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Cryopreservation of spermatozoa from wild-born Namibian cheetahs (*Acinonyx jubatus*) and influence of glycerol on cryosurvival ☆

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Abstract

Sperm cryopreservation is a valuable tool for the genetic management of ex situ populations. This study was conducted to assess: (1) semen characteristics of wild-born cheetahs; and (2) the impact of three types of glycerol influence (duration of exposure, temperature, and method of addition) on sperm cryosensitivity. To evaluate the impact of duration of glycerol exposure, spermatozoa were incubated in Test Yolk Buffer (TYB) with 4% glycerol at ambient temperature (~22 °C) for 15 vs. 60 min before cryopreservation. To evaluate the influence of temperature and method of glycerol addition, spermatozoa were resuspended at ambient temperature either in TYB with 0% glycerol followed by addition of 8% glycerol (1:1 v/v; at ambient temperature vs. 5 °C) or directly in TYB with 4% glycerol. All samples were cryopreserved in straws over liquid nitrogen vapor and evaluated for sperm motility and acrosomal integrity after thawing. Semen samples ($n = 23$; $n = 13$ males) contained a high proportion (78%) of pleiomorphic spermatozoa. Ejaculates also contained a high proportion of acrosome-intact (86%) and motile spermatozoa (78%). Immediately after thawing, a significant proportion of spermatozoa retained intact acrosomes (range, 48–67%) and motility (range, 40–49%). After thawing, incubation in glycerol for 60 min at ambient temperature before freezing decreased ($p < 0.05$) sperm motility and acrosomal integrity at one time-point each (pre-centrifugation and post-centrifugation, respectively). However, method or temperature of glycerol addition had no ($p > 0.05$) impact on sperm cryosurvival. In summary, (1) wild-born cheetahs produce high proportions of pleiomorphic spermatozoa but with a high proportion of intact acrosomes; and (2) resuspension in 4% glycerol, followed by exposure for up to 60 min at ambient temperature, had minimal effect on sperm motility and acrosomal integrity after cryopreservation. Results indicate the feasibility of cryopreserving cheetah spermatozoa under field conditions, providing a user-friendly method to capture and store gametes to enhance genetic management.
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The largest population (~3000) of free-living cheetahs (*Acinonyx jubatus*) is found in Namibia, Africa [20,21]. The wild cheetah officially is recognized as threatened with extinction [3], primarily due to habitat fragmentation and agricultural encroachment [20,21,23]. This disassembly of habitat results in small, isolated populations that are especially vulnerable to loss of gene diversity which in turn causes reproductive compromise and susceptibility to catastrophes, including disease [41,42]. The cheetah is well recognized for its comparatively low genetic variation [25,26] and its difficulty of reproducing in captivity [18]. Interestingly, little is known of its fitness in nature, although all evidence suggests a high incidence of reproductive success in the absence of human perturbations [2]. Most studies of the reproductive physiology of the male cheetah are derived from extensive studies of animals from North American zoos [4,11,32,36,37] and one captive facility in South Africa [38]. Additionally, our laboratory evaluated a few free-living cheetahs ($n = 8$) in the Serengeti ecosystem almost two decades ago [39]. A common denominator to all of these studies has been the observation of an extraordinary incidence of teratospermia, with more than 70% of all ejaculated spermatozoa being malformed.

Spermatozoa from teratospermic males, especially the integrity of acrosomal membranes, are particularly sensitive to the steps of cryopreservation [28,29]. Despite the high proportion of abnormal spermatozoa produced in cheetah ejaculates, there is interest by captive managers in using artificial insemination (AI), preferably with frozen-thawed spermatozoa, as a means of helping manage the ex situ population. A laparoscopic intrauterine AI technique has been developed and is successful (46% pregnancy rate) for producing pregnancies and live cubs in the cheetah following insemination with freshly collected spermatozoa [15]. Such technology, in conjunction with frozen-thawed spermatozoa, would allow (1) the import of spermatozoa from nature to provide new genes to captive populations, (2) overcoming common problems associated with sexual incompatibility between genders, and (3) moving genes (via spermatozoa) from one captive facility to another while eliminating the stress of animal translocation. The development of an organized 'Genome Resource Bank' (GRB) and its advantages have been recognized for the cheetah and other species for years [10,42]. Utilizing frozen cheetah spermatozoa transported from Namibia, Africa, no pregnancies resulted following insemination of $<4 \times 10^6$ motile spermatozoa/AI; however, 50% (3 of 6) of females became pregnant (resulting in 3 litters) following insemination with $6–16 \times 10^6$ motile spermatozoa/AI [14]. Therefore, the biological viability of genome resource banking for cheetah spermatozoa has been proven.

The efficient use of cryopreserved gametes requires a detailed understanding of basic cheetah sperm biology as well as the factors affecting sperm cryosurvival. Our earlier studies of this species largely involved captive-bred cheetahs living in zoos or breeding centers in North America or South Africa. Furthermore, sperm cryopreservation was routinely conducted using the pelleting technique, originally developed in the domestic cat, and involving freezing of spermatozoa on a block of dry ice [13,36]. This approach works well under controlled laboratory conditions, however our overall aim has been to improve sperm cryopreservation methodologies under field conditions, including being able to clearly label cryopreserved samples for the GRB. The pelleting method is incompatible with this goal due to the difficulty of obtaining dry ice in the field and limitations on sample identification (e.g., inability to label individual pellets). Furthermore, storage of sperm pellets in liquid nitrogen tanks provides opportunity for spreading pathogens as samples cannot be completely sealed. We have become particularly interested in the cheetahs of Namibia, Africa, where the highest density of this species has been reported. Here, the environment can consist of rather rigorous conditions, including temperatures that reach 40–45 °C. Further, Namibian cheetahs are often maintained on distant farmlands, making a mobile field laboratory a necessity for collecting and processing sperm samples.

