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Ejaculate traits in the Namibian cheetah (*Acinonyx jubatus*): influence of age, season and captivity

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Abstract. The objective was to examine the influence of animal age, season and captivity status on seminal quality in wild-born cheetahs (*Acinonyx jubatus*) in Namibia, Africa. Animals were divided into three age categories: juvenile (14–24 months; $n = 16$ males, 23 ejaculates); adult (25–120 months; $n = 76$ males, 172 ejaculates); and aged (>120 months; $n = 5$ males, 5 ejaculates). Seasons were categorised into hot–wet (January–April), cold–dry (May–August) and hot–dry (September–December). A comparison between freshly wild-caught ($n = 29$ males, 41 ejaculates) and captive-held cheetahs ($n = 68$ males, 159 ejaculates) was also conducted. Raw ejaculates contained $69.0 \pm 1.1\%$ motile spermatozoa (mean ± s.e.m.) with $73.6 \pm 1.5\%$ of these cells containing an intact acrosome. Overall, $18.4 \pm 0.9\%$ of spermatozoa were morphologically normal, with midpiece anomalies being the most prevalent (~39%) defect. Juvenile cheetahs produced ejaculates with poorer sperm motility, forward progressive status, lower seminal volume and fewer total motile spermatozoa than adult and aged animals. Spermatogenesis continued unabated throughout the year and was minimally influenced by season. Proportions of sperm malformations were also not affected by season. Ejaculates from captive cheetahs had increased volume and intact acrosomes, but lower sperm density than wild-caught counterparts. In summary, Namibian cheetahs produce an extraordinarily high proportion of pleiomorphic spermatozoa regardless of age, season or living (captive versus free-ranging) status. Young males less than 2 years of age produce poorer ejaculate quality than adult and aged males. Because (1) all study animals were wild born and (2) there was little difference between freshly caught males and those maintained in captivity for protracted periods, our results affirm that teratospermia in the cheetah is mostly genetically derived. It also appears that an *ex situ* environment for the Namibian cheetah can ensure sperm quality comparable with that for free-living males.

Additional keywords: Africa, carnivore, felid, seasonality, semen, spermatogenesis.

Introduction

Although Namibia has the world's largest free-living cheetah population (~3000–5500 individuals) (Marker-Kraus and Kraus 1995; Marker-Kraus *et al.* 1996; Hanssen and Stander 2003), the species remains threatened (CITES 2004) as a result of human–carnivore conflicts and substantial habitat loss (Marker-Kraus and Kraus 1995; Marker-Kraus *et al.* 1996). The result has been reduced cheetah home-range size and population fragmentation (Marker-Kraus *et al.* 1996). The worldwide *ex situ* population of cheetahs is composed of ~1350 individuals (Marker 2002), which generally reproduce poorly in captivity (Lindburg *et al.* 1993; Marker-Kraus and Grisham 1993). In contrast, cheetahs living in the Serengeti ecosystem appear to reproduce efficiently in the absence of human perturbations (Caro 1994). Most reproductive failure in captivity has been attributed to suboptimal management (Lindburg *et al.* 1993; Wielebnowski *et al.* 2002), especially given previous observations of similar health, physiological and genetic traits between breeding and non-breeding individuals (Wildt *et al.* 1993). Because only ~18% of all adult cheetahs in North American zoos have ever reproduced (Marker 2002), and owing to high cub mortality (Marker 2002), the worldwide *ex situ* population is not self-sustaining (Marker and O'Brien 1989; Marker 2002). Therefore, zoos traditionally have relied on importing cheetahs from range countries, usually Namibia (Marker-Kraus and Kraus 1995), to bolster their collections.

Most of what we know about the reproductive physiology of the male cheetah has been derived from studying animals living in one large captive facility in South Africa (Wildt *et al.* 1983), many North American zoos (Donoghue *et al.* 1992; Wildt *et al.* 1993; Roth *et al.* 1995; Swanson *et al.* 1996*b*) and one field study of eight wild-caught cheetahs in the Serengeti ecosystem (Wildt *et al.* 1987*b*). From these investigations, it is well established that the cheetah consistently produces high proportions of structurally malformed spermatozoa, usually ~80% of all ejaculated cells (Wildt *et al.* 1983, 1987*b*, 1993; Crosier *et al.* 2006).

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This discovery of teratospermia (which also occurs in certain other felids) has led to detailed investigations into the impact of malformed spermatozoa on reproductive fitness. In brief, we know that deformed felid spermatozoa fail to participate in fertilisation, are biologically compromised at multiple cellular and subcellular levels and that even structurally normal spermatozoa from teratospermic males can be functionally inadequate (see reviews Pukazhenthi *et al.* 2001, 2006*a*).

We have only rudimentary information on what regulates spermatozoa form and function in the cheetah. There is evidence that many unique biological traits, especially teratospermia, can be at least partially attributed to the well-known lack of genetic diversity in the species (O'Brien *et al.* 1983, 1985, 1987). Likewise, extreme teratospermia has been observed in the genetically depauperate Florida panther (Roelke *et al.* 1993) and Asiatic lion (Wildt *et al.* 1987*a*). Nonetheless, there remains a need to examine other potential contributing factors to teratospermia, including age, season and living status. For example, ejaculate quality improves from puberty to adulthood and then decreases during later life for many common domesticated species (Mathevon *et al.* 1998; Eskenazi *et al.* 2003). But, there is a paucity of information concerning the influence of animal age on spermatogenic function in carnivores, with data on circannual influences being only slightly better. Seminal quality does vary throughout the year in the snow leopard (*Uncia uncia*; Johnston *et al.* 1994) and Pallas' cat (*Otocolobus manul*; Swanson *et al.* 1996*a*), with peaks in sperm concentration and structurally normal spermatozoa coinciding with an established breeding season. In contrast, no such seasonal variation exists in the male domestic cat (*Felis catus*; Spindler and Wildt 1999), Siberian tiger (*Panthera tigris*; Byers *et al.* 1990) or clouded leopard (*Neofelis nebulosa*; Wildt *et al.* 1986), despite seasonality in females of these same species (Robinson and Cox 1970; Seal *et al.* 1985; Brown *et al.* 2001). Approximately 70% of cheetah cubs are born on Namibian farmlands from March through July (Marker *et al.* 2003*a*), and given a gestation of 94 days (Brown *et al.* 1996), we can extrapolate to a breeding season of December through April for this geographic region. In terms of impact of a captive environment, zoo-maintained cheetahs produce spermatozoa throughout the year (Wildt *et al.* 1993; Durrant *et al.* 2001). Interestingly, Wildt and colleagues found similar seminal quality for cheetahs in North American zoos, in one captive facility in South Africa as well as from a small cohort of free-living males in Tanzania (Wildt *et al.* 1983, 1987*b*, 1993).

The present study took advantage of the significant cheetah numbers available at the Cheetah Conservation Fund (CCF) in Namibia. Here, staff have access to cheetahs that are captured intentionally on surrounding farmlands because they are considered a threat to livestock. As result of injuries or because animals arrived at the facility as orphans unable to survive on their own, some of these individuals cannot be released and, therefore, must be maintained in captivity. Our overall aim was to establish a reproductive physiology database on male cheetahs of Namibia, comparing males that had been recently captured (free ranging) with those that had been maintained at CCF for a protracted interval. The substantial numbers of animals available over time provided a unique opportunity to examine the impact of age, seasonality as well as free-living versus captivity status on ejaculate quality.

