Abstract: Under the mandate of a Species Survival Plan (SSP), reproductive status was assessed in 128 cheetahs maintained in 18 different institutions in North America. A mobile laboratory research team evaluated cheetahs using anaesthesia, serial blood sampling, electroejaculation (males), and laparoscopy (females). Biomaterials were also collected for parallel studies of genetics, nutrition, and health. There was no mortality, and cheetahs were capable of reproducing naturally after these intense manipulatory examinations. No marked differences were observed in reproductive or endocrine characteristics between proven and unproven breeders. However, males consistently produced teratospermic ejaculates, and cheetah sperm were compromised in conspecific or heterologous in vitro fertilization systems. Structurally abnormal sperm were found to be filtered by the oocyte's zona pellucida. More than 80% of the females were anatomically sound, but morphological and endocrine evidence suggested that ~50% or more of the population may have had inactive ovaries at the time of the examination. Males ranging in age from 15 to 182 months produced spermic ejaculates, but motile sperm numbers/ejaculate and circulating testosterone concentrations were highest in males 60 to 120 months old. Parovarian cysts were observed in 51.5% of female cheetahs, but comparisons between proven and unproven subpopulations revealed that this abnormality likely had no influence on fertility. Fresh luteal tissue not observed in any nonpregnant or nonlactating female, strongly suggesting that the cheetah is an induced ovulator. Overall survey results were discussed in the context of the etiology of reproductive inefficiency, especially with respect to the potential importance of biological versus management factors. Four high priority research areas in cheetah reproductive biology were identified:1) continuous monitoring of ejaculate quality in the extant population, while studying the impact of pleiomorphisms on fertility; 2) determining the potential relationship between libido and androgen production (excretion) in males; 3) confirming the extent of cyclic, or acyclic, ovarian activity in females; and 4) continued development of assisted reproductive techniques for enhancing management. In summary, a multidisciplinary, multi-institutional survey coordinated through the SSP is both possible and useful for generating a physiological and health database beneficial to driving further research and management initiatives.

Reproductive Status of Cheetahs (*Acinonyx jubatus*) in North American Zoos: The Benefits of Physiological Surveys for Strategic Planning

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Under the mandate of a Species Survival Plan (SSP), reproductive status was assessed in 128 cheetahs maintained in 18 different institutions in North America. A mobile laboratory research team evaluated cheetahs using anesthesia, serial blood sampling, electroejaculation (males), and laparoscopy (females). Biomaterials were also collected for parallel studies of genetics, nutrition, and health. There was no mortality, and cheetahs were capable of reproducing naturally after these intense manipulatory examinations. No marked differences were observed in reproductive or endocrine characteristics between proven and unproven breeders. However, males consistently produced teratospermic ejaculates, and cheetah sperm were compromised in conspecific or heterologous in vitro fertilization systems. Structurally abnormal sperm were found to be filtered by the oocyte's zona pellucida. More than 80% of the females were anatomically sound, but morphological and endocrine evidence suggested that ~50% or more of the population may have had inactive ovaries at the time of the examination. Males ranging in age from 15 to 182 months produced spermic ejaculates, but motile sperm numbers/ejaculate and circulating testosterone concentrations were highest in males 60 to 120 months old. Parovarian cysts were observed in 51.5% of female cheetahs, but comparisons between proven and unproven subpopulations revealed that this abnormality likely had no influence on fertility. Fresh luteal tissue was not observed in any nonpregnant or nonlactating female, strongly suggesting that the cheetah is an induced ovulator. Overall survey results were discussed in the context of the etiology of reproductive inefficiency, especially with respect to the potential importance of biological versus management factors. Four high priority research areas in cheetah reproductive biology were identified: 1) continuous monitoring of ejaculate quality in the extant population, while

studying the impact of pleiomorphisms on fertility; 2) determining the potential relationship between libido and androgen production (excretion) in males; 3) confirming the extent of cyclic, or acyclic, ovarian activity in females; and 4) continued development of assisted reproductive techniques for enhancing management. In summary, a multidisciplinary, multi-institutional survey coordinated through the SSP is both possible and useful for generating a physiological and health database beneficial to driving further research and management initiatives.   © 1993 Wiley-Liss, Inc.

INTRODUCTION

While the cheetah is best known by the public for its ability at high speed pursuit, zoo managers best recognize the species as historically difficult to reproduce in captivity. Although cheetahs have been maintained in captivity for millenia, neither ancient Eurasian rulers nor state-of-the-art zoos have yet defined the criteria allowing consistent propagation. In 1989, Marker and O'Brien reviewed cheetah breeding in North American zoos. They determined that, although 44% of institutions attempting to propagate cheetahs succeed, only ~15% of all wild-caught animals reproduce, and only a few cheetahs contribute (disproportionately) to the gene pool. Marker and O'Brien concluded that the North American captive cheetah population is neither self-sustaining nor theoretically "viable," as defined by Soule et al. [1986]. They refused, however, to accept that consistent captive breeding was an impossibility for the species. Rather Marker and O'Brien offered a set of recommendations to the Cheetah Species Survival Planning (SSP) Committee [Grisham, 1988], one of which included greater emphasis on basic research.

In 1989, the Cheetah SSP unanimously concluded that all cheetahs in North American zoos should be managed as a research population, primarily for the purpose of identifying those factors contributing to the reproductive efficiency (or inefficiency) of the species. The SSP established a "Research Council," with members having expertise in reproductive physiology/endocrinology, genetics, behavior, nutrition, and medicine/disease. Research proposals were developed and discussed in a peer-review forum by the combined Council and SSP Committee. A consensus conclusion was that a prerequisite to organized research within any of the 5 disciplines should be a systematic survey of the physiological and health status of the existing population. Because empirical causes of poor reproductive efficiency in the cheetah were unknown, it was only logical to first establish the reproductive and health status of the extant population, information that would be critical for making subsequent research and management recommendations.

We provide here a detailed review of all reproductive/endocrine findings associated with a thorough survey of 128 cheetahs in North American zoos representing 59% of the adult captive population. Parallel information on the incidence of disease/pathologic conditions has been prepared separately [Munson, 1993]. Information on seminal and vaginal bacterial microflora and the relationship among specific bacterial isolates and fertility also has been summarized by Howard et al. [1993]. Combined with other recent and ongoing cheetah studies, this survey has allowed the establishment of one of the most thorough and extensive biological databases ever generated

for an endangered species. For this reason, our second objective is to discuss the structure of the physiological survey and its importance in the context of generating useful data for driving captive management and conservation initiatives. For many species, the interinstitutional development of strategic action plans and the SSP concept [Hutchins and Wiese, 1991] now allow managing single populations, even though individuals are widely scattered among geographically-disparate regions. The key to even more rapid progress is the melding of existing knowledge with more cooperative research initiatives. Therefore, the results of this survey serve as an example of how markedly our understanding of basic species biology can be increased by coordinated, multidisciplinary research, involving multi-institutional participation.

MATERIALS AND METHODS
Survey Structure

The survey was conducted on the basis of a written research protocol approved by the SSP and included in the Cheetah Species Masterplan. A single group of investigators conducted the survey to ensure continuity in data collection and interpretation. Every animal was subjected to a standardized evaluative protocol by the same team of 3 to 5 scientists with extensive cheetah research experience. Team members included 4 reproductive biologists thoroughly familiar with semen assessment, laparoscopic techniques, and endocrine evaluation and one veterinarian with extensive experience in cheetah anesthesia and recovery. Because the anesthesia associated with physiological/health surveys presents a degree of risk, the team veterinarian was essential for providing advice to the local veterinarian(s), when necessary, and for assuring that all data were collected safely and uniformly.

The Cheetah Species Coordinator acted as the liaison between the survey team and each SSP institution. The Coordinator secured permission to conduct each survey at a given host institution and provided potential dates for the team arrival. The Coordinator also requested and organized donations from SSP member institutions and other sources to fund the survey team's travel. Expenses were compensated by 2 sources: 1) direct financial donations to the SSP (Table 1); and 2) in-kind support provided by local institutions and the survey team. For example, zoos commonly paid for meal and hotel expenses either from core budgets or from local contributors. Likewise, the National Zoological Park donated all person-hours of the survey team, and most of the research supplies and expenses were purchased from general grant funds.

At least 6 weeks before planned arrival, the survey team directly contacted each institution's general curator and veterinarian to (1) clarify the objectives of the survey, and (2) ensure that the host institution understood the proposed animal manipulation protocols. Specifics on anesthesia, the time required for evaluating each animal, the exact procedures to be used, and expectations on animal recovery were provided. In turn, the survey team requested the studbook numbers of available males and females and respective health histories. Each host institution also was requested to provide a surgical facility, tilt-table for laparoscopy, miscellaneous surgical supplies and disposables, an anesthetic machine, a centrifuge, a television monitor and video recorder, and 30 square meters of space to be used as a "laboratory." All other

TABLE 1. Sources of financial donations for support of the cheetah survey

Binder Park Zoo
Caldwell Schools, Inc.
Cleveland Metroparks Zoo
Columbus Zoological Gardens
Fort Wayne Children's Zoo
Fossil Rim Wildlife Center
Metropolitan Toronto Zoo
Milwaukee County Zoological Gardens
National Zoological Park's NOAHS Center
Oklahoma City Zoological Park
Rio Grande Zoological Park C.A.T.S. Fund
Roger Williams Park Zoo
St. Louis Zoological Park
Toledo Zoological Gardens
White Oak Plantation
William Hatch School

supplies and equipment (laparoscopy and biopsy instruments, microscopes, compressed CO_2 gas, culture chambers, video camera) were provided by the survey team.

To reduce travel expenses, the survey team usually arranged a 1- to 2-week time-block that allowed visiting multiple institutions in a general region. Survey equipment and supplies were transported in 8 to 12 individual 54 cm × 71 cm × 39 cm reinforced-plastic containers that were shipped as excess baggage on the same airplane as the team members. When appropriate, the team arrived by air transport in a specific geographic location and then radiated to other regional cities, using a rental van or vehicle provided by a host institution. Upon arrival and before proceeding further, the survey team again reviewed all proposed animal manipulations, usually with the curatorial, veterinary, and animal care staff.

The survey was conducted over a 2-year period (inclusive dates: January 15, 1990 to June 21, 1991). Because some genotypes of free-living domestic cats are known to copulate only during the later winter and early spring months [Goodrowe et al., 1989] and because it has been asserted that the cheetah generally has a late winter breeding season [Brand, 1980], the survey was restricted to January through mid-June. This minimized any potential (unknown) seasonal impact upon data interpretation.

Participating Institutions and Cheetah Population Structure

A total of 128 cheetahs (60 males and 68 females) from 18 institutions were evaluated (Table 2). Of these, 12 males (20.0%) and 14 females (20.6%) had reproduced successfully (on the basis of producing live young). The remaining 102 cheetahs had never reproduced, but a portion of these (50.0% of the males; 42.6% of the females) had never been maintained with adult conspecifics of the opposite gender. Therefore, for comparisons of reproductive characteristics between proven versus non-proven breeders, cheetahs with no opportunity to ever mate were omitted from statistical analyses. Surveyed male cheetahs ranged in age from 15 to 182 months (mean 61.2 ± 4.8) whereas females ranged from 16 to 182 months (mean 70.5 ± 5.1). Age distribution on the basis of 24-month intervals is presented in Figure 1.

TABLE 2. Participating institutions and number of male and female cheetahs evaluated per institution

Institution	Males	Females
Binder Park Zoo	2	1
Caldwell Zoo	2	3
Cincinnati Zoo and Botanical Garden	0	1
Cleveland Metroparks Zoo	1	2
Columbus Zoological Gardens	11	11
Fossil Rim Wildlife Center	9	12
Fort Wayne Children's Zoo	2	3
King's Island Wild Animal Habitat	2	1
Louisville Zoological Garden	1	2
Metropolitan Toronto Zoo	2	2
Oklahoma City Zoological Park	3	4
The Phoenix Zoo	4	3
Rio Grande Zoological Park	0	3
St. Louis Zoological Park	3	3
San Antonio Zoological Gardens and Aquarium	1	1
San Diego Wild Animal Park	2	4
Toledo Zoological Gardens	5	2
White Oak Plantation	10	10

With the exception of ~28% of the males being 25 to 48 months of age, all other groups were fairly equally represented (~11 to 23%) across age groups through 10 years; 2 outlier males were >168 months old. Twenty-six and ~28% of surveyed females were 25 to 48 and 73 to 96 months old, respectively, with 2.9 to 15% of the remaining population distributed from the 0 to 24 through the 169 to 192-month age intervals.

