

Natural Versus Chorionic Gonadotropin-Induced Ovarian Responses in the Clouded Leopard (*Neofelis nebulosa*) Assessed by Fecal Steroid Analysis¹

Janine L. Brown,^{2,3} David E. Wildt,³ Laura H. Graham,³ Ann P. Byers^{3,4}
Larry Collins,³ Sean Barrett,⁵ and JoGayle Howard³

Conservation and Research Center,³ National Zoological Park, Smithsonian Institution, Front Royal, Virginia
Conservation Breeding Specialist Group,⁴ Apple Valley, Minnesota
Nashville Zoo,⁵ Joelton, Tennessee

ABSTRACT

The clouded leopard (*Neofelis nebulosa*) is an endangered species difficult to propagate in captivity because of sexual incompatibility between paired individuals. Fecal estradiol (E_2) and progesterone (P_4) metabolites were quantified in 14 females to noninvasively monitor seasonal reproductive patterns and compare ovarian responses to natural mating vs. exogenous gonadotropins. Increased E_2 excretion was associated with behavioral estrus or eCG treatment, whereas elevated P_4 metabolites were observed during natural and hCG-induced pregnant and nonpregnant luteal phases. On the basis of fecal E_2 profiles, duration of the estrous cycle was 24 ± 2 days, with estrus lasting 6 ± 1 days. Mean gestation length was 89 ± 2 days, whereas duration of the nonpregnant luteal phase was 47 ± 2 days. Females experienced a seasonal anestrus during the late summer and early fall. One female demonstrated a lactational anestrus after the birth of three cubs. On nine occasions, clouded leopards ovulated spontaneously (based on elevated fecal P_4 metabolite concentrations) in the absence of mating. Patterns of eCG-stimulated E_2 excretion were similar to those associated with natural estrus and were unaffected by eCG dosage (25, 50, or 100 IU). In contrast, post-hCG P_4 metabolite profiles varied considerably, with responses including anovulation, attenuated luteal P_4 metabolite production, and hyperstimulated luteal function. In some females, compromised luteal function after ovulation induction appeared to be due to the presence of mature CL from previous spontaneous (without copulation) ovulations at the time of gonadotropin treatment. Duration of post-hCG P_4 metabolite excretion was reduced ~40% in these individuals compared to females with no evidence of active luteal activity. In sum, these are the first data describing the ovarian cycle of this endangered species. The information is important because it is based on the longitudinal assessment of multiple females using a completely atraumatic approach, thereby eliminating the potential confounding impact of stress. Data indicate that spontaneous ovulations and the presence of active luteal tissue on the ovary can profoundly affect ovarian responses to exogenous gonadotropin therapy. Therefore, fecal steroid monitoring can serve as a useful adjunct to developing assisted reproduction techniques, especially the hormonal induction of ovulation for planned artificial insemination.

INTRODUCTION

The clouded leopard (*Neofelis nebulosa*) is the only member of its genus and, therefore, taxonomically unique. It is a timorous species inhabiting tropical rain forests of Nepal, southeastern China, peninsular Malaysia, Borneo, Thailand, and Sumatra, and possibly Taiwan, Bangladesh, and India [1–5]. Because it is so elusive in the wild, virtually nothing is known about its physiology or ecology. No accurate census is available for wild populations, although numbers appear to be declining because of habitat destruction and poaching.

Clouded leopards reproduce poorly in captivity, with less than 25% of the North American population (98 males:80 females) having produced offspring, in large part because of intersex incompatibility. Successful propagation generally occurs only when the male and female are paired

at a young (usually prepubertal) age (< 1.5 yr). Introducing unfamiliar adults, even those with long-term olfactory and/or visual contact, often results in the male injuring or killing the female. Ironically, pairs that have consistently produced young now are in a breeding moratorium because of genetic over-representation. The most genetically valuable animals are those that cannot be paired because of the likelihood of mate-induced injury or death. Thus, the management of this species would benefit greatly from using assisted reproductive techniques, such as artificial insemination (AI) and in vitro fertilization (IVF)/embryo transfer (ET). These approaches also offer the possibility of exchanging genetic material (spermatozoa or embryos) between free-living and captive animals to help sustain genetic diversity [6].

To date, successful production of offspring by use of AI has been accomplished for the cheetah (*Acinonyx jubatus*), ocelot (*Felis pardalis*), leopard cat (*Felis bengalensis*), tiger (*Panthera tigris*), puma (*Felis concolor*), and clouded leopard, but pregnancy rates are generally low ($< 10\%$) [7–11]. Of 22 AI attempts in the clouded leopard, only a single pregnancy resulting in the birth of a healthy pair of cubs has resulted [11]. We suspect that this low success rate is due in part to a lack of information on the endocrine status of the

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²Correspondence: Dr. Janine L. Brown, Conservation and Research Center, 1500 Remount Road, Front Royal, VA 22630. FAX: (703) 635-6571.

species as a whole and on the reproductive status of individual females at the onset of gonadotropin therapy preceding AI. Felid AI protocols typically use eCG and hCG to stimulate follicular growth, oocyte maturation, and ovulation. Procedural failure is probably related to a poor maternal environment caused by inappropriate ovarian stimulation. This can occur as a result of too little or too much exogenous gonadotropin or of simply administering the gonadotropin at an inappropriate time in the natural reproductive cycle. The latter can be particularly problematic because many clouded leopards never display overt signs of sexual receptivity. Assisted reproduction success probably will continue to be suboptimal until two objectives are achieved. First, there is a need to determine the reproductive status of females in the captive population. Second, it is necessary to systematically characterize and interrelate normal ovarian responses resulting from natural estrus and mating/pregnancy to those following exogenous hormone treatment.

We recently developed a noninvasive method to assess endocrine activity in felids that effectively measures excreted estradiol (E_2) and progesterone (P_4) metabolites in feces [12]. In the present study, fecal steroid monitoring was used to produce a detailed database for the clouded leopard, including 1) characterizing the reproductive cycle, 2) examining the influence of season on reproductive endocrine patterns, and 3) determining whether steroid excretion profiles differ between pregnancy and nonpregnancy. These profiles were then compared to those produced by ovulation induction and AI to determine the "normality" of ovarian responses to current reproductive manipulation protocols.