Materials and methods

Animals

Ejaculates ($n = 97$ males, $n = 200$ ejaculates) were collected over 10 years, from 1994 to 2003. Cheetahs considered 'wild caught' were those trapped on farmland using previously described procedures (Marker and Dickman 2003) less than 30 days before spermatozoa collection (29 males, 41 ejaculates). The cohort considered 'captive' were those cheetahs that had been held for more than 30 days in outdoor enclosures at CCF (Otjiwarongo; 20.33′S 16.37′E) or at other licensed captive facilities within Namibia. The size of enclosures varied from $\sim 10 \times 10$ m to $\sim 100 \times 100$ m per cheetah. These 68 captive males (159 ejaculates) were 14 months to 17 years old, and all were healthy. Cheetah age was estimated by a thorough examination of tooth wear (Marker and Dickman 2003). At CCF, animals were fed a combination of donkey, horse and game species (2–3 kg of meat on 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., Isando, South Africa). Animals also received organ meat (a mix of heart, liver and/or lung) at least once per week. Cheetahs housed at other captive facilities received a mixture of donkey, horse, cattle and game species (amount and frequency were variable), and calcium and mineral supplements were provided, but at an unknown frequency. For the wild-caught group, electroejaculation was part of a complete biomedical examination that occurred at the site of capture or after animals were translocated to CCF. Whenever appropriate for the animal's well being, these cheetahs were released into the wild after recovery from anaesthesia. All animal procedures were approved by the National Zoological Park's Institutional Animal Care and Use Committee (IACUC).

Anaesthesia, testicular volume, sperm collection and evaluation

Electroejaculation was performed under a surgical plane of anaesthesia induced by an intramuscular injection of tiletamine hydrochloride plus zolazepam (Telazol, Fort Dodge Animal Health, Fort Dodge Laboratories, Fort Dodge, IA or Zoletil, Virbac Laboratories, Carros, France; 4–6 mg kg^{-1}) or a combination of Telazol (2 mg kg^{-1}) or ketamine hydrochloride (10 mg kg^{-1}, Ketaset, Fort Dodge Laboratories) and medetomidine hydrochloride (2 μg kg^{-1}; Domitor, Pfizer, La Jolla, CA, USA). Drugs were administered via an air-pressured darting system while animals were in a holding pen or via a hand syringe after the animals were transferred into a capture box with squeeze mechanism (Wildt *et al.* 1993; Roth *et al.* 1995). If necessary, supplemental ketamine hydrochloride (50–100 mg, intravenous) or Telazol (50–100 mg, intramuscular or intravenous) was given to maintain a desired level of anaesthesia. Once tractable, each animal was weighed using a portable scale, and testicular dimensions were measured using calipers, with the results converted to volume (Howard *et al.* 1990). For electroejaculation, a rectal probe of 1.6 or 1.9-cm diameter was lubricated with KY-jelly

(Johnson & Johnson, Arlington, TX, USA) and inserted into the rectum. Semen was collected in pre-warmed, sterile collection vials using a consistent protocol involving three separate series of increasing voltage (2–5 V) delivered by an electrostimulator (P. T. Electronics, Boring, OR, USA) over 30 min (Wildt *et al.* 1983, 1993; Howard 1993). This standardised semen collection protocol allowed accurate comparisons across groups and time.

A 3-μL aliquot of raw semen from each series was evaluated immediately by phase contrast microscopy (Olympus BX41TF plus warming stage, Olympus Optical Co., Melville, NY, USA) for sperm percentage motility and forward progressive status, a subjective estimate of speed of forward movement (FPS; scale of 0–5, with 0 = no forward movement and 5 = rapid, straightforward motility; Wildt *et al.* 1987*b*; Howard 1993). Sperm acrosomal integrity was assessed using either a modified Coomassie blue staining technique (Larson and Miller 1999) or a rose bengal/fast green stain (Pope *et al.* 1991). For the Coomassie staining, a 20–30-μL aliquot of raw ejaculate was fixed in 500 μL of 4% paraformaldehyde and stored at 4°C until evaluation (as described below). For evaluating sperm morphology, a 20–30-μL sample of raw ejaculate was fixed in 100 μL of 0.3% glutaraldehyde in phosphate-buffered saline (PBS; pH = 7.4; 340 mOsm) and then assessed (see below).

Ejaculate volume was measured from each series and diluted immediately with an equal volume of sterile pre-warmed Ham's F10 culture medium (HF10; Irvine Scientific, Santa Ana, CA, USA) supplemented with 20 mM HEPES, 5% (v : v) fetal calf serum (Irvine Scientific), pyruvate (1 mM), L-glutamine (2 mM); 10 000 IU mL^{-1} penicillin, 10 mg mL^{-1} streptomycin and 20 mg mL^{-1} neomycin (Sigma Chemical Co., St Louis, MO, USA). Upon completion of electroejaculation, samples of comparable sperm percentage motility and FPS were combined. A 10-μL aliquot of the combined diluted sample was placed into a commercial red blood cell counting chamber (Unopette, Becton Dickinson and Co., Franklin Lakes, NJ, USA), and total spermatozoa number within the sample was determined using a haemocytometer (Wildt *et al.* 1983; Howard 1993).

Evaluation of sperm acrosomal integrity

For analysis using a modified Coomassie blue technique, fixed spermatozoa samples were centrifuged (Eppendorf Mini Spin, Eppendorf AG, Hamburg, Germany) for 8 min at 2000*g*, and the supernatant was discarded. Sperm pellets were washed twice with 500 μL of 0.1 M ammonium acetate (pH 9.0), and the pellet was resuspended in ~50 μL of the final wash volume. This suspension was smeared onto a microscope slide and allowed to dry at ambient temperature. Each slide then was flooded with Coomassie stain (Fisher Biotech, Springfield, NJ, USA; 0.220 g Coomassie Blue G-250 in a solution (100 mL final) consisting of 50 mL methanol, 10 mL glacial acetic acid and 40 mL deionised water; Larson and Miller 1999) for 90 s, thoroughly rinsed with deionised water, dried (ambient temperature) and permanently preserved by placing a coverslip over a drop of mounting medium (Krystalon, EM Science, Gibbstown, NJ, USA). Sperm acrosomes were evaluated as intact or non-intact/damaged (Crosier *et al.* 2006). A spermatozoon with an intact acrosomal membrane exhibited a uniform purple staining overlying the acrosomal region. A cell with a non-intact acrosome expressed a clear or patchy staining pattern. From each sample, 200 spermatozoa were assessed individually for acrosomal integrity using brightfield microscopy (1000×).

For evaluating acrosomal integrity using a rose bengal/fast green stain (Pope *et al.* 1991), spermatozoa diluted in HF10 were mixed with the staining solution for 90 s. Briefly, this solution consisted of 1 g (w : v) rose bengal stain (Sigma Chemical Co.) and 1 g (w : v) fast green stain (Sigma Chemical Co.) added to 100 mL buffer (Pope *et al.* 1991; buffer formulated as 60 mL solution consisting of 0.2 M disodium phosphate and 0.1 M citric acid in cell culture water and 40 mL ethanol). After staining, the sperm suspension was smeared onto a glass slide and allowed to air dry, and spermatozoa were examined by bright microscopy (1000×). Spermatozoa with an intact acrosomal membrane appeared blue. Damaged acrosomes were evident by mottled or patchy staining or a visibly loose membrane, whereas spermatozoa with a missing acrosome had a white or pink cap, with no blue staining evident (Pope *et al.* 1991).

