Journal of Andrology, Vol. 22, No. 1, January/February 2001

Semen Characteristics of the Captive Indian Leopard, *Panthera pardus*

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ABSTRACT: Semen samples from 11 Indian leopards (*Panthera pardus*) from 3 different zoos in India were collected by electroejaculation. A computer-aided semen analyzer (CASA) was used for assessing the quality of the semen vis-à-vis sperm motility. The volume of the ejaculate, sperm density, and the number of motile and morphologically normal spermatozoa were found to be 1.57 ± 1.26 mL, 55.78 million ± 38.67 million per mL, 57.05% ± 16.96%. and 71.92% ± 15.32%, respectively. Although the spermatology varied between individuals in the study, Box-Whisker-plot analysis suggested that the distribution was normal ($P > .05$). The ejaculated sperm were cryopreserved after diluting in test-yolk buffer. The post-thaw motility was 32.14% and did not differ at 30 or 60 days after cryopreservation. CASA indicated that the progressive velocity (VSL) of cryopreserved spermatozoa was decreased and, as a consequence, they moved more slowly than the neat (VSL 76.3 μm/sec in neat vs 53.8 μm/sec in cryopreserved spermatozoa) and the trajectories were less planar. However, both cryopreserved and neat spermatozoa penetrated the zona-free hamster oocyte with equal efficiency (79% neat vs 80% cryopreserved). The study also reports application of CASA for feline spermatozoa and provides information for the first time on the spermatology of the Indian leopard. This baseline data could be used in captive breeding programs. The results are compared and discussed with the available information on other felines.

Key words: *Felidae,* semen profile, sperm motility, CASA, cryopreservation.

J Androl 2001;22:25–33

Among the endangered species of the world, cats perhaps make up the largest group. Barring the domestic cat, all other cats are classified as endangered by the Convention on International Trade in Endangered Species (CITES). Of the mega cats, the Indian subcontinent houses the tiger (*Panthera tigris*), the lion (*P leopersica*), and the leopard (*P pardus*), which are endangered (Khoshoo, 1997). Unlike the tiger and lion, the leopard has a remarkable ability to adapt and survive under various climatic conditions in different habitats. This substantiates their wide distribution from Africa to southern Asia. A recent report indicates that their number also is declining in alarming numbers (Daniel, 1996). In order to help their conservation and propagation, apart from management practices, application of assisted reproduction would be necessary for the success of captive breeding programs. This would not only hasten their conservation and propagation but also combat management problems. However, such programs require a basic understanding of reproduction in felines. Few studies are available on the reproduction of felines, including Indian tigers and Asiatic lions (Howard, 1983; Wildt et al, 1983, 1986, 1987a, 1987b; Donoghue et al, 1992; Johnston et al, 1994; Patil et al, 1998; Shivaji et al, 1998). These studies indicate significant variation among feline gamete functions (Roth et al, 1994). The reasons for such variations have been attributed to seasonality or inbreeding (Wildt et al, 1986, 1987a). Although species-specific variation in spermatology (that is, semen volume, pH, total sperm number, sperm concentration, percentage of motile sperm, and percentage of morphologically normal spermatozoa) among felines is not uncommon, ascertaining specific reasons for these variations has been difficult for want of comparative studies in feline species. Successful application of assisted reproductive technology requires understanding of spermatology, endocrinology, and embryology in felines. Furthermore, spermatology is of paramount importance in understanding the reproductive strategy of wild animals and would also help in management of their conservation (Wildt et al, 1995).

The main objectives of this study were to evaluate 13 leopards from 3 zoos in India for their ejaculate characteristics such as semen volume, semen pH, sperm concentration, percentage of motile spermatozoa, and percentage of morphologically normal spermatozoa. In addition, the motility parameters and the fertilizing ability of leopard spermatozoa prior to and after cryopreservation was also monitored.

Supported by Central Zoo Authority (CZA), Ministry of Environment and Forests, Government of India (S.S.).

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Received for publication November 18, 1999; accepted for publication June 21, 2000.

Materials and Methods

Animals

Adult male Indian leopards maintained at the Nehru Zoological Park, Hyderabad (n = 6); Sakkarbaug Zoo, Junnagadh (n = 6); and Nandankanan Zoo, Bhubaneshwar (n = 1), India, served as semen and blood donors. All the animals were kept in cages with access to a kyaal (a fenced exercise area) during the daytime. They were fed with beef and supplemented with adequate amounts of calcium for 6 days per week. Water was available ad libitum. All the studies were performed with the approval of the Institute Ethical Committee and the Government of India.

