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Abstract: Sperm-oocyte interaction in vitro was studied in the cheetah, a species known to produce poor quality ejaculates and to experience low rates of fertility. Twelve female cheetahs were injected (i.m.) with eCG followed by hCG 84 h later. Twenty-four to 26 h post hCG, each was subjected to laparoscopic oocyte aspiration. A sperm motility index (SMI) was calculated for each of 9 cheetah sperm donors that produced ejaculates averaging 41.3 +/- 22.9 x 10(6) motile sperm and 28.4 +/- 4.9% structurally normal sperm. Each ejaculate was used to inseminate cheetah oocytes from 1 or 2 females and salt-stored, domestic cat oocytes. The presence of ovarian follicles (greater than or equal to 1.5 mm in diameter) showed that all females responded to exogenous gonadotropins (range, 11-35 follicles/female). A total of 277 cheetah oocytes was collected from 292 follicles (94.9% recovery; 23.1 +/- 2.2 oocytes/female). Of these, 250 (90.3%) qualified as mature and 27 (9.7%) as degenerate. Of the 214 mature oocytes inseminated, 56 (26.2%) were fertilized, and 37 (17.3%) cleaved to the 2-cell stage in vitro; but the incidence of in vitro fertilization (IVF) varied from 0 to 73.3% (p less than 0.001) among individual males. When oocytes from individual cheetahs (n = 5) were separated into two groups and inseminated with sperm from a male with an SMI greater than 0 after 6 h coincubation versus an SMI = 0 at this time, the mean fertilization rates were 28/44 (63.6%) and 0/37 (0%), respectively (p less than 0.05). Of the 117 domestic cat oocytes coincubated with cheetah sperm, 96.6% contained 1 or more cheetah sperm in the outer half of the zona pellucida (ZP). Although the mean number of cheetah sperm penetrating the outer ZP of the cat oocyte was similar (p greater than 0.05) among all males, there was a positive correlation between the number of sperm reaching the inner half of the ZP and fertilization rate in vitro (r = 0.82; p less than 0.05). Compared to IVF efficiency in the domestic cat and tiger as reported in earlier studies, IVF efficiency in the cheetah is low. Because oocytes from 11 of 12 cheetahs were fertilized in vitro, there is no evidence that the female gamete is incompetent. Although sperm pleiomorphisms may contribute to poor reproductive performance, examination of the data on the basis of individual sperm donors reveals that effective gamete interaction in the cheetah is dictated, in part, by sperm motility.

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Correlation of Sperm Viability with Gamete Interaction and Fertilization In Vitro in the Cheetah (*Acinonyx jubatus*)[1]

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ABSTRACT

Sperm-oocyte interaction in vitro was studied in the cheetah, a species known to produce poor quality ejaculates and to experience low rates of fertility. Twelve female cheetahs were injected (i.m.) with eCG followed by hCG 84 h later. Twenty-four to 26 h post hCG, each was subjected to laparoscopic oocyte aspiration. A sperm motility index (SMI) was calculated for each of 9 cheetah sperm donors that produced ejaculates averaging 41.3 ± 22.9 × 10^6 motile sperm and 28.4 ± 4.9% structurally normal sperm. Each ejaculate was used to inseminate cheetah oocytes from 1 or 2 females and salt-stored, domestic cat oocytes. The presence of ovarian follicles (≥1.5 mm in diameter) showed that all females responded to exogenous gonadotropins (range, 11–35 follicles/female). A total of 277 cheetah oocytes was collected from 292 follicles (94.9% recovery; 23.1 ± 2.2 oocytes/female). Of these, 250 (90.3%) qualified as mature and 27 (9.7%) as degenerate. Of the 214 mature oocytes inseminated, 56 (26.2%) were fertilized, and 37 (17.3%) cleaved to the 2-cell stage in vitro; but the incidence of in vitro fertilization (IVF) varied from 0 to 73.3% ($p < 0.001$) among individual males. When oocytes from individual cheetahs (n = 5) were separated into two groups and inseminated with sperm from a male with an SMI > 0 after 6 h coincubation versus an SMI = 0 at this time, the mean fertilization rates were 28/44 (63.6%) and 0/37 (0%), respectively ($p < 0.05$). Of the 117 domestic cat oocytes coincubated with cheetah sperm, 96.6% contained 1 or more cheetah sperm in the outer half of the zona pellucida (ZP). Although the mean number of cheetah sperm penetrating the outer ZP of the cat oocyte was similar ($p > 0.05$) among all males, there was a positive correlation between the number of sperm reaching the inner half of the ZP and fertilization rate in vitro ($r = 0.82$; $p < 0.05$). Compared to IVF efficiency in the domestic cat and tiger as reported in earlier studies, IVF efficiency in the cheetah is low. Because oocytes from 11 of 12 cheetahs were fertilized in vitro, there is no evidence that the female gamete is incompetent. Although sperm pleiomorphisms may contribute to poor reproductive performance, examination of the data on the basis of individual sperm donors reveals that effective gamete interaction in the cheetah is dictated, in part, by sperm motility.

INTRODUCTION

The cheetah (*Acinonyx jubatus*) is the single surviving species of the holartic pleiostocene genus and is classified as endangered [1, 2]. Overall reproductive performance and health status of the cheetah in captivity is poor; only 15% of wild-caught animals have ever reproduced, and the species experiences a high rate of juvenile mortality and disease susceptibility [1, 3]. For these reasons, the cheetah has been the subject of intense genetic and reproductive studies in our laboratories and those of our collaborators [3–8]. Molecular genetic analyses reveal that fewer than 1% of the loci examined are polymorphic, probably because the species experienced a severe population bottleneck in its recent evolutionary history [3, 4]. The cheetah also produces an unusually high proportion of morphologically abnormal spermatozoa/ejaculate (31.0% to 97.0%) [5, 6] compared to certain other felid species [5, 7, 9, 10].

If teratozoospermia in the cheetah is related to inbreeding depression [3–5], then it is logical that poor reproductive performance may be a partial consequence of the high incidence of defective spermatozoa. In other species, morphologically abnormal sperm are disadvantaged in transport through the female reproductive tract [11–13], suggesting that natural barriers prevent these cells from reaching the site of fertilization. It appears that pleiomorphic sperm from cheetahs are compromised in their ability to penetrate the zona pellucida (ZP) of the domestic cat oocyte [14]. Although ~60% of the ZP-bound sperm contain either midpiece or flagellar defects, only morphologically normal sperm penetrate the inner half of the domestic cat ZP [14]. This suggests that sperm cell integrity or a related characteristic plays a role in successful fertilization in the cheetah. It also is possible that female gametogenesis in the genetically uniform cheetah is suboptimal. To date, reproductive studies of female cheetahs have focused on laparoscopic characterization of ovarian activity [15, 16], hormonal induction of follicular development [8, 15, 16], and artificial insemination [17]. In one study, most cheetahs responded with follicular development after administration of exoge-

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nous gonadotropins, but 0 of 23 females artificially inseminated intracervically or in utero became pregnant, perhaps due to poor semen and/or oocyte quality [17].