Evaluation of sperm morphology

For evaluation of structural morphology, 100 spermatozoa per sample were assessed using phase contrast microscopy (1000×) (Wildt *et al.* 1987*b*; Howard 1993). Spermatozoa were classified as normal or as having one of the following malformations: (1) acrosomal abnormalities that included a missing or damaged acrosomal membrane; (2) head anomalies that included microcephalic, macrocephalic or bi/tri/tetra-cephalic deformities; (3) midpiece abnormalities that involved a bent midpiece with retained cytoplasmic droplet, bent midpiece with no droplet, abnormal midpiece or midpiece aplasia; (4) flagellar defects that included a tightly coiled flagellum, bent flagellum with retained cytoplasmic droplet, bent flagellum with no droplet, bi/tri-flagellate formation, retained proximal droplet or retained distal droplet; and (5) other malformations that involved a spermatid, bent neck, detached head or detached flagellum.

Statistical analysis

To evaluate the influence of animal age on seminal and sperm characteristics, males were divided into three groups: (1) 14–24 months (juvenile, $n = 16$ males, 23 ejaculates); (2) 25–120 months (adult, prime breeding age, $n = 76$ males, 172 ejaculates); and (3) >120 months (aged, $n = 5$ males, 5 ejaculates) (Wildt *et al.* 1993). To examine the impact of season on male traits, time was divided into three categories that best represent the circannual Namibian climate: (1) hot–wet (January–April); (2) cold–dry (May–August); and (3) hot–dry (September–December) (Berry and Louw 1982; Marker *et al.* 2003*a*). To examine the effect of age and season on sperm morphology, data were combined into subgroups as defined earlier: (1) normal spermatozoa; (2) acrosomal defects; (3) head anomalies; (4) midpiece deformities; (5) flagellar malformations; or (6) other defects, including cells that were spermatids or those with a bent neck or detached head or flagellum.

Because all aged animals (>120 months, $n = 5$) were living long-term in captivity, it was not possible to examine the impact of recently wild-caught versus captive status on this age group. Additionally, owing to small group size and because semen collections from animals of the different age groups were not distributed evenly across season, it was not possible to explore for an interaction between season and animal age. There was no interaction ($P > 0.05$) of season and captive status on any ejaculate trait. Therefore, the final statistical model for raw seminal analysis (including sperm morphology) included the main effects of animal age, season and wild-caught versus captive status. Percentage data were subjected to arcsine transformation before analysis. Due to the unbalanced number of observations in main effects categories, all data were evaluated using general linear model analysis-of-variance procedures (SAS 1999). The type III sums of squares estimable function of parameters was used for testing effects and means were considered significantly different at $P < 0.05$. Means were separated using Duncan's multiple-range test, and values are presented as least-squares means ± s.e.m. unless otherwise noted.

Results

Overall ejaculate characteristics and sperm morphology

Of the 97 cheetahs, three (3.1%) were unilaterally cryptorchid, and five males (5.2%) and six of 200 ejaculates (3.0%) were aspermic (i.e. the ejaculates did not contain any spermatozoa). Otherwise, combined testes volume and all seminal traits were similar to those previously described for cheetahs living in a large *ex situ* facility in South Africa (Wildt *et al.* 1983) as well as many North American zoos (Table 1; Wildt *et al.* 1993). This included the unusual finding of a high proportion (~82%) of pleiomorphic spermatozoa per ejaculate.

Because CCF consistently provided a high-quality dietary vitamin and mineral supplement, ejaculate traits at this location were compared to those animals maintained at all other captive facilities where such supplements were inconsistently fed. Cheetahs at CCF produced more ($P < 0.05$) spermatozoa with intact acrosomal membranes (85.7 ± 3.2%) than cheetahs held at counterpart facilities (75.0 ± 4.2%). However, because there were no other differences ($P > 0.05$) in any other ejaculate trait across captive facilities, all remaining data for *ex situ* maintained cheetahs were combined for subsequent analyses.

Table 1. Ejaculate characteristics of wild-born Namibian cheetahs versus captive counterparts in North American zoos

Namibian: $n = 97$ males, $n = 200$ ejaculates. North American: $n = 60$ males, $n = 60$ ejaculates (Wildt *et al.* 1993). Means ± s.e.m. ND, not determined

Trait	Namibian	North American
Total testicular volume (cm^3)	10.2 ± 0.3	13.9 ± 0.4
Seminal volume (mL)	2.1 ± 0.1	1.5 ± 0.1
Sperm motility (%)	69.0 ± 1.1	67.0 ± 2.0
Sperm forward progressive status[A]	3.3 ± 0.1	3.6 ± 0.1
Sperm concentration mL^{-1} ($\times 10^6$)	21.9 ± 1.7	29.3 ± 5.6
Total motile spermatozoa per ejaculate ($\times 10^6$)	36.2 ± 3.2	31.4 ± 5.6
Intact acrosomes (%)	73.9 ± 1.4	ND
Morphologically abnormal spermatozoa (%)	81.6 ± 0.8	78.7 ± 2.0

[A]Scale 0–5, with 5 being the best forward progressive status.

Cheetah sperm morphotypes are depicted in Fig. 1, and the incidences of normal and specific sperm defects are summarised in Table 2. Only ~18% of all spermatozoa per ejaculate were considered to be morphologically normal. Together, spermatozoa with either an abnormal acrosome (Fig. 1*b*) or microcephaly (Fig. 1*c*) comprised ~18% of all anomalies observed (Table 2). Malformations of the sperm midpiece (Fig. 1*f–h*) occurred in the highest frequency, making up ~39% of all deformities in cell shape. Interestingly, spermatozoa with a tightly coiled flagellum (Fig. 1*i*) or at the spermatid stage (Fig. 1*o*) also occurred in a relatively high frequency, each comprising ~7% of total pleiomorphisms observed.

Influence of cheetah age on testes volume and ejaculate traits

Males 25–120 months and >120 months were heavier ($P < 0.05$) than animals 14–24 months (i.e. juveniles), although there was no difference ($P < 0.05$) among age groups in total testicular volume (Table 3). Of the five males that were aged as 14 months, two were aspermic, but three were spermic, producing ejaculates that contained spermatozoa. Compared with the adult and aged groups, the juvenile cats as a group produced less ($P < 0.05$) seminal volume and had lower ($P < 0.05$) motility and FPS scores (Table 3).

Sperm concentration (number mL^{-1}) was almost 2-fold higher in adults compared with juveniles, but this difference was non-significant ($P > 0.05$), probably due to male variance. Because of the greater seminal volume and sperm motility and the tendency for higher sperm concentration, cheetahs in the adult category produced more ($P < 0.05$) total motile spermatozoa per ejaculate than juvenile counterparts. There was no influence ($P > 0.05$) of animal age on percentage of spermatozoa with intact acrosomal membranes or midpiece, flagellar or other structural abnormalities (Table 3). Interestingly, aged animals ejaculated the highest ($P < 0.05$) proportion of normally shaped spermatozoa per ejaculate (~18% more than juveniles and ~14% more than adults). This was largely expressed as more head defects in the juveniles and adults, specifically an increased ($P < 0.05$) incidence of macrocephaly, microcephaly and bi/tri/tetra-cephaly.

