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Sensitivity to Exogenous Gonadotropins for Ovulation Induction and Laparoscopic Artificial Insemination in the Cheetah and Clouded Leopard[1]

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ABSTRACT

Ovarian sensitivity to exogenous gonadotropins was assessed in the cheetah (*Acinonyx jubatus*) and clouded leopard (*Neofelis nebulosa*) to help optimize artificial insemination (AI). Eighteen female cheetahs were used on 29 occasions and were given i.m. injections of 100, 200, or 400 IU eCG and 100 or 250 IU hCG 80 h later. Twenty-three female clouded leopards were treated i.m. on 27 occasions with 25, 50, 75, 100, 200, or 400 IU eCG followed 80 h later with 75, 140, or 280 IU hCG. Ovaries were examined laparoscopically at 43–48 h after hCG in cheetahs and 39–50 h in clouded leopards. All gonadotropin dosages stimulated ovarian activity in both species, but ovulation success and corpus luteum (CL) morphology varied ($p < 0.05$) with treatment. For both species, the highest and intermediate eCG dosages resulted in ovulation in a high proportion (72–100%) of females. The lowest eCG dosage, although capable of stimulating follicular development, compromised ovulation and resulted in few (< 26%) postovulatory females. For each species, small CL (2–4-mm diameter) were observed with the highest and lowest eCG dosage, and large CL (5–8-mm diameter) were associated with intermediate eCG dosages. Aged CL (10–12-mm diameter) were observed in 4 of 23 (17.4%) clouded leopards with no prior male exposure, indicating occasional spontaneous ovulation. Nineteen laparoscopic intrauterine AI procedures were performed in eCG/hCG-treated postovulatory cheetahs. Eighteen AI procedures were conducted in eCG/hCG-treated postovulatory clouded leopards. Six of the 13 cheetahs (46%), all in the 200-IU eCG/100-IU hCG group, became pregnant, in contrast to none of the clouded leopards. This study has revealed differences in ovarian activity in two wild felid species as a result of changes in exogenous gonadotropin dosage. Because of this dose-effect response, this comparative approach is necessary to identify a gonadotropin regimen that can mimic "normalcy." Even then, the relatively high AI success in the cheetah compared to the clouded leopard suggests that factors other than ovarian response can dictate the efficiency of assisted reproduction in this taxon.

INTRODUCTION

Artificial insemination (AI) could assist in managing and conserving all 36 extant wild felid species maintained as reservoir populations in zoos. However, AI would be particularly useful for the cheetah (*Acinonyx jubatus*) and clouded leopard (*Neofelis nebulosa*), which have naturally low levels of genetic variation [1, 2] and poor reproductive success in zoos [3, 4]. To date, we have produced offspring by laparoscopic AI in the cheetah [5], tiger (*Panthera tigris*) [6], puma (*Felis concolor*) [7], leopard cat (*Felis bengalensis*) [8], ocelot (*Felis pardalis*) [9], clouded leopard [10], and snow leopard (*Panthera uncia*) [11].

The cheetah is the only surviving species of the genus *Acinonyx* and is threatened in nature by the rapid spread of agriculture and a declining prey base in Africa. Breeding the species in captivity traditionally has been difficult. Of the ~300 adult cheetahs currently in North America, only 19% of males and 28% of females have reproduced, and many genetically valuable individuals now comprise the national breeding program. The clouded leopard also is taxonomically unique and the only member of the genus *Neofelis*. Virtually nothing is known about this Southeast Asian species in nature, except that it is threatened by native forest destruction and poaching for its beautifully patterned pelt and for bones used in Asian medicines. Clouded leopards demonstrate severe behavioral incompatibility in captivity and a high incidence of male aggression and lethal attacks on females, even those in estrus [4]. The current North American clouded leopard population (~200 individuals) clearly is not self-sustainable [4]. Fewer than 25% of adult males and 37% of adult females have reproduced, and no living males have sired offspring with more than a single female. For these wild felids and others, AI offers enormous potential for managing animals that are difficult to propagate, geographically separated, or require infusions of fresh genetic material.

Early sporadic achievements in using AI in nondomestic felids [12, 13] have been followed recently with substantial progress [14]. One important finding has been that AI success is highly influenced in this taxon by exogenous gonadotropin type, timing, and dosage and by the anatomical site of insemination [5–7, 10, 15, 16]. The anesthesia event itself (necessary for these wild animals) compromises sperm transport in vaginally inseminated felids, a problem that can be circumvented by intrauterine sperm deposition [16]. Anesthetizing animals for AI too early after exogenous gonadotropin administration blocks ovulation, even with use of ovulation-inducing gonadotropins like hCG [16]. Optimal time for AI in felids appears to be immediately after ovulation onset. There also appear to be rather remarkable differences in ovarian response among felid species to eCG and hCG [5, 7, 9–11, 15]. Some dosages result in an excessive number of unovulated follicles [5, 10], whereas others impair ovulation; and both problems appear to be related to inadequate luteal development, which can fail to sustain a pregnancy [7]. Interestingly, the exogenous gonadotropin dosage required to elicit an ovulatory response mimicking normalcy is unrelated to body

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mass [5, 7]. For example, the cheetah and clouded leopard appear to be sensitive to low gonadotropin dosages. Ovulation induction can occur with an eCG dosage of 100–200 IU, similar to that required in the much smaller domestic cat [5, 10, 16]. Finally, earlier studies revealed that ovulation occurs ~38–40 h after eCG/hCG treatment in the cheetah and clouded leopard, which is about 10 h later than in the similarly treated domestic cat [5, 10, 16].

Our long-term goal is to identify and control those factors that will allow AI to become routine for managing rare felids. Here, we examined the level of ovarian sensitivity to exogenous gonadotropins in the cheetah and clouded leopard. Specifically, we evaluated 1) the impact of incremental eCG and hCG dosages and combinations that would induce folliculogenesis and ovulation but minimize unovulated "residual" follicles; 2) the influence of gonadotropin dosage on follicle and corpus luteum (CL) morphology; and 3) the efficiency of intrauterine AI in postovulatory females treated with various eCG/hCG regimens.

MATERIALS AND METHODS

Animals

Cheetahs maintained at six zoological institutions were provided for this study. Eighteen adult females (3–12 yr of age; 29–38 kg BW) and 12 adult males (3–12 yr of age; 28–49 kg BW) were used. Females were housed alone or with other females, and males were maintained alone. All cheetahs were exposed to natural lighting in outdoor enclosures and fed a commercial carnivore diet (Nebraska Brand Feline Diet, North Platte, NE).

Clouded leopards maintained at eight zoological institutions were available for study. Twenty-three females (2–14 yr of age; 11–19 kg BW) and 14 adult males (2–9 yr of age; 12–27 kg BW) were studied. Clouded leopards were housed alone in outdoor enclosures with natural lighting and/or indoor enclosures with artificial light (14L:10D). This species was fed a commercial diet (Nebraska Brand Feline Diet) or whole rodents (3–5 white rats/day), supplemented with an occasional rabbit or chicken.