MATERIALS AND METHODS

Animals

Adult female clouded leopards were maintained at three locations: 1) the Conservation and Research Center (CRC), Front Royal, VA ($n = 3$); 2) the Minnesota Zoological Garden (MZG), Apple Valley, MN ($n = 3$); and 3) the Nashville Zoo, Joelton, TN ($n = 8$). Two of the CRC females (9 and 10 yr of age) were paired with a male, except after the birth of cubs. Both females were captive-born and multiparous but had been maintained on a contraceptive implant (silastic rod containing melengestrol acetate [MGA; Dr. Ed Plotka, Marshfield Medical Research Foundation, Marshfield, WI]) from November 1989 to the onset of the study (February 1992). The third female (3 yr of age) was captive-born, hand-raised, and housed as a singleton. Animals were maintained in indoor enclosures (3.2×5.8 m) containing a nest box and window, connected to an outdoor wire-mesh cage with ~ 23 m² of floor space. Indoor-outdoor enclosures contained tree limbs for climbing and allowed for natural fluctua-

tions in photoperiod. All CRC clouded leopards had visual, olfactory, and auditory contact with conspecific males and females. The MZG study population comprised three females (4 yr of age and nulliparous; 5 yr of age and parous; 13 yr of age and multiparous) that were all captive-born. Females were housed in indoor enclosures (~ 40 m² of floor space) containing tree limbs and a nest box and were exposed to 12 h of artificial fluorescent light/day. All animals were maintained as singletons, but had olfactory and auditory contact with conspecific females. At the Nashville Zoo, clouded leopards averaged 4.8 ± 0.7 years of age (range, 1.5–7 yr) and consisted of nulliparous ($n = 6$) and multiparous ($n = 2$) females. All were captive-born, and five had been hand-raised. Animals were exposed to natural photoperiod and housed as singletons ($n = 5$) or female pairs ($n = 3$) in outdoor enclosures (~ 100 m²) containing perches and natural logs at various heights. All but two females had olfactory and auditory contact with conspecific males and females. At each institution, animals were observed daily for signs of behavioral estrus (lordosis, decreased appetite, increased affective behavior) [13] during routine caretaking procedures.

Fecal Steroid Analyses

Fecal E_2 and P_4 metabolites were extracted from samples as described previously [12]. Samples were dried (Savant Instruments Speedvac Rotary Evaporator; Forma Scientific, Inc., Marietta, OH) and pulverized, and ~ 0.2 g of well-mixed powder was boiled in 5 ml of 90% ethanol:10% distilled water for 20 min. After centrifuging at $500 \times g$ for 10 min, supernatant was recovered, and the pellet was resuspended in 5 ml of 90% ethanol, vortexed for 1 min, and recentrifuged. Both ethanol supernatants were combined, dried completely, and then redissolved in 1 ml methanol. Extractants were vortexed (1 min), placed in an ultrasonic glass cleaner for 30 sec, and revortexed (15 sec). A total of 3000 dpm each of 3H - E_2 and ^{14}C - P_4 (New England Nuclear, Wilmington, DE) was added to each sample before extraction to monitor procedural losses. Samples were diluted (1:40 for E_2 ; 1:800–1:80 000 for P_4) in phosphate buffer (0.01 M PO_4 , 0.14 M NaCl, 0.5% BSA, 0.01% NaN_3) before analysis by validated RIA [12].

The E_2 RIA used an antibody (provided by Dr. Samuel Wasser, Center for Wildlife Conservation, Seattle, WA) raised in rabbits against estradiol-17 β 6-*o*-carboxy-methyl-oxime:BSA [14], 3H -labeled E_2 tracer (New England Nuclear), and E_2 standards. The P_4 RIA was developed in our laboratory and relied upon a monoclonal P_4 antibody produced against 4-pregnen-11-ol-3, 20-dione hemisuccinate:BSA (#331; provided by Dr. Jan Roser, University of California, Davis, CA), an ^{125}I -labeled P_4 tracer (ICN Biomedical, Inc., Costa Mesa, CA), and P_4 standards [12]. Assay sensitivities, based on 90% of maximum binding, were 2 pg/ml and

5 pg/ml for the E_2 and P_4 assays, respectively. Intra- (based on sample values) and inter- (based on 2 control sample values) assay coefficients of variation were < 10% for both assays. All fecal data were expressed on a per-gram dry-weight basis.

Ovulation Induction and AI Procedures

Chorionic gonadotropins (eCG followed by hCG) were injected i.m. without anesthesia 80 h apart to stimulate follicular development and ovulation, respectively. Fecal samples were collected 3–5 times weekly for 10 days before, through 80–105 days after, eCG injection. Electroejaculates for AI were collected under ketamine HCl (15–20 mg/kg, i.m.; Fort Dodge Laboratories, Fort Dodge, IA) anesthesia as described previously [15]. An AC 60-Hz sine-wave electroejaculator with rectal probe (4.5 cm diameter, containing 3 longitudinally positioned electrodes) was used in a regimented sequence consisting of 80 incremental electrical stimuli given in an on-off pattern in 3 series (series 1 and 2 = 30 stimuli/series; series 3 = 20 stimuli) over an ~20-min interval. Each series consisted of repeating sets of 10 stimulations applied at increasing voltages (3–7 volts). Total ejaculate volume and sperm progressive motility were determined after each ejaculation series. Sperm concentration was determined by use of a hemocytometer. Intrauterine AI was performed laparoscopically 44–51 h after hCG injection under ketamine HCl (5–10 mg/kg, i.m) and xylazine (0.5–2 mg/kg, i.m; Rompun, Miles Laboratory, Inc., Shawnee Mission, KS) anesthesia [11].

