data sets generated by computer-assisted semen assessment could be simplified and made more informative by applying pattern analysis. This novel approach is a powerful method for the simplification of comprehensive data as it operates on data sets comprising thousands of individual spermatozoa, each with several descriptors. The computer program is designed to detect natural groupings within large data sets and is not limited by the nature of the distributions involved.

The increased analytical power gained by the application of pattern analysis redirected our attention to the possibility of investigating the influence of genotypic and phenotypic variation on the quality of fresh semen in Mohor gazelles. The underlying hypothesis and justification for this aspect of the study was to investigate the widely held view that semen quality reflects reproductive fitness and is a determinant of sexual selection, especially in situations involving sperm competition (Birkhead, 1995; Gomendio *et al.*, 1998).

Materials and Methods

Animals

The study was conducted using 18 healthy reproductively mature (aged > 2 years) male Mohor gazelles (*Gazella dama mhorr*) from the breeding herd maintained by the Estación Experimental de Zonas Áridas in Almería, south eastern Spain. The animals were kept in isolated enclosures to avoid herd conditioning effects on semen quality, as was shown previously in this species (Holt *et al.*, 1996b). Animals were weighed at the time of semen collection; other data such as age and inbreeding coefficient were available from breeding records.

Semen collection

Semen was collected from the males during February and March 1999 using an electroejaculation procedure

described by Holt *et al.* (1988, 1996b). The only modification was that animals were anaesthetized using halothane gas (1–6% v/v) instead of intravenous ketamine and xylazine. This anaesthetic procedure was introduced as a routine improvement over the previous method. During the electroejaculation procedure, which typically involved < 10 stimulations up to maxima of 4.5–7.0 volts, the maximum voltage ($V_{\max}$) was recorded.

Semen evaluation

Fresh semen was evaluated within 40–60 min of collection, after transportation from the breeding centre to the laboratory. Volume was measured by aspirating the semen with a micropipette; wave motion was scored subjectively using a scale of 0–5 (5 being the most vigorous). The proportion of motile spermatozoa and the proportion of these spermatozoa showing progressive motility were assessed subjectively. Quality of motility was also rated using a 0–5 scale (0: non-motile; 5: highly motile). Sperm concentration was evaluated using a haemocytometer. Aliquots were diluted approximately ten-fold into TES–Tris-buffered media (8 ml distilled water, 0.482 g TES, 0.115 g Tris, 0.04 g glucose, 0.01 g streptomycin) for determination of the proportion of motile spermatozoa, proportion of progressively motile spermatozoa and also to score vigour of motility using a subjective scale (0–5).

Aliquots (5 μ l) of diluted semen were removed for computer-assisted motility assessment (Hobson Sperm Tracker; Hobson Tracking systems, Sheffield; see Holt *et al.* (1996b,c) for procedure and settings). The six sperm motion parameters obtained from the Hobson Sperm Tracker were as follows: (i) curvilinear velocity (VCL): velocity over the total distance moved, including all deviations of sperm head movement; (ii) average path velocity (VAP): velocity over a calculated, smoothed, path, that is, a shorter distance than that used for calculating VCL; (iii) straight line velocity (VSL): velocity calculated using the straight line distance between the beginning and end of the sperm track; (iv) amplitude of lateral head displacement (ALH): the mean value of the extreme side to side movement of the sperm head in each beat cycle; (v) beat cross frequency (BCF): the frequency with which the actual track crosses the smoothed track; and (vi) linearity (LIN): ratio of distances (as a percentage) of straightline tracklength/actual tracklength (this value is 100% for a completely linear track).

Sperm plasma membrane integrity (viability) was assessed by staining aliquots of sperm suspension with eosin–nigrosin (see Tamuli and Watson (1994) for details of eosin–nigrosin solution and Cassinello *et al.* (1998) for details of procedure). Acrosomal integrity was assessed using fluorescein-labelled lectin (*Pisum sativum*; FITC-PSA; Sigma-Aldrich Quimica SA, Madrid); aliquots of sperm suspension were smeared on to glass slides, air dried, fixed in ethanol and stained for 15 min with FITC-PSA (0.1 mg ml⁻¹ in physiological saline). Acrosomal status was categorized as intact, damaged or acrosome missing.