Mammalian spermatozoa (including those from felids) are typically cryopreserved using the permeating cryoprotectant glycerol [9]. The addition of glycerol to spermatozoa before cryopreservation involves equilibration steps, whereby intracellular water exits the spermatozoon across an osmotic gradient, causing initial cell shrinkage, followed by glycerol influx that returns the cell to near original

volume [7]. During glycerol removal, the cell swells as it is exposed to near isotonic conditions and as extracellular water enters the cell faster than glycerol departs [24]. These volume excursions can disrupt both sperm membranes and the motility apparatus [29]. There also may be a toxic effect [22], with protracted glycerol exposure adversely influencing sperm motility, membrane integrity, and overall survival [16]. It also is known (through studies of ram and mouse spermatozoa) that these adverse effects can be affected by temperature, with glycerol supplementation at ambient temperature or after cooling to 5 °C altering the level of osmotic injury [5,16].

Finally, little information is available on cryosensitivity of cheetah spermatozoa. There are sufficient data demonstrating that ejaculates from teratospermic felids are particularly challenging to successfully cryopreserve [29]. In the teratospermic domestic cat, for example, the acrosome is especially sensitive to cooling, osmotic stressors, and the removal of cryoprotectant from the sperm suspension [27–29]. Therefore, the objectives of the present study were to conduct a thorough assessment of semen quality in wild-born cheetahs in Namibia while exploring the efficacy of a two-step straw protocol for sperm cryopreservation. The latter specifically evaluated the influence of (1) length of glycerol exposure at ambient temperature, and (2) temperature and method of glycerol addition on post-thaw sperm motility and acrosomal integrity.

Materials and methods

Animals

Adult, wild-born male cheetahs ($n = 13$; age range 21 months to 14 years) were housed singly or in groups of 2–4 individuals in outdoor enclosures (1 hectare per cheetah) at the Cheetah Conservation Fund (CCF, Otjiwarongo, Namibia) or at other licensed Namibian facilities. Animal age was estimated by a thorough examination of tooth wear [19]. All animals had been held in captivity for a minimum of 1 year and were considered unsuitable for reintroduction to the wild. All animals were brought to the CCF either as orphans unable to hunt on their own or were habituated to captivity as adults at other facilities. No animal was maintained in captivity for a purpose that could potentially affect the study results. At the CCF, cheetahs ($n = 7$ males, $n = 17$ ejaculates) were fed a combination of donkey, horse, and game species (2–3 kg of meat and bone per day; 6 days per week) with daily vitamin and mineral supplementation (8–10 g calcium, 6000–7500 IU vitamin A, 800–1000 IU vitamin D_3, 8–10 IU vitamin E, and 64–80 mg iron; CAL-SUP Powder, Bayer Co.). Animals also received organ meat (mixture of heart, liver, and/or lung) once per week. Animals at other captive facilities ($n = 6$ males, $n = 6$ ejaculates) received a mixture of donkey, horse, cattle, and game species (amount and frequency variable) and dietary supplementation was supplied with unknown consistency.

Semen collection, evaluation, and processing

All animal procedures were conducted following approval of the National Zoo's Institutional Animal Care and Use Committee. Methods for anesthesia and semen collection/evaluation were similar to our previous studies [11,37,38,40]. In brief, a surgical plane of anesthesia was induced with 4–6 mg/kg tiletamine and zolazepam (Telazol; Fort Dodge Laboratories, Fort Dodge, IA) delivered i.m. with an air-pressured darting system. If needed, ketamine hydrochloride (Ketaset; Fort Dodge, 50–100 mg i.v.) was administered during electroejaculation to maintain a surgical plane of anesthesia. Testicular length and width were measured using laboratory calipers and then converted to total testes volume per male [12]. A rectal probe of either 1.6 or 1.9 cm in diameter with three longitudinal electrodes and an electrostimulator (P.T. Electronics, Boring, OR) were used to provide a total of 80 stimuli (at a low voltage of 2–5 V) over a 30 min interval [11,37,38]. Semen was collected in pre-warmed, sterile collection vials, and an aliquot (3 μl) was immediately assessed for sperm percent motility (%M) and forward progressive status (FPS; scale = 0–5 with a five rating equivalent to rapid, straightforward progress; [11]). A 20 μl aliquot of raw semen was fixed in 100 μl of 0.3% glutaraldehyde in PBS (pH, 7.4) for assessing sperm morphology (1000×). Spermatozoa were classified as normal or as having one of the following abnormalities: (1) head abnormalities including microcephalic, macrocephalic, and bi- or tri-cephalic; (2) acrosomal abnormalities including missing or damaged acrosomal membranes; (3) midpiece abnormalities including abnormal or missing midpiece, a bent midpiece with retained cytoplasmic droplet and a bent midpiece with no droplet; (4) flagellar abnormalities includ-

ing tightly coiled flagellum, bent flagellum with retained cytoplasmic droplet, bent flagellum with no droplet, bi- or tri-flagellate, retained proximal droplet and retained distal droplet; and (5) other abnormalities including spermatid and bent neck. A 20 μl aliquot of raw semen was fixed in 500 μl of 4% paraformaldehyde for evaluating acrosomal integrity (as described below). The remainder of each sample was diluted immediately with an equal volume of sterile Ham's F10 culture medium (HF10; Irvine Scientific, Santa Ana, CA) supplemented with 20 mM Hepes, 5% (v:v) fetal calf serum (Irvine Scientific), pyruvate (1 mM), L-glutamine (2 mM), 10,000 IU/ml penicillin, 10 mg/ml streptomycin, and 20 mg/ml neomycin (Sigma Chemical, St. Louis, MO). Sperm concentration was determined using a hemocytometer [11,38]. Diluted samples were centrifuged (Eppendorf Mini-Spin, Hamburg, Germany) for 8 min at 100*g* and the supernatant aspirated from the sperm pellet.

Cryodiluents

For the comparative cryopreservation studies, sperm pellets were resuspended in Test Yolk Buffer (TYB; Irvine Scientific) containing 0% glycerol and diluted further in TYB containing 8% glycerol (v/v; 4% final glycerol concentration) or resuspended directly in TYB containing 4% glycerol. These cryodiluents were prepared using a commercially available 'Freezing Medium-TYB' with 12% glycerol (Irvine Scientific) combined with the commercially available 'Refrigeration Medium-TYB without glycerol.' The modified TYB containing 8% glycerol (v/v) was prepared as a 2:1 mixture of the 'Freezing Medium with 12% glycerol' and 'Refrigeration Medium without glycerol.' The modified TYB containing 4% glycerol (v/v) was prepared as a 1:2 mixture of 'Freezing Medium' and 'Refrigeration Medium.'