A key to assessing reproductive fitness within any population is having access to both proven and non-proven breeders. There is an understandable resistance to subjecting known breeders to the potential risk of anesthesia and reproductive manipulation. Nonetheless, the host institutions provided extraordinary access to these animals; 20.3% of the surveyed population (see below) had produced offspring in the past. Given the relatively few successful breeders in the entire North American population, an excellent sampling was available.

Management Conditions

Discussions with curatorial, veterinary, and animal care staffs revealed major differences in cheetah management protocols among institutions. For example, there were significant variations among zoos in the number, size, and location of enclosure(s) in the exhibit or off-site breeding areas. Some institutions constantly maintained different proportions of males and females together while others consistently reintroduced different males to singleton or cohorts of females. Usually, females were housed individually or in pairs or trios. Every institution provided animals free access to outdoor enclosures ranging from 5.9 to 24,291 square meters in size. All cheetahs were fed a carnivore diet, most often Nebraska Canine Diet (North Platte, NE) with supplements of chicken, beef, rabbit, and/or venison. Usually animals were fasted 1 day/week. Males, when not maintained with females, usually were housed individually. Any given management scheme appeared to be based largely upon personal experience of local staff or anecdotal reports of others. Because of this marked
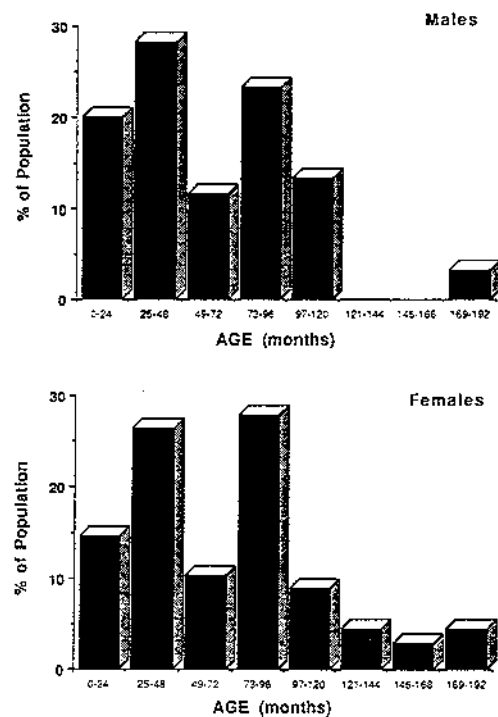
Fig. 1.   Age distribution of surveyed cheetahs.

interinstitutional variability, it was impossible to formally correlate any physiological finding to a specific management factor.

General Protocol and Anesthesia

The systematic physiological and health examination required hands-on access to the animals, which necessitated using a dissociative anesthetic used extensively in previous cheetah studies [Wildt et al., 1987b; Donoghue et al., 1992; Howard et al., 1992]. Males were induced into a surgical plane of anesthesia with Telazol® (A.H. Robins Company, Richmond, VA; 3 to 6 mg/kg estimated body weight, i.m.), which then allowed electroejaculation followed by serial blood sampling. The initial drug dose was delivered by blow-pipe darting or hand-injection. The protocol for females required a 120 min interval of blood sampling (for endocrine evaluation) followed by a laparoscopic evaluation of abdominal cavity content. To achieve this, anesthesia was induced using the same Telazol dosage and delivery approach. To maintain an appropriate level of anesthesia in both males and females, each animal was titrated using supplemental Telazol and/or ketamine hydrochloride (Ketaset, Fort Dodge Laboratories, Fort Dodge, IA). If the level of anesthesia was insufficient early in the procedure, Telazol was given i.m., which produced a medium-duration effect. If the

anesthetic level was inadequate near the end of the procedure, then ketamine HCl was given i.v., which resulted in a relatively short effect. Immediately after collecting the last serial blood sample in females, each cheetah was intubated and maintained on halothane or isofluorane/oxygen anesthesia throughout the laparoscopic examination. Throughout the entire anesthesia interval, the physiological status of all males and females was monitored using electrocardiography and/or pulse oximetry and by making periodic measurements of indirect blood pressure, heart and respiration rate, and rectal temperature. To avoid perturbing endocine data, routine medical procedures (e.g., dental work, nail clipping) were not performed until all blood sampling was completed. At the end of the evaluation, each cheetah was injected i.m. with a long-acting antibiotic as a prophylactic measure, and one liter of lactated Ringers solution was administered by i.v. drip to enhance the renal clearance of the Telazol, and shorten recovery.

Animal Weight and Body Measures

After anesthetic administration and immediately upon becoming tractable, each cheetah was weighed. In addition to providing additional data for the survey, weight information was used to calculate supplemental Telazol dosages and, for females, the dose of gonadotropin releasing hormone (GnRH) needed to test pituitary function (see below). Using a flexible measuring tape, 2 body measures were obtained: 1) chest girth, or the circumference of the chest at a point immediately posterior to the front limbs and over the widest part of the chest; and 2) crown–rump length, or body length from the occipital crest of the skull to the base of the tail.

Testes Measurement, Penile Appearance, and Seminal Analyses

For males, both testes were palpated thoroughly for tone and the absence of abnormalities. Laboratory calipers were used to measure the length and width (to the nearest 0.01 cm) of each testicle. A previously described formula [Howard et al., 1986] was used to calculate combined testes volume (cm^3) for each animal. The penis was extruded from the sheath and examined for anomalies and the presence of a normal complement of spines.

To permit consistent analyses among animals, all males were subjected to a standardized electroejaculation regimen described previously for the cheetah [Wildt et al., 1983a, 1987b, 1988]. In brief, each animal received 80 electrical stimuli of similar voltage (4 to 8 volts) administered over an ~25 min interval. An AC, 60 Hz, sine-wave electroejaculator (P–T Electronics, Boring, OR), and a 1.6 cm in diameter, lubricated rectal probe with 3 longitudinal electrodes were used to deliver the stimuli in 3 series of 30, 30, and 20 stimuli, respectively. A 3 to 5 min rest period was permitted between series.

Semen from each series was collected into a warmed, sterile container (37°C) and analyzed separately from the remaining 2 series/ejaculate. Seminal volume was recorded immediately. All microscopic evaluations of sperm motility were performed on a 37°C microscope stage within 3 to 5 min of ejaculation. Overall sperm motility ratings within each series were measured in raw, undiluted ejaculate. Spermatozoal percent motility and forward progressive motility were evaluated independently by 2 team members, each examining at least 4 separate microscopic fields (400 ×). The 2 overall estimates of motility were averaged and rounded to the nearest whole number. Forward progressive motility was based upon a graded scale of 0 to 5, with 0 repre-

senting no motility and 5 representing steady, rapid forward progression [Wildt et al., 1983a; Howard et al., 1986]. A sperm motility index (SMI), a value that unifies overall percent motility with progressive motility [Howard et al., 1990], was calculated as follows: SMI = [(percent sperm motility) + (sperm progressive motility $\times$ 20)] divided by 2. Using values generated from each series, appropriate calculations were made for total ejaculate volume and sperm motility ratings. Spermatozoal concentration/ml of ejaculate was calculated using a hemocytometer counting procedure [Wildt et al., 1983a, 1987b]. The total number of motile spermatozoa/ejaculate was calculated by multiplying the sperm concentration/ml of ejaculate times the ejaculate volume times the percent sperm motility value.

Gross morphological assessments were made by fixing a 10 μl seminal aliquot in 0.03% glutaraldehyde and later evaluating 200 spermatozoa/ejaculate under phase-contrast microscopy (1,000 $\times$). Structurally-normal and pleiomorphic sperm forms were recorded according to previous criteria [Wildt et al., 1983a, 1987b, 1988; Howard et al., 1986, 1990].

Sperm Function In Vitro

Two assays were used to characterize the viability and functionality of collected cheetah spermatozoa: 1) longevity of sperm motility in vitro, and 2) the zona pellucida (ZP)-intact oocyte penetration assay. In the motility assay, each ejaculate was divided and left untreated or gently diluted 1:1 with Ham's F10 culture medium (Irvine Scientific, Santa Ana, CA) containing 5% fetal calf serum (Irvine Scientific). Diluted semen was centrifuged (300g, 10 min), supernatant discarded, and the sperm pellet resuspended gently in Ham's F10 to a final concentration of 4 $\times$ 10^6 motile sperm/ml. Aliquots were maintained at 38°C in a 5% CO_2 in air, humidified environment and percent sperm motility and progressive motility evaluated at hourly intervals for 7.5 hr. These values then were used to calculate SMI profiles over time for each cheetah.

The oocyte penetration assay took advantage of the ability of the bilayered, domestic cat ZP to be bound and penetrated by heterospecific felid spermatozoa [Howard and Wildt, 1990; Wildt, 1991; Andrews et al., 1992; Wildt et al., 1992b]. In essence, this assay measured the binding/penetration potential of sperm in a type of in vitro fertilization (IVF) system. In the absence of scarce cheetah oocytes for laboratory testing, domestic cat oocytes (commonly obtained from local veterinary clinics) were used to test sperm function. In recent studies, we determined that salt-stored domestic cat ZP were penetrated as efficiently by cheetah spermatozoa as fresh cheetah ZP [Howard et al., 1991a; Donoghue et al., 1992]. In brief, this assay involved recovering domestic cat oocytes from ovaries after ovariohysterectomy. Oocytes were matured in vitro (2 μg/ml FSH, 5 μg/ml LH, and 1 μg/ml estradiol) [Johnston et al., 1989] for 48 hr, rinsed in 0.2% hyaluronidase to remove cumulus, and stored at 4°C in a hypertonic salt solution [0.5 M $(NH_4)_2SO_4$, 0.75 M $MgCl_2$, 0.2 mM $ZnCl_2$, 0.1 mg/ml polyvinylalcohol (PVA), and 40 mM Hepes buffer (pH 7.4)] [Andrews et al., 1992]. In this fashion, oocytes collected and processed in Washington, DC could be transported to all participating institutions for subsequent sperm testing.

Prior to semen collection, oocytes were removed from the salt solution and rinsed twice (1 hr/rinse) in equilibrated (protein-free) Ham's F10. Washed sperm in the motility longevity assay were used for the oocyte penetration assay. Following a

pre-incubation interval of 1 hr at 38°C, a 5 μl aliquot of pre-incubated sperm suspension (2 $\times$ 10^5 motile sperm/ml) was added to a 95 μl drop of equilibrated Ham's F10/5% FCS containing 12 ZP-intact cat oocytes. Sperm were co-incubated with oocytes for 6 hr at 38°C in 5% CO_2 in air. Oocytes then were fixed in 2.0% glutaraldehyde and 2.0% formaldehyde and assessed by differential interference–contrast microscopy (320 $\times$) for ZP penetration. Categories of ZP penetration included: 1) <1/2 ZP, or the percentage of oocytes with sperm in the outer half of the ZP; 2) >1/2 ZP, or the percentage of oocytes with sperm in the inner ZP; or 3) % PVS, or the percentage of oocytes with sperm in the perivitelline space [Howard et al., 1991a; Andrews et al., 1992]. The number and morphology of sperm, both bound to and penetrating the various layers of the ZP, were recorded.

Endocrine Evaluations

For males, blood samples (7 ml each) were obtained by saphenous venipuncture immediately before semen collection was begun, after each electroejaculation series, and at 15, 30, 45, and 60 min after the last electrical stimulus. Thus, a total of 8 samples (56 ml of blood) was collected for hormonal analysis. Mean time intervals from onset of anesthesia through the end of electroejaculation were from pre-electroejaculation to end of Series 1, 8.8 $\pm$ 0.5 min; from end of Series 1 to end of Series 2, 6.9 $\pm$ 0.3 min; and from end of Series 2 to end of Series 3, 7.2 $\pm$ 0.3 min. Because the duration of electroejaculation averaged 24.2 $\pm$ 0.7 min, the blood sampling interval spanned ~90 min. After a 1 hr clotting interval, blood samples were centrifuged (1,000g, 20 min), the serum recovered, stored in individually labelled tubes (-20°C), and later analyzed for serum luteinizing hormone (LH), follicle stimulating hormone (FSH), and testosterone by radioimmunoassay (RIA; see below).