In vitro fertilization (IVF) offers a powerful tool for assessing gamete functionality. We have used an IVF protocol to produce viable-appearing embryos in the domestic cat (*Felis catus*) [18–20], leopard cat (*F. bengalensis*) [9], puma (*F. concolor*) [21] and tiger (*Panthera tigris*) [10]. In each species, the use of eCG stimulates most females to develop ovarian follicles, although the number of resulting oocytes varies among and within species [10, 18–21]. In the domestic cat, the sperm/oocyte co-culture system routinely results in 65–80% of all mature oocytes cleaving in vitro [19, 20]. Likewise, female tigers produce a high percentage of mature oocytes and an IVF rate of ~63%, which may be the consequence of a naturally high incidence of structurally normal, ejaculated spermatozoa [10]. In contrast, fewer ovarian follicles are available for aspiration in the leopard cat, and the oocyte degeneration rate 24 h postinsemination is 5-fold higher than in the domestic cat [9]. A high proportion of aspirated oocytes (31%) in the puma are immature [21], and the overall cleavage rate is only ~9%, which appears related to the remarkably high proportion of pleiomorphic spermatozoa (>90%) per puma ejaculate [21].

This study was a component of a long-term effort to explore the conservation of fertilization mechanisms within the Felidae family [22]. Our general objective was to examine the efficiency of our standard felid IVF system in the teratozoospermic cheetah. We were particularly interested in assessing 1) the normalcy of the cheetah oocyte and 2) the impact of sperm morphology and motility on fertilization as reflected in results from individual males. As another measurement, heterospecific fertilization testing was performed concurrently using a previously developed penetration assay that relied upon salt-stored, domestic cat oocytes [14, 23].

MATERIALS AND METHODS

Animals

The study was conducted in December 1990 (Trial I) at the Fossil Rim Wildlife Center (Glen Rose, TX) and January 1991 (Trial II) at the White Oak Plantation (Yulee, FL). Twelve adult (22 mo to 11 yr of age), nulliparous, female cheetahs were used in oocyte donors (6 in Trial I, 6 in Trial II). Nine adult (2.5–7 yr of age) cheetah males served as sperm donors, 3 in Trial I and 6 in Trial II. Females were housed individually or in pairs, whereas males were maintained individually, all in 335–5 395-square-meter outdoor enclosures. Each animal was fed a carnivore diet (1.4–1.8 kg/day Nebraska Brand Feline Diet, North Platte, NE) daily, and water was available free-choice.

Induction of Ovarian Activity, Laparoscopy, and Oocyte Recovery

Female cheetahs were given gonadotropins without regard to stage of the reproductive cycle. In Trial I, 1 000 I.U. of eCG (Equitech Incorporated, Atlanta, GA) was administered as a single injection (i.m.) via blow-dart delivery to each oocyte donor. In Trial II, all females were treated with a single 500 I.U. dose (i.m.) of eCG. In both trials, the same injection approach was used to give all cheetahs either 500 (n = 6) or 250 (n = 6) I.U. hCG (i.m.; Sigma Chemical Company, St. Louis, MO) 84 h after eCG.

Oocytes were recovered 24–26 h post-hCG using a standard laparoscopic procedure for cheetahs [15, 16], which was modified to allow transabdominal oocyte aspiration [10, 18]. In brief, a surgical plane of anesthesia was induced with Telazol (A.H. Robins Company, Richmond, VA; 5.2–5.8 mg/kg body weight, i.m.). To sustain this plane of anesthesia, each female was intubated and maintained on isoflurane gas/oxygen. Each animal was placed in a supine, head-down position on a surgical table and surgically prepared. A 10-mm-diameter, 180° laparoscope (Richard Wolf Medical Instruments Corp., Rosemont, IL) was inserted into the abdominal cavity through a 2–cm-long skin incision made near the umbilicus. Ovaries were evaluated for number of ovarian follicles (clear structures with a well-defined border measuring 1.5 mm or more in diameter), corpora hemorrhagica (CH), or corpora lutea (CL) according to our previously reported criteria [10, 15]. Follicle diameter was estimated using a 2-mm-diameter Verres needle probe inserted transabdominally [18]. The Verres needle also was used to position and secure the ovary for oocyte aspiration using a procedure described previously for the domestic cat [18], leopard cat [9], puma [21], and tiger [10]. A 22-gauge, 4-cm-long needle attached to size 100 polyethylene tubing (inner diameter, 0.86 mm; Clay Adams, Parsippany, NJ) was rinsed with 2–3 ml of Ham's F-10 medium (Irvine Scientific, Santa Ana, CA) containing 10% fetal calf serum (FCS, Irvine Scientific) and 40 IU heparin/ml of medium. Unless otherwise specified (below), the Ham's F-10 medium was consistently supplemented with 10% FCS. A siliconized collection tube (Terumo Medical Corp., Elkton, MD) was attached to the free end of the polyethylene tubing, and the aspiration system was driven by a vacuum pump (Gast Manufacturing Corp., Benton Harbor, MI). Distinct follicles ≥1.5 mm in diameter were perforated with the needle while gentle negative pressure (100 mm Hg) was applied with the vacuum pump. After follicles from one ovary were aspirated, the collection tube and aspiration needle were replaced and the procedure was repeated for the contralateral ovary. Collection tubes from each animal were emptied into separate plastic culture dishes, which were examined by stereomicroscopy. Each oocyte-cumulus cell complex was evaluated for status and classified as mature, if corona radiata and cumulus oophorus cells were loos-

ened and expanded, or degenerate, if the oocyte appeared abnormal or pale and/or lacked an apparent corona radiata. Oocytes were washed three times in Ham's F-10 under oil and placed in fresh medium (without heparin).

A simplified portable incubation system was used to coculture gametes. Oocytes and/or embryos in culture dishes were placed in a custom culture chamber (5 × 12 × 22 cm) [10], and then one of two airports was attached via a plastic hose to a tank of 5% CO_2, 5% O_2, 90% N_2. To humidify the chamber, the gas was bubbled through a water reservoir. After the chamber was gassed for 2 min, both airports were occluded and the chamber was placed inside a drying oven (LabLine, Melrose Park, IL) maintained at 38°C. The chamber was regassed each time the culture dishes were removed for oocyte/embryo examination and/or washing.

Electroejaculation and Semen Evaluation/Processing

For Trial I, all oocytes collected from an individual female were inseminated with sperm from a given, single male. In Trial II, each ejaculate from 6 individual males was split and used to inseminate oocytes from 2 individual females. The electroejaculation technique was a standardized procedure relying on a rectal probe with three longitudinal electrodes and an AC, 60 Hz sine-wave ejaculator [5–7]. Total ejaculate volume was recorded, and each semen sample was examined immediately (250×) for subjective estimates of sperm percent motility and progressive motility [5–7]. A 10-μl seminal aliquot was used to calculate sperm concentration using a standard hemocytometer method [7]. Detailed sperm morphology evaluations were performed after fixing a 25–μl aliquot in 1% glutaraldehyde and examining 200 individual sperm/male at 1 000× [5–7, 24].

Electroejaculates were diluted in Ham's F-10 medium containing 10% FCS and centrifuged (300 × *g*, 8 min); the supernatant was removed and 100 μl of fresh Ham's F-10 used to resuspend the pellet. Sperm motility and progressive motility were evaluated immediately after resuspension, and the solution was diluted to provide a concentration of 0.5, 1.0, or 2.0 × 10^6 motile sperm/ml.