Study 1: Characterizing seminal traits in the Namibian cheetah

Of the 23 ejaculates, single samples were collected from seven individual males. Of the remaining 16 samples, eight represent two collections from each of four males, and eight represent four collections from each of two males. For all repeat collections, the interval between collections ranged from 17 days to 10 months with all ejaculates ($n = 23$ total) collected over an 18-month period.

Study 2: Influence of duration of glycerol exposure at ambient temperature

Electroejaculates ($n = 4$ males, 8 ejaculates; 2 ejaculates/male) were washed as described above. Sperm pellets were resuspended in TYB containing 0% glycerol (TYB + 0% G) at ambient temperature and divided into five aliquots (Fig. 1A). The control treatment was derived from methods yielding the best cryosurvival of spermatozoa from teratospermic domestic cats (Pukazhenthi, unpublished observations). To determine if cooling vessel would affect post-thaw parameters, samples were cooled to 5 °C either in straws (Veterinary Concepts, Spring Valley, WI and Minitüb, Tiefenback, Germany) or Eppendorf tubes. All samples were cooled in either a standard full-sized refrigerator or in a mobile thermoelectric cooler connected to an automobile 12-V power source (Koolatron P9 Traveler II, Koolatron, Chicago, IL). To determine the impact of glycerol exposure prior to cooling, samples were further diluted 1:1 (v/v) with TYB containing 8% glycerol (TYB + 8% G) at ambient temperature to achieve a 4% final glycerol concentration (v/v; [36]) and then maintained for 15 or 60 min at ambient temperature (~22 °C) before cooling to 5 °C (Fig. 1A).

The treatments were: (1) *Control* (no glycerol at ambient temperature)—washed sperm samples were diluted in TYB + 0% G in Eppendorf tubes and placed in a water bath (300 ml at ambient temperature) and slow-cooled (~3.5 h) to 5 °C. After reaching this temperature, TYB + 8% G was added in three aliquots over a 30-min period (adding 1/4 volume and waiting 15 min, adding 1/4 volume and waiting 15 min and then adding the remaining 1/2 volume). The sample then was loaded into 0.25 ml straws and cryopreserved over liquid nitrogen (LN) vapor using a two-step protocol. Briefly, straws were placed 7.62 cm above the liquid for 1 min, then 2.54 cm above liquid for an additional 1 min and then plunged into the LN; (2) *Glycerol 15 min/straw cool*—TYB + 8% G was added to sperm suspensions in TYB + 0% G in three aliquots at 5-min intervals at ambient temperature. Samples were held in an Eppendorf tube for 15 min at ambient temperature, loaded into straws, slow-cooled to 5 °C and cryopreserved (as described above); (3) *Glycerol 15 min/tube cool*—TYB + 8% G was added to sperm suspensions in TYB + 0% G in three aliquots at 5-min intervals at ambient temperature. Samples were held in an Eppendorf tube for 15 min at ambient temperature, slow-cooled to 5 °C,

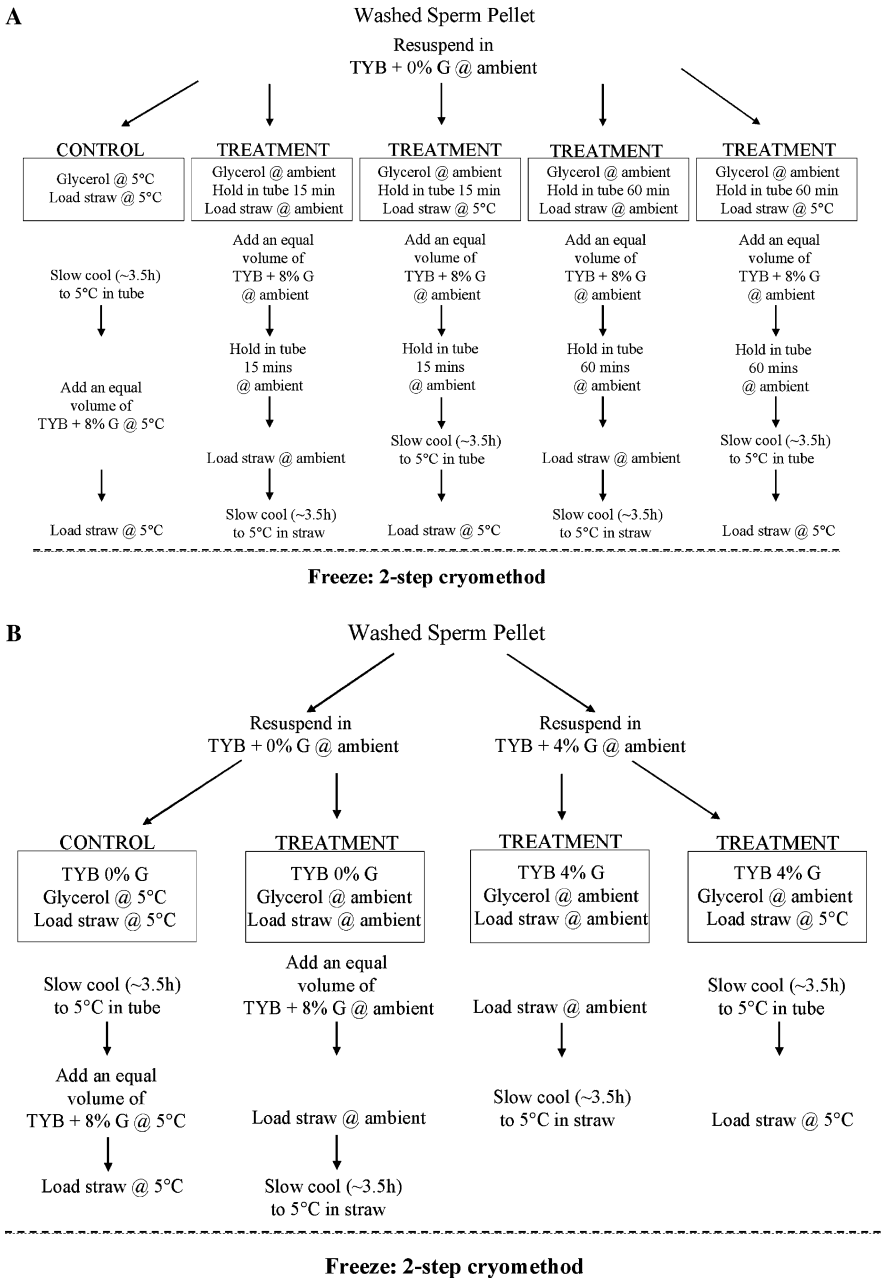

Fig. 1. Schematic diagram of treatment groups for Studies 2 and 3. (A) Study 2: influence of duration of glycerol exposure at ambient temperature. (B) Study 3: influence of temperature and method of glycerol addition.