Serial blood samples from females were used for the purpose of confirming the level of ovarian activity and for analyzing pituitary function. The latter was achieved using a classical hormonal challenge strategy whereby an aqueous bolus of synthetic gonadotropin releasing hormone (GnRH; Cystorelin, Abbot Laboratories, Chicago, IL) was given to induce the pituitary to release both FSH and LH [Wildt et al., 1983b]. Blood samples (7 ml each) from females were obtained by the same route used for males and coincident with the onset of surgical anesthesia. Three consecutive "control" samples (designated the pre-GnRH samples) were obtained at 5 min intervals after which GnRH was injected i.v. Blood samples then were collected at 10 min intervals for an additional 80 min, and a final blood sample was taken immediately after laparoscopy. Thus, 12 samples and 84 ml of blood were obtained from each female for hormonal analysis. Serum was collected and stored as described for the male. All samples were evaluated for LH, FSH, estradiol-17β, and progesterone concentration by RIA.

Radioimmunoassays

Serum LH was quantified using a validated double antibody RIA as described previously [Brown et al., 1991]. The assay utilized a rabbit anti-bovine LH antiserum, ovine LH standards (NIH-LH-S18), and an ovine LH label (LER-1374-A). Binding inhibition curves produced by the displacement of ^{125}I-labelled LH by standard and cheetah serum were parallel. Recovery of 0.031, 0.063, 0.125, 0.25, 0.5, 1.0, or 2.0 ng of ovine LH from cheetah serum was 0.029, 0.065, 0.131, 0.24, 0.54, 1.11, and 1.96 ng respectively after subtraction of endogenous hormone ($y = 0.98 \times$

+ 0.02; r = 0.99). Assay sensitivity, defined as 90% of maximum binding, was 0.15 ng/ml. Inter- and intra-assay coefficients of variation were 8.9 and 6.8%, respectively.

Serum FSH was measured using a double antibody RIA [Brown et al., 1987], which employed a rabbit anti-ovine FSH antiserum, ovine FSH standards (NIADDK-FSH-S8), and an ovine FSH label (LER-1976-A2). Serial dilutions of cheetah serum were parallel to the FSH standard curve. Addition of 2.5, 5.0, 10.0, 20.0, 40.0, or 80.0 ng ovine FSH to cheetah serum resulted in a recovery of 2.6, 4.9, 12.3, 17.9, 39.8, and 78.8 ng respectively after subtraction of endogenous hormone ($y = 0.98 \times + 0.35$; $r = 0.99$). Assay sensitivity was 2.0 ng/ml, and the inter- and intra-assay coefficients of variation were 7.9 and 5.4%, respectively.

Serum estradiol-17β was determined using a solid phase ^{125}I RIA kit (Coat-A-Count®, Diagnostic Products Corporation, Los Angeles, CA). Serial dilutions of cheetah serum pools were parallel to the standard curve. Addition of 10, 30, 100, 300, and 1,000 pg estradiol to cheetah serum resulted in a net recovery of 12, 26, 105, 319, and 1,050 pg respectively after subtraction of endogenous hormone ($y = 1.05 \times - 0.44$; $r = 0.99$). Assay sensitivity was 2.5 pg/ml, and the inter- and intra-assay coefficients of variation were 11.5 and 10.2%, respectively.

Serum progesterone was measured using a double antibody ^{125}I RIA kit (ICN Biomedicals, Costa Mesa, CA). Serial dilutions of cheetah serum pools were parallel to the standard curve. Upon addition of 0.25, 1.0, 2.5, 5.0, 10.0, and 20.0 ng progesterone to cheetah serum, 0.35, 1.03, 2.41, 4.14, 9.97, and 19.71 ng respectively were recovered after subtracting endogenous hormone ($y = 0.99 \times - 0.12$; $r = 0.99$). Assay sensitivity was 0.1 ng/ml, and the inter- and intra-assay coefficients of variation were 9.1 and 6.1%, respectively.

Serum testosterone was measured using a double antibody ^{125}I RIA kit (ICN Biomedicals, Costa Mesa, CA). Binding inhibition curves of cheetah serum and the testosterone standards were parallel. Upon addition of 0.05, 0.125; 0.25, 0.5, 1.25, and 2.5 ng testosterone to cheetah serum, 0.06, 0.13, 0.28, 0.47, 1.23, and 2.69 ng respectively were recovered after subtracting endogenous hormone ($y = 1.06 \times - 0.02$; $r = 0.99$). Assay sensitivity was 0.02 ng/ml, and the inter- and intra-assay coefficients of variation were 8.7 and 7.8%, respectively.

The ^{125}I steroid RIA kits described above were developed for use with unextracted human serum. Analysis of extracted and unextracted cheetah pools (n = 10) yielded similar results. Therefore, samples were assayed unextracted.

Laparoscopy

Immediately after blood sampling, each female cheetah was placed in a supine, head-down position on a surgical table, surgically prepared and draped, and the abdominal cavity insufflated with room air using a hand pump and a 2 mm in diameter Verres needle probe inserted transabdominally (Richard Wolf Medical Instruments Corporation, Rosemont, IL). A 10 mm in diameter trocar-cannula unit followed by a 180° laparoscope (Richard Wolf Medical Instruments Corporation) was inserted into the abdominal cavity through a 2 cm long skin incision made near the umbilicus [Wildt et al., 1981; Phillips et al., 1982]. The laparoscope, attached to an external light source via a fiberoptic cable (Richard Wolf Medical Instruments Corporation), was used to examine the reproductive tract in detail from the bifurcation of uterine body–cornua along the entire length of each uterine horn to the tubo–uterine

junction, ovary, oviduct (encapsulated in the ovarian fimbria), and finally the broad ligament. The Verres needle was used to manipulate the broad ligament, thereby allowing all aspects of each ovary to be viewed, and to examine, count, and measure ovarian follicles, corpora lutea (CL), and parovarian cysts (commonly observed in the species; see Results). A photographic record of the entire reproductive tract of each cheetah was made by means of a video recorder attached to the laparoscope via an endoscopic video camera (Wolf Medical Instruments Corporation).

Other Biomaterials Collected

At the onset of surgical anesthesia, 30 ml of heparinized and 13 ml of nonheparinized blood were obtained for conducting hematology/blood chemistry and genetic analyses by other survey investigators. Aliquots from these samples also were stored for future reference and epidemiology studies. Blood for clinical pathology studies was forwarded to a single analytical laboratory.

During laparoscopy, the remaining abdominal cavity content was scanned, including portions of the gastrointestinal tract, spleen, liver, and gall bladder. To facilitate the ongoing disease/pathology/genetic survey, 3 liver biopsies were obtained from each female during laparoscopy. In brief, this involved inserting a secondary 5 mm in diameter trocar-cannula unit lateral to the midline. The trocar was removed and replaced with a grasping biopsy forceps (Richard Wolf Medical Instruments Corporation) that was used to recover 4 mm^3 sections of hepatic tissue that were stored in Trumps fixative (2 samples) for histopathology or snap-frozen in liquid nitrogen (1 sample) for later genetic or histochemical analysis. Immediately after laparoscopy, a 5 × 10 mm skin biopsy was taken with a scalpel blade from the edge of the midline laparoscope insertion site. This tissue, scheduled for DNA extraction and tissue culture, was stored in appropriate medium, and all materials forwarded via air express to other appropriate survey investigators.

Finally, the disease/pathology survey included culturing for vaginal, seminal, and rectal flora. Therefore, sterile culture swabs were inserted into the vaginal vault, placed in Amies transport medium (with and without charcoal), stored at 5°C, and shipped within 24 to 36 hr to Cornell University Diagnostic Laboratory for analyses. For females, the final evaluation involved finger-palpating the vaginal os and vault immediately after taking the culture to determine normality of size and absence of anomalies. Survey results on disease/pathology, blood hematology/chemistry, and genetics have been summarized by others (see this issue).

Data Presentation and Statistics

Average values are presented as means ± standard errors of the mean (SEM). Hormonal means were compared using Student's t test or Duncan's New Multiple Range tests. All testes and semen characteristics were evaluated using analysis of variance, and correlation coefficients were calculated using a computerized (Statview, BrainPower Inc., Calabasas, CA) program.

RESULTS

Safety and Efficacy of Anesthesia/Manipulatory Procedures

Telazol was a safe and effective anesthetic, allowing electroejaculation (males) and serial blood sampling (males and females). At a Telazol dosage of 4 to 6 mg/kg body weight, cheetahs generally were ataxic within 2 min of injection, and tractable

within 6 to 10 min. Within 4 hr of the end of evaluation, most cheetahs were becoming alert and maintaining a sternal position. Complete recovery from all anesthetic effects usually required 24 hr due to the overall length of the evaluative episode. No animal mortality and minimal post-operative anesthesia morbidity occurred. One female cheetah, with a history of fracturing bones, pivoted sharply during blow-pipe delivery of the anesthetic and sustained a comminuted, proximal femoral fracture. She regained full function of the limb following surgical repair with bone plates and cerclage wiring.

There was no evidence that the anesthesia/evaluation protocol interfered with the ability of any cheetah to later mate, conceive, or produce offspring. To date, 5 of the previously evaluated males and 9 of the females have produced young. Two striking examples illustrated the innocuous effects of the survey. At one institution, an 82 month-old male and a 39 month-old female, neither of which had ever mated or produced young, copulated for the first time 17 days after being evaluated. The female conceived and gave birth to 7 cubs after a normal length gestation. At another institution, an 84 month-old female was subjected to the anesthesia/serial blood sampling/laparoscopy protocol 27 days after being introduced to an adult male. Laparoscopic observation of individual, segmented swellings of the uterine cornua indicated that the female was pregnant. Despite being subjected to the entire evaluative procedure, the female sustained the pregnancy and delivered 3 live cubs 66 days later.

Animal Weight and Body Measures

Male cheetahs ranged in body weight from 27.9 to 51.0 kg (mean 40.2 ± 0.7). All had normal body conformation, except for a single 86 month-old, dwarfish male with a body mass <28 kg. Females ranged from 25.0 to 46.4 kg in body weight (mean 35.0 ± 0.6), and all had normal body conformation. For males, the correlation coefficients between chest girth or crown–rump length and body weight were 0.68 and 0.54, respectively ($P < 0.01$). Likewise, r values for chest girth or crown–rump length and body weight for females were significant (0.52; 0.33, $P < 0.05$).

Testes Volume, Penile Appearance, and Seminal Analyses

Testes volume and seminal characteristics (mean and range values) for the entire survey population (n = 60) and for the proven (n = 12) versus unproven (n = 24) subpopulations are presented in Table 3. Overall, there was no difference ($P > 0.05$) in the volume or tone of the left versus right testis (data not shown). Combined testes volume, however, was correlated to specific ejaculate characteristic including semen volume (r = 0.46; $P < 0.01$), SMI (r = 0.37; $P < 0.01$), motile sperm/ejaculate (r = 0.33; $P < 0.05$), and the proportion of structurally-normal sperm/ejaculate (r = 0.41, $P < 0.01$).

Overall, 59 of the 60 male cheetahs (98.3%) produced sperm in the resulting electroejaculate. The single aspermic male was the 86 month-old cheetah of small body size. Cheetahs previously successful at siring offspring had similar ($P > 0.05$) characteristics to males exposed to females, but failing to produce young (Table 3). Although sperm concentration/ml of ejaculate was almost 2-fold higher in proven versus unproven cheetahs, the difference was nonsignificant, in part, because of individual animal variation.