For AI, an accessory Palmer grasping forcep was inserted 3 cm lateral to the umbilicus to grasp and stabilize the uterine horn [10]. An 18-gauge catheter was inserted transabdominally into the proximal third of each uterine horn, and the stylette was removed and replaced with sterile polyethylene tubing (PE-20; Clay Adams, Parsippany, NJ) containing $\sim 30 \times 10^6$ motile sperm in Ham's F-10 medium (Sigma Chemical Co., St. Louis, MO). The PE tubing was inserted beyond the tip of the catheter into the uterine lumen, and the diluted sperm (100–180 μ l/horn) were expelled. All ovarian structures were recorded at the time of AI.

Longitudinal Endocrine Patterns during Natural and Artificially Induced Cycles

At the CRC and MZG, fecal samples were collected 3–7 times weekly from female clouded leopards for a total of 21 and 17 mo, respectively. During that time, the singleton female at the CRC was induced to ovulate, and AI was performed 46–47 h post-hCG (75 IU) in May 1992 (100 IU eCG) and May 1993 (50 IU eCG) as described above. At the MZG, ovulation induction and AI were performed on all females (75 IU eCG/50 IU hCG) after 15 mo of longitudinal sampling (December 1993). For all MZG females, laparoscopic AI was

conducted 45–47 h post-hCG.

At the Nashville Zoo, two studies were conducted to compare ovarian responses to different eCG doses. In study 1 (February 1992), clouded leopards received i.m. injections of 50 IU ($n = 2$) or 100 IU ($n = 3$) eCG followed 80 h later by 75 IU hCG and laparoscopic AI at 44–48 h post-hCG. In study 2 (May 1993), females received i.m. injections of 25 IU ($n = 3$) or 50 IU ($n = 3$) eCG followed by 75 IU hCG 80 h later. The breeding moratorium imposed by the Clouded Leopard Species Survival Plan prevented AI from being conducted in study 2; however, ovaries were evaluated laparoscopically at 48–51 h post-hCG. Three of the females in study 1 were used again in study 2.

Statistical Analysis

Individual baseline E_2 concentrations were calculated from all samples before and after mating or AI, excluding those associated with the preovulatory E_2 surge (values associated with observed mating or exogenous gonadotropin ovulation induction). The beginning of an E_2 surge was determined by values that exceeded preceding values by 50%. Estrous cycle length was calculated as the number of days between E_2 surges. The period of estrus was based on the number of days E_2 was elevated above baseline. Ovulation was inferred from increased fecal P_4 metabolite excretion. Basal P_4 metabolite concentrations were calculated from values preceding the preovulatory E_2 surge. Postovulatory increases in P_4 excretion were considered significant if values exceeded the mean plus 2 standard deviations of the preceding values and remained elevated for at least 1 wk. Mean P_4 metabolite concentrations during pregnant and nonpregnant luteal phases contained values from the time of observed mating, estrus, or AI to parturition or the return of P_4 excretion to baseline. Weekly or thrice-weekly means were calculated for each individual female and then averaged to provide the respective group means. Differences in preovulatory peak E_2 concentrations or mean P_4 metabolite concentrations during pregnant and nonpregnant luteal phases were determined by a one-way analysis of variance or Student's t -tests.

RESULTS

General Observations

There were no differences ($p > 0.05$) in peak preovulatory E_2 concentrations between pregnant (265 ± 16 ng/g; $n = 3$) and nonpregnant (227 ± 26 ng/g; $n = 24$) clouded leopards for all animals combined (natural and gonadotropin-stimulated; Fig. 1). Similarly, there were no differences ($p > 0.05$) in preovulatory E_2 concentrations between naturally estrous (231 ± 40 ng/g; $n = 8$) and eCG-treated (223 ± 15 ng/g; $n = 16$) females. During the luteal phase, there also were no differences ($p > 0.05$) in overall mean P_4 me-

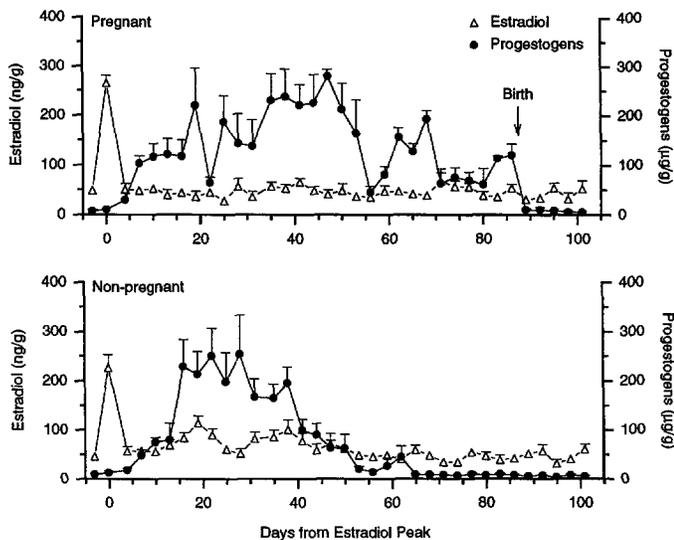


FIG. 1. Mean (\pm SEM) fecal estradiol and progesterone metabolite concentrations in pregnant (top) and nonpregnant (bottom) clouded leopards for all animals combined (natural and gonadotropin-induced). Data from nonpregnant gonadotropin-treated females not demonstrating a post-hCG increase ($p < 0.05$) in progesterone metabolite concentrations were not included. Data are aligned to estradiol peak (Day 0).