Influence of season on ejaculate characteristics

Testicular volume was greater ($P < 0.05$) in free-ranging males during the breeding season (hot–wet, January–April) in Namibia (Fig. 2*a*) than in the cold–dry (May–August) and hot–dry (September–December) seasons. For free-ranging males, testicular volume decreased ($P < 0.05$) by approximately half between the hot–wet (14.1 ± 1.1 cm^3) and the cold–dry seasons (7.9 ± 1.0 cm^3) and remained low for the hot–dry season (8.1 ± 0.9 cm^3) (Fig. 2*a*). In contrast, males that had been maintained long-term in captivity experienced no change ($P > 0.05$) in testes volume between hot–wet (11.1 ± 0.6 cm^3) and cold–dry (10.3 ± 0.7 cm^3) seasons, but did exhibit a decrease ($P < 0.05$)

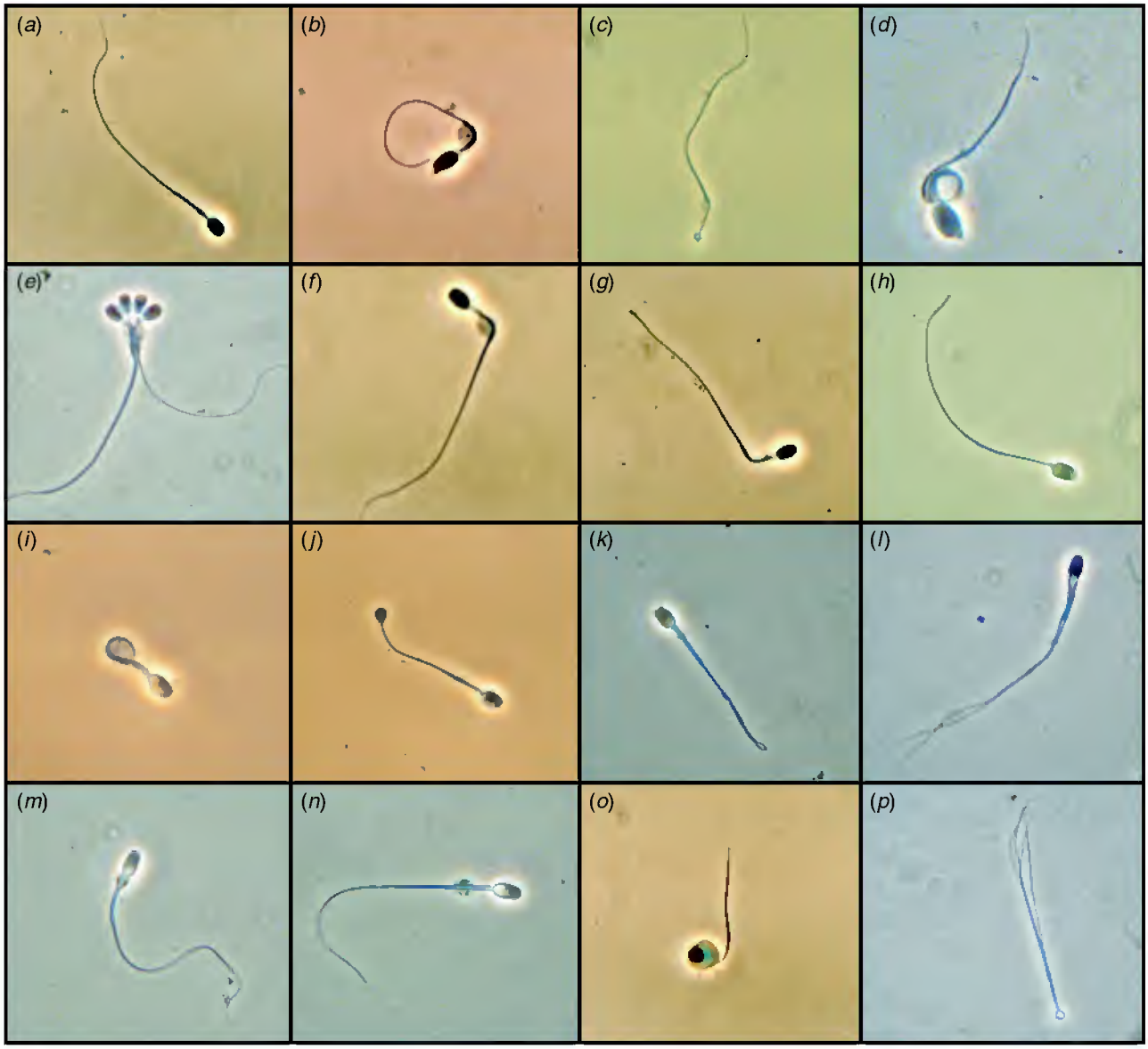

Fig. 1. Morphotypes of cheetah spermatozoa (1000×). (*a*) Spermatozoon with normal morphological structure. (*b–p*) Pleiomorphic cell forms: (*b*) A spermatozoon with an abnormal acrosome. Deformity of the sperm head, including (*c*) microcephaly, (*d*) macrocephaly and (*e*) bi/tri/tetra-cephaly. Midpiece malformations, including (*f*) a bent midpiece with retained cytoplasmic droplet, (*g*) bent midpiece with no droplet, (*h*) midpiece aplasia. Anomalies of the flagellum, including (*i*) a tightly coiled flagellum, (*j*) bent flagellum with retained cytoplasmic droplet, (*k*) bent flagellum with no droplet, (*l*) bi/tri-flagellate, (*m*) retained proximal cytoplasmic droplet and (*n*) retained distal cytoplasmic droplet. (*o*) A spermatid. Other severe developmental abnormalities also were observed, for example, (*p*) a microcephalic tetra-flagellate spermatozoon.

in total testes volume between the cold–dry and hot–dry seasons ($8.0 \pm 0.7\,cm^3$) (Fig. 2*b*). There were minimal effects of time of year on seminal traits, with the only impact being measured on sperm motility and acrosomal integrity. The cold–dry interval favoured increased ($P < 0.05$) sperm motility (over the hot–dry period) and more intact acrosomes (Fig. 3*b*, *f* respectively). In terms of specific sperm structure, there was a tendency for more normal cells to be ejaculated in the hot–wet versus the hot–dry season, although this difference was non-significant ($P = 0.06$; Fig. 4*a*). There were more ($P < 0.05$) head and 'other' (i.e. cells that were spermatids or had a bent neck, detached head or flagellum) anomalies measured in the hot–dry period (Fig. 4*c*, *f* respectively) and more ($P < 0.05$) midpiece malformations during the cold–dry season compared with counterpart times (Fig. 4*d*). Acrosomal (Fig. 4*b*) and flagellar (Fig. 4*e*) pleiomorphisms did not vary with season.

Influence of wild-caught versus captive status on testes volume and ejaculate traits

There was no difference ($P > 0.05$) between wild-caught and captive-held animals in average age, body weight, total testicular volume or sperm motility characteristics (Table 4). Seminal volume in captive-held cheetahs was greater ($P < 0.05$) overall, although the wild-caught animals consistently produced higher ($P < 0.05$) sperm concentrations. The overall result was a similar ($P > 0.05$) number of total motile spermatozoa per ejaculate across groups (Table 4). Although overall percentages of cellular pleiomorphisms were not different ($P > 0.05$), there was an average of 8% fewer ($P < 0.05$) intact acrosomes and ~3% fewer ($P < 0.05$) acrosomal deformities in the recently wild-caught compared with captive-maintained individuals (Table 4). There were no differences ($P > 0.5$) in malformations associated with the head, flagellum or 'other' categories, although there were more ($P < 0.05$) midpiece pleiomorphisms in the free-ranging animals.