All male cheetahs produced high proportions of structurally-abnormal sperm/ejaculate; in general, more than 75% of all sperm in a given ejaculate were pleio-

TABLE 3. Testes volume and seminal traits (mean ± SEM) of surveyed cheetahs

Reproductive trait	All cheetahs (n = 60)	Proven (n = 12)	Unproven[a] (n = 24)
Combined testes volume (cm³)	13.9 ± 0.4	15.7 ± 0.7	13.1 ± 0.5
Semen volume (ml)	1.5 ± 0.1	1.7 ± 0.3	1.5 ± 0.2
Sperm count/ml of ejaculate ($\times 10^6$)	29.3 ± 5.6[b]	50.1 ± 23.3	27.4 ± 4.8
Sperm motility (%)[a]	67 ± 2[b]	66 ± 4	71 ± 3
Sperm progressive status[a]	3.6 ± 0.1[b]	3.5 ± 0.2	3.7 ± 0.1
Sperm motility index[a]	69.1 ± 1.8[b]	67.7 ± 4.3	72.0 ± 2.4
Motile sperm/ejaculate ($\times 10^6$)	31.4 ± 5.6[b]	45.0 ± 18.3	34.0 ± 8.3
Structurally-normal sperm/ejaculate (%)	21.3 ± 2.0[b]	19.6 ± 3.2	23.2 ± 2.9

[a]See text for definition. Twelve males without the opportunity to mate were excluded from the calculations.
[b]Excludes single, aspermic male.

morphic (Table 4). The preponderance of sperm defects was associated with the midpiece (24.8%) and flagellar (30.6%) regions of the cell. However, 9.3% of all sperm heads contained an anomaly, and >35% of the cells were immature as evidenced by the presence of a residual cytoplasmic droplet located proximally or distally on a structurally-normal or abnormal midpiece. There were no differences ($P > 0.05$) in the proportion of normal or specific abnormal sperm forms between the proven versus unproven subpopulations (Table 4).

No anomalies in penile structure were evident, with the exception of a single, 38 month-old, unproven male that presented a 2.3 cm high, spiked papillus located on the penile shaft 3.5 cm from the penis tip. All males had multiple, visible spines along the penile shaft, and electroejaculation elicited full and similar-sized erections among males.

Sperm Function In Vitro

Undiluted cheetah spermatozoa maintained in vitro, under the conditions described in Materials and Methods, experienced a rapid loss in sperm motility over a 2 hr interval (Fig. 2a). Processing the semen by dilution in Ham's F10 containing FCS extended ($P < 0.05$) sperm motility through 7.5 hr. There was no difference ($P > 0.05$) in sperm longevity over time in processed semen from the proven versus unproven subpopulations (Fig. 2b).

Cheetah sperm co-cultured in vitro with salt-stored, domestic cat oocytes were capable of binding to and penetrating domestic cat ZP (Table 5; Fig. 3). Overall, >75% of the oocytes had cheetah sperm entering the outer half of the ZP. However, fewer than 40% of the cat oocytes had cheetah sperm within the inner half of the ZP, and fewer than 6% of the oocytes had sperm within the PVS (Table 5). Detailed examination of the salt-exposed cat oocytes demonstrated the gradual decreasing influence of structurally-abnormal sperm during gamete interaction. Fifty-nine percent of the cheetah sperm bound to the outer ZP and 16% of the sperm within the outer ZP were morphologically abnormal. However, only structurally-normal sperm reached the inner ZP sanctum or the PVS, indicating that this component of the oocyte serves an important "filtering" role in eliminating pleiomorphic spermatozoa. Using inseminates from proven males resulted in ~11% more oocytes with sperm in the inner half of the ZP, but this difference was not significant ($P > 0.05$). The

TABLE 4. Incidence (mean ± SEM) of various types of sperm pleiomorphisms in surveyed cheetahs

Incidence of sperm structure (%)	All cheetahs (n = 59)	Proven (n = 12)	Unproven[a] (n = 23)
Head anomaly			
macrocephalic	0.8 ± 0.2	0.8 ± 0.2	1.0 ± 0.3
microcephalic	4.4 ± 0.9	5.0 ± 1.4	4.7 ± 2.0
bicephalic	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.3
abnormal acrosome	3.7 ± 0.4	4.4 ± 1.0	3.8 ± 0.7
Midpiece anomaly			
abnormal or missing midpiece	0.9 ± 0.2	0.8 ± 0.3	0.4 ± 0.1
bent midpiece with cytoplasmic droplet	21.1 ± 1.1	23.1 ± 2.4	22.0 ± 1.6
bent midpiece without cytoplasmic droplet	2.8 ± 0.4	4.3 ± 1.4	3.0 ± 0.5
proximal droplet	11.0 ± 0.9	12.6 ± 2.0	10.9 ± 1.2
distal droplet	3.0 ± 0.4	3.3 ± 0.7	3.1 ± 0.5
Flagellar anomaly			
lightly coiled flagellum	27.4 ± 2.5	23.9 ± 4.6	23.3 ± 3.3
bent flagellum with cytoplasmic droplet	0.6 ± 0.1	0.3 ± 0.2	1.0 ± 0.3
bent flagellum without cytoplasmic droplet	2.6 ± 0.8	1.5 ± 0.4	3.1 ± 1.9
Total overall mean ± SEM	78.7 ± 2.0	80.4 ± 3.2	76.8 ± 2.9

[a]See text for definition. Twelve males without the opportunity to mate were excluded from the calculations.

percentage of oocytes with PVS sperm was similar ($P > 0.05$) between the proven and unproven populations.

To better illustrate the significance of these results, cheetah co-culture results are also presented in Table 5 in the context of similar data recently collected from normospermic leopard cats [Howard and Wildt, 1990]. Leopard cat sperm were co-incubated with salt-stored domestic cat oocytes under the same conditions as described for the cheetah. For the leopard cat, a higher proportion of oocytes was penetrated at the level of the inner ZP and the PVS (Table 5) than that observed using cheetah sperm. Finally, on a species basis, there were more sperm in the inner ZP and PVS using leopard cat compared to cheetah inseminates (Table 5).

Endocrine Evaluation of Males

Serum LH, FSH, and testosterone concentrations (mean and mean ranges among individual males) for the entire male population are depicted in Table 6. The mean temporal hormone profile during the electroejaculation/blood sampling interval for the entire surveyed population or proven versus unproved subpopulation did not change ($P > 0.05$) over time (data not shown). Figure 4 illustrates the LH, FSH, and testosterone concentrations in 3 representative males demonstrating the temporal secretory nature of the 3 hormones. Occasional episodic spikes of LH activity (usually 1 to 2 surges/~90 min interval, reflecting a 1.5- to 4-fold increase above nadir concentrations) were observed in 23 of 60 (38.3%) males. There was no evidence of distinct FSH pulses. In 47.5% of the males, testosterone tended to decrease (~50%) during the sampling period. However, in the other males, testosterone tended to increase (16.9% of population), increase and then decrease (8.5%), or remain static (27.1%). No statistical relationship was measured between the observed pulses of LH and testosterone, either at coincidental or lagged time points (data not shown).
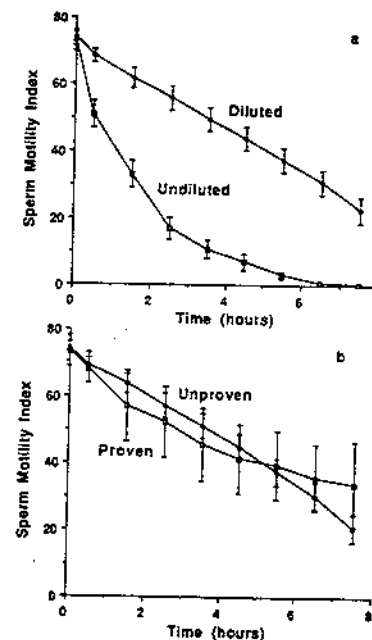
Fig. 2. Sperm longevity in vitro in (a) raw, undiluted cheetah ejaculate versus semen diluted in Ham's F10 culture medium, and in (b) Ham's F10-diluted semen from proven versus unproven breeders.

There were no differences ($P > 0.05$) in either overall mean LH, FSH, or testosterone concentrations (Table 6) or temporal profiles (data not shown) between the proven and unproven subpopulations. However, there was a positive correlation within the overall population between mean circulating testosterone and (1) combined testes volume ($r = 0.33$; $P < 0.01$) and (2) motile sperm/ejaculate ($r = 0.36$; $P < 0.01$), but (3) not percentage structurally-normal sperm ($r = -0.04$; $P > 0.05$). Significant positive correlations were not found ($P > 0.05$) between LH or FSH and these same testicular/ejaculate traits.

Male Results on the Basis of Age

Males at both extremes in the age spectrum produced spermic ejaculates, including a 15-year-old cheetah producing comparatively low testosterone (mean 0.14 ng/ml) that ejaculated ~0.7 × 10^6 total motile spermatozoa. Fourteen of the surveyed animals were 15 to 26 months of age, and all of these males produced motile spermatozoa in the ejaculate.

To examine the impact of age on testes volume, sperm production, the incidence of sperm pleiomorphisms, and circulating testosterone, we arbitrarily categorized males on the basis of 3 age groups: 1) 15 to 25 months (young, subadult, or early-post-pubertal males); 2) 60 to 120 months (fully-adult, presumably prime breed-

TABLE 5. Percent binding and penetration of ZP-intact domestic cat oocytes by sperm from cheetahs and leopard cats

	Cheetah			
	Overall male (n = 44)	Proven (n = 9)	Unproven (n = 20)	Leopard cat[a] (n = 3)
Normal sperm/ejaculate (%)	24.7 ± 2.4	23.9 ± 3.0	23.7 ± 3.2	60.4 ± 0.2
Number of cat oocytes	438	102	192	95
ZP penetration (%)[b]				
<1/2 ZP	77.5 ± 4.8	74.2 ± 10.5	77.6 ± 5.9	90.0 ± 3.3
>1/2 ZP	37.8 ± 4.7	38.4 ± 11.3	26.6 ± 5.3	58.4 ± 3.0
PVS	5.6 ± 2.4	2.9 ± 2.0	2.1 ± 1.0	35.4 ± 5.4
Number of sperm in ZP layers				
<1/2 ZP	7.6 ± 1.2	12.8 ± 4.4	5.1 ± 1.1	13.9 ± 3.1
>1/2 ZP	0.8 ± 0.2	1.3 ± 0.7	0.5 ± 0.2	3.9 ± 0.4
PVS	0.1 ± 0.1	0.03 ± 0.02	0.03 ± 0.02	2.1 ± 0.3

[a]From Howard and Wildt, 1990.
[b]Definitions: <1/2 ZP = % of oocytes with sperm in the outer half of the ZP; >1/2 ZP = % of oocytes with sperm in the inner half of the ZP; PVS = % of oocytes with sperm within the perivitelline space.
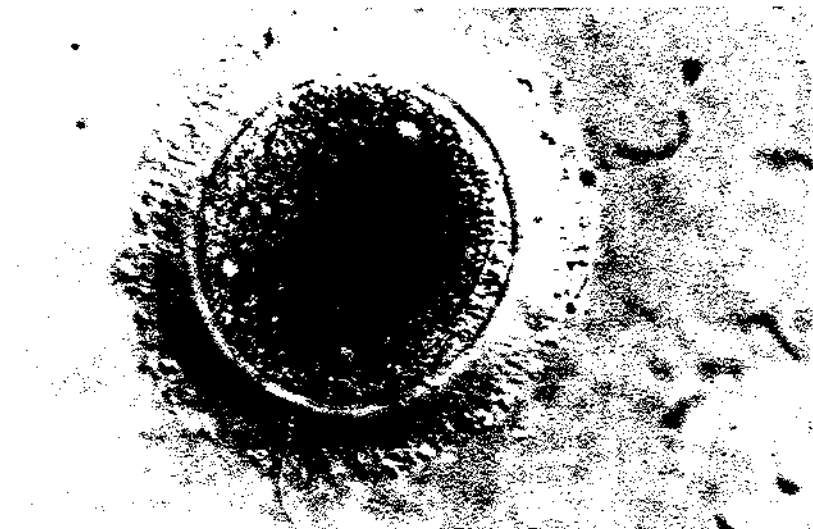

Fig. 3.   Salt-stored, domestic cat oocyte co-cultured with cheetah spermatozoa. Cheetah sperm are bound to and are within the outer and inner layers of the domestic cat ZP.

ing-age males); and 3) 172–182 months (aged adults having reached or approaching reproductive senescence) (Table 7). Both testes volume and the percentage of ejaculated sperm that were pleiomorphic were similar ($P > 0.05$) among age groups. However, mean motile sperm/ejaculate and circulating testosterone concentration increased ($P < 0.05$) with age (from 15 to 25 months through 60 to 120 months).