Collection of Semen

The leopards were anesthetized by intramuscular injection of 200 mg Ketamine and 125 mg Xylazine (Troy Labs, Smithfield, New South Wales, Australia) using a blowpipe or an air pistol. The anesthetized leopards were then laid out on an elevated platform. The entire area surrounding the rectum, testis, and penis was cleaned with water and smeared with a disinfectant. A rectal probe (3 cm in diameter) with 3 longitudinal electrodes smeared with white petroleum jelly, was gently inserted into the rectum of the animal and positioned so that the electrodes of the probe faced the ventral side of the animal. The probe was connected to an electrostimulator (230 V DC, 15 amps sine wave current; Bioelectric Co, Washington, DC). The penis was extended beyond the prepuce and inserted into a 25-mL sterile plastic container for collecting the semen. The stimulatory regime used for electroejaculation of the leopards consisted of a total of 80 stimuli between 2 to 5 V divided into 3 series (Patil et al, 1998; Shivaji et al, 1998). Each stimulus at the desired voltage was given for 2 seconds with a gap of 2 seconds between the stimuli. The animals were rested for 5 minutes between each series of stimuli.

Determination of Semen Characteristics

Ejaculates were collected separately at the end of each series of stimuli, and semen characteristics such as volume, pH, density, and number of motile spermatozoa were determined. Different ejaculates from the same individual were pooled for further studies only if they had similar percentages of sperm motility. The pH of the ejaculates was recorded using pH indicator strips. Sperm number in the ejaculate was counted using a hemocytometer and is expressed as million spermatozoa per milliliter. For this purpose, the semen was diluted in a diluent (50 g of sodium bicarbonate and 10 mL of 35% formaldehyde per liter of distilled water; World Health Organization, 1992).The percentage of motile spermatozoa was determined at 400× magnification using a phase contrast microscope. A small aliquot of the ejaculate was smeared onto clean slides, air-dried, fixed in equal parts of 95% ethanol and ether for 15 minutes, stained with hematoxylin Papanicolaou, dehydrated with a graded ethanol series, and mounted in DPX. About 500 cells were scored under a microscope at 1000× for morphologically normal and pleiomorphic spermatozoa and were expressed as percent of the total number of spermatozoa counted. Aliquots were also stained with chlortetracycline (CTC), a fluorescent dye, to ascertain the morphology of the spermatozoa. For CTC staining, semen was washed free of seminal plasma by centrifugation at 800 × *g*, the sperm pellet suspended in 500 μM CTC in a buffer containing 20 mM Tris and 130 mM NaCl pH 7.8, and the suspension was observed under a fluorescent microscope using a 450 to 490 nm excitation filter (Shivaji et al, 1998).

Cryopreservation of Semen

The protocol used for cryopreservation of leopard semen was essentially similar to that described earlier for the tiger and lion (Patil et al, 1998). Briefly, the semen sample was diluted with an equal volume of Ham F-10 medium maintained at 37°C, spun at 800 × *g* for 10 minutes and, subsequently, the pellet was resuspended in Ham F-10. Aliquots containing 5×10^6 spermatozoa in 150 μL volume were transferred to cryotubes and mixed with an equal volume of test-yolk buffer supplemented with 8% (v/v) glycerol (Mortimer, 1994). The cryotubes were then exposed to liquid nitrogen vapors at a height of 5 to 8 cm from the surface of liquid nitrogen for 45 minutes. Subsequently, the tubes were stored in liquid nitrogen until further use. The cryopreserved semen samples were thawed by plunging the cryotube into a 37°C water bath and assessed for percentage of motile spermatozoa using the motility analyzer.

Motility Analysis of Leopard Spermatozoa

A computer-aided semen analyzer (CASA; HTM-IVOS, Version 10, Hamilton-Thorne Research Inc, Beverly, Mass) was used for the motility analysis of leopard spermatozoa. The parameter setup values used for monitoring the motility of leopard spermatozoa are given in Table 1 and were derived following the guidelines and recommendations of Working and Hurtt (1987) and Yeung et al (1992). In the present study, the motility analyzer was used to determine the sperm number and the number of motile spermatozoa in the semen sample, along with motility parameters such as curvilinear velocity (VCL), progressive velocity (VSL), path velocity (VAP), straightness (STR) and linearity (LIN) of the path, amplitude of lateral head displacement (ALH), and beat cross-frequency (BCF) of a spermatozoon. The detailed definitions of the parameters can be found in our earlier studies (Girija Devi and Shivaji, 1994; Shivaji et al, 1995, 1998; Jayaprakash et al,1997; Peedicayil et al, 1997; Ravinder et al, 1997; Patil et al, 1998; Uma Devi et al, 1999).

Zona-free Hamster Oocyte Penetration Test

The hamster zona-free oocyte penetration assay was performed essentially according to the method described by Yanagimachi (1984). Briefly, female hamsters on Day 1 of the estrous cycle were injected intraperitoneally with pregnant mare's serum gonadotropin (40 IU) followed by human chorionic gonadotropin (hCG; 40 IU) 48 to 72 hours after the first injection. Animals were killed between 16 to 18 hours post-hCG by cervical dislocation, ovaries were removed and transferred to Tyrodes medium with lactate (TL) and HEPES (Bavister, 1981), and oocytes were released by puncturing the ampullary region. The oocytes, along with the cumulus complex, were transferred to a drop of medium containing hyaluronidase (100 μg/mL) and gently pipetted to separate the oocytes from the cumulus mass. The oocytes were then washed three times and individually incubated in TL-HEPES with trypsin (100 μg /mL) until the zona disap-