Exogenous Gonadotropins and Ovarian Assessment

Gonadotropin test dosages for cheetahs were chosen on the basis of our previous observations in this species [5]. Female cheetahs were treated with a single i.m. injection of 400 (n = 5), 200 (n = 18), or 100 (n = 6) IU eCG (Sigma Chemical Co., St. Louis, MO) to stimulate ovarian follicular development (n = number of occasions). Each female was given a single i.m. injection of 250 or 100 IU hCG (Sigma Chemical Co.) 80 h later to induce ovulation. Because of the rarity of the species and to increase sample size, it was necessary to treat seven females (females no. 2, 3, 6, 9, 10, 12, 13) on two occasions with an interval of 8–31 mo between treatments and two females (females no. 4, 5) on three occasions with an interval of 8–13 mo. An interval of > 6 mo between treatments was chosen to prevent producing neutralizing immunoglobulins to eCG/hCG [17].

Female clouded leopards were given a single i.m. injection of 400 (n = 4), 200 (n = 2), 100 (n = 5), 75 (n = 5), 50 (n = 7), or 25 (n = 4) IU eCG (Sigma Chemical Co.) to induce follicle growth (n = number of occasions). Injections (i.m.) with 280, 140, or 75 IU hCG (Sigma Chemical Co.) followed 80 h after eCG. Four clouded leopards (females no. 1, 2, 5, 8) were treated on two occasions with a 9- to 16-mo interval between treatments.

Ovarian activity was assessed laparoscopically 43–48 h after hCG in cheetahs and 39–50 h after hCG in clouded leopards. Our objective always was to anesthetize females for ovarian assessment after the time of expected ovulation (~40 h after hCG in cheetahs; ~38 h after hCG in clouded leopards) because it is well documented that anesthesia compromises ovulation in eCG/hCG-treated domestic cats [5, 10, 16]. All animals were deprived of food for 24 h before laparoscopy. Anesthesia was induced in cheetahs with tiletamine and zolazepam (4–6 mg/kg BW, Telazol; A.H. Robins Co., Richmond, VA) or a combination of ketamine hydrochloride (10 mg/kg, Ketaset; Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (1 mg/kg, Rompun; Haver-Lockhart, Shawnee, KS). Anesthesia was induced in clouded leopards with ketamine hydrochloride (5–15 mg/kg) alone or in combination with xylazine (0.5–2 mg/kg). After tracheal intubation, surgical anesthesia was maintained using isoflurane gas/oxygen inhalation anesthesia. Laparoscopy was performed as previously described [5, 10]. In brief, animals were placed in a head-down, supine position, and a 10-mm-diameter laparoscope (Olympus Corporation, Lake Success, NY) inserted at the abdominal midline was used to examine all aspects of each ovary for preovulatory follicles (flattened or slightly raised, clear areas measuring > 2 mm diameter) and postovulatory CL (opaque, reddish-yellowish structures raised above ovarian surface) [5, 10, 18]. A graduated Verres probe inserted transabdominally was used to precisely measure the size of ovarian structures. Females with at least one CL were classified as "postovulatory," regardless of the number of preovulatory follicles present.

Serum Estradiol-17β and Progesterone Analysis

A blood sample was collected from each female immediately after anesthesia induction and before laparoscopy onset to measure serum estradiol-17β and progesterone by commercially available RIA kits. Serum was collected after a 10-min centrifugation and then stored (−80°C) until analyzed. These specific RIAs had been validated previously for these species [5, 10, 19] by demonstration of 1) parallelism between dilutions of pooled serum extracts and the standard curves and 2) significant recovery of exogenous steroid added to cheetah or clouded leopard serum extracts.

Serum estradiol-17β was determined using a solid-phase ^{125}I RIA kit (Coat-a-Count; Diagnostic Products Corporation, Los Angeles, CA) [10, 19]. Assay sensitivity was 5.0 pg/ml for cheetah serum, and the inter- and intraassay coefficients of variation were 11.5% and 10.2%, respectively. Assay sensitivity was 10.0 pg/ml for clouded leopard serum, and the inter- and intraassay coefficients of variation were < 10.0%. Serum progesterone was determined using a double-antibody ^{125}I RIA kit (ICN Biomedicals, Costa Mesa, CA) [5, 10]. Assay sensitivity was 0.1 ng/ml for both species, and the inter- and intraassay coefficients of variation on the cheetah assays were 9.1% and 6.1%, respectively, and < 10.0% for clouded leopards.

Semen Collection and Processing

For collecting sperm, anesthesia was induced in male cheetahs using tiletamine and zolazepam (3–6 mg/kg BW, Telazol; A.H. Robins Co.) or a combination of ketamine hydrochloride (10 mg/kg, Ketaset; Fort Dodge Laboratories) and xylazine (1 mg/kg, Rompun; Haver-Lockhart). Anesthesia in clouded leopard donors was induced with ketamine hydrochloride (10–15 mg/kg). Semen was collected

TABLE 1. Influence of eCG and hCG on ovarian activity in cheetahs.

Variable	Dosages of eCG/hCG (IU)[a]		
	400/250 (n = 5)	200/100 (n = 18)	100/100 (n = 6)
No. postovulatory females/total no. females (%)	5/5 (100%)[b]	13/18 (72.2%)[b]	1/6 (16.7%)[c]
No. fresh CL/ovulating female	10.4 ± 1.2	8.2 ± 1.4	8.0 ± 0.0
Mean diameter of fresh CL (mm)	3.3 ± 0.1[b]	5.4 ± 0.1[c]	3.3 ± 0.4[b]
No. unovulated follicles/female	3.4 ± 0.5	4.8 ± 0.8	3.3 ± 1.3
Mean diameter of follicles (mm)	3.3 ± 0.2	3.7 ± 0.1	3.6 ± 0.1

[a] n, Number of occasions.
[b,c] Within rows, values with different superscripts differ ($p < 0.05$).

by electroejaculation and was processed for AI according to previous protocols [20, 21]. In brief, an electroejaculator (P.T. Electronics, Boring, OR) and a 1.6-cm diameter rectal probe with 3 longitudinal electrodes were used to deliver 80 electrical stimuli (2–6 volts) in 3 series of 30, 30, and 20 stimuli, respectively.

Semen was examined on a heated (37°C) stage using phase-contrast microscopy for a subjective assessment of sperm percentage of motility (0–100%) and sperm progressive motility (on a scale of 0–5, where 0 = no movement and 5 = rapid forward progression) [20]. Ejaculate volume was determined, and sperm concentration per milliliter of ejaculate was calculated using a hemacytometer procedure [20]. Sperm morphology was assessed after fixing a 5-μl aliquot in 0.3% glutaraldehyde, followed by phase-contrast microscopic examination of 200 sperm/aliquot at ×1000 [20].