Storage of semen at 17°C

Aliquots of semen were diluted to a concentration of 1.0×10^8 spermatozoa ml^{-1} (approximately ten-fold dilution) at 37°C with a diluent based on the zwitterionic buffers TES and Tris (TEST medium) and 5% (v/v) egg yolk. Glycerol was not included, as there was no intention to freeze this semen. Diluted semen was cooled from 37°C to 17°C in a refrigerator for 40 min ($0.5^\circ\text{C min}^{-1}$) and was stored thereafter at 17°C in a thermostatically controlled box (Magapor, Zaragoza). Semen assessments were performed at 0 h (in media lacking egg yolk) and after 24, 48, 72 and 96 h of storage in the egg yolk-containing medium. For assessment, aliquots of diluted semen were rewarmed to 37°C and assessed as described earlier.

Statistical analysis

Sperm motility descriptors obtained from the Hobson Sperm Tracker were used for the multivariate pattern analysis described by Abaigar *et al.* (1999). This procedure uses the pattern analysis statistical computer program (PATN; CSIRO, Canberra) to classify all measured spermatozoa within a data set to a small number of subpopulations. In brief, all spermatozoa measured within the entire experiment, from all animals and at all assessment times, were combined within a single data set (30 511 spermatozoa and six motion descriptors for each). The pattern analysis was then undertaken to group the spermatozoa into a small number of subpopulations (the algorithm suggests the number of subpopulations). Each case in the data set (representing a single spermatozoon) was then categorized as belonging to one of the subpopulations. The relative frequencies of spermatozoa belonging to each subpopulation were then analysed using chi-squared tests or analysis of variance (ANOVA) to evaluate significance where appropriate. The analysis was continued by calculating the relative proportions of each sperm subpopulation within the data sets for each individual animal and each evaluation time for every animal. The figures presented in the paper are derived from this extended analysis.

The effects of phenotypic (age, weight), genetic (inbreeding coefficient) and technical (maximum voltage at semen collection) factors on semen characteristics were analysed by correlation analysis and ANOVAs. Data were log-transformed for ANOVA (Zar, 1984) and used to determine the effects of storage time on semen characteristics. Specific contrasts were examined within the ANOVAs using either orthogonal polynomial coefficients or post-hoc least significant difference tests. The non-parametric Kruskal–Wallis ANOVA was also used for paired comparisons within these data. All data were analysed using Statistica for Windows (Statsoft UK, Letchworth). At least 100 spermatozoa per preparation were assessed in determinations of acrosomal status and viability.

Results

Semen quality: sources of variation in fresh semen

Conventional semen parameters. The mean values and ranges for the semen characteristics of the 18 Mohor gazelles examined in this study are shown (Table 1). The subjective assessments of semen quality indicated that age was correlated inversely with measures of sperm motility (percentage motile spermatozoa: $r = -0.597$, $P = 0.011$; mass activity (log transformed): $r = -0.47$, $P = 0.011$; quality of progression: $r = -0.55$, $P = 0.012$). The maximum voltage used during electroejaculation was positively correlated with the proportion of spermatozoa with normal acrosomes ($r = 0.5$, $P < 0.05$) but not with the proportion of motile spermatozoa. Neither body weight nor inbreeding coefficient was correlated with the measures of semen quality (Table 1).

Sperm subpopulation analysis

Four sperm subpopulations were distinguished by the application of nonhierarchical and subsequent hierarchical classification of 30 511 individual spermatozoa using the six motion descriptors (VCL, VAP, VSL, BCF, ALH and LIN). Summary statistics for these subpopulations are shown (Table 2) and qualitative interpretation of these data is provided below.

Subpopulation 1. These are highly active spermatozoa (high VCL and VAP) but are not progressively motile (low VSL and LIN). BCF and ALH values may be misleadingly low because of the complex track trajectories.