Table 1. *Conditions and parameter values for HTM-IVOS motility analyzer used for the analysis of tiger and lion spermatozoa*

Conditions/parameter set up	
Temperature (°C)	37
Apply sort	No
Frames acquired	30
Frame rate (Hz)	60
Minimum contrast	11
Minimum cell size (pixels)	6
Minimum static contrast	10
Threshold straightness (%)	10
Low VAP cut off (μm/s)	5.0
Medium VAP cut off (μm/s)	15.0
Low VSL cut off (μm/s)	5.0
Nonmotile head size (pixels)	10
Nonmotile head intensity	250
Static head size	0.67–1.68
Static head intensity	1.3–1.7
Static elongation (limits)	0–100
Slow cells motile	YES
Magnification	10×, 1.95
Video source	Camera
Video frequency	60
Bright field	NO
Image type	Phase contrast
Brightness for LED	2214
Chamber depth (μm)	80
Field selection mode	Auto
Minimum track points	15

peared. The zona-free oocytes were washed again and transferred to TALP (Bavister, 1981). Spermatozoal suspension (5×10^6 spermatozoa per mL) was preincubated in TALP-PVA (Bavister and Andrews, 1988) for 3 hours and, subsequently, 100 μL was added to 50 μL of TALP containing 10 zona-free oocytes. The drop was covered with liquid paraffin and further incubated for 3 hours at 37°C in the presence of 5% CO_2. The oocytes were then washed 3 times with TL-HEPES to remove nonspecifically bound spermatozoa and transferred onto a glass slide. The oocytes were fixed with 1% glutaraldehyde, dehydrated with a graded ethanol series, and stained with aceto-orcein. The slides were examined under a phase contrast microscope at 500× magnification to determine the number of oocytes that had spermatozoa and the number of spermatozoa within an oocyte (Shivaji and Bhargava, 1987; Shivaji et al, 1998).

Blood Collection and Radioimmunoassay of Testosterone

Blood samples (10 mL) from leopards were collected from the femoral vein into vacutainer glass tubes (Becton-Dickinson, Franklin Lakes, NJ) and allowed to clot at room temperature. The serum was separated by centrifugation and stored at −20°C until further use. Serum testosterone was assayed according to the manufacturer's instructions using a kit purchased from Amersham Life Sciences, (Buckinghamshire, United Kingdom).

Data Analysis

The data on the semen characteristics were analyzed by Box-Whisker plots (Microcal Origin V5, Microcal Software Inc, Northampton, Mass; and Microsoft Excel, Redmond, Wash) and Student's *t*-test. Data were considered significant if *P* was less than .05. The Shapiro-Wilk W-test was carried out to ascertain whether the distribution of the data was normal (JMP Software, SAS Institute, Inc, Cary, NC).

Table 2. *Semen characteristics and serum testosterone levels of captive leopards (n = 11) from three zoos in India**

Parameter	Mean ± Standard Deviation	
Age (years)	14.35 ± 3.93	(7.25–18.58)
Weight (kg)	60.45 ± 4.99	(50–66.5)
Semen pH	7.39 ± 0.23	(7–7.7)
Ejaculate volume (mL)	1.57 ± 1.26	(0.5–4)
Sperm concentration (×10⁶/mL)	55.78 ± 38.67	(10–142)
Sperm motility (%)	57.05 ± 16.96	(20–90)
Normal spermatozoa (%)	71.92 ± 15.32	(38–90)
Abnormal spermatozoa (%)		
1. Macrocephalic	0.94 ± 0.72	(0.1–2.7)
2. Microcephalic	0.47 ± 0.38	(0.1–1.33)
3. Bicephalic	0.94 ± 1.10	(0–2.8)
4. Amorphous head	2.25 ± 1.81	(0.43–7.6)
5. Tightly coiled tail	13.59 ± 10.41	(1.4–39.28)
6. Bent neck	0.83 ± 0.98	(0.2–3.8)
7. Bent tail	3.01 ± 2.53	(0.2–9.5)
8. Cytoplasmic droplet	0.95 ± 0.67	(0.17–2)
9. Combined defects†	2.66 ± 4.09	(0.17–11.4)
Serum testosterone (pg/mL)	893.77 ± 317.75	(625–1562)

* Values in parentheses indicate the range of variation
† Spermatozoa showing more than one defect

Results

Spermatology

Leopards used in the present study had a mean age of 14.35 ± 3.93 years and the mean body weight was 60.45 ± 4.9 kg (Table 2). No adverse effect after anesthesia or electroejaculation was observed. Eleven out of the 13 leopards varying in age between 7.25 to 18.58 years responded to electrostimulation and the mean volume of the ejaculate was 1.57 ± 1.26 mL (Table 2). The inability to obtain ejaculates from 2 of the leopards born after November 1988 was not related to the age of the animals because leopards that were born before or after 1990 also ejaculated following electrostimulation. In the ejaculates, only 58.88% ± 16.6% of the spermatozoa were found to be motile. Morphologically normal spermatozoa (Figure 1A; Figure 2A) were 70.43% and the majority (13.59%) of the pleiomorphic spermatozoa showed tail abnormalities (Figure 1G and H; Figure 2B through E). Other sperm abnormalities such as head abnormalities (Figure 1D and E; Figure 2B and C), presence of cytoplasmic droplet (Figure 1B), and abnormal midpiece (Figure 1B, C, and F) constituted only a small fraction of the total spermatozoa. The results in Table 2 and Figure 3 indicate