Semen samples were diluted (1:1) with Ham's F-10 medium (Irvine Scientific, Santa Ana, CA) containing 5% heat-treated fetal calf serum (Irvine Scientific; for cheetah semen) or 5% heat-treated (56°C, 30 min) male clouded leopard serum (for clouded leopard semen). Diluted semen was centrifuged (300 × *g*, 10 min), the supernatant was discarded, and the sperm pellet was resuspended gently with 200–300 μl of fresh medium. Samples were maintained at room temperature in darkness until AI. Immediately before insemination, samples were assessed for total volume, sperm concentration, and sperm percentage of motility and progressive motility. Because total sperm number per ejaculate varied markedly, and because felids naturally produce comparatively low sperm concentrations [20], no attempt was made to standardize the number of sperm inseminated. As a result, all sperm recovered after processing were deposited in utero.

Laparoscopic AI

Females determined to be postovulatory by the presence of at least one CL were inseminated using a laparoscopic intrauterine AI procedure adapted previously to both species [5, 10]. After confirmation that ovulation had commenced, an accessory grasping forceps (Richard Wolf Medical Instruments Corp., Rosemont, IL) was inserted through the abdominal wall to stabilize the uterine horn. Each horn was cannulated with an 18-gauge, 5-cm-long feline indwelling catheter (Sovereign, Sherwood Medical, St. Louis, MO) inserted transabdominally into the proximal third of the lumen. After stylette removal, polyethylene tubing (PE 10; Intramedic, Clay Adams, Parsippany, NJ) attached to a 30-gauge needle and a 1-ml syringe containing the processed sperm was inserted through the catheter into the uterine lumen. Each horn was inseminated with 100–150 μl of sperm suspension. Females were subjected to ultrasound or radiography 40–70 days later to diagnose pregnancy.

Statistical Analysis

Average values are presented as means ± SEM. Differences in the mean numbers of unovulated follicles and CL, sizes of follicles and CL, types of CL, and serum concentrations of estradiol-17β and progesterone observed among gonadotropin dosage groups were compared by analysis of variance, followed by the Duncan's New Multiple Range tests. The proportions of females ovulating and with different CL types were compared using chi-square analysis. Correlation coefficients between serum hormone data and gonadotropin dosages or number of follicles or CL were calculated on a portable calculator.

RESULTS

Ovarian Response to Gonadotropins in Cheetahs

Ovarian activity and the proportion of females ovulating after eCG/hCG were influenced ($p < 0.05$) by gonadotropin treatment (Table 1). More than 70% of the cheetahs ovulated in the 200- and 400-IU eCG groups compared to only one of the six females treated with the 100-IU dosage. In ovulating females, however, the number of fresh CL was similar ($p > 0.05$) among gonadotropin dosages (Table 1). The mean diameter of fresh CL was influenced ($p < 0.05$) by eCG dosage, with the 200-IU eCG dosage resulting in larger CL than the 100- or 400-IU dosages (Table 1). Although laparoscopic assessments of ovarian status were conducted after the time of expected ovulation (~40 h post-hCG), unovulated residual follicles were observed after each treatment (Table 1). However, their number and size were unrelated ($p > 0.05$) to gonadotropin dosage (Table 1).

Dosage of eCG also influenced ($p < 0.05$) cheetah CL morphology (Table 2). Two distinct CL types were detected: 1) small (2–4 mm in diameter) fresh CL (Fig. 1A); and 2) large (5–8 mm in diameter) fresh CL (Fig. 1B). All cheetahs treated with 400 IU eCG had small CL compared to only 46.2% of females given 200 IU ($p < 0.05$; Table 2). Further, the only ovulating cheetah given 100 IU eCG produced small CL. The number of small CL per ovulating female was at least 4-fold higher ($p < 0.05$) in cheetahs receiving the highest (400 IU) or lowest (100 IU) eCG dosage than in females given 200 IU (Table 2). Conversely, the intermediate dosage of 200 IU eCG produced large CL in all ovulating cheetahs. Only one female in the 400-IU eCG group produced the large CL.

There was variation within individual females in response to consecutive gonadotropin treatments. One female (no. 5) treated on three occasions with 400/250, 200/100,

TABLE 2. CL morphology and AI efficiency in postovulatory cheetahs treated with eCG and hCG.

Variable	Dosages of eCG/hCG (IU)[a]		
	400/250 (n = 5)	200/100 (n = 13)	100/100 (n = 1)
Types of CL[b]			
No. females with small fresh CL/no. ovulating females (%)	5/5 (100%)[c]	6/13 (46.2%)[d]	1/1 (100%)[c]
No. small fresh CL/ovulating female	9.8 ± 1.8[c]	2.1 ± 0.8[d]	8.0 ± 0.0[c]
No. females with large fresh CL/no. ovulating females (%)	1/5 (20.0%)[c]	13/13 (100%)[d]	0/1 (0%)[c]
No. large fresh CL/ovulating female	0.6 ± 0.6[c]	6.1 ± 0.9[d]	0.0[c]
Postovulatory AI			
No. pregnant females/total no. inseminations (%)	0/5 (0%)[c]	6/13 (46.2%)[d]	0/1 (0%)[c]

[a] n, Number of occasions.
[b] Two types of fresh CL were observed: 1) small CL (2–4 mm in diameter), and 2) large CL (5–8 mm in diameter).
[c,d] Within rows, values with different superscripts differ ($p < 0.05$).

and 200/100 IU eCG/hCG (13-mo interval between the first and second treatment; 8-mo interval between the second and third treatment) ovulated during the first episode but failed to ovulate during the second and third episodes. Three other females (no. 10, 12, 13) produced 3–15 CL after treatment with 200 IU eCG and no ovulations after the low (100 IU) eCG dosage on a second occasion. However, the response in other cheetahs treated on multiple occasions was unrelated to consecutive treatments over time. For example, two females (no. 2 and 4) produced similar numbers of CL (6–12/female) after 400 IU eCG on the first occasion and 200 IU eCG on a second occasion. Further, three females (no. 4, 6, 9) given two repeated treatments of 200 IU eCG at an interval of 8–31 mo produced a high number of CL (4–17/female) during each episode.

Ovarian Response to Gonadotropins in Clouded Leopards

Fresh CL and/or mature-appearing ovarian follicles (Fig. 2) were observed in all clouded leopards regardless of gonadotropin dosage (Table 3). However, the occurrence of ovulation depended on eCG dosage (Table 3). Most females (80–100%) ovulated following 50–400 IU eCG (Table 3). A minimal dosage of 50 IU eCG was needed for ovulation to occur in a high proportion of females. Residual unovulated follicles (1–15/female) were observed in all 27 test episodes with one exception (one female treated with 50 IU eCG/75 IU hCG with 3 fresh CL only). Number and size of these follicles were unrelated ($p > 0.05$) to gonadotropin regimen (Table 3).