Insemination of Cheetah Oocytes, Assessment of Fertilization, and Spermatozoal Longevity In Vitro

In Trial I, mature oocytes from an individual female were divided randomly into two groups and inseminated with a 100-μl aliquot of a diluted suspension containing either 0.5 or 1.0 × 10^5 motile sperm. In Trial II, mature oocytes from an individual female were divided randomly into two groups and inseminated with either 1.0 × 10^5 or 2.0 × 10^5 motile sperm. Ten eggs or fewer/100-μl sperm drop were placed in a 35 × 10-mm culture dish into a 5% CO_2, 5% O_2, 90% N_2 humidified culture chamber at 38°C as described previously [20]. As a parthenogenetic control, one or more representative mature oocytes from each collection were cultured under these conditions without sperm. Eighteen to 20 h post-insemination, all oocytes were removed from the incubator and washed three times in a 0.2% hyaluronidase solution (Type 1-S, from bovine testes; Sigma) for 3 min, returned to the incubator in 100-μl drops of fresh Ham's F-10 under oil, and examined 30 h after insemination for evidence of fertilization.

Fertilization criteria were based on the presence of 2 polar bodies, 2 pronuclei, or cleavage to at least the 2-cell stage [18]. Oocytes with more than 2 nuclear structures within the cytoplasm were considered polyspermic. Oocytes failing to cleave in Trial I were incubated with Hoechst #33342 (H342; bisbenzamide; Sigma; a DNA-specific fluorescent stain [18]), counterstained with 0.1% Trypan blue (Sigma; 0.09 mg/ml, 15 min, 23°C) for 1–2 min, and then examined using differential interference contrast (DIC) and fluorescence optics (250× and 400×). Oocytes failing to cleave in Trial II were fixed in a 2% glutaraldehyde/2% formaldehyde solution, stored at 4°C, evaluated for sperm penetration within 5 days, and then stained with H342 and assessed for fertilization. Using DIC microscopy (400× and a micromanipulator-maneuvering pipette [14, 23, 25], each oocyte was examined for number of spermatozoa bound to the ZP; sperm heads < halfway through the ZP (<1/2 ZP); and sperm heads > halfway through the ZP (>1/2 ZP). The percent penetration for < 1/2 ZP and > 1/2 ZP was defined as the number of oocytes with sperm penetrating the specific layer divided by the total number of oocytes inseminated × 100.

Embryos cleaving to the 2-cell stage or greater were assigned a quality grade [26]. In brief, embryos of good-to-excellent quality were perfectly symmetrical (or only slightly asymmetrical), spherical, and uniformly dark. Embryos were classified as fair or poor in quality if they were partially or severely degenerating, pale in color, or contained lyzed blastomeres. After initial quality grading at 30 h post-insemination, embryos were transferred into fresh Ham's F-10 medium and examined for stage of development 48 and 72 h after insemination.

To determine the longevity of sperm cell motility in vitro, drops containing 100 μl of the diluted sperm suspension were maintained under the IVF conditions without oocytes for 6 h after the insemination time point. A 5-μl sample from each sperm drop was assessed every hour for sperm percent motility and forward progressive motility (based on the type of forward movement of the sperm cell; 0 = no movement to 5 = steady, rapid forward progression). These values were used to calculate a sperm motility index (SMI) [24], an overall evaluation of sperm motility characteristics; SMI = [sperm % motility + (forward progressive motility × 20)] ÷ 2.

Salt-Stored Domestic Cat Oocyte Preparation, Insemination, and Evaluation of ZP Penetration by Cheetah Spermatozoa

Fresh domestic cat ovaries were obtained from local veterinary clinics and maintained ex situ in PBS at 4°C for 1–

TABLE 1. Ovarian activity, oocyte recovery, and oocyte quality results in female cheetahs treated with eCG/hCG.

Trial	Animal no.	Ovarian activity on day of aspiration	No. of oocytes				
			Collected	Mature	(%)	Degenerate	(%)
I	1[ac]	31 follicles	31	28	(90.3)	3	(9.7)
I	2[ac]	31 follicles	29	19	(65.6)	10	(34.4)
I	3[ac]	15 follicles	13	13	(100.0)	0	(0.0)
I	4[ad]	28 follicles	26	23	(88.5)	3	(11.5)
I	5[ad]	31 follicles, 3 CH	30	30	(100.0)	0	(0.0)
I	6[ad]	17 follicles	17	14	(82.3)	3	(17.6)
II	7[bc]	11 follicles, 1 CL	11	11	(100.0)	0	(0.0)
II	8[bc]	29 follicles	26	25	(96.2)	1	(3.8)
II	9[bc]	23 follicles	21	21	(100.0)	0	(0.0)
II	10[bd]	24 follicles	24	24	(100.0)	0	(0.0)
II	11[bd]	35 follicles	35	28	(80.0)	7	(20.0)
II	12[bd]	17 follicles	14	14	(100.0)	0	(0.0)

[a]Received 1000 I.U. eCG.
[b]Received 500 I.U. eCG.
[c]Received 250 I.U. hCG.
[d]Received 500 I.U. hCG.

4 h before processing. Ovaries were placed into Eagle's Minimum Essential Medium (Eagle's MEM; Sigma) supplemented with 5% fetal calf serum (FCS) and punctured repeatedly with a 22-gauge needle to release cumulus-oocyte complexes. Oocytes with homogeneously dark vitelli, tightly compacted corona radiata, and cumulus cell masses were washed three times in fresh medium and cultured (38°C; 5% CO_2, 5% O_2, 90% N_2) for 48 h in 100-μl drops of Eagle's MEM containing 0.23 mM pyruvate, 5% FCS, and 1 μg/ml ovine FSH (NIADDK-oFSH-17 AFP-6446C); 1 μg/ml ovine LH (NIADDK-oLH-25 AFP-5551B); and 25 μg/ml estradiol-17β (Sigma) [27]. To remove cumulus cells after maturation, oocytes were transferred to Eagle's MEM containing 0.2% hyaluronidase for 15 min (38°C) and then were mechanically pipetted. Oocytes were washed and transferred to the salt storage solution [23, 28] consisting of 0.5 M $(NH_4)_2SO_4$, 0.75 M $MgCl_2$, 0.2 mM $ZnCl_2$, 0.1 mg/ml polyvinylalcohol, and 40 mM HEPES buffer (pH 7.4). Salt-stored cat oocytes were maintained at 4°C until the day of insemination, when they were rinsed and incubated in IVF conditions consisting of Ham's F-10 medium supplemented with 5% FCS in a 38°C, 5% CO_2, 5% O_2, 90% N_2 environment [19, 20] for 1 h, then transferred to fresh drops and cultured for an additional 1 h. For each male cheetah in Trial II, 10 salt-stored oocytes were placed in a 100-μl drop of Ham's F-10 containing either 1.0×10^5 or 2.0×10^5 motile sperm/ml under washed paraffin oil. Following a 6 h co-incubation, oocytes were fixed in a 2% glutaraldehyde/2% formaldehyde solution, stored at 5°C, and then evaluated for sperm-ZP interaction using DIC optics as described above for cheetah oocytes.