TABLE 6. Circulating concentrations of LH, FSH, and testosterone in adult cheetahs over an ~90 min interval of anesthesia/electroejaculation and blood sampling

	LH (ng/ml)	FSH (ng/ml)	Testosterone (ng/ml)
Overall concentration (mean ± SEM; n = 60)	0.64 ± 0.05	5.33 ± 0.59	0.34 ± 0.05
Mean range concentration among males[a] (n = 60)	0.15–1.51	2.50–26.81	0.02–1.89
Minimum individual value measured	0.015	2.5	0.02
Maximum individual value measured	2.4	29.2	2.54
Mean (± SEM) concentration for proven subpopulation (n = 12)	0.64 ± 0.12	6.03 ± 1.66	0.35 ± 0.13
Mean (± SEM) concentration for unproven subpopulation (n = 24)	0.67 ± 0.07	7.55 ± 2.12	0.85 ± 0.46

[a]Mean lowest value among all males to mean highest value among all males.
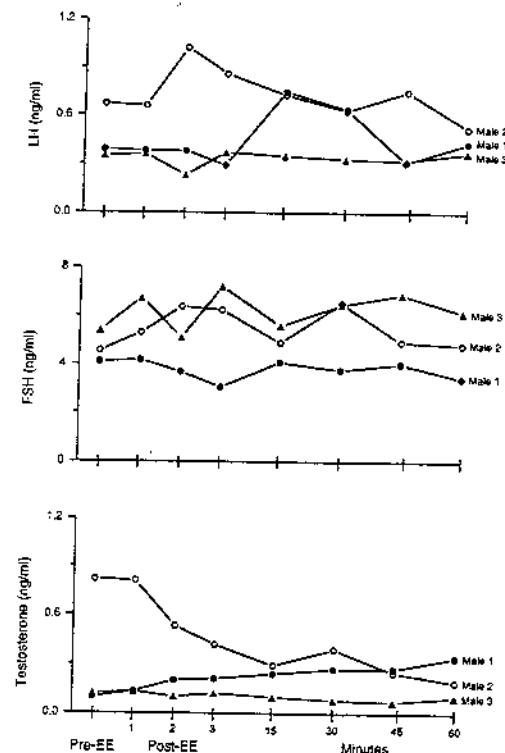

Fig. 4.   Circulating LH, FSH, and testosterone concentrations in 3 adult cheetahs during anesthesia, and before, during, and for a 60-min period after an electroejaculation episode.

There were no differences ($P > 0.05$) in mean LH or FSH between the 15 to 25-month and 60 to 120-month-old groups. In contrast, the 2 aged males produced few sperm/ejaculate and baseline LH and testosterone concentrations.

TABLE 7. Relationship of testes size, ejaculate characteristics, and circulating LH, FSH, and testosterone in male cheetahs of different age groups*

	Cheetah age (months)		
	15 to 25 (n = 12)	60 to 120 (n = 26)	172 to 182 (n = 2)
Testes volume (cm³)	11.5 ± 0.7	14.5 ± 0.6	14.3 ± 0.5
Motile sperm/ejaculate (× 10⁶)	12.6 ± 5.1[a]	37.2 ± 9.7[b]	0.5 ± 0.3[c]
Pleiomorphic sperm/ejaculate (%)	85.1 ± 3.7	79.1 ± 2.5	95.5 ± 2.5
Mean LH (ng/ml)	0.8 ± 0.1	0.7 ± 0.1	0.3 ± 0.0
Mean FSH (ng/ml)	6.8 ± 0.9	7.5 ± 1.1	6.0 ± 4.4
Mean testosterone (ng/ml)	0.1 ± 0.0[a]	0.4 ± 0.1[b]	0.2 ± 0.2[a,b]

*Values are means ± SEM. Row values with different superscripts are different ($P < 0.05$).

Endocrine Evaluation of Females

GnRH administration elicited an increase ($P < 0.05$) in both circulating LH and FSH above pretreatment nadir concentrations in all females (Fig. 5a). Elevations of LH were detected at 10 min after GnRH injection with mean peak concentrations (3.1 ± 0.21 ng/ml) achieved at 30 min, after which LH was sustained for ~80 min before gradually declining. The post-GnRH rise in FSH was not as sharp as observed with LH, but modest (~2-fold) elevations ($P < 0.05$) were evident at 10 min after injection with a sustained peak (~14 ng/ml) achieved within 30 min. There was no difference ($P > 0.05$) in the temporal profiles of LH (Fig. 5b) or FSH (Fig. 5c) between the proven and unproven female subpopulations.

Mean concentrations of circulating LH, FSH, estradiol-17β, or progesterone (pre-GnRH) did not vary ($P > 0.05$) either among individuals in the overall female population (excluding the 2 luteal females) or between proven versus unproven subpopulations (Table 8). In the context of parallel studies involving exogenous gonadotropin stimulation of adult cheetahs for IVF [Donoghue et al., 1992], the absolute estradiol-17β values presented in Table 8 were comparatively low, suggesting minimal estrogen secretion. Figure 6 depicts LH, FSH, estradiol-17β, and progesterone profiles in 3 cheetahs, one with no follicular or luteal activity (female #1), one with distinct ovarian follicles (female #2), and one with prominent CH (female #3). In these cheetahs (and counterparts with similar ovarian activity), LH and FSH profiles responded in a similar temporal fashion to GnRH. Circulating estradiol-17β and progesterone concentrations generally were basal and static in most females. However, the temporal profiles demonstrated by female #2 and #3 (Fig. 6) illustrated how both estradiol-17β and progesterone can vary markedly within individuals, even during a relatively brief time. Thus, these data indicated the importance of multiple blood sampling, even in an acute situation, for accurately evaluating endocrine status.

Female Reproductive Organs

Any female having a normal-appearing uterine body, uterine cornuae, ovaries, oviducts, fimbriae, vaginal os, and vault with no gross abnormalities of the reproductive tract or other abdominal organs was declared reproductively–anatomically sound. Fifty-seven of 68 (83.8%) females met these criteria. Those cheetahs found to be reproductively unsound included: 1) 2 (2.9%) aged females (138 and 108 months of age, respectively) with degenerate/fibrous ovaries; 2) 1 (1.5%) female (175
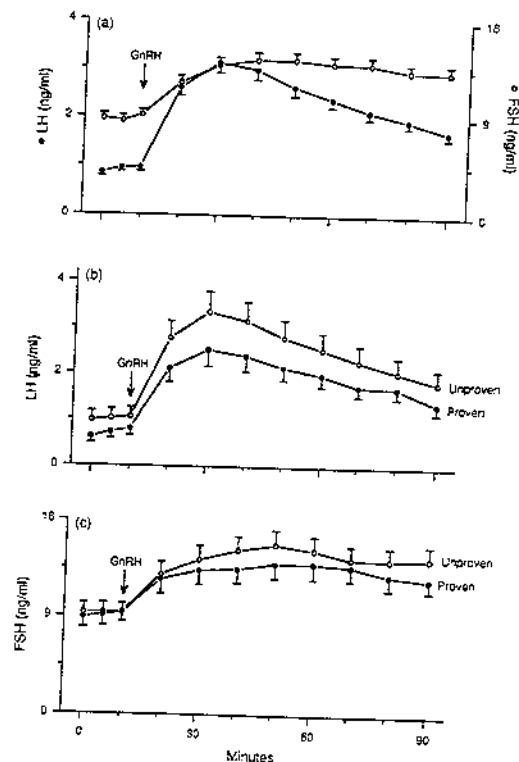
Fig. 5. Mean (± SEM) LH and FSH responses in adult female cheetahs treated with 1 μg/kg body weight GnRH. a represents profiles for the entire surveyed population, whereas b and c represent LH and FSH profiles, respectively, in proven versus unproven subpopulations.

months) with a single ovary; 3) 1 (1.5%) female (80 months) with a unilateral ovarian adhesion to adjacent omentum; 4) 1 (1.5%) female (86 months) with an infantile reproductive tract that included both ovaries and the uterine cornuae; 5) 1 (1.5%) female (152 months) with an ~5 to 6 cm long mass within the left uterine horn; 6) 4 (5.9%) females (106, 76, 96, and 96 months) with an abnormally small vaginal os; and 7) 1 (1.5%) female (60 months) with severe, degenerative hepatic disease.

The most common unusual feature of the cheetah reproductive tract was the presence of parovarian cysts existing as single or multiple fluid-filled pockets located within the tissues of the ovarian fimbria, immediately lateral to the proximal aspect of the ovary and usually adjacent or in close proximity to the oviduct. Based on more detailed, histological studies by Munson [1993], the presence of parovarian cysts was not indicative of anatomical unsoundness. Of the 68 surveyed females, 35 (51.5%) had parovarian cysts (mean 3.7 ± 0.4 cysts/female), including 10 of 14 (71.4%) of the proven breeders compared to 25 of 54 (46.3%) of the unproven cheetahs. On 66% of the occasions when parovarian cysts were observed, their

TABLE 8. Basal concentrations of LH, FSH, estradiol-17β, and progesterone (pre-GnRH challenge) in adult female cheetahs without detectable luteal tissue*

	LH (ng/ml)	FSH (ng/ml)	Estradiol-17β (pg/ml)	Progesterone (ng/ml)
Overall mean (± SEM) concentration (n = 66)	0.91 ± 0.06	8.99 ± 0.51	7.49 ± 0.89	0.21 ± 0.01
Mean (± SEM) range concentration among females[a] (n = 66)	0.10–2.07	2.50–25.90	2.50–48.09	0.10–0.81
Minimum individual value measured	0.1	2.0	2.5	0.1
Maximum individual value measured	2.4	27.6	61.7	2.87
Mean (± SEM) concentration for proven subpopulation (n = 13)	0.71 ± 0.12	9.09 ± 0.87	6.46 ± 0.49	0.23 ± 0.05
Mean (± SEM) concentration for unproven subpopulation (n = 28)	1.00 ± 0.11	9.55 ± 0.88	8.84 ± 1.52	0.24 ± 0.03

*Excludes 2 females that had distinct CH or CL.
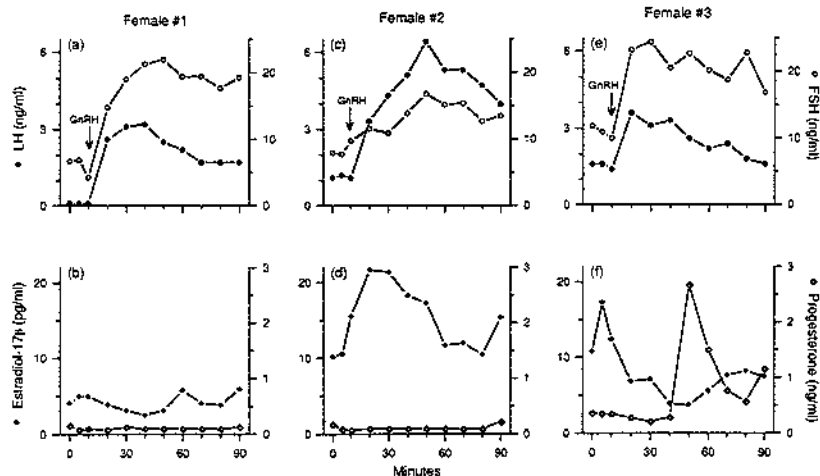[a]Mean lowest value among all females to mean highest value among females.

Fig. 6.   Circulating LH, FSH, estradiol-17β, and progesterone profiles among individual cheetahs demonstrating no follicular activity (a,b), distinct ovarian follicles (c,d), or fresh CH (e,f). Arrows on LH/FSH panels indicate time of GnRH injection.

presence was bilateral. The overall range in cyst diameter was 2.0 to 35.0 mm, with an overall mean of 9.4 ± 1.2 mm. Parovarian cysts were present in all age groups, although the condition was less prevalent (P < 0.05) in females 48 months of age or less (Fig. 7).