metabolite concentrations between pregnant ($144 \pm 13 \mu\text{g/g}$) and nonpregnant ($145 \pm 19 \mu\text{g/g}$; $n = 17$) clouded leopards (Fig. 1). For gonadotropin-treated, nonpregnant females, only data from those demonstrating post-hCG increases ($p < 0.05$) in P_4 concentrations were included in Figure 1. Of 16 ovulation induction attempts, post-eCG increases in fecal E_2 occurred in all animals; however, subsequent increases ($p < 0.05$) in P_4 metabolite excretion were observed on only nine occasions. Although responses varied, overall mean P_4 metabolite concentrations were similar ($p > 0.05$) between natural ($129 \pm 13 \mu\text{g/g}$; $n = 8$) and gonadotropin-induced ($155 \pm 29 \mu\text{g/g}$; $n = 8$) nonpregnant luteal phases. Mean gestation length (from day of observed mating or preovulatory E_2 surge to birth) was 89 ± 2 days for three pregnancies (86, 89, and 93 days). In contrast, the duration of the nonpregnant luteal phase was about half ($p < 0.05$) that of pregnancy (47 ± 2 days; range, 35–58 days; Fig. 1). On the basis of sequential E_2 profiles, the duration of the estrous cycle was 24 ± 2 days (range, 14–43 days; $n = 35$) with estrus lasting 6 ± 1 days (range, 1–10 days; $n = 45$).

Longitudinal Evaluations

Fecal steroid metabolite profiles for clouded leopards at the MZG and CRC are presented in Figures 2 and 3, respectively. In Figure 2, regular episodic increases in E_2 excretion were observed in two individuals (Fig. 2, A and B), with only sporadic cyclic activity observed in the third (Fig. 2C). All three of these females were maintained under constant

artificial lighting, and there was no evidence of seasonal suppression of ovarian activity. In contrast, females depicted in Figure 3 were exposed to natural fluctuations in photoperiod and clearly expressed less ovarian cyclic activity during the summer and early fall. In one female, two nonpregnant luteal phases followed E_2 surges between February and July (Fig. 3A). Ovarian quiescence then was observed from August to November, followed by two nonpregnant luteal phases and a pregnancy beginning in March of the next year. During the first month of gestation, fecal P_4 metabolite concentrations increased ~ 40 -fold and then declined to almost baseline by Day 26. Concentrations increased again before decreasing at about 50 days of gestation. Although remaining elevated above baseline until after parturition, P_4 metabolite concentrations were considerably lower than during the first two-thirds of gestation. A single cub was born after 93 days of gestation but was rejected and removed after 2 days for hand-rearing. Within 1 mo, the female resumed cycling and experienced another nonpregnant luteal phase. Figure 3B depicts three E_2 surges from early April through late May, the latter followed by a nonpregnant luteal phase increase in fecal P_4 metabolite excretion. No E_2 activity occurred until late October, after which the female became pregnant. Elevated fecal P_4 metabolite concentrations were sustained throughout gestation, except for a brief decline at about Day 55. Three cubs were born after an 86-day gestation followed by 9 mo of suppressed ovarian activity while the cubs nursed (lactational anestrus).

Because most felid species are considered induced ovulators [6, 16, 17], nonpregnant luteal phase increases in fecal P_4 excretion usually are attributed to nonfertile matings. This perhaps was the case for the paired females where six nonpregnant luteal phases (44 ± 2 days, range, 35–49 days) were observed (Fig. 3, A and B). However, some singleton females (Figs. 2B and 3C) demonstrated both induced (via exogenous gonadotropins) and spontaneous ovulations. For the Figure 3C singleton, elevated P_4 was measured at study onset, indicating that a spontaneous ovulation had occurred before sample collection was initiated. E_2 surges were observed from March to May, with ovulation induction and AI performed in May 1992 resulting in a 56-day nonpregnant luteal phase. At the time of laparoscopy, 12 unovulated ovarian follicles and 5 CL were present. There was little ovarian activity from September to mid-December, after which a spontaneous ovulation occurred followed by a 43-day luteal phase. Another AI was performed in May 1993, but a spontaneous ovulation apparently had preceded hormonal induction as demonstrated by the elevated P_4 at the time of eCG administration. Twelve follicles and 3 CL were counted, the latter being large (12 mm diameter), mushroom-shaped, and vascularized compared with normal fresh CL (4–6 mm diameter) identified during earlier AI attempts.