Table 2. Incidence of structurally normal and abnormal spermatozoa in ejaculates from wild-born Namibian cheetahs
$n = 90$ males, 177 ejaculates

Trait	Mean ± s.e.m.
Morphologically normal spermatozoa (%)	18.4 ± 0.8
Morphologically abnormal spermatozoa (%)	81.6 ± 0.8
Abnormal acrosome	9.8 ± 0.5
Microcephalic	8.3 ± 0.6
Macrocephalic	0.8 ± 0.1
Bi/tri/tetra-cephalic	0.4 ± 0.1
Bent midpiece with droplet	30.3 ± 0.9
Bent midpiece without droplet	4.5 ± 0.3
Midpiece aplasia	0.7 ± 0.1
Abnormal midpiece	3.3 ± 0.3
Tightly coiled flagellum	7.5 ± 0.6
Bent flagellum with droplet	1.6 ± 0.2
Bent flagellum without droplet	1.8 ± 0.2
Bi/tri-flagellate	0.3 ± 0.1
Proximal cytoplasmic droplet	3.6 ± 0.3
Distal cytoplasmic droplet	1.0 ± 0.1
Spermatid	6.9 ± 0.6
Bent neck	0.2 ± 0.1
Detached head and/or flagellum	0.6 ± 0.1

Discussion

From an ecological, genetic and physiological perspective, the cheetah is one of the world's most thoroughly studied wildlife species (Caro 1994; Wildt *et al.* 2001; Marker and Dickman 2003; Marker *et al.* 2003*a*, 2003*b*, 2003*c*). The cheetah's uniqueness also has been frequently cited, especially a well known lack of gene diversity and subsequent influence on health and reproductive fitness (O'Brien *et al.* 1983, 1985, 1987). The species continues to be highly popular for zoo exhibition, which is a challenge because of the non-self-sustainability of captive populations (Marker 2002). As a result, there has been a call in North America to establish regional breeding centers (Grisham *et al.* 2005) as well as reinvigorate past multidisciplinary research studies (Wildt and Grisham 1993).

The cheetah does deserve more basic research attention because of its interesting inherent traits and remaining biological mysteries, including the question – what drives testicular function and seminal quality, including the odd but consistent finding of teratospermia? Since its detection more than 20 years ago

Table 3. Influence of cheetah age on testes volume and ejaculate traits
Least-squares means ± s.e.m.

Variable	Juvenile (14–24 months)	Adult (25–120 months)	Age (>120 months)
Number of males	16	76	5
Number of ejaculates	23	172	5
Bodyweight (kg)	37.7 ± 1.3[a]	43.7 ± 0.5[b]	44.0 ± 2.4[b]
Total testicular volume (cm^3)	8.7 ± 0.7	10.0 ± 0.3	10.2 ± 1.6
Seminal volume (mL)	0.7 ± 0.3[a]	2.2 ± 0.1[b]	2.3 ± 0.6[b]
Sperm motility (%)	56.8 ± 3.3[a]	69.9 ± 1.4[b]	79.2 ± 6.7[b]
Forward progressive status[A]	2.9 ± 0.1[a]	3.4 ± 0.1[b]	3.7 ± 0.3[b]
Sperm concentration mL^{-1} ($\times 10^6$)	12.7 ± 4.9	25.1 ± 2.2	19.3 ± 10.6
Total motile spermatozoa per ejaculate ($\times 10^6$)	7.1 ± 9.3[a]	42.2 ± 4.2[b]	23.5 ± 20.2[a,b]
Intact acrosome (%)	67.6 ± 4.9	73.0 ± 1.8	87.6 ± 9.4
Morphologically normal spermatozoa (%)	14.3 ± 2.3[a]	18.1 ± 1.1[a]	32.6 ± 5.2[b]
Morphologically abnormal spermatozoa (%)			
Acrosomal defects	10.2 ± 1.4	9.1 ± 0.6	10.6 ± 3.6
Head defects	13.4 ± 1.5[a]	9.9 ± 0.7[a,b]	3.9 ± 3.9[b]
Midpiece defects	41.2 ± 2.4	45.1 ± 1.1	40.6 ± 6.2
Flagellar defects	11.8 ± 1.9	10.7 ± 0.8	7.7 ± 4.8
Other defects[B]	9.7 ± 1.4	7.0 ± 0.6	4.4 ± 3.6

[A]Scale 0–5, with 5 being the best forward progressive status. [B]Other abnormalities include a spermatid, bent neck, detached head or detached flagellum. [a,b]Within rows, values with different superscripts differ at $P < 0.05$.

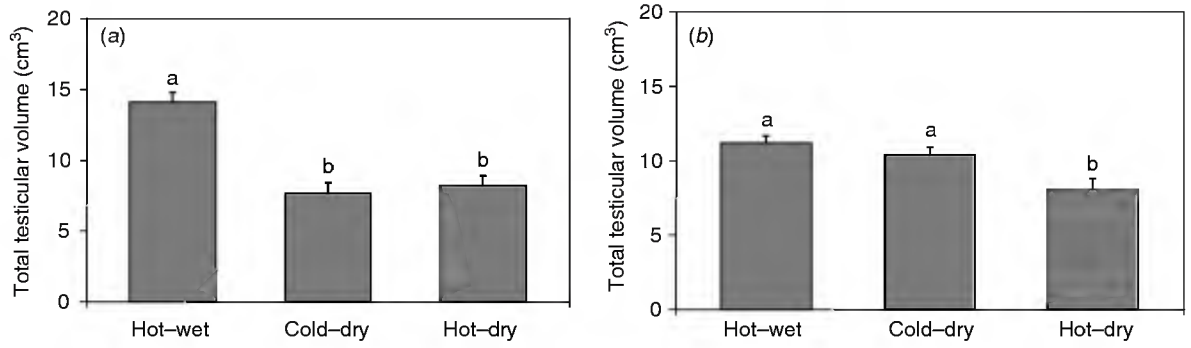

Fig. 2. Influence of season (hot–wet, January–April; cold–dry, May–August; and hot–dry, September–December) on total testicular volume for (*a*) wild-caught versus (*b*) captive-held male cheetahs. All values are least-squares means ± s.e.m. Within each animal group, values with different superscripts differ ($P < 0.05$).