With the exception of the one female described above with a uterine mass of unknown etiology, the uterine anatomy of the remaining females appeared normal. The average diameter of the uterine horn was 8.4 ± 0.4 mm (range, 4 to 17 mm). The appearance of the oviduct coursing through the fimbria appeared invariant among cheetahs, and the mean diameter was 3.8 ± 0.1 mm (range, 2 to 7 mm).

Three general ovarian shapes (categories) were identified. In 41 of 68 (60.3%) cheetahs, both ovaries were oblong egg-shaped, and well-rounded on all medial and
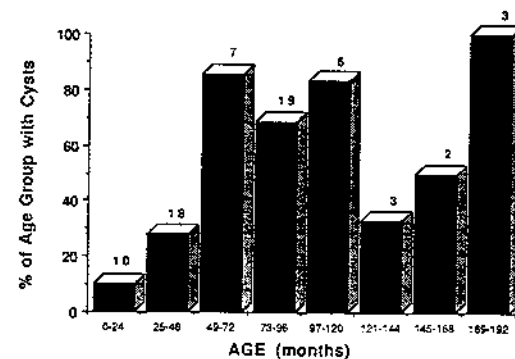
Fig. 7.   Incidence of parovarian cysts in cheetahs within different age groups. The number above each bar represents the number of animals.

lateral aspects (Type I; Fig. 8). In 13 of 68 (19.1%) females, one or both ovaries failed to be universally rounded on the medial and lateral aspects and, instead, appeared slightly flattened, usually along the entire medial length (Type II; Fig. 8). In 14 of 68 (20.6%) cheetahs, one or both ovaries lacked a well-rounded shape, were flattened on both the medial and lateral aspects, and subjectively appeared to have substantially less overall mass (Type III) than Type I or II gonads. In cheetahs with Type II or III ovaries, the condition was bilateral in 63.6% and 78.6% of the cases, respectively; in the remaining animals, the contralateral ovary was always classified as Type I.

Four different structures were observed on the ovaries: 1) follicles (clear, flat areas with or without a distinct border with the surrounding ovarian stroma); 2) corpora hemorrhagica (CH) (fresh ovulation sites, bright red in appearance, 4 to 8 mm in diameter, and raised ~4 mm above the ovarian surface); 3) CL (ovulation sites at least 48 hr of age that were orange-yellowish in coloration, 6 to 7 mm in diameter, and raised 1 to 2 mm above the ovarian surface); and 4) luteal scars (flat yellow spots, 2 mm or less in diameter). Follicles were categorized into 3 size groups, <2 mm, ≥2 mm but <4 mm, and ≥4 mm. In general, follicles ≥2 mm in diameter contain those oocytes most capable of fertilizing in vitro [Donoghue et al., 1992], whereas, based upon previous laparoscopy data [Wildt et al., 1981], follicles ≥4 mm in diameter are believed to be fully mature. Using these criteria, 33.3% of the overall population had ovaries with only indistinct or distinct follicles that were <2 mm in diameter. In contrast, 66.7% of the females had ovaries with distinct follicles ≥2 mm in diameter, but only 22.7% of the population had follicles that appeared fully mature (≥4 mm). Four CH were observed in a singleton, 71 month-old female (1.5%) that still was nursing 4 cubs 6 months post-parturition (mean progesterone concentration, 0.95 ng/ml). This cheetah was maintained only with cubs and had not been exposed to a male since giving birth. Four CL were detected in an 84 month-old female (1.5%) subjected to laparoscopy during the first trimester of pregnancy (mean progesterone, 73.9 ng/ml). One or more luteal scars were present in 13 (19.1%) cheetahs, 5 having experienced pregnancy and parturition 7 to 42 months before laparoscopy, and 8 females that had never produced young.
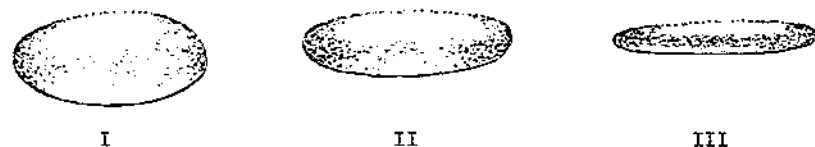
Fig. 8. Three common ovarian shapes identified in cheetahs.

There was no difference ($P > 0.05$) in mean circulating LH, FSH, or estradiol-17β (pre-GnRH) among cheetahs with ovaries producing any of the 3 sizes of follicles (Table 9). Likewise, there was no difference ($P > 0.05$) between the proven and unproven breeder subpopulations with respect to the mean number of follicles < or ≥2 mm or >4 mm in diameter (Table 9).

Thirteen of 14 (92.9%) proven breeder cheetahs had bilateral Type I ovaries, compared to 65.5% of the unproven subpopulation ($P < 0.05$). Of the cheetahs with bilateral Type III ovaries, the majority (53.5%) produced only follicles <2 mm in diameter. In contrast, of the 338 total follicles observed that were ≥2 mm in diameter, 256 (75.7%) were observed in females with Type I ovaries. Likewise, with one exception, all observations of CH, CL, or luteal scars were made from females only with bilateral Type I ovaries.

Female Results on the Basis of Age

LH and FSH responses to GnRH were compared among young (16 to 59 months), prime breeding age (60 through 120 months), and aged (>120 month) cheetah groups, but no differences were found ($P > 0.05$) (data not shown).

Young cheetahs (age range, 16 to 59 months) had a higher incidence ($P < 0.05$) of Type II and III ovaries than females ≥60 months of age (Table 10). Over 80% of females in prime reproductive age (60 to 120 months) had uniformly well-rounded ovaries (Type I) compared to <35% of younger animals. Likewise, a higher ($P < 0.05$) proportion of cheetahs <60 months of age produced only follicles <2 mm in diameter compared to older counterparts (Table 10). Most cheetahs with luteal scars (>80%) were 60 months or older. However, 1 female 32 months of age and another 51 months old had 2 and 1 distinct luteal scars, respectively, indicating that these unproven females had ovulated earlier. There were no differences ($P > 0.05$) in mean circulating (pre-GnRH) concentrations of LH, FSH, estradiol-17β, or progesterone among cheetahs in the 16 to 59, 60 to 120, or >120 month age groups (data not shown). The oldest cheetah surveyed (182 months) had 5 distinct follicles on the ovaries.

DISCUSSION
Feasibility, Value, and Safety of Physiological Surveys

The inability to consistently propagate any species in captivity can inevitably be traced to an inadequate database from which to systematically resolve the problem. Frequently, zoos have failed to recognize the need to take an organized research approach for dealing with specific management challenges. Certainly, one of the many advantages to an SSP program is the inherent emphasis on research, and the

TABLE 9. Ovarian activity versus mean (± SEM) circulating hormone concentrations (pre-GnRH) in adult cheetahs, including proven versus non-proven breeders*

	Inactive, or follicles <2 mm	Follicles ≥2 mm, <4 mm	Follicles ≥4 mm
Number of cheetahs with	22	29	15
Mean number of[a]	9.3 ± 1.4	8.2 ± 1.1	3.4 ± 0.7
Mean LH (ng/ml)	0.82 ± 0.11	1.02 ± 0.1	0.83 ± 0.13
Mean FSH (ng/ml)	8.69 ± 0.77	8.33 ± 0.66	10.55 ± 1.37
Mean estradiol-17β (pg/ml)	5.87 ± 0.43	9.01 ± 1.62	7.12 ± 0.66
Mean progesterone (ng/ml)	0.19 ± 0.02	0.25 ± 0.03	0.19 ± 0.01
Mean number for:			
proven* (n = 12)	9.0 ± 6.0	9.3 ± 3.4	3.6 ± 0.7
unproven (n = 31)	13.6 ± 2.0	6.2 ± 1.4	3.6 ± 1.1

*Excludes 2 females that had distinct CH or CL.
[a]For females producing that specific ovarian structure.

TABLE 10. Distribution of ovary type, follicle type, and size and presence of luteal scars in adult female cheetahs of different ages

Cheetah age	Ovary type			Percent of population with			
	I	II	III	Inactive, or follicles <2 mm	Follicles ≥2 mm, <4 mm	Follicles ≥4 mm	Luteal scars
16 to 59 (n = 29)	31.0	34.5	34.5	48.2	40.7	11.1	6.9
60 to 120 (n = 33)	80.6	6.5	12.9	25.8	51.6	22.6	22.6
>120 (n = 8)	87.5	12.5	0.0	12.5	25.0	62.5	50.0

interinstitutional collaboration. Not only does an SSP strategy reaffirm that research provides the only hope for generating problem-solving data, but, frequently, the SSP is the only avenue ensuring actual access to an endangered species. Of course, sound scientific judgments can be made only on the basis of adequate sample sizes. For many large-sized species, zoos usually maintain too few animals from which to make statistical conclusions. Because SSPs manage regional (rather than local) populations, it is possible to consider all or a portion of that population useful for attacking specific management problems. Such was the precedent-setting decision made by the Cheetah SSP in 1989. Perhaps, as important as generating new information for the cheetah was determining that it was both feasible and practical to conduct a continent-wide, highly manipulatory survey of an endangered species.

The amount of cooperation mandated for this survey by the SSP Coordinator and survey team was not trivial. At institutions maintaining large numbers of cheetahs, the survey team frequently required 4 or more days, which demanded extensive time and active participation commitments by curatorial, animal care, and veterinary staff. To minimize expenses and time of the survey team, it also was not unusual for host institutions to dedicate staff and facilities to weekend efforts. We view the extraordinary willingness of institutions to participate and provide unselfish access to valuable collection specimens, as an index of emerging enthusiasm for more organized approaches to solving single species conservation problems.

This survey also confirmed the technical feasibility of translocating a mobile laboratory team and some rather sophisticated technology among zoos. There are alternatives, one being the movement of animals to a central location for evaluation and another being the collection of data by multiple research teams. The former is unacceptable from both a practical and humane perspective, and the latter poses problems in data interpretation. As one example, it would be impossible to compare semen characteristics for a regional population on the basis of measures made by independent laboratories because different institutions use different techniques and quantifiers of ejaculate quality. Therefore, one of the primary values of this ever-expanding cheetah data base is that all information on reproduction, genetics, disease/pathology, and nutrition has been (and continues to be) collected using standardized procedures. Such continuity will ensure accuracy in answering questions about fundamental biology, as well as eventually determining the etiology of poor reproductive performance.

Sharing expertise and mobilizing laboratory technology also avoids the need for *all* zoos to replicate incredibly expensive research programs, laboratories, and equipment. Given current fiscal constraints and the precarious population size for many rare species, then interinstitutional research cooperation and the active sharing of expertise among zoos is the most efficient strategy for studying why some species thrive in captivity and others do not. Although the concept of zoos collaborating certainly is not new, the idea of moving personnel, sophisticated laboratory equipment, and technology among institutions is relatively novel. Particularly exciting are recent findings that even delicate gamete culture systems can be moved from zoo to zoo, allowing the development of IVF [Miller et al., 1990; Donoghue et al., 1990], artificial insemination [Howard et al., 1992], and genetic resource banking [Rall et al., 1991; Wildt, 1992; Wildt et al., in press] systems.

Historically, there has been a reluctance within the zoo community to use highly manipulative procedures involving anesthesia on rare wildlife species, unless for medical reasons. However, major advances in zoo veterinary medicine have minimized the risks associated with manipulative approaches for many species. The absence of mortality or any lasting adverse effects to the 128 animals in the present, intense physiological survey attests to the safety of this research approach for the cheetah. The key to this success was due largely to extensive pre-survey experience with all manipulatory procedures, including anesthesia, electroejaculation, and laparoscopy. It also was mandatory that there be a high level of communication and interdisciplinary cooperation, especially among the veterinary participants and the curators, animal care, and research staffs.