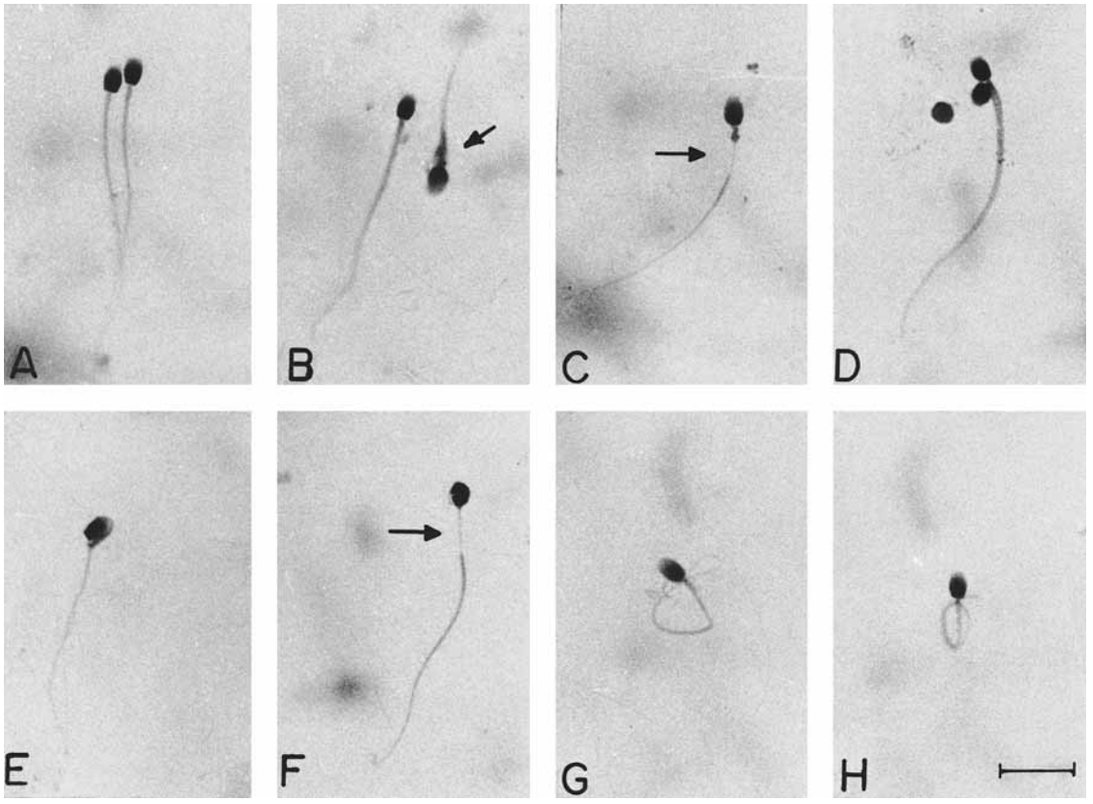


Figure 1. Bright field photomicrographs of spermatozoa of leopard stained with Papanicolau (1000×). **(A)** Two spermatozoa with normal morphology; **(B)** a spermatozoon with a cytoplasmic droplet (arrow); **(C)** a spermatozoon with an abnormal midpiece (arrow); **(D)** a bicephalic spermatozoon; **(E)** a spermatozoon with a bent neck; **(F)** a spermatozoon with an abnormal head and midpiece; **(G** and **H)** spermatozoa with coiled tail. Scale = 10 μm.

that the ejaculate volume, percentage of motile spermatozoa, sperm concentration, and percentage of morphologically normal spermatozoa vary from animal to animal. But based on the Shapiro-Wilk W-test for normality for ejaculate volume (P = .1285), percentage motile spermatozoa (P = .2549), sperm concentration (P = .4148), and percentage of morphologically normal spermatozoa (P = .2968), it was obvious that the differences in these parameters between individuals was not significant because the P value was greater than .05, thus indicating normal distribution.

It may be worthwhile to mention that the Indian leopard is still breeding successfully in the wild, and therefore captive breeding programs have been taken up only in a small number of animals and only in some zoos. For instance, out of the 13 leopards studied from 3 zoos, only 1 of them has been used for breeding and was of proven fertility. The semen characteristics of this 11.4-year-old animal (semen pH 7.5, ejaculate volume 2.3 mL, sperm concentration 90 × 10^6/mL, motile sperm 60%, and normal spermatozoa 83%) was also observed to be comparable to the average values reported in Table 2.