Statistics

Mean values are presented as ± SEM. Number of follicles and oocytes collected were compared between eCG/hCG treatment groups, and insemination concentrations were measured by Student's *t*-test [29]. Chi-square analysis [29] was used to compare the proportion of oocytes eventually classified as mature, degenerate, or fertilized. Differences in sperm penetration were determined by analysis of variance [29]. Correlation coefficients were calculated for number of sperm penetrating the ZP versus fertilization rates.

RESULTS

Neither eCG nor hCG dose affected ($p > 0.05$) ovarian follicle or oocyte recovery number or overall oocyte quality and fertilizability in vitro (data not presented). Likewise, the number of sperm used/inseminate had no influence ($p > 0.05$) on subsequent IVF rates. Therefore, the data from the gonadotropin dosage and sperm concentration treatment groups were pooled for more detailed analyses.

Following eCG/hCG treatment, the ovaries of all 12 cheetahs contained distinct preovulatory follicles (range in number of follicles ≥ 1.5 mm in diameter, 11–35/female [Table 1]). CH were observed on the ovaries of Female #5 at the time of laparoscopy (26 h post-hCG), indicating that ovulation had begun before all follicles could be aspirated. A single CL of unknown origin was observed on one ovary of Cheetah #7, the female producing the fewest follicles (Table 1). This CL, which was raised slightly above the ovarian surface, was yellowish-orange and appeared to be advanced in age rather than to have been formed as a result of the recent gonadotropin injections. Overall, females produced an average of 24.3 ± 2.1 follicles. A total of 277 oocytes was collected from 292 aspirated follicles (94.9% recovery) for a mean of 23.1 ± 2.2 oocytes/female. Of the total, 250 (90.3%) oocytes were classified as mature and 27 (9.7%) as degenerate. The proportion of degenerate oocytes among females ranged from 0–34.4%, but half the donors produced only mature oocytes (Table 1).

Overall mean ejaculate characteristics for male cheetahs were as follows: ejaculate volume, 1.1 ± 0.2 ml; sperm cell concentration, 40.6 ± 21.1 × 10^6/ml; motile sperm/ejac-

TABLE 2. Percentages of sperm structural abnormalities observed for individual male cheetahs.

Description	Male no.								
	1	2	3	4	5	6	7	8	9
Morphologically abnormal sperm (%)	58.0	77.5	87.5	51.5	61.0	80.0	92.5	54.0	82.0
Abnormal head shape	0.5	4.0	8.0	2.5	1.0	1.5	0.0	4.0	2.5
Abnormal acrosome	0.0	2.0	3.5	0.5	1.0	4.5	5.5	7.0	3.0
Abnormal/missing midpiece	0.0	4.5	1.0	0.5	0.5	0.0	1.5	1.0	3.5
Tightly coiled flagellum	4.5	24.0	29.0	4.5	15.5	29.0	22.0	8.0	16.5
Bent midpiece with cytoplasmic droplet	15.0	22.5	31.0	22.0	16.0	28.0	30.5	13.0	30.0
Bent midpiece without cytoplasmic droplet	3.0	3.5	2.5	2.0	2.0	3.5	4.0	1.0	3.0
Proximal/distal cytoplasmic droplet	31.0	6.0	3.5	18.5	21.5	10.5	24.0	18.0	19.5
Bent flagellum	0.0	2.5	9.0	1.0	2.0	2.0	5.0	1.5	4.0
Other	4.0	8.5	0.0	0.0	1.5	1.0	0.0	0.5	0.0

ulate, 41.3 ± 22.9 × 10^6; sperm percent motility, 74.4 ± 3.6%; sperm forward progressive motility, 3.8 ± 0.2; SMI, 75.1 ± 3.7; percent morphologically normal sperm/ejaculate, 28.4 ± 4.9%. Seminal characteristics for individual sperm donors varied widely; however, all males consistently produced high proportions of pleiomorphic spermatozoa (range, 51.5–92.5%) (Table 2). The most prevalent sperm cell abnormalities were a bent midpiece (overall mean, 23.9%), tightly coiled tail (16.5%), proximal droplet (15.5%), or abnormal acrosome (5.0%) (Table 2).

Fifty-six of the 214 mature oocytes inseminated (26.2%) fertilized in vitro; of these 37 (17.3%) cleaved to at least the 2-cell stage. Hoechst staining revealed that 19 uncleaved oocytes contained 2 polar bodies (Fig. 1) or 2 pronuclei. Of the cleaved embryos, 36 were classified as excellent (24.3%) or good (73.0%) in quality according to our rating criteria. Parthogenetic cleavage was not observed in any of 19 control oocytes.

The range in IVF rate for the 6 males used in Trial II was comparable to that found in Trial I (Table 3). However, during Trial I, we observed few sperm bound or penetrating cheetah oocytes. For this reason, we decided to determine the actual number of sperm penetrating the ZP of both cheetah and domestic cat oocytes in Trial II. Of the 75 cheetah oocytes and 117 cat oocytes co-incubated with cheetah sperm, 75.5 ± 15.0% and 96.6 ± 4.0%, respectively, contained 1 or more cheetah sperm in the outer half of the ZP. Additionally, the mean number of sperm penetrating this region of the ZP was similar ($p > 0.05$) among all males (13.3 ± 6.6 sperm/cheetah oocyte; 10.6 ± 2.5 sperm/cat oocyte). There was a positive correlation between the number of sperm reaching the inner half of the ZP of either cheetah ($r = 0.99$; $p < 0.001$, Fig. 1a) or cat ($r = 0.82$; $p < 0.05$) oocytes (Table 4) and fertilization rate in vitro. More ($p < 0.05$) sperm from the 3 cheetah males producing the highest IVF rate #4, 5, 6) reached the inner half of cheetah (mean, 5.3 ± 1.6) or cat (mean, 2.2 ± 1.0) ZP than sperm from males #7, 8, and 9 (mean, 0.3 ± 0.1; 0.3 ± 0.1, respectively; Figs. 1b). Additionally, insemination with ejaculates from the 3 males (#4, 5, 6) producing IVF embryos resulted in more ($p < 0.05$) cheetah oocytes (94.1 ± 4.8%) and cat oocytes (59.7 ± 12.0%) containing sperm within the inner ZP than insemination with sperm from Males #7, 8, and 9 (7.5 ± 4.0% of cheetah oocytes; 24.4 ± 10.1% of cat oocytes). Sperm from only the 2 males producing the highest IVF rates (#4 and 5) were observed to have penetrated into the perivitelline space (PVS) of domestic cat oocytes (Table 3).

The number of ZP-penetrated oocytes as well as the number of sperm in the inner ZP appeared unrelated to the number of abnormal sperm forms within individual inseminants. For example, Male #8 produced comparatively high proportions of structurally normal sperm (Table 2) but no embryos (Tables 3 and 4). In contrast Males #2, 3, and 6 produced more than 77% structurally defective sperm (Table 2), yet IVF rates in these males ranged from 17.9–42.9% (Table 3). There also appeared to be no relationship between the type of sperm abnormality observed and the ability of sperm to fertilize in vitro. It might be expected that the most severe deformities listed in Table 2 would be the ones to compromise normal penetration (i.e., abnormal acrosome) or motility (i.e., missing midpiece and tightly coiled flagellum). Yet males with high proportions of sperm with a damaged acrosome (#3, 6), a deranged midpiece (#2), or a tightly coiled flagellum (#2, 3, 6) were capable of fertilizing oocytes at a rate comparable to or higher than other sperm donors.