loaded into straws and cryopreserved; (4) *Glycerol 60 min/straw cool*—TYB + 8% G was added to sperm suspensions in TYB + 0% G in three aliquots at 5-min intervals at ambient temperature. Samples were held in an Eppendorf tube for 60 min at ambient temperature, loaded into straws, slow-cooled to 5 °C and cryopreserved; and (5) *Glycerol 60 min/tube cool*—TYB + 8% G was added to sperm suspensions in TYB + 0% G in three aliquots at 5-min intervals at ambient temperature. Samples were held in an Eppendorf tube for 60 min, slow-cooled to 5 °C, loaded into straws and cryopreserved. All samples were frozen in straws containing 90–120 µl of sperm suspension with a final sperm concentration of either 30 or 50×10^6 motile spermatozoa/ml and a final glycerol concentration of 4% (v/v).

Study 3: Influence of temperature and method of glycerol addition

Electroejaculates ($n = 11$ males, 15 ejaculates) were evaluated as described above. Two of the donors were the same individuals as used in Study 2. Sperm suspensions in HF10 were divided into two aliquots and washed by centrifugation (100*g*; 8 min). Sperm pellets were resuspended immediately in either TYB + 0% G or in TYB + 4% glycerol (TYB + 4% G) at ambient temperature. Each of these two aliquots then was split in half to produce four treatments (Fig. 1B).

The treatments were: (1) *Control* (8% G at 5 °C)—The aliquot was resuspended in TYB + 0% G, placed in an Eppendorf tube and slow-cooled (~3.5 h) to 5 °C. After reaching 5 °C, an equal volume of TYB + 8% G was added to the sample in three aliquots over a 30 min period (as described in Study 2). The sample was loaded into straws and cryopreserved (as above); (2) *8% G ambient/straw cool*—The aliquot was resuspended in TYB + 0% G, diluted (1:1) with TYB + 8% G at ambient temperature in three aliquots at 5-min intervals. The sample was loaded into straws, slow-cooled to 5 °C and cryopreserved; (3) *4% G ambient/straw cool*—The aliquot was resuspended directly in TYB + 4% G, immediately loaded into straws, slow-cooled to 5 °C and cryopreserved; and (4) *4% G ambient/tube cool*—The aliquot was resuspended directly in TYB + 4% G, placed in an Eppendorf tube, slow-cooled to 5 °C, loaded into straws and cryopreserved. All samples from this study were frozen at a final concentration of 30×10^6 motile spermatozoa/ml in straws containing 90–120 µl and a final glycerol concentration of 4% (v/v).

Post-thaw evaluation

Individual straws were thawed (1 or 2 straws per treatment) for 10 s in air followed by 30 s in a 37 °C water bath. Straw contents were emptied into a sterile Eppendorf tube, and samples were evaluated immediately for % M and FPS. For assessment of acrosomal integrity, 8–10 µl of each sample was fixed in 500 µl of 4% paraformaldehyde. Samples then were diluted in HF10 (150 µl per straw), assessed for % M and FPS and 20 µl removed for assessment of acrosomal integrity (see below). Thawed sperm suspensions in HF10 then were centrifuged for 8 min at 100*g*, supernatant was aspirated from each pellet and resulting sperm pellets were resuspended in 200 µl HF10 in a drop-wise fashion. Washed samples were evaluated following centrifugation for % M and FPS and at hourly intervals for 4 h. Over the time course of assessments, samples were held in Eppendorf tubes protected from light and held at a constant ambient temperature. At each time-point of evaluation, a 20 µl aliquot of the sperm suspension was fixed in 500 µl of 4% paraformaldehyde for assessment of acrosomal integrity.

Evaluation of acrosomal integrity

Sperm samples fixed in 4% paraformaldehyde were centrifuged for 8 min at 2000*g* and the supernatant discarded. Pellets were washed twice with 500 µl of 0.1 M ammonium acetate (pH 9.0) and the pellet resuspended in approximately 50 µl of the ammonium acetate solution. An aliquot of this suspension was smeared onto microscope slides and allowed to dry at ambient temperature. Thereafter, slides were flooded with Coomassie stain (0.22% Coomassie Blue G-250, Fisher Biotech, Springfield, NJ, in 50% methanol, 10% glacial acetic acid and 40% deionized water; [17]) for 90 s, rinsed with deionized water, dried at ambient temperature and permanently preserved by placing a coverslip over a drop of mounting medium (Krystalon, EM Science, Gibbstown, New Jersey). For each sample, 200 spermatozoa were assessed individually for acrosomal integrity using bright field microscopy at 1000× (Fig. 2) and categorized as intact, damaged or non-intact. Briefly, cheetah spermatozoa with intact acrosomal membranes exhibited a uniform blue staining overlying the acrosomal region. Spermatozoa with non-intact or damaged acrosomes displayed a clear area overlying the acrosomal region or a patchy staining pattern. Spermatozoa with an abnormal acrosomal membrane often showed evidence of a knobbed-membrane structure (Fig. 2).