Levels of Testosterone in the Semen of Captive Leopards

The mean testosterone concentration in the leopards from the Indian zoos was 894 ± 317 pg/mL of serum (Table 2) and varied from 625 to 1562 pg/mL (Figure 3E). The distribution of the data was normal (P = .5708) and the interassay and intra-assay coefficients were less than 15%.

Cryopreservation of Spermatozoa

Semen samples with mean initial motility of 59.37% ± 8.63% (range 50%–70%) from 6 different leopards were cryopreserved. The post-thaw motility in all the semen samples decreased to a mean of 32.14% ± 9.14% (range 20%–45%). The difference between the neat and post-thaw sperm motility was significant ($P < .05$), and all the motile spermatozoa exhibited progressive motility (Figure 4).

CASA

Motility parameters of leopard spermatozoa prior to and after cryopreservation were monitored (Table 3). The results indicate a significant decrease in VAP, VSL, BCF,

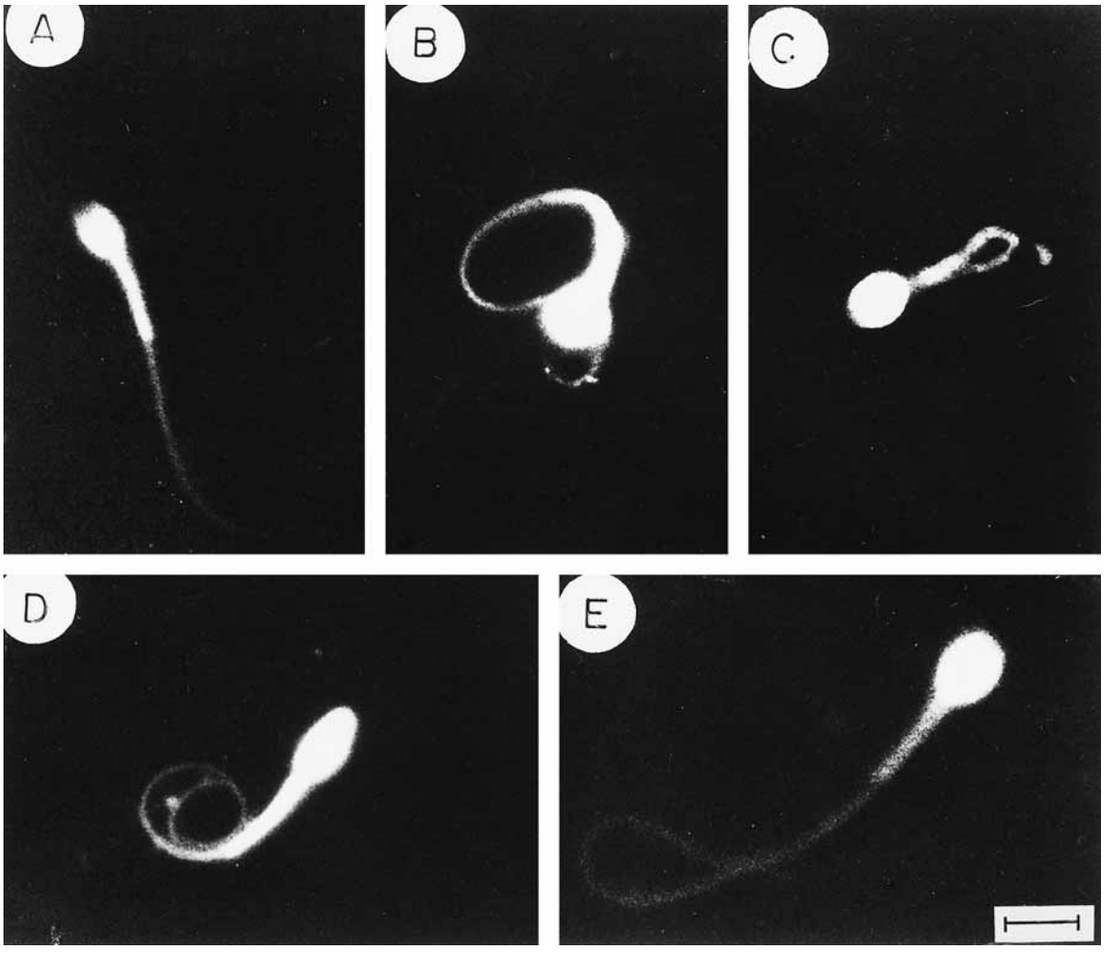


Figure 2. Fluorescence photomicrographs of spermatozoa of leopard stained with chlortetracycline (1000×). **(A)** A normal spermatozoon; **(B)** a spermatozoon with a round head and coiled tail; **(C)** a spermatozoon with a round head and exhibiting coiling of the midpiece; **(D)** a spermatozoon with the end piece tightly coiled; **(E)** a spermatozoon with the end piece partially coiled. Scale = 5 μm.

and LIN and increase in ALH in cryopreserved spermatozoa. Simultaneously, it was also observed that the trajectories of the post-thaw spermatozoa were slower, more curved, less planar, and more zig-zag (Figure 4). Furthermore, the spermatozoa that were not cryopreserved covered a longer distance than the cryopreserved ones.