Subpopulation 2. These are poorly progressive (low VSL) but highly active spermatozoa (high VAP). Trajectories must be generally straighter than subpopulation 1 as LIN is significantly higher.

Subpopulation 3. This population of spermatozoa shows most progressiveness (high VSL and LIN) and notably also the most active flagellar beat (high BCF). VAP is relatively low compared with VCL, indicating that many trajectories must be complex and non-linear. More than 50% of the spermatozoa in the data set were assigned to this subpopulation.

Subpopulation 4. This subpopulation contained spermatozoa displaying non-linear and circular trajectories. Although highly active (high VCL) their non-linearity and poor progressiveness was evident as low VSL and very high ALH. The ALH value is likely to be anomalous, owing to the complexity of the trajectories and difficulties in determining the average path.

There were significant differences in the distributions of the four sperm subpopulations in fresh semen across the 18 males sampled (chi-squared = 2577.6, degrees of freedom = 54, $P < 0.0001$; Fig. 1). The proportion of spermatozoa classified as subpopulation 3 (the most active and progressive) ranged from 40% to 68%, and the proportion identified as

Table 1. Semen characteristics of Mohor gazelles

Characteristic	Mean $\pm$ SD ($n = 18$) ^a	Range
Volume (μ l)	1351.3 $\pm$ 893.4	510–3994
Sperm concentration ($\times 10^6$ ml ⁻¹)	1068.8 $\pm$ 706.7	117–3220
Wave motion ^b	4.05 $\pm$ 0.82	2.5–5.0
Motility (%)	80.0 $\pm$ 16.9	30–95
Progressive motility (%)	59.5 $\pm$ 19.7	20–90
Quality of progressive motility (0–5 scale) ^c	4.22 $\pm$ 0.55	3–5
Plasma membrane integrity (viability)	77 $\pm$ 16.8	36–93
Acrosome integrity		
Intact (%)	64.7 $\pm$ 21.6	18–91
Damaged (%)	17.9 $\pm$ 12.4	3–56
Absent (%)	16.8 $\pm$ 22.1	1–79

^aOne ejaculate was assessed from each animal.

^bWave motion scored on a scale 0–5, where 5 indicates vigorous motion.

^cOn a scale 0–5, where 5 indicates rapid, linear movement.

Table 2. Motility descriptors for four Mohor gazelle sperm subpopulations after pattern analysis

Subpopulation	<i>n</i>	%	VCL	VAP	VSL	BCF	ALH	LIN
1	8151	26.7	131.3 $\pm$ 29.8 ^a	129.7 $\pm$ 29.9 ^a	10.6 $\pm$ 14.9 ^a	0.08 $\pm$ 1.1 ^a	0.02 $\pm$ 0.46 ^a	7.95 $\pm$ 7.8 ^a
2	2445	8.0	76.6 $\pm$ 13.2 ^b	75.7 $\pm$ 13.4 ^b	8.5 $\pm$ 6.2 ^b	0.02 $\pm$ 0.44 ^a	0.02 $\pm$ 0.4 ^a	11.35 $\pm$ 8.3 ^b
3	16789	55.0	131.7 $\pm$ 28.6 ^a	46.1 $\pm$ 23.2 ^c	26.8 $\pm$ 20 ^c	6.05 $\pm$ 4.73 ^b	13.1 $\pm$ 11.2 ^b	20.3 $\pm$ 14.56 ^c
4	3126	10.2	168.1 $\pm$ 36.3 ^c	19.2 $\pm$ 11.9 ^d	13.6 $\pm$ 6.7 ^d	2.35 $\pm$ 1.66 ^c	81.6 $\pm$ 42.9 ^c	8.3 $\pm$ 5.9 ^d

Values are mean $\pm$ SD.

VCL: curvilinear velocity; VAP: average path velocity; VSL: straight line velocity; BCF: beat cross frequency; ALH: amplitude of lateral head displacement; and LIN: linearity.