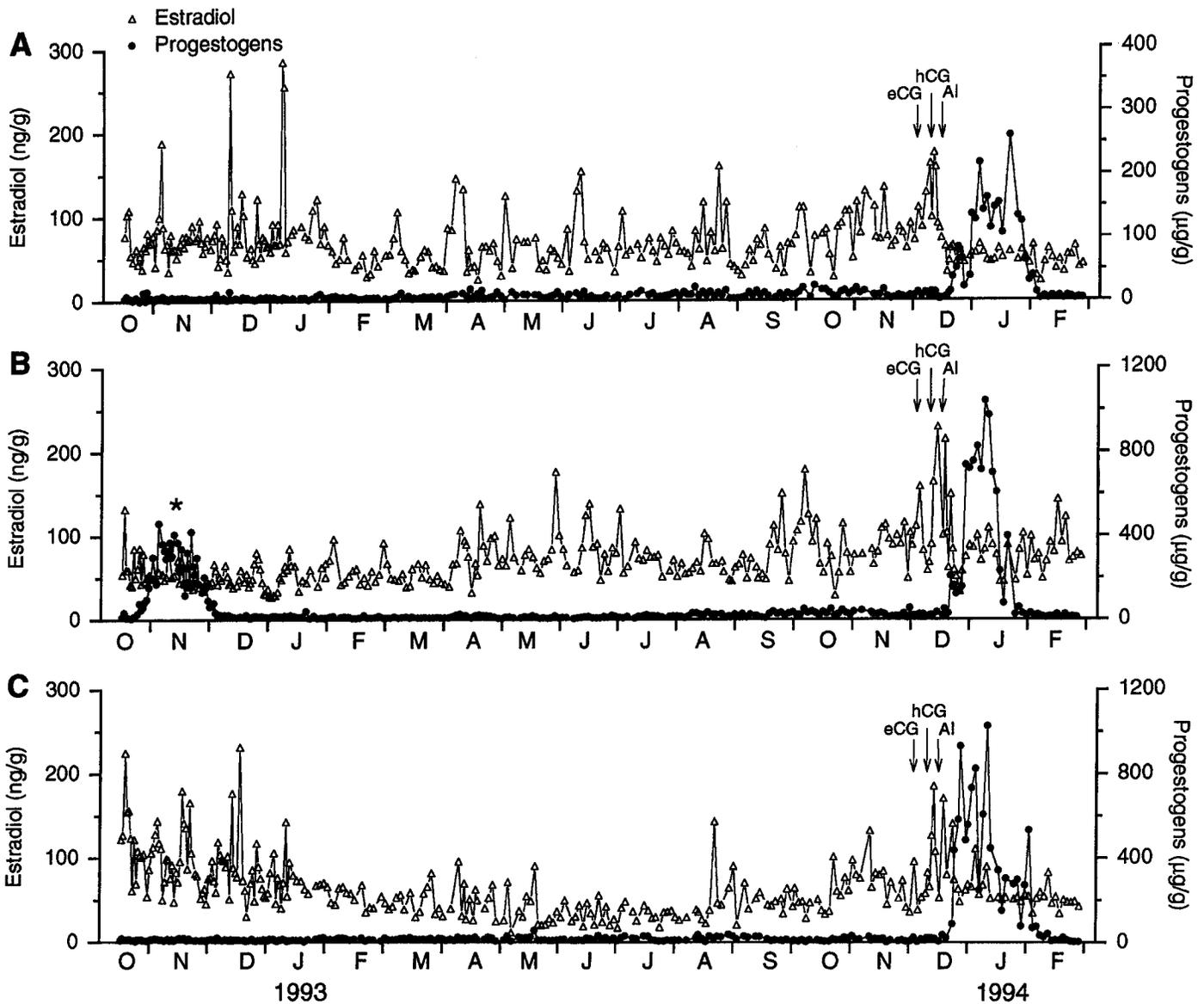


FIG. 2. Individual longitudinal profiles of fecal estradiol and progesterone metabolite concentrations in singleton clouded leopard females housed under artificial fluorescent lighting (12L:12D). Each animal was administered 75 IU eCG and 50 IU hCG i.m. and artificially inseminated 45–47 h post-hCG in December. *Increased fecal progesterone metabolite concentrations resulting from spontaneous (without copulation) ovulation and nonpregnant luteal phase.

The three MZG singletons responded to exogenous gonadotropins with increased E₂ and subsequent P₄ metabolite excretion, but none became pregnant after AI (Fig. 2). In two of these females (Fig. 2, B and C), overall mean and peak P₄ metabolite concentrations following hCG were greater ($p < 0.05$) than those observed in either the third female (Fig. 2A) or the other gonadotropin-stimulated females (Fig. 3C-5), or in clouded leopards ovulating after natural mating (Fig. 3, A and B). For these females, 3, 5, and 5 CL (1–3 unovulated follicles each) were observed at laparoscopic AI, and nonpregnant luteal phases lasted 59, 45, and 53 days (Fig. 2, A-C, respectively).

Impact of Ovulation Induction Therapy

In study 1, steroid excretory profiles after gonadotropin administration and AI varied considerably among individuals (Fig. 4). In general, E₂ concentrations increased 3- to 5-fold after eCG administration with no differences ($p > 0.05$) in peak levels between doses of 50 vs. 100 IU. In the female that became pregnant (Fig. 4A), increased P₄ metabolite excretion (5- to 80-fold above baseline) was sustained throughout the 89-day gestation period. At the time of laparoscopic AI, 0 follicles and 5 fresh CL were present. In two females failing to become pregnant, 4- to 6-fold increases in P₄ metabolite concentrations were apparent (with occa-