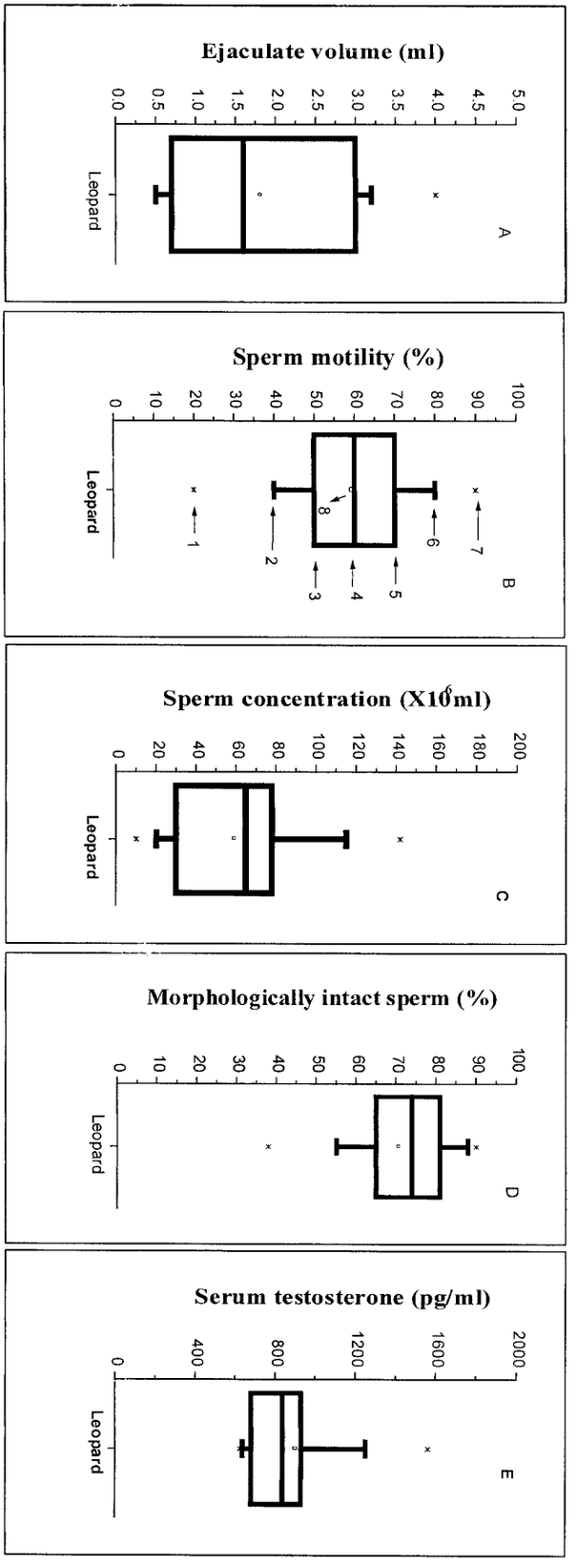
Zona-Free Hamster Egg Penetration Assay

Spermatozoa from both neat and cryopreserved semen penetrated 79% (33 out of 42) and 80% (29 out of 36) of the zona-free oocytes, respectively. The number of spermatozoa penetrating a single oocyte was higher when the neat semen sample was used (24.29 ± 0.9 spermatozoa per oocyte) compared with the cryopreserved semen sample (13.22 ± 4.02 cryopreserved spermatozoa per oocyte).

Discussion

The present study provides baseline data on the semen characteristics of the Indian leopard, *P pardus*. The studies indicate that the anesthetic doses and the ejaculatory regime used for other feline species can reasonably be extended to the Indian leopards. Further, the anesthetic dose was sufficient to maintain a surgical plane of anesthesia and adequate to perform procedures of electroejaculation and blood collection. Furthermore, it did not induce any postoperative symptoms in the animals used and it also demonstrated that ejaculated semen could be cryopreserved and that cryopreserved semen penetrated zona-free oocytes with an efficiency similar to that of neat semen.

The quality of the electroejaculates was very good with the majority of the leopards exhibiting good spermatozoal



concentrations ($>50 \times 10^6$ spermatozoa per mL), motility (>50%), increased number of morphological normal spermatozoa (>70%), and excellent oocyte penetration ability (~80%). Although there was variation in the volume of ejaculate among individuals, the Shapiro-Wilk W-test indicated that the distribution was normal (Figure 3) and the differences were not significant ($P > .05$). Earlier studies have also indicated a great degree of individual variation with respect to ejaculate volume, sperm concentration, percentage of sperm motility, and percentage of sperm with abnormal morphology in other feline species (Wildt et al, 1983, 1986, 1987a, 1987b; Roelke et al, 1993; Johnston et al, 1994). In some felines, ejaculate characteristics such as ejaculate volume, sperm concentration, and percentage of motile spermatozoa correlated with seasonality (Wildt et al, 1986; Johnston et al, 1994). However, in the present study, no tangible comparison of the influence of the season on semen characteristics could be made.