^{abcd}Different superscripts indicate significant differences within columns ($P < 0.05$). Raw data are shown but statistical comparisons were calculated after logarithmic transformations.

subpopulation 2 ranged from 0.65% to 18.6%. Subpopulation 1 varied from 18% to 39% and subpopulation 4 from 3.2% to 25.1%. The relative proportions of sperm subpopulations 2 and 4 were not correlated ($r = 0.103$, $P = 0.335$); however, all the other combinations of subpopulations were significantly correlated ($P < 0.0002$).

Exploration of the factors determining the relative proportions of sperm subpopulations in the ejaculates revealed the following relationships. The proportion of sperm subpopulation 2 in fresh ejaculates was negatively correlated with maximum voltage used during electroejaculation ($V_{\max}$) ($r = -0.875$, $P < 0.0001$; Fig. 2a). The frequency of sperm subpopulation 4 in fresh ejaculates was positively correlated with $V_{\max}$ ($r = 0.727$, $P < 0.005$; Fig. 2b). Body weight was negatively correlated with the proportion of subpopulation 4 spermatozoa in the fresh ejaculates ($r = -0.59$, $P < 0.005$; Fig. 3). Extraction of the partial correlations, that is, the extent to which each variable independently contributes to the regression equation, revealed that subpopulation 4 was independently correlated with $V_{\max}$ and body weight. Moreover, body weight and $V_{\max}$ were not correlated with each other.

Partial correlation coefficients were: subpopulation 4 versus body weight ($r = -0.67$, $P = 0.002$; and subpopulation 4 versus $V_{\max}$ ($r = 0.755$, $P < 0.0001$).

None of the sperm subpopulation frequencies were correlated with age or inbreeding coefficient. However, as the range of inbreeding coefficients was mostly very restricted (16 of 18 animals within the range 0.064–0.126, and only 2 of 18 with higher values, 0.314 and 0.207), this correlation analysis could not be considered valid.

Effects of dilution and storage at 17°C on sperm quality

All sperm characteristics were affected by storage, but most of these effects were only significant over relatively long time scales (Fig. 4a,b,c). A significant reduction in plasma membrane integrity (eosin–nigrosin stain) was evident only after 72 h ($P < 0.001$); a significant reduction in the proportion of intact acrosomes became apparent only after 96 h ($P < 0.001$). In contrast, subjectively assessed motility was almost halved within the first 24 h of storage ($P < 0.005$) and a further significant decrease occurred between 48 h and 72 h ($P < 0.01$).

Fig. 1. Relative proportions of sperm subpopulations 1–4 in fresh ejaculates from 18 Mohor gazelles. Subpopulation 1 (▨): highly active but non-progressive spermatozoa. Subpopulation 2 (□): highly active but not progressive spermatozoa: however, more linear than subpopulation 1. Subpopulation 3 (▩): rapid, linear and highly active spermatozoa. Subpopulation 4 (■): poorly progressive and non-linear, active spermatozoa.

The modification of sperm motility during storage at 17°C was detected in some detail by the use of computer-assisted sperm analysis and sperm subpopulation analysis. The curve for the decrease in sperm subpopulation 3 (Fig. 5b) resembled the curve for the subjectively assessed motility. However, there was only a small and non-significant decrease over the first 24 h. A significant decrease in this parameter became detectable after 48 h storage ($P < 0.001$); thereafter the proportion continued to decrease, reaching approximately 30% after 96 h.

Sperm subpopulation 1 behaved very differently (Fig. 5a); the proportion of spermatozoa displaying this type of highly active but non-linear motion was consistently about 23% for the first 24 h of storage. However, this proportion increased significantly to reach approximately 35% ($P < 0.05$) between 24 h and 48 h of storage; thereafter it decreased linearly during the next 48 h of storage (48 h versus 72 h, $P < 0.05$; and 72 h versus 96 h, $P < 0.01$). Sperm subpopulation 4 represented a minority of the overall number of motile spermatozoa, showing nonsignificant fluctuations of about 7–15% during the first 48 h of storage. The proportions of this subpopulation then decreased significantly from 48 h onwards ($P < 0.01$; Fig. 5c).