Gonadotropin treatment also influenced ($p < 0.05$) clouded leopard CL morphology (Table 4). The same CL phenotypes (small and large) and relative diameters as seen in cheetahs were observed. Clouded leopards given the highest (200 or 400 IU) and lowest (25 IU) eCG dosages produced only small CL, whereas 75–100% of females treated with 50 to 100 IU produced large CL (Table 4). In comparison, however, the number of large CL (1.7–2.8/female) was lower in clouded leopards (Table 4) than in cheetahs (6.1/female; Table 2). A third CL type consisting of mature luteal tissue (Fig. 3) was detected in four clouded leopards (females no. 5, 8, 20, 23). These CL were large

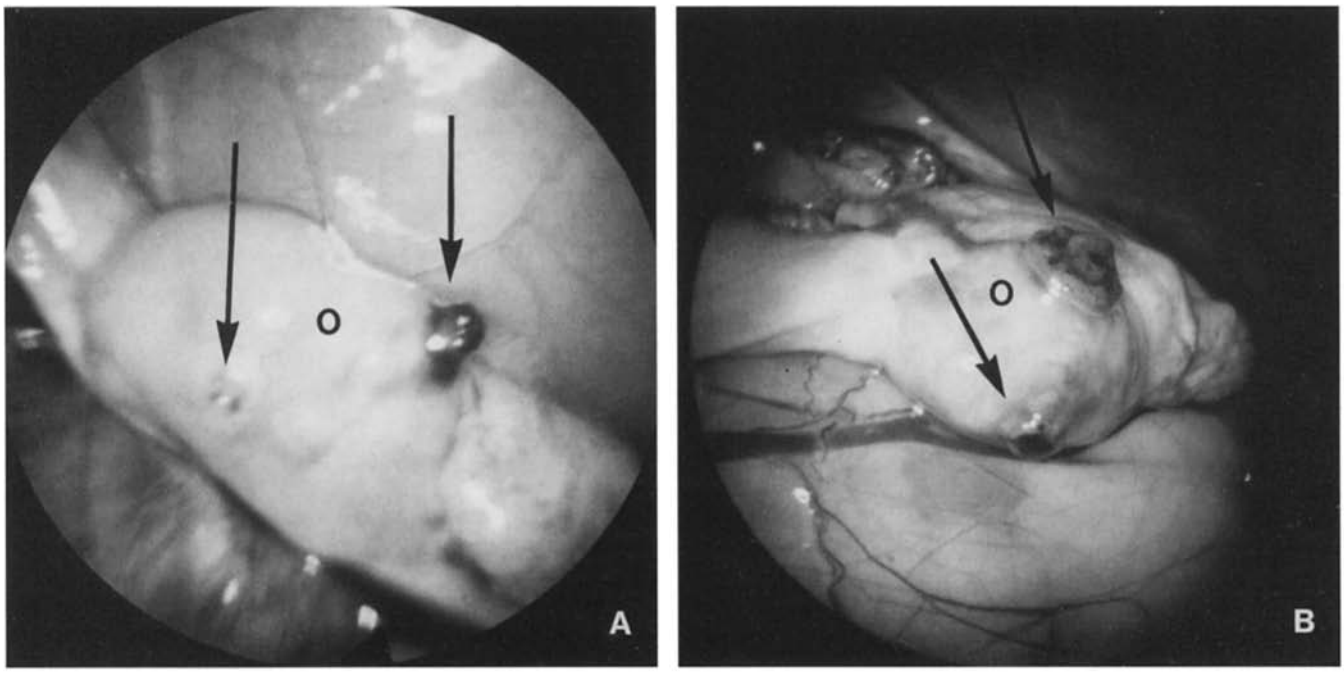

FIG. 1. Cheetah ovaries (O) each containing one of two types of fresh CL after gonadotropin stimulation. **A**) The small CL (arrows) were 2–4 mm in diameter. **B**) The large CL (arrows) were 5–8 mm in diameter. Magnification can not be determined.

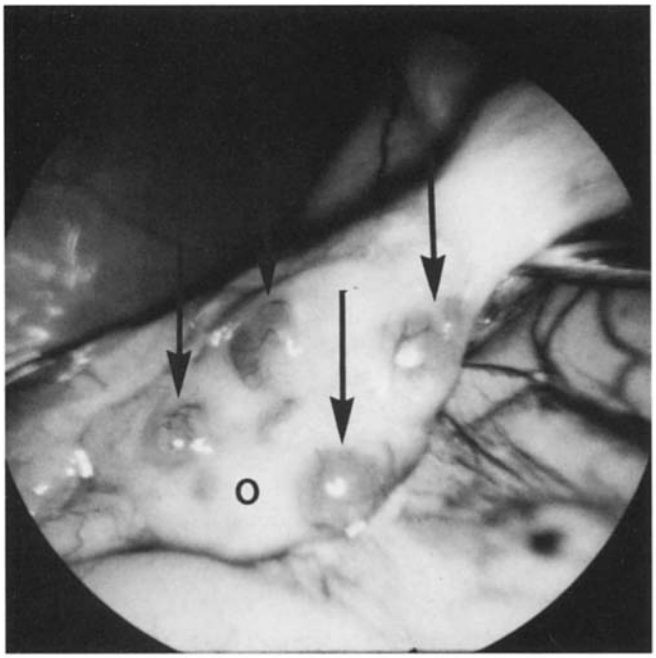

FIG. 2. Clouded leopard ovary (O) containing unovulated, residual follicles (arrows) measuring > 2 mm in diameter.

spherical structures (10–12 mm in diameter, 8–10 mm in height) with extensive surface vascularization. These aged CL were presumed to have formed before exogenous gonadotropin stimulation, occurring as a result of spontaneous ovulation in these females housed as singletons.

Circulating Hormone Concentrations

Serum estradiol-17β concentrations at the time of laparoscopic ovarian assessment were not influenced ($p > 0.05$) by gonadotropin dosage in cheetahs ($r = 0.2$) or clouded leopards ($r = 0.3$). Mean estradiol-17β concentrations were 14.4 ± 1.3 pg/ml in cheetahs (range, 9.0–37.2 pg/ml) and 18.1 ± 1.5 pg/ml in clouded leopards (range, 10.0–39.1 pg/ml). In both species, there was no relationship ($p > 0.05$) in serum estradiol-17β concentration and the number of residual unovulated follicles (cheetah, $r = 0.3$; clouded leopard, $r = 0.2$) and CL number (cheetah, $r = 0.1$; clouded leopard, $r = 0.1$).