Statistical analysis

Correlations between the percentages of structurally normal spermatozoa and those with an intact acrosomal membrane in raw ejaculates were performed using Pearson's correlation coefficient [34]. To determine the relationships between the percentages of normal spermatozoa in raw ejaculates with the proportion of acrosome-intact spermatozoa at each evaluation time post-thawing, data for percentage of spermatozoa with intact acrosomes (% IA)

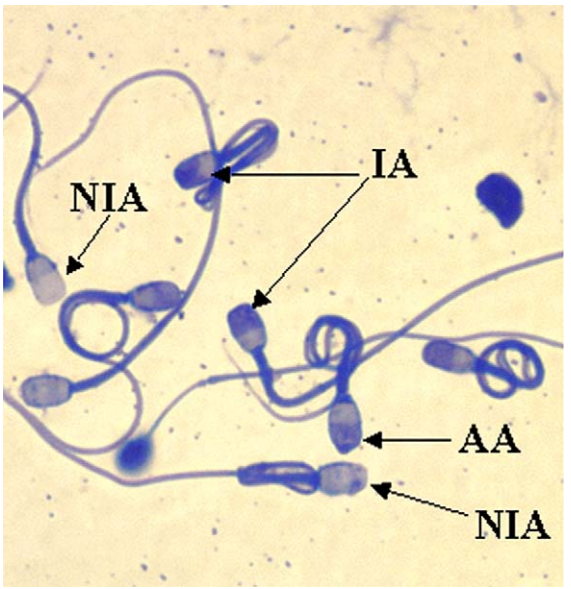

Fig. 2. Coomassie blue staining of cheetah spermatozoa for assessment of acrosomal integrity (1000×). Spermatozoa with intact acrosomes (IA) exhibited a uniform purple staining overlying the acrosomal region. Spermatozoa with non-intact acrosomes (NIA) displayed a clear or patchy staining pattern. Also depicted is a spermatozoon with an abnormal (knobbed) acrosome (AA).

were combined for each animal across treatments. Correlations were considered significant at $p < 0.05$.

For evaluation of treatment effects, all data for % M and % IA were arcsine transformed before analysis. The final statistical model included the main effects of treatment and the covariate of percent normal spermatozoa in the raw ejaculate on % M, % IA and FPS. When a significant *F*-statistic was found, means were separated using Duncan's multiple-range test. Results are reported as least-squares means ± SEM, and means were considered statistically different at $p < 0.05$. To determine treatment differences, data were analyzed by General Linear Model procedures of SAS [33]. Repeated measures analysis [34] was used to determine differences within treatments in the rate that % M and % IA changed over time. Within a given treatment, values were considered different from the previous time-point at a level of $p < 0.05$.

Results

Study 1: Characterizing seminal traits in the Namibian cheetah

Characteristics of the 23 raw ejaculates are depicted in Table 1. Donor location influenced some seminal traits. Cheetahs housed at the CCF had a lower ($p < 0.05$) seminal volume (3.2 ± 0.4 ml) and higher ($p < 0.05$) sperm density ($24.6 \pm 3.3 \times 10^6$/ml) than cheetahs sampled at other captive sites (means, 5.3 ± 0.6 ml and $8.4 \pm 5.5 \times 10^6$/ml, respectively). There were no other differences in raw semen characteristics between these two sources of donors. When these findings were compared to historical data from zoo-maintained cheetahs in North American zoos [37], overall results were similar (Table 1).

The type and frequency of sperm malformations detected in the ejaculates of Namibian cheetahs are summarized in Table 2. The most prevalent morphological defects were a spermatozoon with a bent midpiece with retained cytoplasmic droplet (Fig. 3B) or an abnormal acrosome (Fig. 3C). Together these abnormalities comprised more than 40% of the structural defects observed (Table 2). The proportion of various types of pleiomorphisms also was similar to historic data collected for cheetahs living

Table 1
Ejaculate characteristics of wild-born Namibian[a] and captive North American cheetahs[b]

Trait	Namibian cheetahs			North American cheetahs
	Means ± SEM	Minimum	Maximum	
Total testes volume (cm^3)	9.2 ± 0.4	5.8	12.7	13.9 ± 0.4
Seminal volume (ml)	3.7 ± 0.4	0.6	6.8	1.5 ± 0.1
Sperm concentration ($\times 10^6$/ml)	20.4 ± 3.1	3.5	66.0	29.3 ± 5.6
Sperm motility (%)	78.0 ± 1.4	70.0	90.0	67.0 ± 2.0
Sperm forward progressive status (FPS)*	3.7 ± 0.1	3.0	4.0	3.6 ± 0.1
Total motile sperm ($\times 10^6$)	49.8 ± 8.7	10.3	170.8	31.4 ± 5.6
Morphologically normal sperm (%)	21.7 ± 2.4	5.0	45.0	21.3 ± 2.0
Intact acrosomes (%)	86.3 ± 1.6	68.0	97.5	ND

ND, not determined.
[a] $n = 13$ males, 23 ejaculates.
[b] $n = 60$ males, 60 ejaculates; [37].
* FPS scale = 0–5 with 5 being the most rapid, straightforward progression.

Table 2
Average percentage (±SEM) of normally and abnormally shaped spermatozoa in electroejaculates from wild-born Namibian[a] and captive North American cheetahs[b]

Trait	Namibian cheetahs	North American cheetahs
Normal sperm	21.7 ± 2.4	21.3 ± 2.0
Abnormal sperm	78.3 ± 2.4	78.7 ± 2.0
Macrocephalic	0.6 ± 0.2	0.8 ± 0.2
Microcephalic	5.4 ± 0.9	4.4 ± 0.9
Bi/tri-cephalic	0.5 ± 0.2	0.4 ± 0.1
Abnormal acrosome	10.5 ± 1.5	3.7 ± 0.4
Abnormal or missing midpiece	4.6 ± 0.8	0.9 ± 0.2
Tightly coiled flagellum	5.6 ± 1.5	27.4 ± 2.5
Bi/tri-flagellate	0.3 ± 0.1	ND
Bent midpiece with droplet	30.0 ± 2.2	21.1 ± 1.1
Bent midpiece without droplet	4.0 ± 0.5	2.8 ± 0.4
Bent flagellum with droplet	1.1 ± 0.4	0.6 ± 0.1
Bent flagellum without droplet	1.5 ± 0.3	2.6 ± 0.8
Proximal droplet	6.3 ± 1.1	11.0 ± 0.9
Distal droplet	0.8 ± 0.2	3.0 ± 0.4
Bent neck	0.3 ± 0.1	ND
Spermatid	6.0 ± 1.0	ND

ND, not determined.
[a] $n = 13$ males, 23 ejaculates.
[b] $n = 60$ males, 60 ejaculates; [37].