Sperm subpopulation 2 (poorly progressive and non-linear) was initially very small in fresh semen but increased significantly ($P < 0.005$) after dilution and storage for 24 h. Sperm subpopulation 2 then remained constant until again decreasing significantly ($P < 0.005$) after 96 h.

Discussion

A previous study of sperm motility in the Mohor gazelle using computer-assisted sperm analysis and a multivariate statistical analysis technique (pattern analysis; Abaigar *et al.*, 1999) showed that this combination yields a considerable amount of biological information. Abaigar *et al.* (1999) demonstrated that functionally distinct sperm subpopulations existed within samples of cryopreserved gazelle spermatozoa and a parallel study undertaken using boar spermatozoa confirmed that the subpopulations differed in their physiological status. As semen preservation and artificial insemination studies are currently in progress for facilitating genetic management and conservation of the Mohor gazelle (Holt *et al.*, 1996b), the opportunity to interpret sperm motility data in terms of reproductive fitness also arises.

In the present study our previous findings that four sperm subpopulations could be identified within samples of gazelle semen were confirmed. Group values for the individual computer-assisted sperm analysis-derived descriptors provide an indication of the type of motion behaviour shown by each subpopulation. Physiological interpretation of the numerical data is thereby facilitated. Thus, in the present study it was evident that subpopulation 3 represented the most progressive spermatozoa, probably those which most attract the attention of a subjective observer. The other sperm subpopulations had highly non-linear patterns of

Fig. 2. Frequency of sperm subpopulations (a) 2 and (b) 4 in fresh ejaculates from Mohor gazelles. Frequencies correlated with the maximum voltages ($V_{\max}$) used during electroejaculation (subpopulation 2 versus $V_{\max}$: $r = -0.875$, $P < 0.0001$; subpopulation 4 versus $V_{\max}$: $r = 0.727$, $P < 0.005$). Dashed lines represent 95% confidence limits of the regression line.

motility, but differed in trajectory and vigour. Thus, subpopulation 1 was characterized by highly vigorous but non-linear motion, possibly representing a form of hyper-activated or uncoordinated motility. Populations 2 and 4 differed from each other in terms of vigour and shape of trajectory. The combination of high VCL and extremely low VAP values, coupled with remarkably high ALH values, indicate that population 4 spermatozoa are typified by wide lateral excursions of the sperm head. The positive correlation of these spermatozoa with $V_{\max}$ indicates that these cells may have been deleteriously affected by the electrical field and, thus, may be degenerative.

A reasonable interpretation of this analysis is that the various subpopulations represent spermatozoa in different physiological states. Spermatozoa are known to change their behaviour in response to treatments and storage, and, therefore, it is likely that the pattern analysis is detecting the subtleties of this process. They could also represent spermatozoa originating from different parts of the epididymis, although this is unlikely. A possible sequence

Fig. 3. Frequency of sperm subpopulation 4 in fresh ejaculates from Mohor gazelles correlates negatively with body weight ($r = -0.59$, $P < 0.005$). Dashed lines represent 95% confidence limits of the regression line.

for the progression of spermatozoa through the subpopulation structure can be constructed. It is proposed that subpopulation 3, which contains most spermatozoa, represents activated spermatozoa that would normally be detected by a subjective observer estimating motility. During storage, some of these spermatozoa convert to behaviours classified as populations 1 and 2, possibly through loss of metabolic integrity and coordination. As subpopulation 4 increases when subpopulation 2 decreases and is highest when semen quality is low, it is likely to represent a late stage of degeneration.