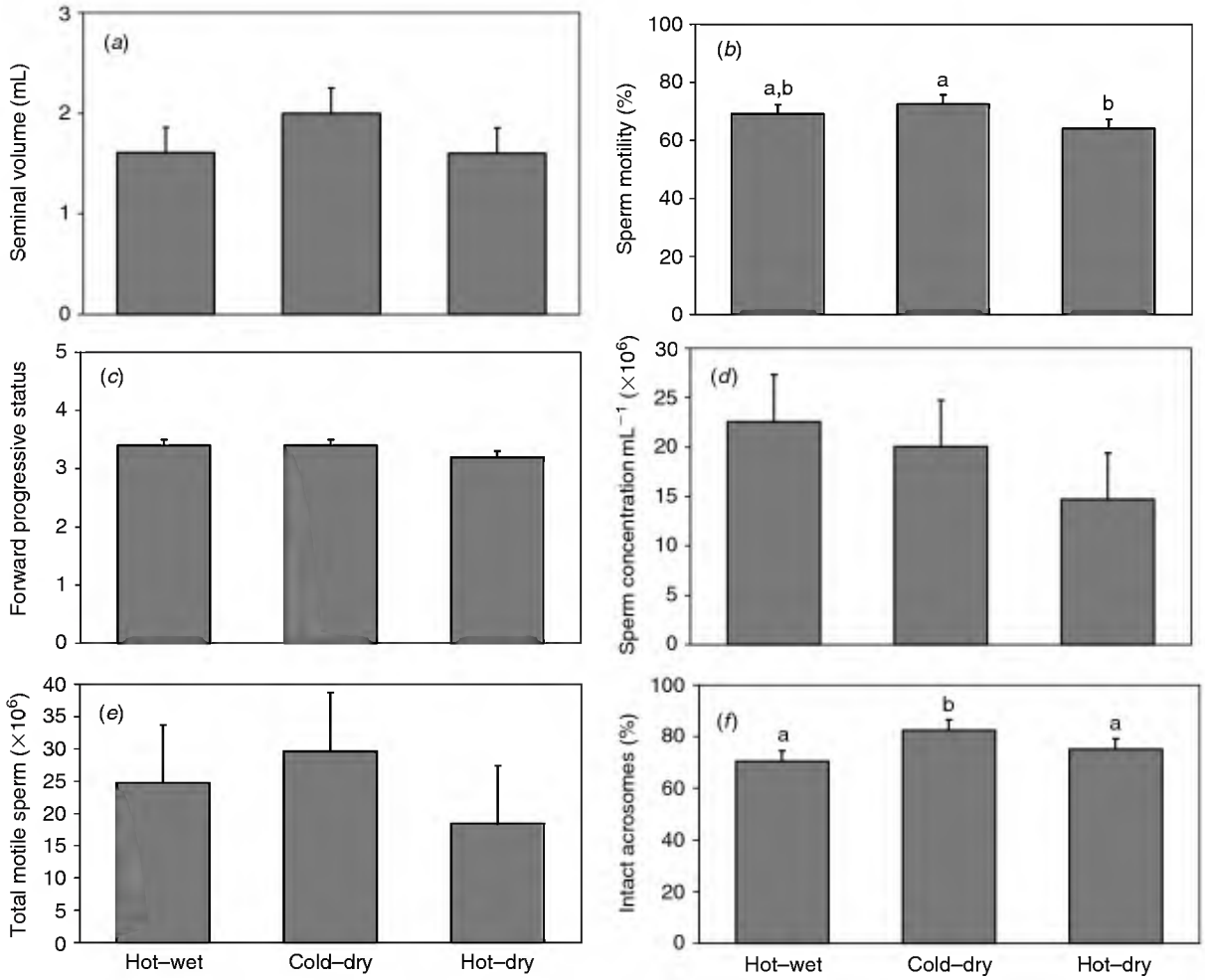

Fig. 3. Influence of season (hot–wet, January–April; cold–dry, May–August; and hot–dry, September–December) on cheetah raw ejaculate characteristics: (*a*) seminal volume, (*b*) sperm motility, (*c*) forward progressive status, (*d*) sperm concentration, (*e*) total motile spermatozoa and (*f*) intact acrosomes. All values are least-squares means ± s.e.m. Within each trait, values with different superscripts differ ($P < 0.05$).

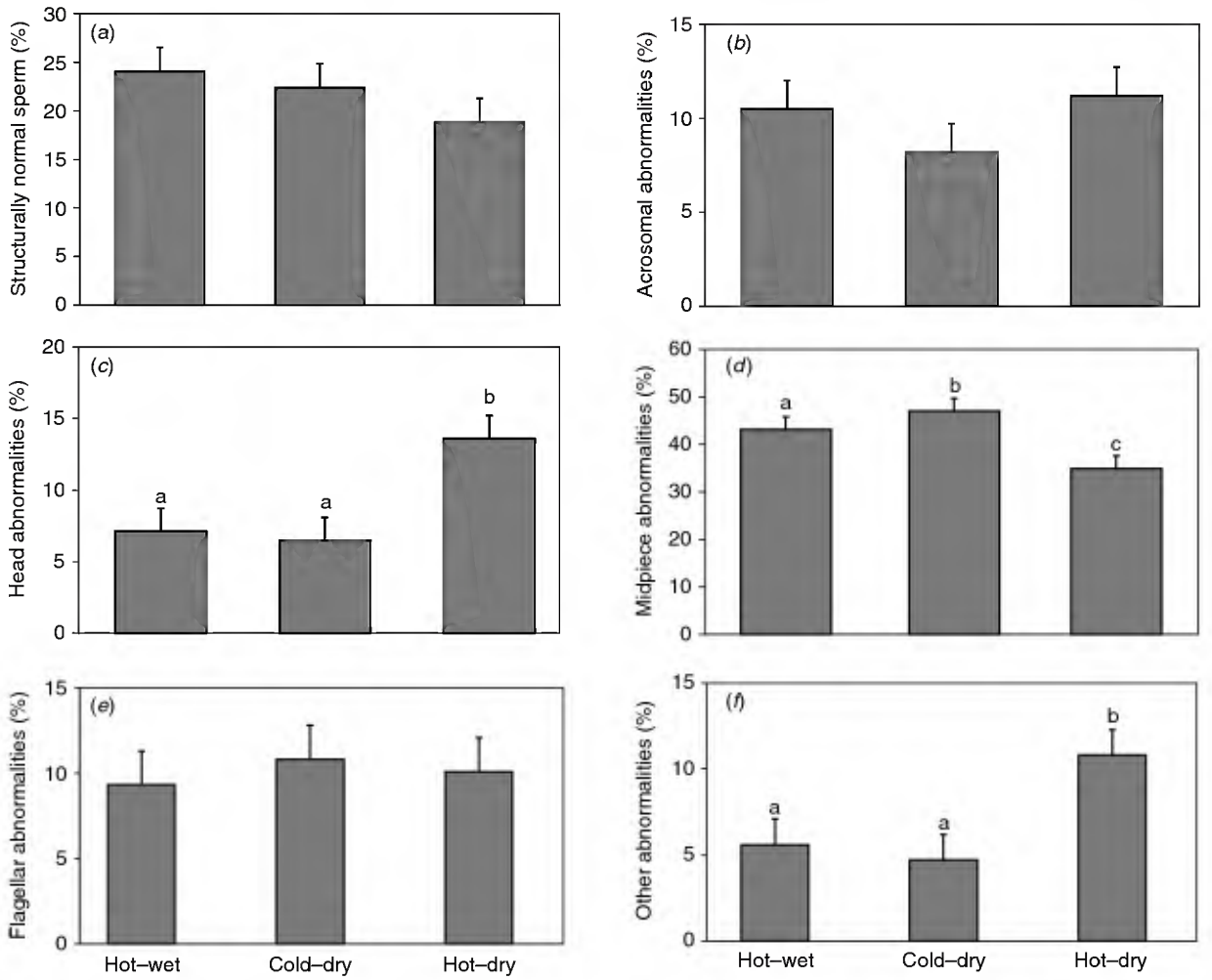

Fig. 4. Influence of season (hot–wet, January–April; cold–dry, May–August; and hot–dry, September–December) on cheetah sperm morphology: (*a*) normal versus anomalies involving the (*b*) acrosome, (*c*) head, (*d*) midpiece, (*e*) flagellum as well as (*f*) other malformations. All values are least-squares means ± s.e.m. Within each trait, values with different superscripts differ ($P < 0.05$).

(Wildt *et al.* 1983), much has been learned about this unique trait in felids. We know that these malformed cells do not participate in fertilisation, and even normal spermatozoa from teratospermic donors are dysfunctional at subcellular and biochemical levels (see reviews, Pukazhenthi *et al.* 2001, 2006*a*). What this means for reproductive fitness is unclear. Poor semen quality and a high incidence of abnormal spermatozoa are commonly observed in many of the 37 felid species (Pukazhenthi *et al.* 2006*b*). Specifically, species lacking in genetic diversity produce fewer normal sperm cells compared with their genetically variant counterparts (Pukazhenthi *et al.* 2006*b*). For four species in particular, the Florida panther (Roelke *et al.* 1993), Asiatic lion (Wildt *et al.* 1987*a*), domestic cats bred as models of human disease (Pukazhenthi *et al.* 2006*b*) and the cheetah (O'Brien *et al.* 1983, 1985, 1987), reduced genetic diversity can be strongly linked with teratospermia, and all four of these species demonstrate reduced fertility (Wildt 1994). It is, however, worth noting that compared with many other mammals, the cheetah produces comparatively few motile, structurally normal spermatozoa, yet can impregnate a female with a single mating (Lindburg *et al.* 1993). Therefore, perhaps one could even assert that the cheetah is exquisitely reproductively efficient. However, before such a hypothesis can be tested, it is necessary to better understand factors, such as age, season and living status that could potentially influence sperm form and function.