Circulating progesterone concentrations also were unrelated ($p > 0.05$) to gonadotropin dosage in either species (cheetah, $r = 0.5$; clouded leopard, $r = 0.5$). Progesterone was baseline (≤ 1 ng/ml) in all cheetahs but one (female no. 1, Table 5) that produced 3 small and 3 large CL (4.4 ng/ml). Serum progesterone appeared to be more variable in ovulating clouded leopards (range, 0.1–73.5 ng/ml); this variability was largely linked to the presence of fresh versus aged CL. Females with older, mature CL had higher circulating progesterone (mean, 51.1 ± 10.8 ng/ml; range, 31.9–73.5) than did females with only fresh CL (mean, 1.5 ± 0.4 ng/ml; range, 0.1–7.8). Serum progesterone was baseline (≤ 1 ng/ml) in 14 of the 20 occasions in which only fresh and no aged CL were observed. On the remaining six occasions in which only fresh CL were detected, progesterone ranged from 2.3 to 7.8 ng/ml. The size of fresh CL (small vs. large) in clouded leopards did not influence ($p > 0.05$) serum progesterone concentrations. In females having only recent ovulations, circulating progesterone was similar ($p > 0.05$) between individuals with only small CL (2.0 ± 0.9 ng/ml) and those having at least one large CL (1.2 ± 0.4 ng/ml).

Artificial Insemination of Cheetahs

Nineteen AI procedures were conducted on 14 postovulatory cheetahs after treatment with 400 IU eCG/250 IU hCG (n = 5), 200 IU eCG/100 IU hCG (n = 13), or 100 IU eCG/100 IU hCG (n = 1) (Tables 2 and 5). Ten females (no. 1, 3, 5, 8, 10, 11, 12, 13, 14, 16) were inseminated once and three females twice (at an interval of 8 mo for female no. 9, 13 mo for female no. 2, and 31 mo for female no. 6). The remaining female (no. 4) was inseminated on three occasions (13 mo after the first treatment and again 8 mo after the second). Within individuals, repeated gonadotropin treatment did not compromise ovarian response (Table 5). The same gonadotropin dosage evoked similar ovarian activity between episodes.

Mean ejaculate characteristics for the 12 cheetah sperm donors were as follows: ejaculate volume, 1.4 ± 0.2 ml; sperm concentration, 22.7 ± 5.3 × 10^6/ml; sperm percentage of motility, 75.6 ± 1.6%; and sperm forward progressive motility, 3.7 ± 0.1. All males produced low percentages of morphologically normal spermatozoa (mean, 18.9 ± 2.2%; range, 3.5–34.0%), with the predominant malformations being an abnormal acrosome (9.0%), coiled flagellum (10.2%), bent midpiece (22.2%), bent flagellum (17.7%), or cytoplasmic droplet (12.3%).

Mean interval between hCG administration and AI was 45.9 ± 0.3 h (range, 43.5–48.0 h) (Table 5). No pregnancies occurred in postovulatory cheetahs in the high (400 IU) or low (100 IU) eCG dosage groups. Pregnancies occurred only in cheetahs given 200 IU eCG/100 IU hCG and with ovaries containing large CL (Table 2, 5). Six of the 13 (46.2%) inseminations resulted in a pregnancy and subsequent birth (one to four cubs; mean, 2.0 ± 0.4 cubs/litter) after a 91- to 95-day gestation. Cheetahs becoming pregnant were inseminated with twice the number of motile

TABLE 3. Influence of eCG and hCG on ovarian activity in clouded leopards.

	Dosages of eCG/hCG (IU)[a]				
Variable	200/140 or 400/280 (n = 6)	100/75 (n = 5)	75/75 (n = 5)	50/75 (n = 7)	25/75 (n = 4)
No. postovulatory females/total no. females (%)	6/6 (100.0%)[b]	4/5 (80.0%)[b]	4/5 (80.0%)[b]	7/7 (100.0%)[b]	1/4 (25.0%)[c]
No. fresh CL/ovulating female	5.3 ± 1.3[b]	4.5 ± 1.0[b,c]	4.0 ± 0.6[b,c]	2.6 ± 0.6[c]	3.0 ± 0.0[c]
Mean diameter of fresh CL (mm)	3.7 ± 0.1[b]	5.1 ± 0.3[c]	5.4 ± 0.2[c]	5.6 ± 0.3[c]	3.7 ± 0.3[b]
No. unovulated follicles/female	6.2 ± 2.2	7.2 ± 2.5	5.2 ± 2.3	5.1 ± 1.7	8.5 ± 2.2
Mean diameter of follicles (mm)	4.6 ± 0.2	4.9 ± 0.3	4.8 ± 0.2	4.1 ± 0.1	4.0 ± 0.1

[a] n, Number of occasions.
[b,c] Within rows, values with different superscripts differ ($p < 0.05$).

TABLE 4. CL morphology and AI efficiency in postovulatory clouded leopards treated with eCG and hCG.

	Dosages of eCG/hCG (IU)[a]				
	200/140 or 400/280 (n = 6)	100/75 (n = 4)	75/75 (n = 4)	50/75 (n = 7)	25/75 (n = 1)
Types of CL[b]					
No. females with small fresh CL/no. ovulating females (%)	6/6 (100%)[c]	3/4 (75.0%)[c,d]	2/4 (50.0%)[d,e]	3/7 (42.9%)[d,e]	1/1 (100%)[c]
No. small fresh CL/ovulating female	5.3 ± 1.4[c]	2.8 ± 1.0[c,d]	1.2 ± 0.8[d]	0.9 ± 0.6[d]	3.0 ± 0.0[c]
No. females with large fresh CL/no. ovulating females (%)	0/6 (0%)[c]	3/4 (75.0%)[d]	4/4 (100%)[d]	6/7 (85.7%)[d]	0/1 (0%)[c]
No. large fresh CL/ovulating female	0.0[c]	1.7 ± 0.6[d]	2.8 ± 0.9[d]	1.7 ± 0.4[d]	0.0[c]
Postovulatory AI					
No. pregnant females/total no. inseminations (%)	0/6 (0%)	0/4 (0%)	0/4 (0%)	0/3 (0%)	0/1 (0%)

[a] n, Number of occasions.
[b] Two types of fresh CL types were observed: 1) small CL (2–4 mm in diameter); and 2) large CL (5–8 mm in diameter). Mature, aged CL (10–12 mm in diameter) also were observed in certain females (data not shown).
[c,d,e] Within rows, values with different superscripts differ ($p < 0.05$).

sperm (15.8 ± 3.8 × 10^6) as were nonpregnant females (8.0 ± 2.4 × 10^6). Of the four cheetahs inseminated on multiple occasions, two produced a pregnancy after the second (female no. 2) and third (female no. 4) AI procedure, and another (female no. 6) produced two pregnancies after both the first and second AI (Table 5). Of the 12 cubs produced by AI, five (41.7%) died. Four of these deaths were related to stillbirth or dystocia. The fifth cub died of pneumonia during hand-rearing at 19 days of age. The remaining seven cubs were born without complications and were mother-reared.