This interpretation would help to explain the strong relationships found between $V_{\max}$ and the frequencies of both subpopulations 2 and 4. The present observations revealed that as the maximum stimulatory voltage increased, the incidence of spermatozoa classified as subpopulation 4 also increased. This result implies that higher voltages cause deterioration of semen quality and, therefore, supports the view that subpopulation 4 represents degenerating cells. Conversely, the incidence of subpopulation 2 spermatozoa decreased as higher voltages were used, possibly indicating that these are the cells, already displaying non-linear but vigorous motion, that are affected detrimentally by the stronger electrical fields. One possible explanation for these effects is that the direct action of electrical fields upon spermatozoa or accessory fluids stimulates the production of deleterious reactive oxygen species. Evidence that this does indeed happen as a result of electroejaculation has been reported from studies on the collection of spermatozoa from men with spinal cord injury (Rajasekaran *et al.*, 1994). Other studies of human spermatozoa have confirmed the detrimental effects of electrical fields on sperm motility and viability (Sikka *et al.*,

Fig. 4. Estimates of (a) plasma membrane integrity, (b) proportion of motile spermatozoa and (c) proportion of intact acrosomes in relation to storage time in semen samples from Mohor gazelles. Values are mean $\pm$ SEM ($n = 18$ animals at each time point).

Fig. 5. Relative proportions of sperm subpopulations 1, 3 and 4 from Mohor gazelles in relation to storage time. Values are mean $\pm$ SEM ($n = 18$ males at each time point). The proportion of subpopulation 1 is significantly higher after 48 h storage compared with after 24 h storage ($P < 0.05$) and is significantly lower after 96 h storage compared with 72 h ($P < 0.01$).

1994). Moreover, a study of sperm motility in domestic cats (Dooley and Pineda, 1986) demonstrated a weak correlation ($P < 0.1$) with the electroejaculation voltage. It should be noted that studies showing the absence of deleterious effects of electroejaculation on semen quality or in comparison to natural ejaculates have also been reported (for review see Watson, 1990).

The negative correlation between body weight and incidence of subpopulation 4 spermatozoa, which was

detectable independently of the voltage effects, indicates that heavier animals tend to produce a greater proportion of highly motile spermatozoa. This conclusion supports earlier findings in the Mohor gazelle, which showed that deleterious effects of inbreeding on semen quality (expressed as mean VSL) were compensated for in heavier animals (Holt and Abaigar, 1996). Holt and Abaigar (1996) measured sperm motility using computer-assisted sperm analysis after thawing and subsequently dilution of the semen samples in

a modified Tyrode's medium (Abaigar *et al.*, 1999). Spermatozoa from animals with low inbreeding coefficient (< 0.1 ; $n = 8$) had higher mean velocity (VSL), linearity and straightness than did those with inbreeding coefficients > 0.1 ($n = 4$). The deleterious effects of inbreeding were apparent only in animals whose body weight was lower than the median (62 kg). In the present study, which used a different group of males, the median body weight was 65 kg.

The validity of applying pattern analysis to sperm subpopulation studies has been demonstrated by Abaigar *et al.* (1999). Abaigar *et al.* (1999) demonstrated that subpopulations of boar spermatozoa can be detected within single ejaculates, and that they differ in their responses to physiological stimulators such as bicarbonate ions and caffeine (Harrison *et al.*, 1996). Some spermatozoa respond within 2 min of stimulation, whereas others do not. Furthermore, it was shown that the frequencies of responsive and unresponsive spermatozoa differed among individual animals. These changes in sperm subpopulation structure within semen samples were easily detected by the pattern analysis, thus confirming the power of the technique. The assessment technique used in the present study involved dilution of the gazelle spermatozoa in a simple organic buffer solution that did not include bicarbonate ions or any other stimulators. However, the motility of gazelle spermatozoa appears to be fully activated after ejaculation without any requirement for further stimulation. Nevertheless, differences between the motion of individual cells must be determined by the efficiency of the protein kinase/phosphatase cascades that control motility and their interactions with other cellular components. Some of the significant components have been identified in other species and genetic studies of mice and other species have attributed sperm dysfunction to specific gene defects or alterations that are carried through the generations by mechanisms involving non-Mendelian inheritance (Herrmann *et al.*, 1999). Therefore, the differences among subpopulations of gazelle spermatozoa in the fresh ejaculates might be partly attributable to functional variability arising through the specific combinations of sperm-related genes carried by each animal. Moreover, as a consequence of the genetic mosaicism that results from meiosis, clones of spermatozoa derived from separate spermatocytes undoubtedly possess different gene combinations that could lead to significant differences in sperm motility.