The factor appearing most related to oocyte penetration and fertilization in vitro was sperm motility over time. The SMI profiles for individual males over the 6-h incubation period following insemination of oocytes in Trial II are depicted in Figure 2. The SMI for males failing to produce any embryos in vitro (#7–9) deteriorated rapidly whereas the SMI for the 2 males with the highest IVF rate (#4, 5) was sustained throughout the 6-h co-culture interval (Fig. 2, Table 4). The SMI profile for Male #6 as well as this donor's IVF rate was intermediate among the other males. The overall correlation between mean SMI at 6 h and IVF was high (0.90; $p < 0.05$).

A retrospective analysis also revealed that half the oocytes from Females #7–9, 11, and 12 had been inseminated with sperm from males with an SMI > 0 at 6 h of co-culture

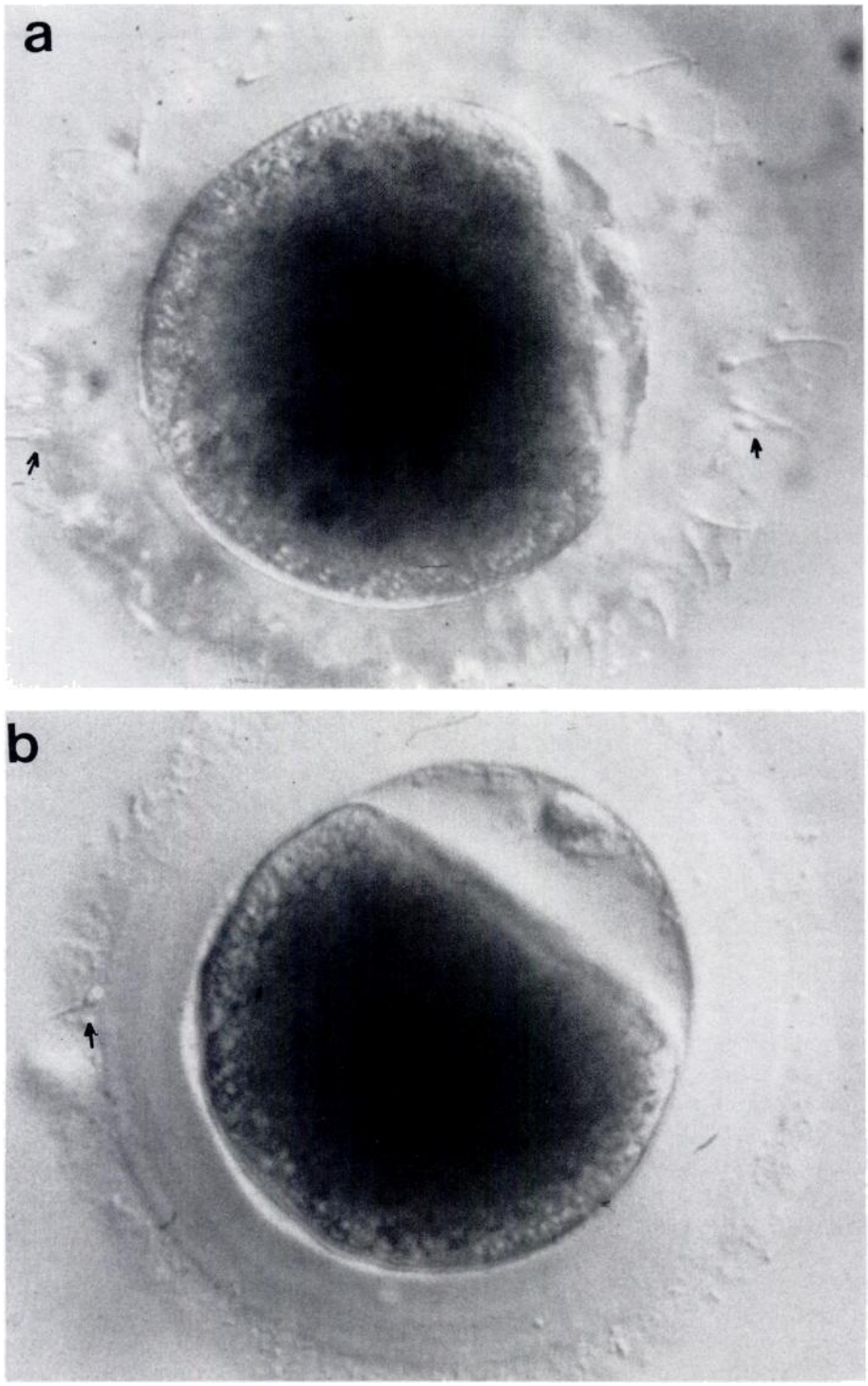

FIG. 1. Penetration of conspecific oocytes by cheetah spermatozoa. (a) Single cell oocyte co-cultured with sperm from Male #4. Arrows indicate numerous sperm penetrating the ZP. (b) Oocyte with single polar body co-cultured with sperm from Male #9. Arrow indicates a single sperm bound to the ZP.

(Males #4–6), whereas the remaining oocytes were incubated with sperm having an SMI = 0 at this same time (Males #7–9) (Table 5). When oocytes were co-incubated with high SMI (>0) sperm, 28/44 (63.6%) oocytes fertilized compared to 0/37 (0%) with SMI = 0 sperm.

A total of 25 and 19 cheetah embryos were cultured for 48 and 72 h, respectively. At 48 h, 24 of 25 (96.0%) embryos either contained 4 blastomeres (n = 7) or were at the 8-

TABLE 3. Semen characteristics and IVF results for individual male cheetahs.

Trial	Male no.	Ejaculate volume (ml)	No. sperm/ml ejaculate ($\times 10^6$)	No. motile sperm ejaculate ($\times 10^6$)	Sperm motility %	Sperm motility Status[a]	Sperm motility SMI[b]	Morphologically normal sperm (%)	No. oocytes fertilized no. inseminated (%)
I	1	0.85	97.0	66.0	80	4.0	80	42.0	5/59 (8.5)
I	2	0.62	7.0	2.6	60	3.5	65	22.5	14/37 (37.8)
I	3	0.21	8.0	0.9	55	3.0	58	12.5	6/14 (42.9)
II	4	1.60	28.2	36.1	80	4.5	85	48.5	11/15 (73.3)
II	5	1.26	201.0	227.2	90	4.5	90	39.0	15/24 (62.5)
II	6	0.99	3.0	2.1	70	3.0	65	20.0	5/28 (17.9)
II	7	0.86	24.2	16.6	80	4.0	80	7.5	0/12 (0.0)
II	8	1.23	12.0	12.6	85	4.5	88	46.0	0/19 (0.0)
II	9	2.24	5.0	7.7	70	3.0	65	18.0	0/6 (0.0)

[a]Speed of progression on a 0 to 5 scale (0 = no forward movement; 5 = rapid, linear progression).
[b]Sperm motility index = [sperm % motility + (sperm progressive motility × 20)] ÷ 2 at the time of initial evaluation.

cell stage of development (n = 17) (Table 6). By 72 h, 52.6% were at the 16-cell stage.