Incidences of abnormal spermatozoa are reported to be high in felines. In some feline species the percentage of morphologically abnormal spermatozoa was very high (greater than 70%) as in the Florida panther (Roelke et al, 1993), snow leopard (Johnston et al, 1994), cheetah (Wildt et al, 1983, 1987b, 1988), leopard, and puma (Wildt et al, 1988), and this may be a characteristic feature of these animals compared with the domestic cat, tiger, lion, leopard cat, and clouded leopard, in which the percentage was less than 50% (Wildt et al,1986, 1987a; Brown et al,1989; Howard and Wildt,1990; Donoghue et al,1992; Howard et al, 1993; Shivaji et al,1998). In the Indian leopard, about 30% of the spermatozoa were morphologically abnormal and the predominate abnormality was the result of coiling of the tail of the spermatozoon (13.59%). This is comparable to the Chinese leopard, the cheetah, and the puma cats, which showed, respectively, 35.6%, 22%, and 20% of spermatozoa with tightly coiled tails (Wildt et al, 1987b, 1988). The reasons for the high incidence of morphologically abnormal spermatozoa in Felidae is not known but has been attributed to genetic and hormonal etiologies because increased homozygosity could have an adverse effect on spermatozoal morphology (Wildt et al, 1987b, 1988). An increase in genetic ho-

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Figure 3. Box-Whisker plots depicting variation in semen ejaculate volume **(A)**, percentage of motile spermatozoa **(B)**, sperm concentration **(C)**, percentage of morphologically normal spermatozoa **(D)** and serum testosterone concentration **(E)** in the 11 leopards. Each Y column of data is represented as a separate box. The horizontal lines in the box denote the 25th, 50th, and 75th percentile values (arrows 3 to 5, respectively). The error bars denote the 5th and 95th percentile values (arrows 2 and 6). The 2 symbols below the 5th percentile error bar denote the zero and 1st percentile values (arrow 1). The 2 symbols above the 95th percentile error bar denote the 99th and 100th percentile (arrow 7). The square symbol in the box denotes the mean of the column of data (arrow 8).

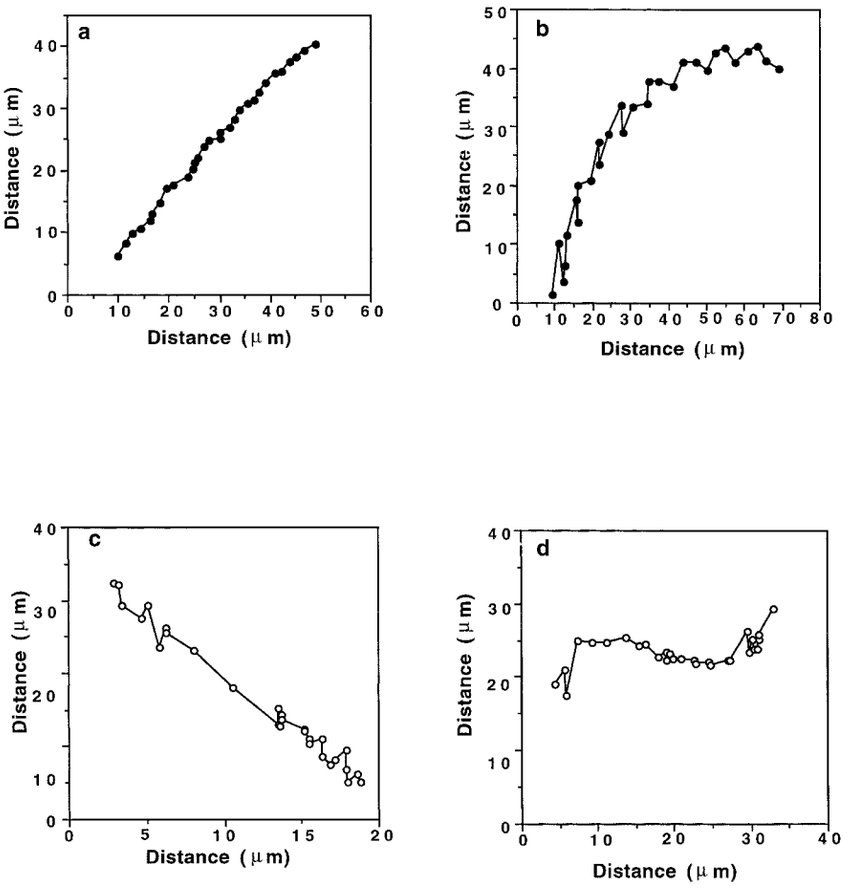

Figure 4. Motility track of a leopard spermatozoon prior to **(a** and **b)** and after cryopreservation **(c** and **d)** recorded directly on the HTM-IVOS motility analyzer. The spermatozoa exhibit linear **(a** and **c)** or curved **(b** and **d)** trajectories. The closed circles along the motility track represent the position of the head and the solid line the actual track of the spermatozoon. Note that compared with the cryopreserved spermatozoa the noncryopreserved spermatozoa have covered a greater distance in the same tracking time.