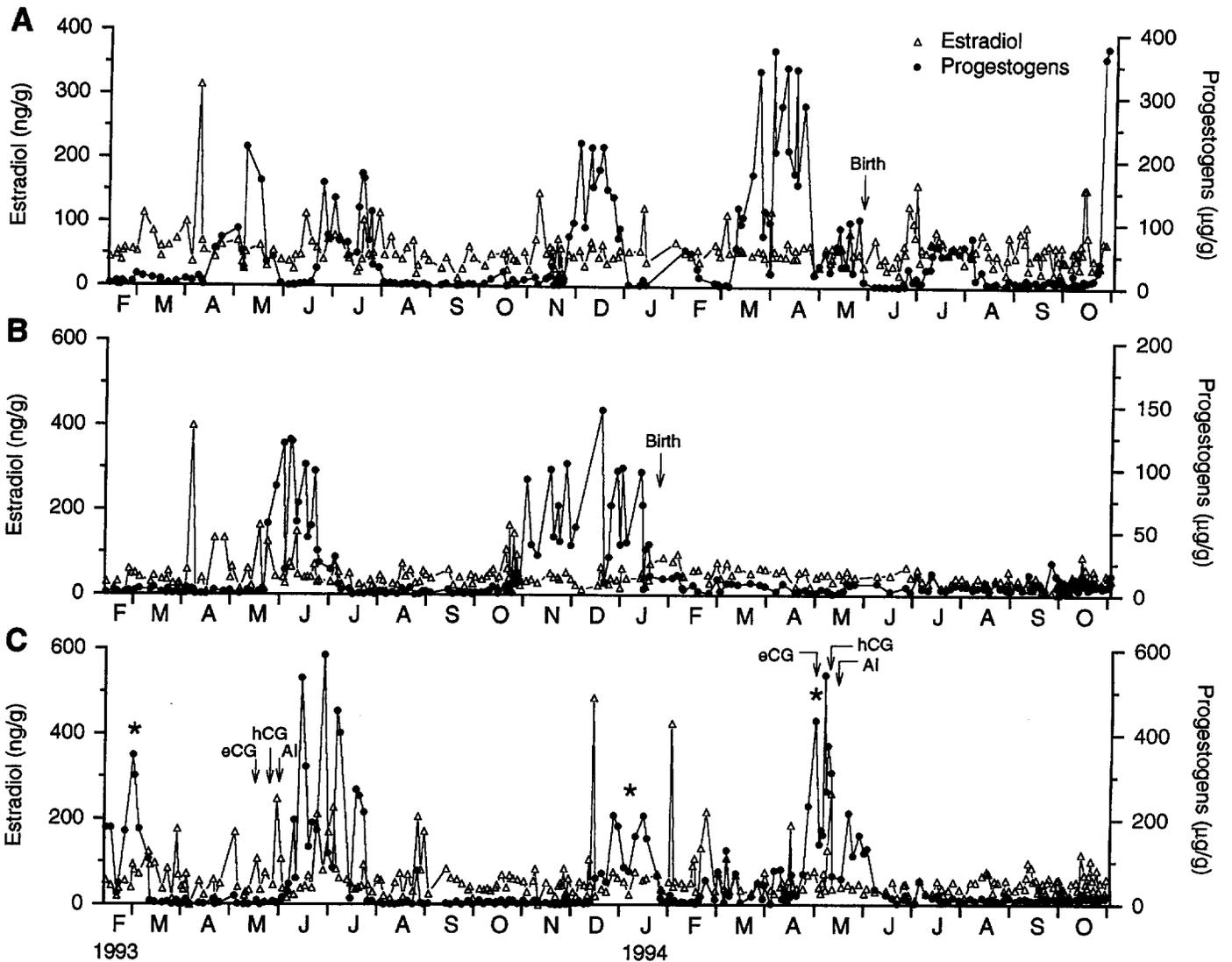


FIG. 3. Individual longitudinal profiles of fecal estradiol and progesterone metabolite concentrations in clouded leopards exposed to natural fluctuations in photoperiod. Females in panels A and B were each paired with a male. Female in panel C was housed as a singleton and subjected to ovulation induction and artificial insemination 46–47 h post-hCG (75 IU) in May 1992 (100 IU eCG) and May 1993 (50 IU eCG). *Increased fecal progesterone metabolite concentrations resulting from spontaneous (without copulation) ovulation and nonpregnant luteal phase.

sional spikes up to 30-fold) for 40–50 days after the gonadotropin-induced E_2 surge (Fig. 4, B and C); however, overall mean P_4 metabolite concentrations were lower ($p < 0.05$) than those depicted in Figures 1–3. At the time of AI, 3 follicles/7 CL and 1 follicle/5 CL were observed as shown in Figure 4, B and C, respectively. A second increase in fecal P_4 metabolite excretion was observed in Figure 4B, possibly the result of a spontaneous ovulation. In Figure 4D, P_4 metabolite concentrations were elevated several days before eCG was given, again probably due to a spontaneous ovulation. Although 6 fresh CL were present at laparoscopy, 2 mature CL also were evident, and the subsequent luteal phase was abbreviated. The individual depicted in Figure 4E had only 2 unovulated follicles and, on the basis of a

lack in P_4 elevation, ovulation did not occur.

Study 2 revealed two distinct responses to ovulation induction therapies. Three females responded comparatively normally, with several fresh CL (2–3/animal, 4–6 mm diameter) and post-hCG increases in P_4 metabolite excretion lasting ~50 days (Fig. 5A). In the other three females, CL (2–4/animal) were large (10–15 mm diameter), mushroom-shaped, vascularized, and presumably the result of pretreatment ovulations (i.e., spontaneous, without copulation) similar to those described in Figures 3C and 4, C and D. The P_4 metabolite profile also differed considerably, with concentrations elevated initially and then declining precipitously after hCG (Fig. 5B). These differential responses were

DISCUSSION

These data represent the first hormonal description of reproductive patterns in the clouded leopard and provide important information on ovarian responses to exogenous gonadotropins that should help improve current AI protocols. On the basis of fluctuations in fecal E₂ concentrations, the estrous cycle of the clouded leopard was ~24 days in length with estrus lasting ~6 days. While the duration of estrus results agree with previous behavioral estimates (6 ± 4 days) [13], the mean duration of the reproductive cycle was about 6 days shorter than previously reported, although there was an extraordinary range between periods of sexual receptivity (30 ± 14 days) in that study. This, in part, demonstrates the utility of fecal metabolite monitoring for assessing reproductive status because behavioral cues often are difficult to detect in this timorous, nocturnal species.

Steroid excretory patterns in clouded leopards exposed to natural fluctuations in photoperiod provided evidence of seasonality, with decreased reproductive activity observed in the late summer and early fall. These data concur with estimates of frequency of behavioral estrus and retrospective analyses of birth records that suggest the highest incidence of reproductive activity occurs from October through February [13, 15]. We previously observed a seasonal influence on testosterone secretion in male clouded leopards using a conventional blood sampling approach, with the highest concentrations observed during winter in North America [18]. In contrast, females housed indoors and exposed to 12 h of artificial light per day displayed no obvious