DISCUSSION

These results demonstrated the feasibility of adapting the domestic cat IVF system to the teratozoospermic cheetah, the outcome being that 26.2% of all inseminated oocytes fertilized, and 37 (17.3%) formed cleaved embryos. The data also illustrated that it was possible to generate embryos capable of developing in vitro from a species that has resisted routine natural propagation in captivity. Results suggested that IVF eventually may have a useful role in captive breeding of this endangered species. But more importantly, the data provide new information on the absolute impact of sperm pleiomorphisms and motility on gamete interaction. Overall, there was a nonsignificant relationship between numbers or types of structurally abnormal sperm inseminated and IVF success, but a positive correlation between sperm motility rating and sperm-oocyte interaction. Likewise, the fertilization of at least some oocytes from every female inseminated with sperm from 2 donors clearly demonstrated that IVF success in the cheetah was regulated more by sperm than by oocyte integrity. If an organic fertility problem contributes to poor reproductive performance in this species, all evidence suggests that the etiology is more male- than female-related.

Single injections of eCG and hCG were as effective for provoking follicular development and intrafollicular oocyte maturation as a similar hormone treatment given to domestic cats [18–20], leopard cats [9], pumas [21], and tigers [10]. In a previous study, female cheetahs ovulated after repeated injections of FSH-P and hCG [17], but the viability of the oocytes was not measured. There was no evidence from the present study that the tested dosages of eCG or hCG influenced the number of follicles recruited, the number or quality of the oocytes recovered, or IVF. There was wide variability in follicular response to a given eCG/hCG regimen, a finding that was consistent with observations made in similarly treated domestic cats [18], leopard cats [9], pumas [21], and tigers [10]. However, there was remarkable uniformity in number and maturational status of recovered oocytes among individual cheetahs. Eight of 12 donors produced more than 20 oocytes, and most of these (>90%) met our subjective criteria for maturity. In this respect, these results mimicked recent data from a study of eCG/hCG-challenged tigers in which more than 82% of aspirated, follicular oocytes were mature [10]. From a structural perspective, cheetah oocytes appeared grossly similar to domestic cat [18], leopard cat [9], puma [21], and tiger [10] oocytes. The cytoplasm was darkly pigmented, perhaps as a result of the high concentration of intracellular lipid characteristic of carnivore oocytes [30].

Only 17.3% of all cheetah oocytes inseminated in vitro cleaved; this was 44–55% less than the incidence measured in normospermic domestic cats [19, 20] or tigers [10] using the same IVF system. The incidence of fertilization appeared to be male-specific, that is, a high proportion of oocytes fertilized when coincubated with sperm from certain

TABLE 4. Fertilization of cheetah oocytes and inner ZP penetration of cheetah and domestic cat oocytes by individual males.

Male no.	Cheetah oocytes: No. cheetah oocytes fertilized/no. inseminated (%)	Cheetah oocytes: No. penetrated >1/2 ZP/no. inseminated (%)	Cheetah oocytes: Mean no. sperm/oocyte	Domestic cat oocytes: No. cat oocytes penetrated >1/2 ZP/no. inseminated (%)	Domestic cat oocytes: Mean no. sperm/oocyte
4	11/15 (73.3)[a]	4/4 (100.0)[a]	7.3 ± 1.4[a]	14/16 (87.5)[a]	4.7 ± 0.9[a]
5	15/24 (62.5)[a]	19/19 (100.0)[a]	7.3 ± 0.9[a]	13/24 (54.2)[b]	1.2 ± 0.3[b]
6	5/28 (17.9)[b]	14/17 (82.4)[a]	1.4 ± 0.2[b]	9/24 (37.5)[b]	0.6 ± 0.2[c]
7	0/12 (0.0)[c]	2/12 (16.7)[b]	0.3 ± 0.2[c]	8/20 (40.0)[b]	0.5 ± 0.3[c]
8	0/19 (0.0)[c]	1/17 (5.9)[b]	0.5 ± 0.4[c]	6/18 (33.3)[b]	0.4 ± 0.2[c]
9	0/6 (0.0)[c]	0/6 (0.0)[c]	0.0[d]	0/15 (0.0)[c]	0.0[d]

[abcd]Values within columns with different superscripts differ ($p < 0.05$).

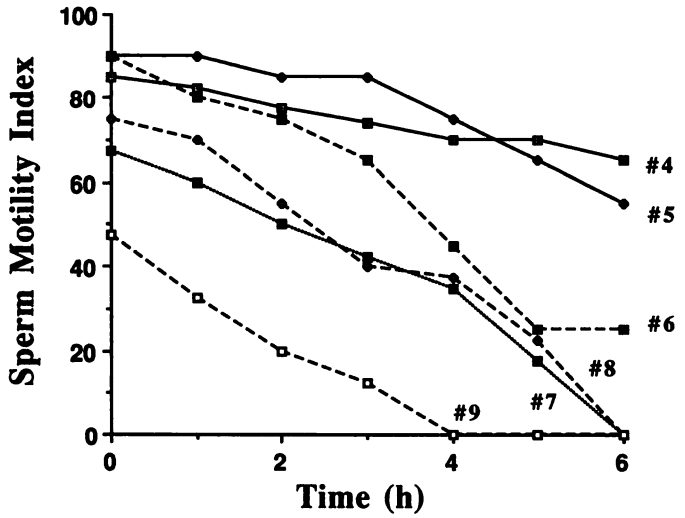

FIG. 2. Sperm motility index (SMI) values for individual cheetahs over a 6-h interval; time 0 = time of insemination.

males but not others. To assess this phenomenon further, we retrospectively examined 2 potential contributors to the variability measured among males, incidence of teratozoospermia and sperm motility (as measured by motility longevity in vitro). The seminal characteristics of the 9 sperm donors confirmed our earlier observations [5–7] that this species consistently produces more than 60% pleiomorphic spermatozoa/electroejaculate. Nevertheless, it was difficult to identify a clear relationship between any proportion of abnormal sperm or a specific sperm structural abnormality and IVF rate. Part of this problem perhaps was attributable to the limited number of cheetahs available and the finding that essentially all males were producing many structurally defective sperm. Because none of the cheetahs produced ejaculates containing normal sperm in the ranges reported for normospermic domestic cats [19, 20] and tigers [10], it was impossible to accurately measure the impact of sperm pleiomorphisms in the cheetah. The influence of sperm defects is more clear in other felid species. For example, co-culturing ZP-intact domestic cat oocytes with structurally normal sperm from either normospermic or teratozoospermic cat semen results in more sperm binding and ZP penetration when sperm from normospermic males are used [25]. In vivo-matured domestic cat oocytes also are less likely to form cleaved embryos when coincubated with normal cat sperm from teratozoospermic (51.8%) compared to normospermic (82.9%) ejaculates [31]. Our interpretation of these findings, taken together is that even structurally normal sperm from teratozoospermic males are inherently compromised in ability to fertilize in vitro. Nonetheless, it also was expected that spermatozoa from the teratozoospermic cheetah would be capable of fertilizing oocytes in vitro for at least two reasons. First, the cheetah as a species is not entirely infertile, although only about 15% of all wild-caught animals in North American zoos have ever reproduced [1]. An ongoing, parallel survey of reproductive characteristics in captive cheetahs has revealed that proven breeder males (n = 12) consistently produce structurally abnormal sperm numbers (~80%) no different from those of unproven counterparts (n = 24; ~75%) [D.E. Wildt, J.L. Brown, M. Bush, M.A. Barone, K. Cooper, Grisham, and J.G. Howard, unpublished data]. Secondly, there is at least one other felid species, the puma, that produces an extraordinarily high proportion of pleiomorphic sperm (>90%) and still is capable of IVF (25.0% cleavage rate) [7, 21, 32]. Therefore, although sperm from teratozoospermic male felids can interact with oocytes in vitro to form fertilized and even cleaved embryos, IVF efficiency appears compromised.