Artificial Insemination of Clouded Leopards

Eighteen AI procedures were conducted in 15 postovulatory clouded leopards given 200 or 400 IU eCG/140 or 280 IU hCG (n = 6), 100 IU eCG/75 IU hCG (n = 4), 75 IU eCG/75 IU hCG (n = 4), 50 IU eCG/75 IU hCG (n = 3), or 25 IU eCG/75 IU hCG (n = 1) (Tables 4 and 6). Four females (no. 16, 17, 18, 20) treated with 50 IU eCG/75 IU hCG were postovulatory; however, no insemination was conducted because of the unavailability of sperm donors. Twelve females were inseminated once and three females (no. 1, 2, 5) twice (at a 9- to 16-mo interval). Females no. 1 and 2 were treated with 400 IU eCG during the first occasion and 200 IU eCG during the second occasion, whereas female no. 5 was given 100 IU eCG on the first and 50 IU eCG on the second occasion (Table 6).

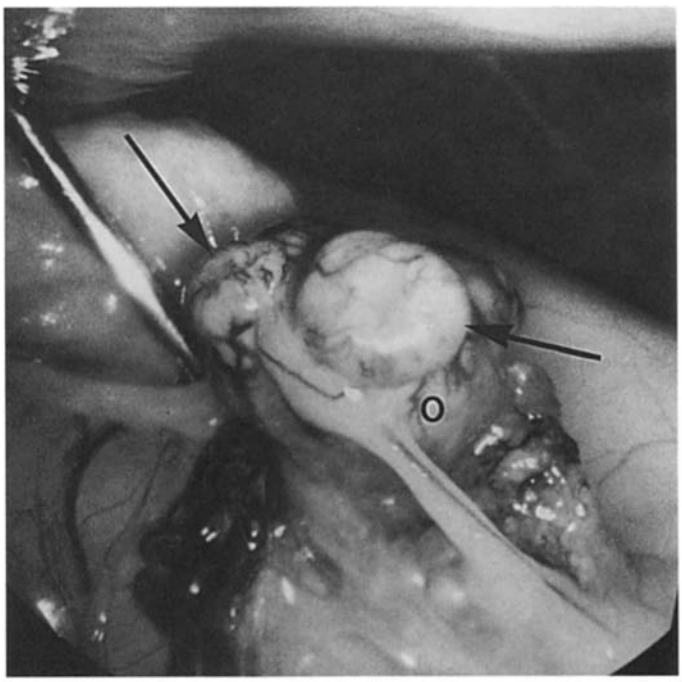

FIG. 3. Clouded leopard ovary (O) containing mature, aged CL (arrows) in a spontaneously ovulating female. Aged CL were spherical structures (10–12 mm in diameter, 8–10 mm in height) with extensive surface vascularization.

Mean ejaculate characteristics for the 14 clouded leopard sperm donors were as follows: ejaculate volume, 0.9 ± 0.1 ml; sperm concentration, 35.7 ± 4.3 × 10^6/ml; sperm percentage of motility, 77.3 ± 2.4%; and sperm forward progressive motility, 3.9 ± 0.1. All males produced low proportions of structurally normal spermatozoa (mean, 23.0 ± 4.3%; range, 2–64%), with most of the pleiomorphic forms being abnormal acrosome (22.4%), bent midpiece (14.5%), or cytoplasmic droplet (14.5%) deformities.

Mean interval from hCG to AI was 45.1 ± 0.6 h (range, 39.0–49.5 h), with females receiving 2.5 × 10^6 to 43.2 × 10^6 motile spermatozoa in utero (Table 6). Despite the presence of fresh CL and the use of relatively high numbers of motile spermatozoa, no pregnancies were produced.

DISCUSSION

Reliable and consistent success using assisted reproduction depends on understanding those factors influencing the hormonal control of folliculogenesis and ovulation. Whole-animal studies of this kind are crucial for generating sufficient data to eventually allow techniques like AI to become routine for managing endangered species. Both the cheetah and clouded leopard are propagated in North American and European zoos under the auspices of an organized genetic management plan. Cooperating institutions breed animals on the basis of genetic value in order to maintain maximal genetic diversity in the regional population. The result is a need to move animals between zoos for breeding. We assert that it would be less expensive and more humane to move sperm rather than living animals from one location to another. Although the concept is easily advocated, it actually is quite difficult to apply assisted reproduction techniques in wildlife, largely because of species-specific phenomena [14]. Simply put, reproductive processes in humans

TABLE 5. Ovarian responses and inseminate traits of postovulatory cheetahs inseminated in utero.

Female no.[a]	eCG/hCG dosage (IU)	Time (h post hCG)	No. unovulated follicles	No. CL[b] (small, large)	Sperm motility (%)	Sperm progressive motility[c]	Normal sperm (%)	Motile sperm inseminated (× 10^6)	Pregnancy
1	400/250	45.0	4	6 (3, 3)	40	2.5	33.0	1.5	–
2a	400/250	43.5	2	12 (12, 0)	65	4.0	19.0	5.4	–
3	400/250	47.0	3	13 (13, 0)	50	3.0	8.0	7.3	–
4a	400/250	44.0	5	11 (11, 0)	60	4.5	14.5	6.7	–
5	400/250	46.0	3	10 (10, 0)	65	3.0	15.0	1.6	–
2b	200/100	46.0	2	12 (0, 12)	70	4.0	22.5	14.2	+ (4 cubs)
4b	200/100	44.0	2	6 (0, 6)	50	3.5	16.5	0.4	–
6a	200/100	45.0	5	5 (0, 5)	85	4.0	32.5	3.4	+ (2 cubs)
8	200/100	45.5	7	10 (4, 6)	50	4.0	24.0	1.4	–
9a	200/100	45.0	2	4 (0, 4)	90	4.5	31.0	11.5	–
4c	200/100	47.0	0	9 (0, 9)	60	3.0	8.5	7.6	+ (2 cubs)
10	200/100	45.5	9	3 (1, 2)	50	3.0	14.0	7.6	–
11	200/100	47.5	11	1 (0, 1)	70	3.5	5.5	17.1	–
12	200/100	47.0	5	13 (3, 10)	60	3.0	12.0	21.2	+ (1 cub)
13	200/100	47.0	1	15 (8, 7)	55	3.5	22.0	19.5	+ (1 cub)
9b	200/100	47.0	1	17 (7, 10)	50	3.0	34.0	13.5	–
16	200/100	48.0	5	4 (0, 4)	70	2.5	20.0	4.3	–
6b	200/100	47.5	0	8 (5, 3)	60	3.5	24.0	28.6	+ (2 cubs)
14	100/100	45.0	3	8 (8, 0)	60	3.0	3.5	9.4	–

[a] Females used on multiple occasions are represented by a letter (a, first occasion; b, second occasion; c, third occasion); there was an interval of 8–31 mo between successive trials.
[b] Total number of fresh CL; numbers in parentheses represent number of small CL and large CL.
[c] Based on a scale of 0–5 (0, no movement; 5, rapid forward progression).

and livestock are vastly different from those in cheetahs and clouded leopards. Therefore, a basic research approach always is necessary to develop reliable techniques but usually is complicated because few endangered species are available for fundamental studies. Thus, one of the important characteristics of this study was the relatively large number of animals made available by generous collaborating institutions. This allowed determination of the relative responsiveness of the two species to various dosages of exogenous gonadotropins, eventually identifying a gonadotropin regimen in one species (cheetah) that allowed offspring to be produced with some consistency. Nonetheless, interesting findings also were made with respect to CL phenotype and the inability of clouded leopards to become

TABLE 6. Ovarian responses and inseminate traits of postovulatory clouded leopards inseminated in utero.