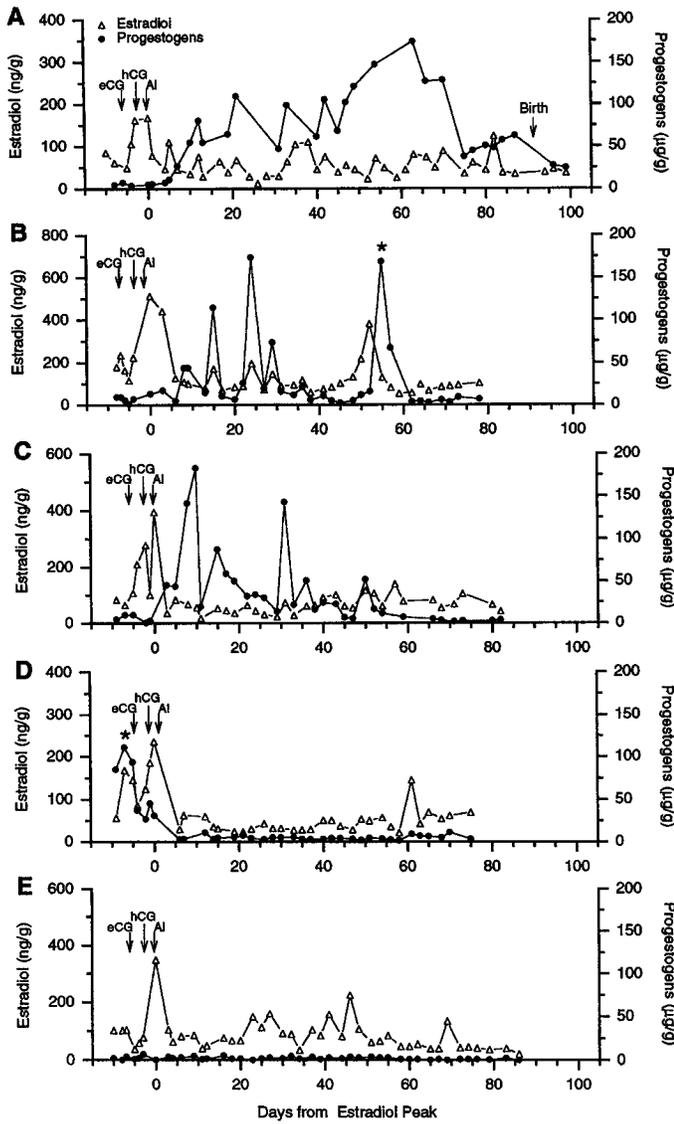


FIG. 4. Individual profiles of fecal estradiol and progesterone metabolite concentrations in clouded leopards subjected to ovulation induction and artificial insemination. Females received i.m. injections of 100 IU (A, B, C) or 50 IU (D, E) eCG followed 80 h later by 75 IU hCG and artificial insemination 44–48 h post-hCG. All data are aligned to estradiol peak (Day 0). *Increased fecal progesterone metabolite concentrations resulting from spontaneous (without copulation) ovulation and non-pregnant luteal phase.

unrelated to eCG dose (25 vs. 50 IU) since normal and abnormal luteal activity was observed at both doses. Of the three females that ovulated spontaneously, one was housed as a singleton, and the other two were maintained in female pairs. One of the ovulating females in study 2 also had ovulated spontaneously in study 1 (Fig. 4D). In contrast to P₄ surges, eCG-stimulated E₂ surges were similar (*p* > 0.05) in magnitude between the two groups (Fig. 5).

Overall, there was no correlation (*r* = 0.21; *p* > 0.05) between CL number at laparoscopy and overall mean fecal P₄ metabolite concentrations for all hormonal treatment groups.

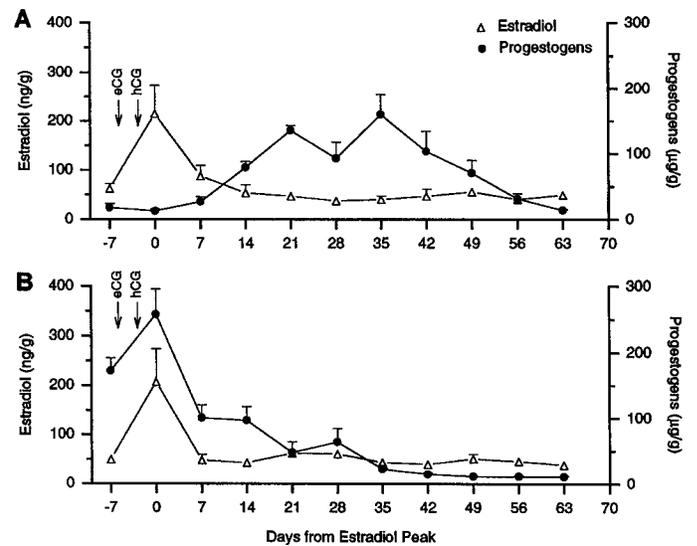


FIG. 5. Mean (± SEM) fecal estradiol and progesterone metabolite profiles in clouded leopards displaying two responses to ovulation induction. A) Normal ovarian response to eCG/hCG treatment (n = 3). B) Initially elevated fecal progesterone metabolite concentrations and presence of mature ovarian CL at laparoscopy (48–51 h post-hCG) suggested these females ovulated spontaneously (without copulation) prior to hormone treatment (n = 3). All data are aligned to estradiol peak (Day 0).

seasonal patterns in steroid metabolite excretion. These findings mimic observations in the domestic cat that queens are seasonally polyestrous in response to natural photoperiod but cycle year round when maintained under 12–14 h of artificial light per day [17, 19, 20]. Melatonin and possibly prolactin appear to be involved in mediating seasonal anestrus in the domestic cat, since concentrations of both hormones are inversely proportional to day length, and administering melatonin effectively suppresses ovarian activity [21]. It is likely that photoperiod plays at least a partial role in modulating reproductive activity in the clouded leopard, as has been suggested for the tiger [22] and snow leopard (*Panthera uncia*) [23]. However, a seasonal impact on reproduction is not universal within the Felidae family, as lions (*Panthera leo*) [24], leopards (*Panthera pardus*) [25], and pumas [26] maintained under natural light show no evidence of seasonal E_2 secretion or estrual activity.

During pregnancy, P_4 metabolite concentrations generally increased ~25-fold above baseline and peaked about mid-term. However, it was not uncommon for each female to experience 1–2 conspicuous decreases in P_4 metabolite excretion within the first 60 days of gestation. Although P_4 metabolite concentrations almost always were above baseline, this pattern differs qualitatively from that of the domestic cat, in which circulating P_4 peaks mid-term and then declines gradually until parturition [27]. In the lion, Briggs et al. [28] observed a marked decline in serum P_4 concentrations at mid-gestation, but concentrations quickly recovered until after parturition, similar to concentrations depicted for the animal represented in Figure 3B. However, in the other individuals (Fig. 3A; Fig. 4A), P_4 metabolites remained elevated until 1–2 mo before parturition, then declined to levels about midway between peak and baseline concentrations. An even more unusual hormonal pattern during pregnancy has been described recently for the cheetah [29]. Although elevated above baseline, fecal P_4 concentrations fluctuated considerably for the first 2 mo, declined to baseline for about 5 days, and then resumed an erratic, episodic pattern until parturition. The physiological significance of the P_4 declines consistently measured in these diverse felid species is unknown. While perhaps reflecting normal fluctuations in circulating steroid concentrations, they also may represent a shift in steroid production from the CL to the placenta. In the domestic cat, placental P_4 production increases in late gestation and may support pregnancy in the absence of ovaries after Day 45 [30, 31].