Despite this finding, the absolute incidence of total or specific sperm abnormalities was an unreliable index of predicted IVF success in the cheetah. Although increasing the concentration of sperm/inseminate in men producing high proportions of abnormal sperm increases IVF success [33, 34], increasing sperm concentration (from 1.0 to 2.0 × 10^5 motile sperm inseminated) did not alter fertilization rates in the cheetah. A more effective indicator in this study was sperm motility over time. Low or no fertilization resulted when oocytes were inseminated with sperm from males whose motility deteriorated to zero by 6 h of co-culture. However, when sperm motility was maintained for at least 6 h after insemination, the proportion of oocytes fertilizing (66.7%) was similar to IVF rates for domestic cat (80.0%) [19] and tiger (69.4%) oocytes [10] under the same culture conditions. A retrospective study evaluating the effect of human seminal traits on IVF revealed that sperm motility is the single most important factor dictating fertilization success [33]. In that study, sperm motility ratings of >50% at the time of insemination resulted in 75.8% of all oocytes fertilizing as compared to 49.1% when the motility was at 30–49% and complete fertilization failure when motility was <20%. It seems reasonable to speculate that sperm from teratozoospermic ejaculates may lack robust motility. Therefore, although we were unable to specifically relate pleiomorphic sperm number and IVF rate, another easily monitored trait—sperm motility longevity— accurately predicted IVF success. Similarity in results among the var-

TABLE 5. Comparative fertilization results for individual cheetahs whose oocytes were inseminated with sperm from 2 males.

Oocyte donor no.	Total oocytes inseminated	Insemination male no. and no. oocytes fertilized no. inseminated (%)[a]	Insemination male no. and no. oocytes fertilized/no. inseminated (%)[b]
#9	17	#4, 7/9 (77.8)	#7, 0/8 (0.0)
#12	10	#4, 4/6 (66.7)	#7, 0/4 (0.0)
#8	20	#5, 9/11 (82.0)	#8, 0/9 (0.0)
#11	23	#5, 6/13 (46.2)	#8, 0/10 (0.0)
#7	11	#6, 2/5 (40.0)	#9, 0/6 (0.0)

[a]Males with SMI > 0 at 6 h of co-culture.
[b]Males with SMI = 0 at 6 h of co-culture.

TABLE 6. Culture rate and development of IVF cheetah embryos.

Culture interval	No. embryos	Stage of development			
		2-cell	4-cell	8-cell	16-cell
30 h	37	13 (35.1)	20 (54.1)	4 (10.8)	—
48 h	25	—	7 (28.0)	17 (68.0)	1 (4.0)
72 h	19	—	2 (10.5)	7 (36.8)	10 (52.6)

ious felid species thus far studied and from humans affirms the utility of the Felidae taxon as a model system for more detailed studies concerning the impact of male factors on fertilization and early embryogenesis.

It was important that our concurrent findings for salt-stored, domestic cat oocytes confirmed those for conspecific oocytes. Among all categories of males, similar numbers of cheetah sperm bound to and penetrated the outer layer of the cat ZP. However, the proportion of cat oocytes and the number of sperm within the inner layer of the ZP were increased in males with the highest SMI profiles. The perivitelline space (PVS) contained no sperm from the males failing to produce fertilized conspecific oocytes in vitro. However, in 9 oocytes inseminated with sperm from 2 males producing the highest SMI profiles, 1 to 3 sperm had advanced completely through both ZP layers into the PVS. Binding and penetration of the ZP are prerequisites to fertilization, and ZP penetration assays have provided important predictive information on sperm capacitation and fertilization potential in humans [35–37]. We recently demonstrated the utility of the salt-stored domestic cat oocyte as an indicator of sperm capacitation in the domestic cat [23], leopard cat [23], and cheetah [14]. The present study demonstrated a clear relationship between the incidence of salt-stored, cat oocyte penetration and conspecific IVF and sperm viability. Although it remains to be proven that this technique could be useful for predicting fertility in this or other felid species, results were encouraging, especially in the context of comparable human studies. Most recently, Franken et al. [37] effectively used human oocytes, microbisected into hemispheres and placed in co-culture with sperm, to test ZP binding capacity in men with high rates of teratozoospermia. Certainly, the availability of a functional testing system will facilitate understanding the fundamental aspects of gamete physiology as well as the testing of fertility potential in rare felid taxa. If artificial breeding is ever to become a useful propagative approach for these species, then most recovered oocytes will be needed for IVF purposes, not for assessing sperm function or male fertility. In these cases, the availability of a heterologous system would be of enormous benefit. To date, it is apparent that the oocytes of many species in the Felidae family lack a mechanism for excluding ZP penetration of heterologous felid spermatozoa.

Although it is necessary to test the biological competence of these IVF-generated cheetah embryos in vivo on a large scale, we were encouraged by the ability of cleaved embryos to advance readily in culture to the 16-cell stage of development. The rate of growth in Ham's F-10 was comparable to that measured in domestic cat [19, 20] and tiger [10] embryos maintained in the same medium and culture conditions. Further cross-species comparative studies are planned, and it will be especially interesting to determine whether in vitro-cultured cheetah embryos can develop to the blastocyst stage under our conditions, as seen for the domestic cat [19, 20] and tiger [10].

In summary, these results provide an improved and more focused understanding of those factors that potentially influence sperm-oocyte interaction in the Felidae family. Our use of the teratozoospermic cheetah, and a rapidly developing database for taxinomically-related felid species illustrate the importance of sperm quality, especially motility and structural characteristics, in achieving IVF.