Female no.[a]	eCG/hCG dosage (IU)	Time (h post hCG)	No. unovulated follicles	No. CL[b] (small, large)	Sperm motility (%)	Sperm progressive motility[c]	Normal sperm (%)	Motile sperm inseminated (× 10^6)	Pregnancy
1a	400/280	39.0	8	1 (1, 0)	60	3.0	9.0	20.6	–
2a	400/280	44.5	3	8 (8, 0)	65	4.0	18.0	2.5	–
3	400/280	46.5	2	5 (5, 0)	65	3.5	30.0	8.8	–
4	400/280	44.5	1	10 (10, 0)	80	4.0	49.5	14.3	–
1b	200/140	39.0	15	2 (2, 0)	50	3.0	26.0	40.0	–
2b	200/140	42.5	8	6 (6, 0)	60	4.0	3.5	28.1	–
5a	100/75	45.5	12	3 (0, 3)	80	4.0	41.5	19.0	–
7	100/75	46.0	6	3 (3, 0)	70	3.0	39.5	19.6	–
8	100/75	46.0	1	5 (3, 2)	65	4.0	11.5	15.2	–
9	100/75	45.0	3	7 (5, 2)	60	4.0	3.5	30.9	–
10	75/75	45.5	8	3 (0, 3)	65	4.0	3.0	14.2	–
12	75/75	47.0	1	5 (0, 5)	40	3.5	42.0	8.7	–
13	75/75	47.0	3	5 (3, 2)	80	4.0	7.0	11.0	–
14	75/75	44.5	1	3 (2, 1)	70	3.5	27.0	5.6	–
5b	50/75	46.5	12	1 (1, 0)	80	3.5	64.0	43.2	–
15	50/75	45.5	0	3 (1, 2)	50	3.0	18.0	9.4	–
16	50/75	47.5	10	1 (0, 1)	No AI				
17	50/75	49.0	6	2 (0, 2)	No AI				
18	50/75	48.0	3	2 (0, 2)	No AI				
19	50/75	48.5	2	3 (0, 3)	70	3.5	18.5	14.7	–
20	50/75	50.0	3	6 (4, 2)	No AI				
22	25/75	49.5	2	3 (3, 0)	80	3.0	2.0	6.6	–

[a] Females used on multiple occasions are represented by a letter (a, first occasion; b, second occasion); there was an interval of 9–16 mo between successive trials; four females (nos. 16, 17, 18, 20) were not inseminated due to unavailability of sperm sources.
[b] Total number of fresh CL; numbers in parentheses represent number of small CL and large CL. Mature, aged CL also were observed in females no. 5b (n, 3 aged CL) and no. 20 (n, 4 aged CL).
[c] Based on a scale of 0–5 (0, no movement; 5, rapid forward progression).

pregnant despite the presence of fresh CL and the intrauterine deposition of motile spermatozoa.

The aggressive nature of wild felids largely dictated our decision to use eCG as the hormone of choice for stimulating follicular activity. Earlier cheetah studies determined that FSH was effective for stimulating ovulation without causing ovarian hyperstimulation and residual follicular development [18]. However, because of its short biological half-life, FSH typically is administered in a series of daily injections, which can highly stress wild animals. In contrast, it is well known that a single eCG injection elicits significant follicular activity in felids, in part because of a prolonged circulatory persistence, recently found to approach 120 h in the domestic cat [22]. The disadvantage of eCG in combination with hCG is a second wave of postovulatory follicular development and a secondary rise in circulating estradiol-17β [16, 23, 24], which may alter oviductal/uterine conditions and disrupt fertilization or embryo transport/development [25–31]. The extent of the latter problem in the cheetah and clouded leopard is unknown, but clearly both species produced significant numbers of unovulated residual follicles. We recently determined that even hCG given alone to an anestrual domestic cat is highly folliculogenic, in part because of its persistence in circulation for at least 96 h [22]. Further, cats given eCG alone and then mated with males produce a number of unovulated follicles at ovariohysterectomy 6 days later that is similar to the number in naturally estrual, copulating counterparts [32]. All of this suggests that perhaps the unovulated follicles usually observed in eCG/hCG-treated felids actually result from the hCG component. For these reasons and because of its efficacy after a single i.m. injection, we continue to advocate eCG as a practical exogenous gonadotropin for wild felids.

Of particular interest was the level of sensitivity in the cheetah and clouded leopard to eCG/hCG. Female responsiveness was dose-dependent, but more importantly, both the cheetah and clouded leopard were more sensitive to eCG than previously studied species. For example, although 3–8 times larger in body weight, the cheetah and clouded leopard require about the same eCG dosage (50–200 IU) as the domestic cat to elicit comparable follicular growth [16]. By contrast, we recently determined that the ocelot (*Felis pardalis*), despite being half the size of the clouded leopard, requires 500 IU eCG (10 times the clouded leopard dosage) to mimic similar ovarian follicular activity [9]. It is clear that eCG dosage is uncoupled to felid body mass, with the differences in species sensitivity perhaps being evolutionarily based (ocelots originate in Latin America whereas cheetahs originate in Africa/Asia and clouded leopards in Asia). Regardless, these results re-emphasize the fundamental differences in reproductive mechanisms, even among related species in the same family.

Our comparison of various gonadotropin dosages was a useful strategy for determining the minimum effective eCG/hCG dosage that would cause adequate follicular development and ovulation but not ovarian hyperstimulation. We determined in the cheetah and clouded leopard that insufficient eCG dosages produced follicles incapable of ovulating in response to an hCG stimulus, despite high hCG dosages (75–100 IU) being used. The etiology of the low ovulation success may have been associated with the effects of eCG on ovarian LH receptors during follicular induction and maturation. LaPolt et al. [33] demonstrated that eCG increases the number of ovarian LH receptors, with peak receptor content observed several days after eCG. The lowest eCG dosages in the cheetah (100 IU) and clouded leopard (25 IU) may have been insufficient for inducing numbers of LH receptors sufficient for the biochemical and physical events of ovulation. Interestingly, a similar ovulation threshold has been observed in pumas treated with 100 IU eCG and 100 IU hCG [7].

Results also revealed the presence of two CL phenotypes in the cheetah and clouded leopard, with presence perhaps influenced by gonadotropin dosage. Although the highest eCG/hCG dosage produced ovulations in all females, the resulting CL appeared small and underdeveloped, similar to those observed in females treated with the lowest, least-effective dosage. In contrast, although the intermediate gonadotropin dosages generally resulted in fewer postovulatory females, CL observed after these dosages were large-size. The significance of this remains unknown because circulating progesterone concentrations were similar in females producing large versus small CL. However, the large CL described here were morphologically similar to those observed in cheetahs that had ovulated after natural estrus and mating [19]. Luteal function in such females appears to be adequate to sustain embryo development and implantation in the cheetah, because all six AI pregnancies achieved here, as well as in a previous study [5], occurred in females producing large CL. Conversely, none of the eCG/hCG-treated cheetahs with only small CL became pregnant after insemination. Obviously, there is a need to more clearly understand the significance of these phenotypic variations in terms of function and importance, especially since the presence of large CL had no effect on pregnancy success in the clouded leopard.