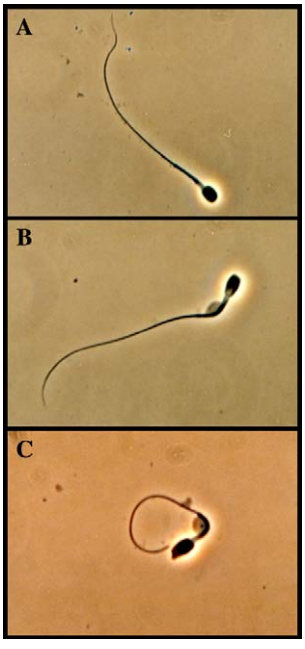

Fig. 3. Common morphological forms of spermatozoa in ejaculates obtained from wild-born cheetahs in Namibia (1000×). (A) Structurally normal spermatozoon; (B) spermatozoon with a bent midpiece and retained cytoplasmic droplet; and (C) spermatozoon with an abnormal acrosome.

in North American zoos (Table 2). For all raw ejaculates, there was a positive correlation ($r = 0.66$, $p = 0.001$) between the percentage of spermatozoa with normal morphology and % IA (Fig. 4).

Study 2: Influence of duration of glycerol exposure at ambient temperature

There was no effect ($p > 0.05$) of cooling samples in straws compared with cooling in tubes for the two treatments held in 4% glycerol for 15 min at ambient temperature on % M, % IA or FPS post-thaw at any time-point. Similarly, there were no differences in these post-thaw values between samples from the two treatments held in 4% glycerol at ambient temperature for 60 min. Therefore, for final analysis and presentation, data from the two treatments exposed to glycerol for 15 min were combined into one overall treatment group (glycerol exposure for 15 min, hereafter referred to as GE 15 min). Similarly, data from the two treatments exposed to glycerol for 60 min were combined into one overall treatment group (glycerol exposure for 60 min, or GE 60 min).

There was no effect ($p > 0.05$) of glycerol exposure duration on % M or % IA immediately after thawing (Table 3). Following dilution in HF10 (pre-centrifugation), % M decreased ($p < 0.05$) in samples from the GE 60 min treatment compared to controls. There was no difference ($p > 0.05$) between treatments in the % IA at this time. However, after centrifugation, the proportion of spermatozoa with intact acrosomal membranes in the GE 60 min treatment decreased ($p < 0.05$) compared to controls (Table 3). There was no effect of treatment ($p > 0.05$) at any other time of evaluation on the percentage of motile spermatozoa or spermatozoa with intact acrosomal membranes. Similarly, there were no differences ($p > 0.05$) between treatments on sperm FPS at any time-point of evaluation (data not shown).

For all treatment groups, the percentage of motile spermatozoa decreased ($p < 0.05$) immediately after thawing compared to raw samples (Table 3). For control samples, % M decreased ($p < 0.05$) again only between centrifugation and 1 h post-thaw. For post-thaw samples from the GE 15 min treatment, dilution in HF10 increased ($p < 0.05$) sperm % M. The percent motile spermatozoa then decreased ($p < 0.05$) as a result of centrifugation and decreased ($p < 0.05$) between 2 and 3 h post-thaw (Table 3). For samples from the GE 60 min treatment, % M decreased ($p < 0.05$) between 1 and 2 h and decreased ($p < 0.05$) again between 3 and 4 h after thaw.

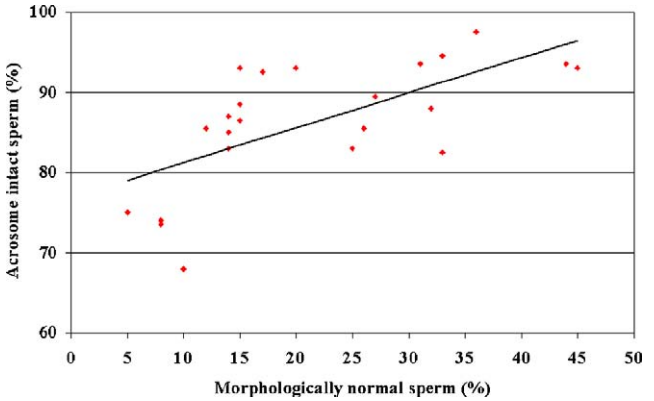

Fig. 4. Relationship ($r = 0.66$, $p = 0.001$) between percent structurally normal sperm and percent intact acrosomal membranes in raw cheetah ejaculates.

Table 3
Influence of duration of glycerol exposure at ambient temperature on sperm motility (% M) and intact acrosomal membranes (% IA)

	Control		Glycerol exposure 15 min		Glycerol exposure 60 min	
	% M	% IA	% M	% IA	% M	% IA
Raw ejaculate	73.8 ± 2.3	81.7 ± 2.5	73.8 ± 2.3	81.7 ± 2.5	73.8 ± 2.3	81.7 ± 2.5
Immediate post-thaw	49.4 ± 2.9†	53.1 ± 2.7†	44.4 ± 2.1†	50.2 ± 1.9†	42.8 ± 2.1†	48.1 ± 1.9†
Post-dilution in HF10						
Pre-centrifugation	53.8 ± 2.2^{a}	37.7 ± 2.4†	51.3 ± 1.5^{†,a,b}	39.8 ± 1.7†	47.5 ± 1.5^{b}	37.4 ± 1.7†
Post-centrifugation	51.9 ± 3.8	33.1 ± 1.7^{c,†}	43.8 ± 2.7†	30.0 ± 1.2^{c,d,†}	44.4 ± 2.7	27.5 ± 1.2^{d,†}
1 h post-thaw	45.0 ± 3.2†	29.2 ± 1.7†	42.5 ± 2.3	27.1 ± 1.2	41.6 ± 2.3	24.1 ± 1.3†
2 h post-thaw	40.0 ± 2.8	30.3 ± 1.8	38.4 ± 2.0	26.8 ± 1.2	37.2 ± 2.0†	25.0 ± 1.2
3 h post-thaw	36.9 ± 2.4	27.2 ± 1.7	31.9 ± 1.7†	25.7 ± 1.2	34.7 ± 1.7	24.1 ± 1.2
4 h post-thaw	33.8 ± 2.7	29.4 ± 1.7	28.8 ± 1.9	26.4 ± 1.2	27.8 ± 1.9†	24.3 ± 1.2

Values represent least square means ± SEM ($n = 4$ males; $n = 8$ ejaculates).
[a,b] Within rows, % M values with different superscripts differ at $p < 0.05$.
[c,d] Within rows, % IA values with different superscripts differ at $p < 0.05$.
[†] Within treatment, values with different superscripts differ from the previous time at $p < 0.05$.

