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Comparative Aspects of Steroid Hormone Metabolism and Ovarian Activity in Felids, Measured Noninvasively in Feces[1]

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ABSTRACT

Noninvasive fecal assays were used to study steroid metabolism and ovarian activity in several felid species. Using the domestic cat (*Felis catus*) as a model, the excretory products of injected [^{14}C]estradiol (E_2) and [^{14}C]progesterone (P_4) were determined. Within 2 days, 97.0 ± 0.6% and 96.7 ± 0.5% of recovered E_2 and P_4 radioactivity, respectively, was found in feces. E_2 was excreted as unconjugated estradiol and estrone (40%) and as a non-enzyme-hydrolyzable conjugate (60%). P_4 was excreted primarily as non-enzyme-hydrolyzable, conjugated metabolites (78%) and as unconjugated pregnenolone epimers. A simple method for extracting fecal steroid metabolites optimized extraction efficiencies of the E_2 and P_4 excretion products (90.1 ± 0.8% and 87.2 ± 1.4%, respectively). Analysis of HPLC fractions of extracted fecal samples from the radiolabel-injected domestic cats revealed that E_2 immunoreactivity coincided primarily with the unconjugated metabolized [^{14}C]E_2 peak, whereas progestogen immunoreactivity coincided with a single conjugated epimer and multiple unconjugated pregnenolone epimers. After HPLC separation, similar immunoreactive E_2 and P_4 metabolite profiles were observed in the leopard cat (*F. bengalensis*), cheetah (*Acinonyx jubatus*), clouded leopard (*Neofelis nebulosa*), and snow leopard (*Panthera uncia*). Longitudinal analyses demonstrated that changes in fecal E_2 and P_4 metabolite concentrations reflected natural or artificially induced ovarian activity. For example, severalfold increases in E_2 excretion were associated with overt estrus or exogenous gonadotropin treatment, and elevated fecal P_4 metabolite concentrations occurred during pregnant and nonpregnant (pseudopregnant) luteal phases. Although overall concentrations were similar, the duration of elevated fecal P_4 metabolites during pseudopregnancy was approximately half that observed during pregnancy. In summary, steroid metabolism mechanisms appear to be conserved among these physically diverse, taxonomically related species. Results indicate that this hormone-monitoring approach will be extremely useful for elucidating the hormonal regulatory mechanisms associated with the reproductive cycle, pregnancy, and parturition of intractable and endangered felid species.

INTRODUCTION

Of the 37 extant felid species, all but the domestic cat are considered endangered in at least a portion of their natural range [1]. Most felid species reproduce poorly in captivity, a problem attributed to behavioral incompatibilities, captivity stress, inappropriate husbandry, or a pervasive loss of gene diversity [1–3]. Causes of female reproductive failure are challenging to diagnose because historically it has been difficult to measure complex endocrine interactions involved in controlling estrous activity, ovarian function, and conception. Nevertheless, characterizing these endocrine norms is essential for assessing reproductive competence, identifying those individuals with fertility problems, and determining whether assisted reproduction (artificial insemination, in vitro fertilization, and/or embryo transfer) is necessary. Recent efforts to supplement natural breeding have focused on using assisted reproductive techniques in captive management programs to 1) ensure reproduction between genetically valuable but behaviorally incompatible pairs; 2) eliminate the risks associated with animal transportation; and 3) ultimately preserve gene diversity [4–6]. Thus, there is a critical need to develop strategies to accurately assess endocrine status in nondomestic felids while providing a fundamental database that is lacking for this relatively large taxonomic group.