Circulating estradiol concentrations at the time of laparoscopic ovarian examination (39–50 h post-hCG) were not correlated to the presence of residual, unovulated ovarian follicles. Despite observation of significant numbers of follicles (~3–9 follicles/female) in most cheetahs and clouded leopards, estradiol concentrations averaged 14–18 pg/ml, comparable to values measured in the anestrus domestic cat [34, 35]. Peripheral progesterone concentration at the time of ovarian assessment was slightly more informative about luteal activity, but even by 39–50 h after hCG, serum progesterone remained near baseline (≤ 1 ng/ml) in most cheetahs and clouded leopards, similar to that in the early postovulatory domestic cat. Serum progesterone commonly remains at baseline for 24–48 h after natural or gonadotropin-induced ovulation in domestic cats, and then progesterone secretion gradually increases to peak concentrations approximately 5 days postovulation [35–37]. These observations also agree with endocrine profiles now being generated through the measurement of hormonal metabolites in voided feces [38–41]. Fecal progesterone metabolite concentrations remain at nadir for several days after ovulation in the domestic cat, cheetah, and clouded leopard, with peak levels detected 5–10 days later [38–41]. In the present study, circulating progesterone concentrations were higher than 1 ng/ml in only a single cheetah and six clouded leopards. Interestingly, four of these females were treated with the highest (400 IU) eCG dosage, suggesting either ovarian hyperstimulation and/or premature luteinization of follicles [42–44]. Substantially higher serum progesterone concentrations (31–73 ng/ml) were detected in four clouded leopards with obviously aged ovarian CL. Using fecal hormone metabolite monitoring, we recently determined that 6 of 14 clouded leopard females (43%) housed singly or with other females spontaneously ovulated, on the basis of increased and sustained progesterone excretion [39]. The present

study also clearly confirmed that this species can ovulate in the absence of male exposure. In contrast, no aged CL were observed in cheetahs, which affirms recent fecal hormonal monitoring in this species indicating that spontaneous ovulation rarely occurs [41]. Overall, the limited usefulness of sporadic serum hormone values reconfirms the power of fecal hormone monitoring for identifying reproductive status (noninvasively) as well as for enhancing assisted reproduction. Because the presence of luteal tissue can compromise ovulation induction and progesterone secretion following exogenous gonadotropins [39], the ability to diagnose elevated progesterone will be important to improving the efficiency of AI.

AI was much more efficient in the cheetah than in the clouded leopard, despite observations of fresh CL and use of comparable quality spermatozoa for insemination in clouded leopards. It is particularly encouraging that pregnancies can be produced in cheetahs by AI despite the production of ~80% malformed spermatozoa by males. We now know that structurally abnormal felid spermatozoa are incapable of participating in fertilization because of delayed and problematic capacitation [45], inability to penetrate the bilayered zona pellucida [45, 46], and compromised protein tyrosine phosphorylation associated with the cascade of events leading to the acrosome reaction [46]. Therefore, if a mean AI dosage of 16 million motile spermatozoa resulted in pregnancy in cheetahs and ~80% of these were abnormally shaped, then fertilization occurred in the presence of relatively few (~3 million) motile, normally structured spermatozoa. The fact that cheetahs that became pregnant were inseminated with almost twice the number of motile spermatozoa than were nonpregnant counterparts suggests that total sperm number was important to achieving pregnancy success. However, for clouded leopards, there was no effect of sperm number inseminated (3–43 million) on ability to become pregnant. Nonetheless, in an earlier trial, we produced a clouded leopard pregnancy by depositing 88 million motile spermatozoa laparoscopically in utero [10]. This female, treated with 100 IU eCG/75 IU hCG, produced 5 large CL and gave birth to two healthy offspring after an 89-day gestation.

Part of the reason that differences in AI efficiency in this study were probably unrelated to residual follicle development is that both species had significant numbers of these unovulated follicles. Although a hyperfollicular condition could alter gamete transport, fertilization, or implantation, we have no evidence that these events are compromised in the eCG/hCG-treated cat. Four of the cheetahs becoming pregnant in this study had 1–5 residual follicles. It is possible that the species difference in AI efficiency was related to ovarian activity at the time of eCG/hCG treatment. We know from a nationwide survey that only 22% of 68 adult cheetahs (from 18 institutions) examined laparoscopically had any evidence of mature follicular activity [19]. More recently, a longitudinal evaluation of reproductive activity using fecal steroid monitoring revealed that 25% of cheetahs fail to show any ovarian estrogen production, and all cheetahs demonstrate obvious periods of anestrus ranging from 2 to 5 mo in duration [41]. We suspect that this could explain in part why cheetahs appear to be highly responsive to exogenous gonadotropins: prolonged reproductive inactivity allows the ovaries and perhaps the uterus to be highly sensitive to eCG/hCG stimulation. In contrast, recent fecal hormone-tracking studies of clouded leopards reveal much more consistent ovarian activity throughout the year, with few or no anestrual periods [39]. Thus, exogenous gonadotropins compete with or are confounded by endogenous endocrine rhythms in clouded leopards. When this situation is combined with occasional spontaneous ovulations, it becomes much more difficult to achieve a consistent, optimal ovarian response in the clouded leopard using exogenous gonadotropins.

Clearly, these results have demonstrated that these endangered species are highly sensitive to eCG and hCG. However, although fresh spermatozoa were deposited in utero at a time coincident with the presence of fresh CL in both species, the cheetah was far more likely to become pregnant than the clouded leopard. Because there were few remarkable differences in ovarian response between the two species in this study, it must be assumed that other factors beyond those assessed here regulate pregnancy success. It is obvious that sperm quality, although important, is not the dictating force, because cheetahs inseminated with predominantly malformed spermatozoa became pregnant. However, the impact of sperm number appears to be worth pursuing because cheetahs were more likely to become pregnant when inseminated with more spermatozoa. Perhaps another important factor is luteal function, given the significant differences observed in CL phenotype and the finding that the ovaries of all pregnant cheetahs had large CL. However, this phenomenon is difficult to study in endangered species, and it may be more useful to focus on differences in reproductive/endocrine rhythms and dynamics over time between the two species. The inconsistent ovarian activity and frequent anestrus displayed by cheetahs may be advantageous for assisted reproduction due to a high responsiveness to a novel gonadotropin stimulus. Therefore, testing the hypothesis that pregnancy success from AI can be enhanced by first suppressing all ovarian activity before exogenous gonadotropin therapy is a high priority.

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