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REFERENCES

1. Marker L, O'Brien SJ. Captive breeding of cheetah (*Acinonyx jubatus*) in North American Zoos (1871–1986). Zoo Biol 1989; 8:3–16.
2. Conventional on International Trade in Endangered Species of Wild Flora and Fauna, part of the Endangered Species Act of 1984, CFR, part 23.
3. O'Brien SJ, Roelke ME, Marker L, Newman A, Winkler CW, Meltzer D, Colly L, Everman J, Bush M, Wildt DE. Genetic basis for species vulnerability in the cheetah. Science 1985; 227:1428–1434.
4. O'Brien SJ, Wildt DE, Bush M. The cheetah in genetic peril. Sci Amer 1986; 5:84–92.
5. Wildt DE, Bush M, Howard JG, O'Brien SJ, Meltzer D, van Dyk A, Ebedes H, Brand DJ. Unique seminal quality in the south African cheetah and a comparative evaluation in the domestic cat. Biol Reprod 1983; 29:1019–1025.
6. Wildt DE, O'Brien SJ, Howard JG, Caro TM, Roelke ME, Brown JL, Bush M. Similarity in ejaculate-endocrine characteristics in captive versus free-ranging cheetahs of two subspecies. Biol Reprod 1987; 36:351–360.
7. Wildt DE, Phillips LG, Simmons LG, Chakraborty PK, Brown JL, Howard JG, Teare A, Bush, M. A comparative analysis of ejaculate and hormonal characteristics of the captive male cheetah, tiger, leopard, and puma. Biol Reprod 1988; 38:245–255.
8. Goodrowe KL, Graham J, Mehren KG. Stimulation of ovarian activity and oocyte recovery in the caracal (*Felis caracal*) and cheetah (*Acinonyx jubatus*). J Zoo Wildl Med 1991; 22:42–48.
9. Goodrowe KL, Miller AM, Wildt DE. In vitro fertilization of gonadotropin-stimulated leopard cats (*Felis bengalensis*) follicular oocytes. J Exp Zool 1989; 252:89–95.
10. Donoghue AM, Johnston LA, Seal US, Armstrong DL, Tilson RL, Wolf P, Petrini K, Simmons LG, Gross T, Wildt DE. In vitro fertilization and embryo development in vitro and in vivo in the tiger (*Panthera tigris*). Biol Reprod 1990; 43:733–744.
11. Krzanowska H. The passage of abnormal spermatozoa through the utero-tubule junction in the mouse. J Reprod Fertil 1974; 38:81–90.
12. Nestor A, Handel MA. The transport of morphologically abnormal sperm in the female reproductive tract of mice. Gamete Res 1984; 10:119–125.
13. Saacke RG, Bame JH, Karabinus DS, Mullins KJ, Whitman S. Transport of abnormal spermatozoa in the artificially inseminated cow based upon accessory spermatozoa in the zona pellucida. In: Proceedings of 11th international congress on reproduction and artificial insemination, Dublin, Ireland. 1988; 3:292 (abstract).
14. Howard JG, Barone MA, Bush M, Wildt DE. A heterologous salt-stored zonae pellucidae assay for assessing sperm capacitation and the impact of teratospermia in the cheetah (*Acinonyx jubatus*). J Androl 1991; (suppl):101 (abstract).

15. Wildt DE, Platz CC, Seager SWJ, Bush M. Induction of ovarian activity in the cheetah (*Acinonyx jubatus*). Biol Reprod 1981; 24:217–222.
16. Phillips LG, Simmons LG, Bush M, Howard JG, Wildt DE. Gonadotropin regimen for inducing ovarian activity in captive wild felids. J Am Vet Med Assoc 1982; 181:1246–1250.
17. Wildt DE, Schiewe MC, Schmidt PM, Goodrowe KL, Howard JG, Phillips LG, O'Brien SJ, Bush M. Developing animal model systems for embryo technologies in rare and endangered wildlife. Theriogenology 1986; 25:33–51.
18. Goodrowe KL, Wall RJ, O'Brien SJ, Schmidt PM, Wildt DE. Developmental competence of domestic cat follicular oocytes after fertilization in vitro. Biol Reprod 1988; 39:355–372.
19. Johnston LA, Donoghue AM, O'Brien SJ, Wildt DE. Culture medium and protein supplementation influence in vitro fertilization and embryo development in the domestic cat. J Exp Zool 1991; 257:350–359.
20. Johnston LA, Donoghue AM, O'Brien SJ, Wildt DE. Influence of temperature and gas atmosphere on in vitro fertilization and embryo development in the domestic cat. J Reprod Fertil 1991; 92:377–382.
21. Miller AM, Roelke ME, Goodrowe KL, Howard JG, Wildt DE. Oocyte recovery, maturation and fertilization in vitro in the puma (*Felis concolor*). J Reprod Fertil 1990; 88:249–258.
22. Wildt DE. Fertilization in cats. In: Dunbar BS, O'Rand M, (eds.), A Comparative Overview of Mammalian Fertilization. New York: Plenum Publishing Corp., 1991; 299–328.
23. Andrews JC, Howard JG, Bavister BD, Wildt DE. Sperm capacitation in the domestic cat (*Felis catus*) and leopard cat (*Felis bengalensis*) studied with a salt-stored zona pellucida penetration assay. Mol Reprod Devel 1992; (in press).
24. Howard JG, Brown JL, Wildt DE. Teratospermic and normospermic domestic cats: Ejaculate traits, pituitary-gonadal hormones and improvement of sperm viability and morphology after swim-up processing. J Androl 1990; 11:204–215.
25. Howard JG, Bush M, Wildt DE. Teratospermia in domestic cats compromises penetration of zona-free hamster ova and cat zonae pellucidae. J Androl 1991; 12:36–45.
26. Schiewe MC, Schmidt PM, Wildt DE. A comparative study of mouse embryo freeze preservation including the examination of a thermoelectric cryogenic device. Cryobiology 1987; 24:238–246.
27. Johnston LA, O'Brien SJ, Wildt DE. In vitro maturation and fertilization of domestic cat follicular oocytes. Gamete Res 1989; 24:343–356.
28. Boatman DE, Andrews JC, Bavister BD. A quantitative assay for capacitation: Evaluation of multiple sperm penetration through the zona pellucida of salt-stored hamster eggs. Gamete Res 1988; 19:19–29.
29. Steel RD, Torrie JH. Principles and Procedures of Statistics. New York: McGraw-Hill; 1960.
30. Guraya SS. A histochemical analysis of lipid yolk deposition in the oocytes of cat and dog. J Exp Zool 1965; 160:123–136.
31. Howard JG. Semen evaluation and sperm function in the domestic cat. In: Proceedings of the annual meeting of the Society of Theriogenology. 1991; 215–217.
32. Barone MA, Howard JG, Roelke ME, Wildt DE. The unique ejaculate and hormonal characteristics of the Florida Panther. In: Proceedings of the annual meeting of the Society of Theriogenology. 1991; 354–357.
33. Mahadevan MM, Trounson AO. The influence of seminal characteristics on the success rate of human in vitro fertilization. Fertil Steril 1984; 42:400–405.
34. Wolf DP, Byrd W, Dandekar P, Quigley MM. Sperm concentration and the fertilization of human eggs in vitro. Biol Reprod 1984; 31:837–848.
35. Burkman LJ, Coddington CC, Franken DR, Kruger TF, Rosenwaks Z, Hodgen GD. The hemizona assay: development of a diagnostic test for the binding of human spermatozoa to the human hemizona pellucida to predict fertilization potential. Fertil Steril 1988; 49:688–693.
36. Oehninger S, Coddington CC, Scott R, Franken DR, Burkman LJ, Acosta AA, Hodgen GD. Hemizona assay (HZA): assessment of sperm dysfunction and prediction of in vitro fertilization outcome. Fertil Steril 1989; 51:665–671.
37. Franken DR, Kruger TF, Menkveld R, Oehninger S, Coddington CC, Hodgen GD. Hemizona assay and teratozoospermia: increasing sperm insemination concentrations to enhance zona pellucida binding. Fertil Steril 1990; 54:497–503.