Male cheetahs in North American zoos are generally believed to reach sexual maturity (i.e. produce spermic ejaculates) by 13 to 16 months of age (Wildt *et al.* 1993). In our study, every male cheetah in Namibia that was 15 months or older produced a spermic ejaculate. There were six total aspermic ejaculates produced by five individuals. Two of the aspermic males were wild caught simultaneously (thus, were likely siblings), were estimated to be 14 months, and one of these was cryptorchid. One aspermic animal was a CCF resident, approximately 19 months old at the time of first examination and had been recovered from a captive facility with two female siblings approximately 7 months

Table 4. Influence of wild-caught versus captive status on testes volume and ejaculate traits
Least-squares means ± s.e.m.

Variable	Free ranging	Captive held
Number of males	29	68
Number of ejaculates	41	159
Animal age (years)	3.7 ± 0.4	4.0 ± 0.2
Bodyweight (kg)	41.8 ± 0.9	41.7 ± 1.2
Total testicular volume (cm^3)	9.6 ± 0.6	9.7 ± 0.8
Seminal volume (mL)	1.5 ± 0.3[a]	2.0 ± 0.2[b]
Sperm motility (%)	68.0 ± 3.4	69.3 ± 2.5
Forward progressive status[A]	3.4 ± 0.1	3.3 ± 0.1
Sperm concentration mL^{-1} ($\times 10^6$)	23.8 ± 5.2[a]	14.4 ± 3.9[b]
Total motile spermatozoa ($\times 10^6$)	27.5 ± 9.9	21.0 ± 7.4
Intact acrosomes (%)	72.2 ± 4.7[a]	80.1 ± 3.6[b]
Morphologically normal spermatozoa (%)	21.1 ± 2.6	22.3 ± 2.1
Morphologically abnormal spermatozoa (%)		
Acrosomal defects	8.6 ± 1.6[a]	11.4 ± 1.3[b]
Head defects	9.7 ± 1.7	8.5 ± 1.4
Midpiece defects	45.1 ± 2.7[a]	39.6 ± 2.2[b]
Flagellar defects	9.2 ± 2.1	10.9 ± 1.7
Other defects[B]	6.5 ± 1.6	7.6 ± 1.3

[A]Scale 0–5 with 5 being the best forward progressive status. [B]Other abnormalities include a spermatid, bent neck, detached head or detached flagellum. [a,b]Within rows, values with different superscripts differ at $P < 0.05$.

earlier. This trio had been living in poor conditions for about 5 months (in a ~10 × 10 m enclosure with a diet of carrion and game meat) before translocation to CCF. This earlier suboptimal environment may have delayed physiological development, especially since this male thrived at CCF and began producing spermic ejaculates beginning at ~26 months of age. The fourth male was captive held, contributed two aspermic ejaculates and was unilaterally cryptorchid. There is no physiological explanation for the aspermic ejaculate from the fifth individual, a seemingly healthy, wild-caught animal of approximately 4.5 years of age.

Overall, there was remarkable consistency between testes, seminal and sperm traits observed in Namibian cheetahs (wild caught versus captive held) compared with previous metrics measured in cheetahs at a large South African facility (De Wildt Cheetah Breeding Center; Wildt *et al.* 1983), at 18 zoos throughout North America (Wildt *et al.* 1993) and for eight free-ranging males in the Serengeti ecosystem (Wildt *et al.* 1987*b*). All of these populations were essentially subjected to the exact same methodologies. Collectively, it was apparent that the unusual reproductive physiological characteristics of the male cheetah were highly conserved across geographically disparate free-living and captive environments. However, we noted the condition of cryptorchidism for the first time, with 3.1% of all Namibian males having a single retained testis. Low levels of genetic diversity in other felids, such as the Florida panther, also have been reflected in cryptorchidism, with bilateral testis retention occurring in unusually high proportions in that subspecies (Roelke *et al.* 1993).

Three important findings resulted from the age comparisons, the first being that young males of 14–24 months are producing inferior quality semen compared to adult counterparts. In fact, adult cheetahs were producing 6-fold more motile spermatozoa per ejaculate. Although appearing to be adult size, these juvenile males averaged 6 kg less in body mass. The relationship between sperm numbers in juveniles, libido and fertility is also unclear, and it would be worthwhile to expand studies to more clearly understand the pubertal transition zone for this species. Of particular interest is determining the variability in sperm production onset and its relationship with reproductive behaviours and endocrine profiles. The latter now can be measured non-invasively via serial monitoring of fecal hormonal metabolites (Brown *et al.* 1996, 2001). Interestingly, it is known that cheetah females can become pregnant by artificial insemination with as few as 3.4×10^6 motile spermatozoa (Howard *et al.* 1997), which is only half that measured in the young males in our study. Therefore, most young cheetahs, even at slightly more than 1 year of age, theoretically could be fertile on the basis of total motile spermatozoa produced.

The second important age-based finding was the absence of differences in the percentage of sperm pleiomorphisms or specific morphotypes between the juvenile and adult groups. This demonstrated that teratospermia is not age dependent in the cheetah, thereby supporting the notion that this condition likely has genetic origins (O'Brien *et al.* 1983, 1985). The unusually high percentage of normal spermatozoa in the aged group was inexplicable, but was probably due to the many fewer total ejaculates ($n = 5$) compared to a combined 195 for the other two age categories. In terms of specific malformations, the only differences were observed for head deformities, with minor variation between young and adult cheetahs owing to a ~3% reduction in head pleimorphisms, which declined another 6% for the small population of aged males. This may or may not be directly related to fertilising ability because our earlier biomedical survey of captive cheetahs in North American zoos found no difference in the incidence of sperm pleiomorphisms between proven or unproven breeders (Wildt *et al.* 1993). The third age-related point was that males as old as 17 years (captivity-maintained) and 6.5 years (oldest free-ranging individual studied) were producing spermic ejaculates of a quality comparable with that used to produce pregnancies by artificial insemination (Howard *et al.* 1997). Thus, we can conclude that older captive-held males continue to have reproductive potential, which is interesting because fecundity of zoo-maintained females peaks from 3 to 10 years of age (Marker 2002).

There are no data on age limits for males mating in the wild, and studbook records indicate that zoo-held males have bred successfully from 2 to 15 years of age (Marker 2002). Clearly, mechanisms exist in the cheetah to ensure protracted male reproductive function and fertility. There are also lessons here for *ex situ* management. The lower quality ejaculates of young males may be incentive to delay breeding these individuals until at least 2 years of age, especially if they are behaviourally immature. In contrast, the detection of high-quality spermatozoa in older males indicates that these individuals (which may not breed naturally) could still be a source of germplasm for genome resource banking and assisted breeding (Wildt *et al.* 1997). Nonetheless, it would be interesting (and probably prudent) to study the functional viability of spermatozoa from aged males.

Until this study, there were no data on seasonal influences on testes volume or seminal quality in cheetahs. Longitudinal monitoring of hormonal metabolites has revealed episodic and occasionally prolonged periods of anoestrus in cheetahs housed in North American zoos, a finding unrelated to seasonality (Brown *et al.* 1996). More recently, it has been discovered that these acyclicity periods appear to be due to reproductive suppression induced by females sharing enclosures (Wielebnowski *et al.* 2002), perhaps a remnant of species asociality normally displayed in nature. But overall, zoo-maintained female cheetahs appear to exhibit reproductive activity and produce cubs (albeit sporadically) throughout the year.