Conventional methods for obtaining normative endocrine data in domesticated animals have relied upon analysis of serially collected blood samples. This approach is impractical for most nontractable and stress-susceptible wildlife species, including felids. Noninvasive methods like measuring hormone metabolites excreted in urine or feces are potentially attractive alternatives. During the last decade, longitudinal monitoring of excreted estradiol (E_2) and progesterone (P_4) metabolites has proven effective for characterizing estrous cycles, pregnancy, and seasonal patterns of reproduction in a host of primate, ungulate, and equid species [7–17]. Before longitudinal studies of hormone metabolites can be initiated for a new species, the route by which amounts of metabolites are excreted must first be identified. Because many felid species void urine by spraying, collecting urine from a pooled source is impossible. Fortunately, E_2 metabolism studies in the domestic cat (*Felis catus*) have demonstrated that > 95% of this steroid is excreted in feces [18,19]. Furthermore, several preliminary re-

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ports have suggested that E_2 and/or P_4 metabolites are quantifiable in nondomestic felid feces (cheetah, *Acinonyx jubatus* [20–22]; tiger, *Panthera tigris* [21]; lion, *P. leo* [21]; caracal, *F. caracal* [21]; serval, *F. serval* [23]; bobcat, *F. rufus* [23]).

Our objectives were to extend these observations in detail by 1) identifying the predominant fecal P_4 metabolites in domestic cat feces while studying immunoreactive E_2 and P_4 metabolite profiles in other nondomestic felid species; 2) validating extraction and RIA techniques for measuring fecal E_2 and P_4 metabolites; and 3) demonstrating the utility of these techniques for longitudinally monitoring ovarian function and pregnancy in the leopard cat (*F. bengalensis*), cheetah, clouded leopard (*Neofelis nebulosa*), and snow leopard (*P. uncia*). These species represent the four genera within the family Felidae [24] and have diverse morphology/morphometry and natural geographic origins. The leopard cat female generally weighs less than 5 kg and is indigenous to India, Burma, Thailand, and Indo-China. Free-living cheetahs (females, 25–50 kg BW) now exist only in Africa, whereas the clouded leopard (16–23 kg) inhabits regions of the Himalayas, southern China, Taiwan, and various islands of Indonesia. The snow leopard (largest of our study group, 40–75 kg) is indigenous to central Asia, including regions of Russia, Mongolia, China, Nepal, India, and Pakistan.

MATERIALS AND METHODS

Radiolabel Studies

Adult domestic cats were maintained individually in stainless steel cages (1 × 0.8 × 1 m). Two anestrous females received i.m. injections of 3 μCi [^{14}C]E_2 (~56 mCi/mmol; New England Nuclear, Boston, MA) in 2.0 ml 0.9% NaCl. One month later each female (and an additional female) was administered 3 μCi [^{14}C]P_4 (~56 mCi/mmol; New England Nuclear) combined with 100 μg unlabeled P_4. Syringes were sonicated for 30 sec immediately before isotope injection, and 50 μl was removed and counted in a Beckman LS5801 counter with 10 ml scintillation fluid (Ultima Gold, Packard Instruments, Meriden, CT) to calculate total administered radioactivity. After isotope administration, syringes were rinsed with ethanol, and the residual radioactivity was counted and subtracted from the preinjection total. Urine was collected from litter pans containing a plastic nonabsorbable litter (A.J. Buck, Owings Mills, MD) that allowed urine to pass through while retaining fecal material. Fecal and urine samples were collected (if present) and frozen at −20°C at ~2-h intervals for 5 days postinjection. Litter pans were cleaned, and the litter was replaced daily. Aliquots of thawed samples (0.5 g feces, 0.5 ml urine) were counted for total radioactivity. Fecal samples were first solubilized by boiling in 1 ml 100% ethanol, and the entire homogenate was counted. Each fecal sample was divided into ~10 aliquots and counted separately in 20 ml scintillation fluid until the "H" number (a measure of quench) for each vial was < 120 (value at which little or no quench is observed for labeled steroid added to sample). The sum of the counts for all vials was added to generate total dpm/g sample.