Reproductive/Endocrine Status of Males

Semen and testes characteristics measured in adult cheetahs were consistent with earlier reports involving isolated institutional populations in North America or southern Africa, or from males free-living in the Serengeti ecosystem [Wildt et al., 1983a, 1987b, 1988]. Overall ejaculate quality and the ability of cheetah sperm to perform in in vitro bioassays were considerably inferior to results from other felid species like the normospermic domestic cat and leopard cat [Howard et al., 1991b; Wildt et al., 1988, 19982a,b]. However, particularly encouraging was the high proportion of cheetahs (>90%) producing spermic ejaculates, and the finding that testes and seminal characteristics were similar in proven versus unproven breeders. There

was no clear physiological marker distinguishing males that had previously sired young from those that had not. Proven breeder cheetahs had just as many pleiomorphic sperm/ejaculate as unproven breeders, which could be interpreted to mean that the latter are not necessarily "sperm failures." Rather, if males are contributing to reproductive inefficiency, then the cause may be more related to behavioral inadequacy or libido dysfunction, both of which may be associated with husbandry, an endocrine imbalance, or some other undefined problem.

An alternative explanation is that the high proportions of pleiomorphic sperm are not "normal" for either proven or unproven breeders and are related directly to low reproductive efficiency. For this to be true, the existence of teratospermia must be known to affect gamete interaction and establishment of a pregnancy. Based on a well-established relationship between inbreeding and sperm morphology in laboratory and livestock species, we have asserted from previous studies [O'Brien et al., 1983, 1985; Wildt et al., 1983a, 1987b, 1992a] that the extreme loss of genetic diversity in the cheetah is the most logical explanation for its poor ejaculate quality. Further, using an array of bioassays and a variety of felid species, we have consistently observed an inverse relationship between the incidence of sperm abnormalities and sperm fertilizing ability in vitro. Particularly important are recent data demonstrating that even structurally-normal sperm from teratospermic domestic cats are compromised in ability to penetrate the oocyte ZP and achieve embryo cleavage in vitro [Howard et al., 1991b]. But this argument also holds for the cheetah, because, compared to normospermic populations of domestic cats, leopard cats, and tigers, sperm viability and IVF rates in the cheetah are vastly inferior [Wildt et al., 1992a,b].

Donoghue et al. [1992] recently reported more detailed evidence to support the contention that sperm quality affects fertilization in the cheetah. In that study, IVF success was measured among individual male cheetahs used to inseminate a homogeneous population of cheetah oocytes. Half the males failed to fertilize any oocytes, whereas cleavage rates for some males exceeded 70%. All males produced high proportions of pleiomorphic sperm; the only difference was that sperm from cheetahs achieving IVF were able to sustain motility in vitro longer than sperm from males failing to fertilize. But the present survey clarified the issue even further, because pleiomorphic cheetah sperm, although capable of binding to the oocyte ZP, were incapable of penetrating the inner ZP and reaching the cytoplasm (the final step to fertilization). Therefore, there are clear data indicating that sperm motility *combined* with sperm morphology plays a role in regulating gamete interaction in the cheetah.

Nonetheless, our survey confirmed that breeding activity and conception can occur in cheetahs despite more than 75% of the sperm in a given ejaculate being structurally abnormal. The threshold limit at which sperm quality becomes so poor that natural fertility is affected post-mating is unknown for the cheetah. However, some Florida panthers and Asian lions producing more than 90% sperm pleiomorphisms/ejaculate have been confirmed sterile on the basis of actual breeding observations with numerous females [Miller et al., 1990; Wildt et al., 1987a; Wildt, in press]. It is possible that ejaculate quality in most cheetahs is just below a threshold, where sterility, even among the few males interested in copulating, will become the norm. In fact, the survey revealed that there were 5 males producing at least 90% structurally-abnormal sperm, and none of these had ever produced offspring, despite having the opportunity to mate. This should be cause for concern, and serve as

motivation to continue managing the population to avoid further inbreeding depression.

Endocrine activity as determined by short-term sampling of circulating LH, FSH, and testosterone was not different between proven and unproven male breeders. Compared to other felid species, cheetahs consistently secrete less testosterone [Wildt et al., 1988], so it is tempting to correlate poor reproductive performance to an inadequacy in the production of this hormone. Present data cannot support this assertion, although if androgens are driving male aggressiveness and libido, then it would be reasonable to speculate that males producing nadir testosterone (0.1 ng/ml or less) concentrations (~28% of the population) would have little hormonal incentive to mate. Using present information, it is impossible to make any further inferences simply due to the limitations of the survey protocol. Unfortunately, we have measured endocrine status only during a very brief window in the life history of each cheetah. Circulating hormones can be notoriously erratic and admittedly we were forced to collect blood samples during anesthesia, an event well known to perturb gonadal hormone secretion. Perhaps this limitation can be circumvented in future studies by using noninvasive methods like fecal steroid excretion. It remains possible that this comparatively low testosterone production by the cheetah as a species is related to ejaculate quality. Recently, Howard et al. [1991b] reported that serum LH and FSH concentrations were comparable, but testosterone was significantly depressed, in terato- versus normospermic domestic cats. Because testosterone plays a major role in spermatogenesis, it may well be that there is a direct correlation in the cheetah between consistently low testosterone and overall poor semen quality.

Reproductive/Endocrine Status of Females

GnRH challenge testing demonstrated that there were no differences among proven and unproven female cheetahs in pituitary sensitivity or the ability to acutely produce LH or FSH. The temporal profiles and the absolute concentrations of hormones produced mimicked earlier findings in cheetahs [Wildt et al., 1983b] as well as more recent studies of leopards, tigers [Brown et al., 1988], and African lions [Brown et al., in press]. Because every female responded with a measurable increase of LH and FSH after GnRH treatment, there was no apparent evidence of pituitary dysfunction.

The reproductive tract of most females was anatomically sound, and the few structural abnormalities observed, like degenerate ovaries in aged females, were not unusual for a population of this size. However, finding an adult-aged female with only a single ovary and another with an infantile reproductive tract was uncommon. Despite performing laparoscopy in well over 1,000 individuals representing 15 different felid species, we have never before observed those specific anomalies. Likewise, we have not routinely observed parovarian cysts in other felid species, certainly not to the extent observed here, where 50% of the surveyed population was affected. In a parallel study, Munson [1993] has examined the precise etiology and potential relevance of the cysts and has concluded that these fluid-filled pockets are generated within segmented remnants of the Wolffian duct. Although a majority of the cysts are located immediately adjacent to the oviduct, Munson has concluded that neither their size nor orientation likely compress the oviductal lumen. Likewise, even in the presence of cysts >3 cm in diameter, we failed to observe any abnormal effect on the ability of the ovary to be enveloped by the ovarian fimbria. Because multiple, large-

sized cysts were observed in females producing cubs as recently as 12 months before examination, we concluded that the presence of parovarian cysts in cheetahs likely is of no consequence.

Unlike in the male cheetah, where the majority of sperm are known to have both structural and functional problems, there is no such evidence for oocyte dysfunction in the female. In a recent IVF trial, in which oocytes from individual females were divided randomly and inseminated in vitro with sperm from different males, at least some oocytes from all females became fertilized [Donoghue et al., 1992]. Such oocytes routinely are collected from ovarian follicles ≥2 mm in diameter. For this reason, it is encouraging that ~67% of the surveyed population had at least 1 ovarian follicle of this size. However, there were lines of evidence suggesting that the majority of the surveyed females were demonstrating minimal, if any, active ovarian cyclicity at the time of the survey. First, it is not unusual to observe small, distinct follicles of ~2 mm in diameter on the ovaries of any felid species at any time of the estrous cycle, including in animals not demonstrating overt estrus. Waves of ovarian follicular development and regression occur continuously, including throughout gestation [Goodrowe et al., 1989; Wildt, 1991]. Second, in a previous study in which we induced follicular activity and eventually ovulation in the cheetah with exogenous gonadotropins, ovarian preovulatory follicles were at least 4 mm in diameter. However, only 15 cheetahs (22.7% of the population) had ovaries containing this sized follicle. Third, we identified 3 distinct ovarian shapes including 2 types in which the ovary was not well-rounded, appearing almost partially atrophic. To our knowledge, there are no empirical or even anecdotal data on ovarian shape characteristics or changes over time among the Felidae species. In general, domestic cats that are monitored longitudinally via laparoscopy and are known to be demonstrating cyclic reproductive activity retain a normal size, well-rounded ovary. The partially or wholly flattened appearance of the Type II and III categories defined here for the cheetah was more characteristic of ovaries seen in prepubertal domestic cats or cats that were acyclic due to natural fluctuations in season. A similar phenomenon may be occurring in the cheetah as fewer total distinct follicles were observed in females with predominantly Type II and III ovaries, further suggesting that these females were not reproductively active. Finally, most of the cheetahs in the surveyed population were producing extremely low serum estradiol-17β concentrations, often approaching the lower sensitivity of the RIA. Although these data were based upon a limited number of blood samples collected during an acute interval, the overall mean estradiol-17β concentration was about half that measured in females scheduled for IVF and previously treated with exogenous gonadotropins [Donoghue and Brown, unpublished data]. A few of the surveyed cheetahs (n = 7) produced circulating estradiol-17β in excess of 10 pg/ml, and, in general, those females producing more estrogen had greater ovarian activity. For example, 1 female with a total of 3 preovulatory follicles (3, 5, and 6 mm in diameter, respectively) was producing a mean of 12.5 pg/ml estradiol-17β (range, 10 to 23.6 pg).

Of course, an alternative explanation was that distinct follicles of 2 mm in diameter were normal-sized Graafian follicles for the cheetah. Because 66.7% of the surveyed population had ovaries containing follicles of this size, perhaps a substantial proportion of the cheetahs in fact were demonstrating active ovarian cyclicity. This scenario seemed unlikely, primarily because Graafian follicles in the much smaller domestic cat generally are ≥2 mm in diameter [Wildt and Seager, 1980]. It was also

remotely possible that most cheetahs were reproductively active, and we coincidentally made all evaluations during a nadir in the ovarian cycle. Given (1) the number of cheetahs examined, (2) that the reproductive cycle is estimated to be ~10 to 20 days in duration [Eaton and Craig, 1973; Bertschinger et al., 1984; Asa et al., 1992], and (3) that large preovulatory follicles remain visible on the ovaries for at least 4 to 6 days (~20 to 25% of the cycle as in the domestic cat), then more large-sized follicles should have been discovered, if females were truly reproductively active. All of these questions should and can be resolved from future studies (see below).

New Information on Cheetah Reproductive Biology

A by-product of the survey was a substantial increase in our fundamental understanding of reproductive processes in the cheetah. For example, the longevity bioassay confirmed that the duration of cheetah sperm motility in vitro was brief unless raw ejaculate was diluted immediately in tissue culture medium. This finding allowed us to determine that cheetah sperm were capable of binding and penetrating heterologous (domestic cat) oocytes in vitro, which in turn allowed the discovery that morphologically-abnormal sperm were filtered by the oocyte's ZP. The existence of this mechanism no doubt prevents the oocyte from being fertilized by a pleiomorphic spermatozoon that may or may not have a normal DNA complement. Finding that cheetah sperm actively interact with cat oocytes in vitro confirmed earlier observations from our laboratory that felid oocytes have failed to develop a mechanism for excluding penetration by a "foreign" felid spermatozoon [Howard and Wildt, 1990; Andrews et al., 1992]. This discovery has been beneficial because domestic cat oocytes, collected from ovariohysterectomy material and stored in a hypertonic salt solution, now can be used to test sperm function in many felid species. Thus, we have another semen evaluation tool that can be moved from zoo to zoo. We now rely extensively on this specific sperm function assay to study the ability of structurally-normal versus -abnormal sperm to participate in fertilization [Wildt et al., 1992b]. Likewise, knowing that sperm and oocytes from different felid species readily interact in vitro allowed developing an oocyte "rescue" system, an in vitro culture procedure in which immature oocytes can be salvaged from the ovaries of felids that have died or undergone ovariohysterectomy for medical reasons [Johnston et al., 1989, 1991]. Lastly, results from the 2 sperm function assays developed here were invaluable in developing assisted (artificial) breeding strategies for the cheetah. Knowing (1) how to sustain cheetah sperm motility in vitro, and (2) that cheetah sperm and domestic cat oocytes interact in vitro, allowed conducting of a recent conspecific cheetah IVF study [Donoghue et al., 1992]. A greater understanding of basic laboratory procedures for maintaining sperm viability in vitro also recently led to the first cheetah litters produced by artificial insemination [Howard et al., 1992; Howard, unpublished data].