Physiological evidence of lactational anestrus was provided by a clouded leopard with suppressed ovarian activity for several months postpartum during a time of observed nursing. In contrast, a female that rejected her cub resumed ovarian cyclicity within 1 mo. Lactation typically produces a postpartum anestrus in the domestic cat, with a return to estrus occurring 2–8 wk after weaning [27, 32]. However,

resumption of estrous activity within 7 days has been observed when kittens are removed 1–2 days postpartum [32].

Length of the nonpregnant luteal phase in the clouded leopard was about half that of pregnancy (~45 days). Except for duration, however, there were no other obvious qualitative or quantitative differences in P_4 metabolite excretion between these two reproductive states. Similar data have been reported for the domestic cat, where nonpregnant luteal phases resulting from sterile matings or hormonal/mechanical stimulation last only about one-half to two-thirds the length of pregnancy with no other difference in the patterns or concentrations of circulating P_4 concentrations [16, 17, 20]. Limited endocrine data are available for nondomestic felid species, but the occurrence of short-lived, nonpregnant luteal phase increases in serum P_4 concentrations have been reported in the snow leopard [23], lion [24, 28], leopard [25], and puma [26], and in fecal P_4 metabolite concentrations in the cheetah [29]. Where comparative data are available, no quantitative differences in P_4 production are observed between pregnant and nonpregnant females [24, 26, 28, 29]. Because the duration of the nonpregnant luteal phase appears to be about half that of pregnancy, it may be possible to use fecal P_4 metabolite analyses to diagnose pregnancy, but only after mid-gestation [12].

Two females paired with males had received implants of silastic rods containing MGA, which were removed just before fecal sample collection was initiated. MGA is a widely used contraceptive given to zoo-maintained animals to prevent pregnancy in genetically over-represented individuals [33–37]. The long-term impact of this treatment had not been examined until the recent discovery of a relationship between MGA use and increased uterine and mammary gland hyperplasia and neoplasia in several felid species [35–37]. The clouded leopards studied here had had the implants for ~2.5 yr, and both females expressed clear increases in E_2 excretion within 4–8 wk of implant removal. However, despite displaying distinct nonpregnant luteal phases, neither became pregnant until after the second breeding season postimplant removal, suggesting that more studies on the influence of this contraceptive on fertility are needed. Fecal steroid metabolite analysis in combination with systematic breeding attempts will be valuable for examining such a cause/effect relationship.

One unexpected finding was that several clouded leopards ovulated spontaneously in the absence of mating. Of 14 individuals studied to date, spontaneous ovulations occurred on at least nine occasions in 6 individuals (3 singletons; 3 paired with another female). This contrasts somewhat with the general belief that nondomestic felid species are induced ovulators, like the domestic cat, and require a mating or similar stimulus for ovulation to occur [16, 17, 20]. Indeed, no spontaneous increases in circulating P_4 occurred after E_2 surges in blood samples collected 1–3 times weekly

for 3–12 wk from tigers [22], pumas [26], or snow leopards [23]. However, intermittent increases in serum P_4 concentrations lasting 1–6 wk were observed in two leopard females housed together, but not singly, suggesting that female-to-female contact induced ovulation [25]. In lions, luteal phase increases in serum P_4 also have been observed in females housed together, and in at least one female housed alone [28, 38]. Taken together, these data suggest that ovulatory mechanisms vary within the Felidae family and may be regulated to a greater or lesser extent by species-specific and even individual-specific responses to physical and/or psychosocial stimuli. It is clear that, while still being primarily induced ovulators, at least some felid species also occasionally exhibit nonmating-induced ovulations.

From a comparative perspective, information integrating estrous cycles, seasonality, and ovulatory responses provides an important database for studying reproductive mechanisms within the Felidae family. On a practical basis, these analyses could provide a critical foundation for making better management decisions. For example, it appears that most females examined demonstrated evidence of ovarian cyclicity and that this activity can be maintained throughout the year in the presence of artificial light. It also appears that the poor responsiveness of some clouded leopards to gonadotropin treatment for AI is due, in part, to the presence of active luteal tissue from prior ovulations at the time of hormonal treatment. The duration of fecal progesterone excretion in females with ovaries containing older, mature CL was reduced significantly compared to that in females with no evidence of active luteal activity. From these results, it is clear that more studies are needed to critically examine potential problems associated with spontaneous ovulations in the context of using assisted reproduction in this species. In cases of infertility, it also may be necessary to separate paired animals several months before scheduling AI to assure the absence of endogenous CL resulting from sterile matings. Alternatively, developing additional treatments to suppress endogenous ovarian activity might improve exogenous gonadotropin responses. For example, pretreating females with GnRH analogues to produce a temporary state of hypogonadotropic hypogonadism has been used successfully in human assisted reproduction programs [39, 40].

Natural habitat destruction and fragmentation mandate that rare species be maintained in captivity as reservoirs of genetic diversity. However, because most wild felid species reproduce poorly in zoos, maintaining genetically viable populations in geographically disparate regions also depends on our ability to reliably use assisted reproduction technologies as propagation management tools [6, 41]. We believe that this study illustrates the utility of noninvasive monitoring of ovarian activity as one means of understanding the impact of various pharmacological manipulations on AI success. We also predict that this approach will facilitate

the reliable use of an entire host of assisted technologies for contributing to conservation of ex situ, and perhaps even in situ, populations of endangered species.

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