Table 3. *Comparison of the motility parameters of leopard spermatozoa from neat semen and a cryopreserved semen sample**

Motility Parameters	Neat Semen (n = 146)	Cryopreserved Semen Sample (n = 188)
Path velocity (μm/s)	82.1 ± 36.3[a]	63.3 ± 17.9[b]
Progressive velocity (μm/s)	76.3 ± 36.6[a]	53.8 ± 20.8[b]
Curvilinear velocity (μm/s)	124.2 ± 40.6[a]	114.3 ± 25.7[a]
Lateral head displacement amplitude (μm)	3.9 ± 1.1[a]	5.3 ± 1.7[b]
Beat cross-frequency (Hz)	44.1 ± 7.4[a]	31.7 ± 11.7[b]
Straightness (%)	87.7 ± 11.9[a]	83.3 ± 18.3[a]
Linearity (%)	57.1 ± 18.6[a]	47.5 ± 15.7[b]

* Values represent mean ± standard deviation. Different superscripts indicate that the means are significantly different ($P < .05$) as determined by Student's *t*-test

mozygosity may be a consequence of inbreeding (Czaker and Mayr, 1984). It is worthwhile to mention that the 4 North Chinese leopards studied by Wildt et al (1988), and which exhibited a high percentage of abnormal spermatozoa in semen were all born in captivity. Furthermore, in the snow leopards, the number of abnormal spermatozoa varied with the season and endocrine status (Johnston et al, 1994). The testosterone level in the present study was 894 pg/mL and is comparable to the North Chinese leopard (Wildt et al, 1986), but was lower than that observed for the lions (1850 pg/mL) and tigers (1720 pg/mL) of the Indian zoos (Shivaji et al, 1998) and greater than that observed for the cheetah (300 to 540 pg/mL; Wildt et al, 1988). Despite these differences in testosterone levels, the number of motile and normal spermatozoa in the Indian leopard has been high, indicating that the hormonal quantity is probably a characteristic feature of the species. Furthermore, it is worthwhile to mention that semen and hormonal data are not an index of the fertility status or viability of spermatozoa, and that the role of

morphologically abnormal spermatozoa in fertilization is debatable (Howard and Wildt, 1990).

The ejaculated spermatozoa were cryopreserved using the test-yolk buffer supplemented with 8% glycerol (pH 7.4). The post-thaw motility of the spermatozoa was 32.14% and it was independent of the quality of the initial motility. This is the first study on the motility parameters of leopard spermatozoa both from neat and cryopreserved semen. From the results it is obvious that spermatozoa after cryopreservation are slower due to a decrease in the velocity parameters, VAP and VSL, and the frequency with which the tail beats (BCF). A significant decrease in the linearity of the trajectory (LIN) and increase in ALH was also observed, and this may account for the trajectories being curved in spermatozoa from cryopreserved semen samples (Figure 3). Lion and tiger spermatozoa following cryopreservation also exhibited curved trajectories and traveled a shorter distance in unit time compared with normal spermatozoa (Patil et al, 1998). Nonetheless, the cryopreserved and the neat spermatozoa were equally capable of penetrating denuded hamster oocytes. However, the number of cryopreserved spermatozoa that penetrated an oocyte was significantly less compared with spermatozoa from a neat semen sample (13 vs 24). This is not surprising, as loss of quality after freeze-thaw has also been reported for other feline spermatozoa (Johnston et al, 1991). Reports also indicate that snow leopard spermatozoa failed to penetrate the inner layer of the zona pellucida of the domestic cat oocytes but successfully penetrated homologous eggs (Roth et al, 1994; Wildt et al, 1995). It is also worthwhile to mention that although experiments with zona-free hamster oocytes provide important information about the penetration ability of spermatozoa, they do not mimic the in vivo event involving zona-intact oocytes of homologous species. Such an experiment for evaluation of semen of wild cats is extremely difficult because protocols and regimes for induction of ovulation, collection of oocytes, and so on would need to be standardized. But this is not impossible and has been achieved by Roth et al (1994).

The present studies form the first report on the spermatology of the Indian leopard, which is vulnerably endangered. Semen parameters such as volume, sperm density, and the number of motile and morphologically normal spermatozoa varied between individuals. Furthermore, it is demonstrated that cryopreserved spermatozoa are motile and capable of penetrating zona-free hamster oocytes. In addition, we have demonstrated application of CASA to test the quality of the spermatozoa in wild animals. The data acquired could facilitate the identification of leopards with the best semen profiles for captive breeding programs.

Acknowledgments

Our thanks to Mr S. C. Sharma, Member Secretary, CZA for his constant support and encouragement. Our special thanks to T. G. Cooper for critically evaluating the manuscript. The authors express thanks to the directors and staff of the Nehru Zoological Park Hyderabad, Nandan Kanan Zoo, and Bhuvaneshwar and Sakkarbagh Zoo, Junagadh for their help and cooperation. K. Santosh Kumar and C. C. Sudha Kumari assisted during the initial stages of this work. We also thank P. Ramesh for his help in data computation. Meticulous secretarial assistance by N. Rajani is gratefully acknowledged.

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