The lack of fresh luteal tissue on the ovaries of almost all cheetahs strongly suggested that the species was an induced ovulator (i.e., 1 or more copulations were required to elicit an appropriate neurohormonal response causing ovulation). If cheetahs were spontaneous ovulators (i.e., ovulation occurred independent of mating and only after a spontaneous discharge of gonadal estrogen followed by pituitary LH), then at least some surveyed females should have had fresh CH or CL on the ovaries. However, the latter were observed in only 2 individuals, inexplicably a non-mated female nursing cubs, and a female during the first trimester of pregnancy. It was

impossible to conclude with total authority that the cheetah was an induced ovulator, for two reasons. First, if the majority of females were reproductively inactive, as asserted above, then it was logical that no luteal tissue was observed. Second, a recent estrous cyclicity study of the cheetah, based upon vaginal cytology, reveals that females occasionally spontaneously ovulate [Asa et al., 1992]. In this context, the cheetah resembled the domestic cat, a species confirmed largely to be an induced ovulator, but which occasionally ovulates spontaneously [Goodrowe et al., 1989]. It is rather remarkable that no definitive studies have been done in the vast majority of felid species to resolve the question of induced versus spontaneous ovulation. The few studies to date illustrate inconsistencies in the taxon. For example, based upon laparoscopic observations and/or serial blood sampling and hormonal analyses, the jaguar [Wildt et al., 1979], tiger [Seal et al., 1985], and puma [Bonney et al., 1981] are considered induced ovulators. In contrast, distinct elevations in circulating progesterone after clear preovulatory estradiol-17β surges have been reported in both lions [Schmidt et al., 1979] and leopards [Schmidt et al., 1988] housed with conspecific females but in the absence of males. These findings have been interpreted to suggest that the latter 2 species either are induced to ovulate by the mere presence of cage-mates, or they are spontaneous ovulators. From opportunistic laparoscopy studies, we occasionally have observed fresh luteal tissue on the ovaries of clouded leopards, tigers, and lions housed alone or without a male [Wildt and Bush, unpublished observations]. As in the domestic cat [Goodrowe et al., 1989], it appears that cheetah CH eventually regress to form distinct, yellow luteal scars that remain visible on the ovaries for months. Presence of these scars is advantageous because they reveal which females have ovulated over the last several months. Many of the females with luteal scars were those previously confirmed pregnant and delivering young. However, it was interesting that luteal remnants were visible in 8 unproven females, 4 of which had never had the opportunity to mate. This probably supports Asa et al.'s [1992] contention that the cheetah occasionally ovulates spontaneously.

Wrogemann [1975] first suggested that both male and female cheetahs reach sexual maturity between 13 and 16 months of age. We observed that all male cheetahs ≤26 months of age, including males as young as 15 months, produce motile sperm in the ejaculate. Additionally, of the 11 females examined in the same age group, 5 (45.4%) had distinct ovarian follicles (≥2 mm in diameter). However, based upon other reproductive characteristics, including testosterone concentrations in males and circulating estradiol-17β and ovarian shape in females, it was doubtful that any of these individuals was truly sexually mature in terms of complete gonadal function or libido. For example, males by 15 months of age essentially had achieved adult body size, while having testes volumes near those of prime breeder males (>60 months of age). However, peripheral testosterone concentrations in these young cheetahs did not approach the higher levels measured in older counterparts, and, because testosterone supports spermatogenesis, this likely explained the differences in motile sperm produced/ejaculate between the two age groups. Likewise, assuming that circulating testosterone also was driving sexual aggressiveness and libido, this age-specific difference in hormone secretion may explain why young cheetahs, although producing some sperm in the ejaculate, had never bred, even though provided the opportunity.

It was interesting that there was no relationship between age and percentage of pleiomorphic sperm/ejaculate. Abnormal sperm forms were as prevalent in young as in older cheetahs. This finding was similar to that recently reported for young versus

old African lions isolated in the Ngorongoro Crater in Tanzania, and known to be genetically-depauperate [Brown et al., 1991; in press]. Previous studies have demonstrated that Crater lions, regardless of age, have poorer sperm quality than outbred, free-living counterparts in the adjacent Serengeti ecosystem [Wildt et al., 1987a; Brown et al., 1991; in press]. In contrast, in the latter population there appears to be an indirect and significant correlation between increasing age and the proportion of pleiomorphic sperm/ejaculate. Together, these findings seem to suggest that as genetic diversity within a population decreases, there is an eventual loss in the relationship between age and sperm structural integrity. In genetically-monomorphic populations like the cheetah and Ngorongoro Crater lion, inbreeding depression probably circumvents the potential advantages that increasing age may exert on improved sperm structure, and, instead, most cells remain pleiomorphic.

Although the age at which most cheetahs become reproductively senescent is unknown, most managers of the species usually expect little breeding activity from females older than 9 years [Marker and O'Brien, 1989]. Therefore, it was rather surprising to discover individual cheetahs ≥10 and as old as 15 years with functioning gonads. All of these males continued to produce sperm, and some females had normal-appearing ovaries with distinct follicles. Because circulating gonadal hormones were basal in these cheetahs, it was unlikely that any of these animals would breed naturally. Nonetheless, given recent advances in assisted reproductive technology [Donoghue et al., 1992; Howard et al., 1992], it may be possible to use these older animals as gamete donors for artificial insemination or IVF.

Overall Perspective and Directions for the Future: Emphasis on Biology Versus Management

Theoretically, there are two extreme viewpoints on the possible cause(s) of reproductive inefficiency in the North American cheetah population. One extreme could defend the view that the species is physiologically normal, and that the key to consistent reproductive success will be as simple as identifying some unknown management factor that will promote reproductive success. The alternative is that the historical loss in species genetic diversity has resulted in such a pervasive loss of reproductive vigor that routine propagation is unlikely. In this scenario, the genetic blueprint for extinction has been drawn, and we as managers will continue to struggle using an ever-smaller pool of breeders.

Paradoxically, our survey data supported both viewpoints. It may well be that one or (more likely) a series of husbandry factors can be altered, thereby enhancing the proportion of males and females in the captive population that will reproduce. Survey evidence supportive of this view comes from the similarity in ejaculate, ovarian activity, and hormonal patterns between the proven and unproven breeders. Likewise, as described earlier in Materials and Methods, there were striking differences among institutions in the type of management schemes used. The 1989 review of Marker and O'Brien reveals that certain institutions are much more successful at propagating cheetahs than others. It was beyond the scope of our study to identify institutional traits that could potentially contribute to physiological differences among cheetahs. Cheetahs simply were maintained in environments too varied to allow sound scientific comparisons among institutions. Nonetheless, we scanned the data for potential variations in reproductive characteristics on the basis of individual zoos. In fact, when average sperm number was compared among 6 different zoos main-

taining 3 to 10 males each, the inter-institutional variation was profound; for example, the mean number of motile sperm/ejaculate ranged from a low of $10.0 \pm 8.6 \times 10^6$ for one institution to $65.8 \pm 32.3 \times 10^6$ for another!

Likewise, we produced considerable evidence demonstrating the almost universal existence of some rather novel physiological characteristics that may be contributing directly or indirectly to poor reproductive performance. From a reproductive physiologist's perspective, it is impossible to argue that extraordinary proportions of ejaculated pleiomorphic sperm are normal, especially considering that teratospermia is a leading cause of infertility in humans and domesticated species [see review, Howard et al., 1991b]. Certainly, the presence of these deformed sperm cannot be enhancing reproductive potential. In fact, laboratory assays associated with the survey conclusively demonstrate that these structurally-defective sperm are incapable of fertilization. This knowledge combined with parallel information on the compromised ability of cheetah sperm to penetrate heterologous and conspecific oocytes in vitro leads us to be highly suspicious of sperm functionality in this species. There is little doubt that this characteristic is more related to genetics than management, primarily because free-living cheetahs also produce high proportions of structurally-defective sperm [Wildt et al., 1987b]. There also is little evidence of mature ovarian follicular activity in adult cheetahs, a finding that could be related to novel genetic characteristics of the species or to a suboptimal captive environment. Finally, the survey revealed that it was not unusual to observe that "successful" institutions frequently had only 1 or 2 breeding males, whereas other males were "disinterested." If management and environment play such a crucial role in male reproductive performance, then "successful" institutions commonly should have many rather than singleton proven breeder males.

The mission of any survey is to produce useful data for generating new theories and direction for the future. Such was the case with the physiological survey of cheetahs. We have eliminated certain areas that likely need no further attention. For example, most males are spermatogenic, and structurally-normal sperm are capable of fertilizing oocytes. A high incidence of cryptorchidism, common in the genetically-compromised Florida panther [Miller et al., 1990; Barone et al., 1991] is not evident in the cheetah. Likewise, females have normal pituitary responsiveness, and most appear anatomically sound. Our results, combined with parallel data collected by Munson [1993], also suggest that the low rates of fertility are unrelated to any specific pathologic etiology.

From a reproductive biologist's perspective, there are 4 high priority research areas where new questions now require answers. First, precisely how serious is the threat of teratospermia in cheetahs to subsequent reproductive performance? Will the incidence of structurally-abnormal sperm increase, if we continue to rely on an ever-decreasing pool of breeders, and, if so, is there a threshold of sperm pleiomorphisms that inevitably results in sterility? Also, are we absolutely sure that these rather unique seminal characteristics are not perturbed over time by season or the captive environment? These issues now can be addressed using a 3-dimensional approach. For example, because we have identified male cheetahs that are producing pleiomorphic sperm numbers in excess of the overall population mean, we can use a conspecific IVF system to actually measure sperm function. Further, we need to continuously monitor ejaculate characteristics within the overall North American population, especially when male offspring are generated from line-bred or inbred

physiology, the highest priorities are (1) studying the impact of pleiomorphic sperm, (2) establishing if there is a hormonal basis to libido differences among males, (3) determining if females are cycling longitudinally, and (4) continuing development of assisted reproductive technologies.

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Comparative Evaluation of Seminal, Vaginal, and Rectal Bacterial Flora in the Cheetah and Domestic Cat

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To determine the status and potential impact of microorganisms on reproductive health, bacterial cultures were evaluated from cheetah seminal, vaginal, and rectal swabs and the results compared to those from clinically healthy, domestic cats. Aerobic bacteria were isolated in the semen from 26 of the 40 (65.0%) cheetahs and 25 of the 27 (92.6%) domestic cats. Gram-negative organisms predominated in the electroejaculates of both species, accounting for >70% of the total bacterial isolates. The most common seminal organism in both species was hemolytic Escherichia coli. Bacteria were isolated from vaginal samples obtained from 49 of the 67 (73.1%) cheetahs and 46 of the 49 (93.9%) domestic cats. Gram-negative organisms dominated, representing >63% of the vaginal bacteria, and again hemolytic E. coli was the most prevalent isolate in both species. None of the cheetah or domestic cat vaginal cultures contained Mycoplasma spp. or Ureaplasma spp. Numerous gram-negative and gram-positive bacteria were identified in rectal cultures of 73 cheetahs and 60 domestic cats, but hemolytic E. coli clearly was the most common isolate. Within each species, a comparison between electroejaculates that were positive vs. negative for hemolytic E. coli growth revealed no differences in sperm concentration, sperm motility ratings, or the proportion of structurally abnormal spermatozoa. Neutrophils were not detected in any of the 67 felid ejaculates, and the presence of seminal hemolytic E. coli was unrelated to fertility, on the basis of past ability to sire young or fertilize oocytes in vitro. Vaginal cytologic evaluations in both the cheetah and domestic cat indicated that hemolytic E. coli was not associated with a pathologic inflammatory response. Overall fecundity and proven ability to produce young were similar between females producing positive or negative vaginal cultures for E. coli. These findings indicate that commensal bacteria exist in the reproductive tract of the