In Namibia, it has been found that ~70% of wild cheetah cubs are born from March to July (Marker *et al.* 2003*a*), a period that corresponds to the most rainfall, plentiful grass and forage cover (for hiding offspring) and the greatest amount of prey in the form of ungulate newborns (Estes 1991). In contrast, only 5% of cheetah cubs are born from October to December (Marker *et al.* 2003*a*), a time period occurring during the driest and hottest time of the year with least cover and prey. Backdating with a gestation of 94 days (Brown *et al.* 1996), most matings must occur during the hot–wet season of January to April. However, in general, there was little evidence of seasonal fluctuation in cheetah seminal traits, although it was interesting that combined testes volume was significantly higher in free-living males during the hot–wet interval compared to other times of the year. The testes volume advantage for the breeding season in wild males was not observed in the cheetahs maintained *ex situ*. This could mean that captivity may have ameliorated a seasonal variation in testes volume; however, the significance of the mass of these organs in felids remains vague (Howard 1993). In a previous study, testes volume was related to seminal quality (Wildt *et al.* 1993), and testes size may indeed correlate with the need for adequate interstitial tissue that ensures appropriate androgen production; this would be another justification to investigate hormonal metabolite profiles in males throughout the year.

Of course, it made sense that testes volume peaked for wild-caught males in Namibia's hot–wet season when most breeding occurs. Regardless, there were no profound impacts of season on resulting seminal metrics. Overall, cheetahs (free ranging or in captivity) produced spermatozoa of fairly consistent quality throughout the year. But several observations were noteworthy, including an 8% decline in sperm motility from the cold–dry to the hot–dry period. The latter interval in Namibia can be composed of many weeks of zero rainfall and ambient daytime temperatures of 40–46°C. Such conditions may evoke 'stress' that could account for decreased gamete motility, as observed for other species (Meyerhoeffer *et al.* 1985; Flowers 1997) as well as the usual increases in head deformities and the presence of spermatids in the ejaculate. Regardless, it was clear that the cheetahs of Namibia are spermatogenic throughout the year, producing few changes in seminal traits and, thereby, being similar to the domestic cat (Spindler and Wildt 1999), Siberian tiger (Byers *et al.* 1990) and clouded leopard (Wildt *et al.* 1986) and vastly different from the highly seasonal Pallas' cat (Swanson *et al.* 1996*a*).

In general, there were few differences in seminal quality between free-living and captive-held cheetahs in Namibia, indicating that the maintenance environment did not substantially influence male reproductive physiology. Of course, the enclosures at CCF were large and situated in native, *Acacia* shrub habitat. But seminal characteristics in these cheetahs and their wild counterparts in this range country were indistinguishable from those for the species collected during a large survey of North American zoos (Wildt *et al.* 1993). So clearly, the management protocols used by the North American Cheetah Species Survival Plan are adequate, at least for eliciting normal male reproductive physiology traits. These data should not be interpreted to mean that the cheetah is immune to poor *ex situ* management, especially a compromised diet.

It is well documented that nutritionally complete diets are essential for normal reproduction in felids (Lewis *et al.* 1989), and that a calcium supplement contributes to enhanced ejaculate quality (Morais *et al.* 2002; Swanson *et al.* 2003; Howard and Allen 2007). Seventy-five per cent of the wild cheetah's diet comes from consuming the young of large antelopes, including the eland (*Taurotragus oryx*), kudu (*Tragelaphus strepsiceros*), red hartebeest (*Alcelaphus buselaphus*) and gemsbok (*Oryx gazella*), as well as the adults and young of the smaller steenbok (*Raphicerus campestris*) and duiker (*Cephalophus monticola*) (Marker *et al.* 2003*c*). Because of the cheetah's comparatively weak jaw structure, most bones cannot be crushed except the cartilaginous ends of ribs and small portions of the skeleton. Therefore, little bone actually is consumed (Phillips 1993; van Valkenburgh 1996) in contrast to substantial quantities of muscle that is high in protein, but deficient in calcium and vitamins, especially vitamins A, D_3 and E (Lewis *et al.* 1989; Howard and Allen 2007). Organ meat (i.e. liver and heart) is high in vitamins (Lewis *et al.* 1989) and is likely the main source of vitamins and minerals in the cheetah's natural diet. To mimic this, cheetahs at CCF received 2–3 kg of skeletal muscle meat plus a daily dietary supplement of calcium, vitamins A, D_3, E and iron as well as organ meat at least once weekly. This rigorous dietary protocol was the likely reason for some of the improved sperm characteristics (especially in acrosomal integrity) compared with other captive facilities in Namibia. This finding was consistent with that of Swanson *et al.* (2003), who found improved sperm quality in endemic Latin American feline species after improving the diet with a vitamin and mineral supplement. Improvements in ejaculate quality (especially sperm concentration) were also detected by Howard and Allen (2007) after vitamin/mineral supplementation to meat diets in the puma (*Puma concolor*) and numerous species maintained in zoological institutions in Thailand, including the leopard cat (*Prionailurus bengalensis*), golden cat (*Catopuma temmincki*) and fishing cat (*Prionailurus viverrinus*).

Together, this new information offers an improved understanding of the reproductive physiology of male cheetahs living in Namibia. Clearly, age influenced seminal quality, but spermatogenesis continued unabated throughout the year and was minimally affected by season or whether the males lived freely or in captivity. This new knowledge has importance for improving management of cheetahs *ex situ* that, in turn, has value for sustaining wild populations largely by avoiding animal removals from the wild. Despite decades of studies, the cheetah cannot be propagated consistently in captivity. We now know that this

problem is unrelated to physiological deficiencies, but rather to still unknown biocomplexities associated with inadequate captive environments that fail to promote normal reproductive behaviours. Certainly, social factors, including dominance, reproductive suppression and no doubt sexual partner preferences, are involved (Lindburg *et al.* 1993; Caro 1994; Wielebnowski *et al.* 2002). These variables and how they contribute to cheetah reproduction require further attention so that *ex situ* breeding programs can be consistently successful. It is the hope that this strategy will reduce the need to import wild Namibian cheetahs as a resource for zoos.

Although captive cheetahs serve an important role as 'ambassadors' and a resource for basic research (Wildt *et al.* 2001), it would be more prudent to advance these populations as real-life insurance for wild counterparts. This would include creating self-sustaining populations that best reflect cheetahs living in nature (behaviourally, genetically and physiologically). However, *ex situ* populations also need to be exhibited and promoted in ways that inspire the public to support *in situ* conservation. There also are other advantages to directly linking *ex situ* and *in situ* cheetah populations. For example, cheetahs in North American zoos have already benefited from advances in artificial insemination, including with frozen–thawed spermatozoa imported from Africa (Wildt *et al.* 1997). Additionally, a 'field-friendly' method for cryopreserving cheetah spermatozoa, even under field conditions in Namibia, has been developed and is helping the creation of a cheetah genome resource bank (Crosier *et al.* 2006). Such frozen repositories containing spermatozoa, tissues, blood products and DNA have utility for basic and applied research including protecting existing genetic diversity, moving genes among populations (via artificial insemination) and disease surveillance. A cheetah genome resource bank now exists at CCF, with biomaterials available for a diversity of scientific disciplinary studies. Such novel approaches, including more investigations of wildlife physiology, should be pursued because more integrated knowledge is essential to combating escalating threats to wild predators.

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