As expected, compared with raw ejaculates, % IA decreased ($p < 0.05$) in all treatments immediately after thaw (Table 3). Within each treatment, the % IA decreased ($p < 0.05$) compared to each previous time of assessment from immediately after thawing through post-centrifugation. The % IA did not decrease ($p > 0.05$) further for samples from the GE 15 min treatment. The % IA for samples from the control treatment and the GE 60 min treatment decreased ($p < 0.05$) again only between centrifugation and 1 h post-thaw (Table 3).

Study 3: Influence of temperature and method of glycerol addition

There was no effect of treatment ($p > 0.05$) on % M or % IA for any time-point of evaluation after samples were thawed (Table 4). There was no effect of treatment ($p > 0.05$) on FPS immediately after thawing in TYB up to 1 h post-thaw (data not shown). However, 2 h after thawing, samples from the 8% G ambient treatment had a decreased FPS value ($p < 0.05$; 2.6 ± 0.1) compared to controls (2.8 ± 0.1) or samples from the 4% G/straw cool treatment (2.8 ± 0.1).

The % M decreased ($p < 0.05$) in all treatments immediately after thaw compared with raw samples (Table 4). However, in the control and 8% G ambient treatments, there was an increase ($p < 0.05$) in % M upon dilution in HF10. Surprisingly, centrifugation did not affect ($p < 0.05$) sperm motility in any treatment. For each treatment, the % M decreased 10–12% from 1 h through 4 h post-thaw (Table 4).

As expected, the % IA decreased ($p < 0.05$) in all treatments immediately after thawing compared to raw ejaculates (Table 4). At each time of evaluation,

Table 4
Influence of temperature and method of glycerol (G) addition prior to cooling (ambient temperature; AT) on sperm motility (% M) and intact acrosomal membranes (% IA)

	Control							
	Slow G addition 5 °C tube cool in 0% G		Slow G addition AT straw cool in 4% G		Direct G addition AT straw cool in 4% G		Direct G addition AT tube cool in 4% G	
	% M	% IA	% M	% IA	% M	% IA	% M	% IA
Raw ejaculate	80.4 ± 1.7	88.4 ± 1.9	80.4 ± 1.7	88.4 ± 1.9	80.4 ± 1.7	88.4 ± 1.9	80.4 ± 1.7	88.4 ± 1.9
Immediately post-thaw	40.4 ± 2.1†	65.4 ± 1.6†	40.4 ± 2.1†	62.6 ± 1.6†	43.9 ± 2.2†	67.1 ± 1.7†	43.2 ± 2.1†	62.6 ± 1.6†
Post-dilution in HF10								
Pre-centrifugation	44.5 ± 2.1†	58.5 ± 1.8†	48.4 ± 2.1†	55.3 ± 1.9†	45.1 ± 2.2	55.7 ± 1.9†	44.5 ± 2.1	54.7 ± 1.8†
Post-centrifugation	45.6 ± 2.2	46.3 ± 1.8†	45.3 ± 2.2	44.4 ± 1.8†	45.7 ± 2.4	44.0 ± 1.9†	42.8 ± 2.3	41.7 ± 1.8†
1 h post-thaw	42.0 ± 2.0	39.8 ± 1.5†	38.8 ± 2.0†	39.4 ± 1.5†	39.4 ± 2.1	40.5 ± 1.6†	36.6 ± 2.0†	36.6 ± 1.5†
2 h post-thaw	36.5 ± 2.1†	39.1 ± 1.6	34.3 ± 2.1	37.0 ± 1.6	34.1 ± 2.2	37.5 ± 1.6	34.0 ± 2.1	33.6 ± 1.6†
3 h post-thaw	32.4 ± 2.1†	35.5 ± 1.3†	33.8 ± 2.1	35.9 ± 1.3	32.9 ± 2.2	38.0 ± 1.3	30.6 ± 2.1†	35.7 ± 1.3
4 h post-thaw	29.4 ± 2.1	38.5 ± 1.7†	28.3 ± 2.1†	37.1 ± 1.7	28.6 ± 2.2†	37.6 ± 1.8	24.4 ± 2.1†	34.0 ± 1.7

Values represent least squares means ± SEM (11 males, 15 ejaculates).
AT (Ambient temperature; 20–25 °C).
† Within treatment, values with different superscripts differ from the previous time at $p < 0.05$.

the % IA within each treatment decreased at an identical rate ($p < 0.05$) immediately after thawing up to 1 h post-thaw. The % IA did not decrease ($p > 0.05$) further for samples from the 8% G ambient or the 4% G/straw cool treatments. The % IA decreased ($p < 0.05$) between 2 and 3 h post-thaw and again between 3 and 4 h post-thaw for samples from the control treatment (Table 4). For samples from the 4% G/tube cool treatment, the % IA decreased ($p < 0.05$) again only between 1 and 2 h post-thaw (Table 4).

For all samples from each individual animal after thaw (all treatments combined at each time point), there was a positive correlation between the percent of spermatozoa with normal morphology in raw ejaculates and % IA in samples evaluated immediately post-thaw in TYB ($r = 0.47$, $p = 0.02$), upon resuspension in HF10 ($r = 0.48$, $p = 0.02$), after centrifugation ($r = 0.43$, $p = 0.04$) and 2 h post-thaw ($r = 0.43$, $p = 0.04$).

Discussion

This study represents the first systematic effort to characterize ejaculates from wild-born cheetahs in Namibia and to develop effective field-friendly sperm cryopreservation techniques. Although cheetahs are one of the most charismatic felids, little effort has been directed towards understanding the fundamental cryobiological properties of their spermatozoa. Interestingly, cheetahs represent a unique challenge as a typical ejaculate routinely contains >70% abnormally-shaped spermatozoa [30,38–40]. Nonetheless, cryopreservation of cheetah spermatozoa under various conditions of glycerol exposure in this study resulted in improved post-thaw recovery of both sperm acrosomal integrity and motility compared with values previously reported for cheetah spermatozoa [36]. To our knowledge, this represents the first systematic cryopreservation of spermatozoa from wild-born cheetahs living in Africa.