It was not possible to evaluate separate effects of inbreeding in relation to semen quality in the present study. However, studies of Cuvier's gazelles (*G. cuvieri*), which are also bred and managed at EEZA, demonstrated that detrimental effects of inbreeding upon sperm morphology could be detected (Roldan *et al.*, 1998; Gomendio *et al.*, 2000). These authors also pointed out that since the individuals in their study population are all exposed to the same environmental conditions, differences among individuals are likely to have a genetic basis. The same argument is applicable to the Mohor gazelles used in the

present study as they are kept in precisely the same location and under the same management.

Any explanation that invokes a genetic basis has to predict that sperm subpopulation structure cannot be affected by age. This prediction is consistent with the results of this study. However, age effects were detected using subjective observations of motility. This finding demonstrates that the trained observer and the computer-assisted sperm analysis system are detecting subtly different aspects of motility. Although the computer-assisted sperm analysis system can measure the proportions of motile and non-motile spermatozoa, this facility was not used in the present study, whereas a subjective observer would almost involuntarily include this aspect of the sample in the assessment.

The sperm storage method tested in the present study was reasonably satisfactory provided the storage time did not exceed 48 h; neither sperm plasma membrane integrity nor acrosomal damage deteriorated significantly during this period. The proportion of motile spermatozoa decreased abruptly during the first 24 h of storage and continued to decrease more gradually thereafter; this assessment could only be obtained subjectively.

However, the use of computer-assisted sperm analysis and pattern analysis to analyse temporal changes in sperm behaviour during storage provided some surprising insights into the storage process. The most progressively motile sperm subpopulation (subpopulation 3) decreased steadily during storage, halving in 72 h, whereas subpopulation 1 underwent an increase during the initial 48 h, before decreasing. This finding could be explained if the subpopulations represent sequential changes in sperm behaviour associated with prolonged storage. As subpopulation 1 is highly active but non-progressive, the increase at 48 h may indicate subtle deterioration of sperm motility control mechanisms, with consequential loss of co-ordination and implications for reduced fertility. After 48 h there was a marked decrease in the proportions of all spermatozoa represented within the individual animal data sets; to some extent this is an artefact of the analysis and indicates that for future experiments involving storage the absolute number of spermatozoa sampled at each time point should be equal.

The aim of short-term storage is to prevent deleterious cellular changes and to inhibit natural developmental processes such as capacitation. Decreasing the temperature is one way of achieving this, as the activity of metabolic pathways and degradative enzymes is reduced. The zwitterionic TES-Tris buffer used in this experiment provides a beneficial environment for sperm storage and cryopreservation (Salamon and Maxwell, 1995a,b), presumably by avoiding inadvertent stimulatory effects such as might be obtained in the presence of inorganic ions.

The opportunity to conduct experiments with semen from a species such as the Mohor gazelle arises infrequently because procedures such as electroejaculation are invasive and require general anaesthesia. In the present study it was impossible to undertake a larger and more comprehensive experiment. The decision to undertake semen storage at

17°C and not, for example, at 5°C was based on the knowledge that a full experiment could not be conducted. It was also a judgement based on correspondence with scientists (J. Smith, AgResearch, Hamilton; personal communication) studying similar questions in agricultural species such as sheep, who suggested that avoidance of membrane permeabilization by exposure to the lower temperature should be beneficial. To date, it has not been possible to test the fertility of the stored semen satisfactorily as techniques for artificial insemination and synchronization of oestrus are still under development. However, this is a good illustration of the need for studies of reproduction to be wide-ranging and integrated to make full use of the benefits offered by reproductive technologies.

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