Although cheetah ejaculates were first characterized in the early 1980s [38,39], there has been no other effort to document ejaculate traits of wild-born animals. Interestingly, the ejaculate characteristics in the present study were comparable to previous reports on both captive cheetahs in North American zoos [37] and in East Africa [39]. Production of high proportions of abnormal spermatozoa has been linked to reduced genetic diversity in this species [25,26] as well in other felid groups including some lion populations [1] and one subspecies of puma, the Florida panther, which have low levels of genetic variation [31]. While the quality of spermatozoa from Namibian cheetahs appeared similar to that for other cheetah populations [37,39], there were some interesting locational differences, specifically in reference to higher sperm concentrations from animals at the CCF compared to other licensed farms. We suspect that this variation is due to improved nutritional status of cheetahs held at the CCF who receive daily mineral and vitamin supplements in addition to high-quality meat. It has been established that diet can influence seminal quality in wild felids held in captivity [35], and nutritional impact usually is on sperm numbers rather than sperm morphology.

While developing convenient methodology for use under field conditions, it was expected that prolonged exposure of cheetah spermatozoa to glycerol at ambient temperature would markedly reduce sperm motility and acrosomal integrity post-thawing. Survival of mouse spermatozoa exposed to 0.8 M glycerol at ambient temperature is doubled if the length of exposure is reduced from 20 to 1–5 min [16]. Interestingly, there was little detriment to cheetah sperm motility or acrosomal integrity post-thaw resulting from glycerol exposure (0.4 M) at ambient temperature for up to 60 min. Permeability of glycerol at ambient temperature is relatively high [16], and it may be that damage induced by glycerol entering sperm cells at ambient temperature is only slightly confounded by prolonged exposure. Regardless, this unexpected result was favorable for field-friendly cheetah sperm cryopreservation systems where samples may need to be maintained at ambient temperature in glycerol for extended time periods.

During addition of permeating cryoprotectants such as glycerol, cells undergo dehydration causing shrinkage as water leaves the cells, followed by a re-establishment of cell volume as both water and cryoprotectant enter the cells [8,16]. This movement of glycerol and water across sperm membranes creates osmotic stress resulting in membrane damage [7], which may be reduced by adding glycerol in a stepwise manner [16]. An optimal cryoprotectant is one that will permeate cell membranes in the shortest time causing the least amount of volume excursion during both its addition and removal [8]. Interestingly, there were no differences between addition of glycerol in a single aliquot or in multiple steps on cheetah spermatozoa post-thaw motility or acrosomal integrity. Under field conditions usually encountered in Namibia, addition of glycerol at ambient temperature before initiation of the cooling process prevents the changes in sample temperature that occur when adding glycerol to spermatozoa after the sample has reached 5 °C. These temperature changes result primarily from limited refrigeration facilities, thereby requiring that samples be removed from the refrigerated environment for stepwise glycerol addition and briefly be exposed to the ambient environment. Addition of glycerol at ambient temperature before the cooling process prevents such temperature changes and, therefore, also prevents possible damage to the acrosomal membranes induced by temperature flux while creating a more technically consistent field methodology.

Interestingly, upon combining post-thaw values for all treatments in the present study, the proportion of spermatozoa with intact acrosomes decreased from 50–65% immediately post-thawing to 25–40% by 1 h after glycerol removal. This is in comparison with minimal loss of motility over the same time period (40–49% and 37–45%, respectively). This suggests that, compared to sperm motility, cheetah sperm acrosomes were more susceptible to cryopreservation-induced damage. Based on the decline in acrosomal integrity following dilution of the cryoprotectant and centrifugation, it appeared that cheetah spermatozoa were susceptible to both osmotic damage and centrifugal forces. It has been previously reported that the proportion of structurally normal spermatozoa in a raw ejaculate may be directly related to overall sperm function due to a negative association between abnormal sperm morphology and acrosomal function [6]. Indeed, there was a strong positive correlation between the percentage of normal spermatozoa and the proportion of these cells with intact acrosomal membranes in raw ejaculates. This relationship carried over post-thaw in that there was a strong relationship between the percentage of spermatozoa with intact acrosomes at four time points post-thaw (immediately post-thaw, before and after centrifugation and 2 h after thawing) with the proportion of normal spermatozoa in the raw ejaculate.

Methods for cheetah sperm cryopreservation, such as those developed in the present study, have future applications in assisted breeding programs. Reproduction in captive cheetahs is so inconsistent that ex situ populations are not self-sustaining [18]. Recently, managers have pursued the use of techniques such as artificial insemination using frozen-thawed spermatozoa for propagation of animals. Cheetah cubs have been produced in the past using cryopreserved spermatozoa for artificial insemination [14,42]. Sperm samples used to produce those litters yielded post-thaw values of ~45% sperm motility and ~40% intact acrosomal membranes [14]. Interestingly, the new field-friendly methods developed in the present study match or exceed the values for sperm motility (40–50%) and intact acrosomes (50–60%) of cryopreserved spermatozoa proven to be biologically viable.

In conclusion, we confirmed that wild-born cheetahs living in captivity in Namibia consistently produced high proportions of pleiomorphic spermatozoa, similar to that observed for cheetahs in North American zoos and one free-living population in

East Africa. Although all raw ejaculates contained high levels of malformed spermatozoa, intact acrosomes predominated, exceeding 86% of all cells. Furthermore, even when exposed to glycerol at ambient temperature for up to 60 min followed by cryopreservation, ~50–60% of thawed cheetah spermatozoa retained acrosomal integrity and ~40–50% of spermatozoa retained motility. However, compared to sperm motility, cheetah sperm acrosomes appeared more susceptible to cryopreservation-induced damage with 25–40% remaining intact by 1 h after glycerol removal. Nonetheless, these post-thaw values were comparable to that of inseminates that have been used in this species to produce offspring [14,42]. Therefore, we assert that the knowledge gained in this study was valuable for developing new field-friendly protocols for the cryopreservation of cheetah spermatozoa, samples that can be added to the growing genome resource bank for this species [42] and used for global genetic management.

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