After extraction of radioactive steroid metabolites from fecal material (see below), extracts were taken to dryness, resuspended in 1 ml PBS, and then extracted with 10 volumes of diethyl ether to separate conjugated (aqueous phase) from unconjugated (organic phase) steroid forms. Residual aqueous samples (0.5 ml) were enzymatically hydrolyzed with 50 μl β-glucuronidase/aryl sulfatase (5 000 Fishman U/40 000 Roy U, respectively; Boehringer-Mannheim Corp., Indianapolis, IN) as described by Shille et al. [19] and then extracted with 10 volumes of diethyl ether to separate enzyme-hydrolyzable (organic phase) from nonhydrolyzable (aqueous phase) conjugate forms.

Fecal Extraction

A method for extracting fecal steroid metabolites from cat feces was simplified from a technique described by Wasser et al. [16, 25] for the baboon. Fecal samples were dried with a Savant Instruments Speedvac Rotary Evaporator (Forma Scientific, Inc., Marietta, OH) and pulverized, and 0.1–0.2 g of powder was boiled in 5 ml of 90% ethanol:distilled water for 20 min. After being centrifuged at 500 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 to free particulates adhering to the vessel wall, and then vortexed briefly (15 sec). The major modifications from Wasser et al. [16, 25] involved boiling feces in 5 ml of 90% ethanol instead of 10 ml of 100% ethanol, and the removal of two dichloromethane extraction steps. A total of 3000 dpm each of [^{3}H]E_2 and [^{14}C]P_4 (New England Nuclear) was added to each fecal sample before extraction to monitor recovery using a quench curve compensation program. Samples were diluted (1:40 for E_2; 1:800–1:80 000 for P_4) in assay buffer (0.01 M PO_4, 0.14 M NaCl, 0.5% BSA, 0.01% sodium azide) before analysis by RIA.

Other Fecal Extraction Tests

Several variations of the above extraction method were evaluated to determine the benefit of 1) extracting with dichloromethane to remove lipids; 2) boiling feces in ethanol containing 20, 30, 40, or 50% water; 3) boiling once versus twice in 90% ethanol; 4) boiling in 5 vs. 10 ml of 90% ethanol; and 5) boiling in 90 vs. 100% methanol.

Because drying and pulverizing fecal samples substantially increased preparation time (24–48 h), two experiments were conducted to determine whether samples could

be analyzed wet. First, the distribution of steroids within fecal samples from the cheetah, clouded leopard, and snow leopard (n = 4 animals/species) was determined by dividing each unmixed sample into six sections and then removing 1 g from each section for E_2 and P_4 analysis. For comparative purposes, the remaining material from each section was then dried, and ~0.2 g of well-mixed fecal powder was analyzed for E_2 and P_4. Second, the correspondence in E_2 and P_4 concentrations between matched wet (1 g) and dry (0.2 g) samples from cheetahs (n = 4 females; 19 samples each) and clouded leopards (n = 3 females; 26–56 samples) was also determined. Because samples were unavailable, similar wet/dry comparisons were not conducted in the leopard cat or snow leopard.

RIAs

The E_2 RIA was based on the technique of Risler et al. [13] with a sensitivity of 2 pg/ml at 90% of maximum binding. The P_4 RIA was developed in our laboratory and relied upon a monoclonal P_4 antibody (produced against 4-P-11-ol-3, 20-dione hemisuccinate:BSA) 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. The monoclonal antibody cross-reacted 100% with P_4, 96% with 5α-pregnane-3β-ol-20-one, 36% with 5α-pregnane-3α-ol-20-one, 15% with 5β-pregnane-3β-ol-one, 15% with 17β-hydroxyprogesterone, 13% with pregnenolone, 7% with 5β-pregnane-3α-ol-20-one, 5% with 5β-pregnane-3α,17α-diol, 20α-one, and < 1% with pregnanediol-3-glucuronide, androstenedione, testosterone, E_2, estrone (E_1), estriol, 21-hydroxyprogesterone, 20α-hydroxyprogesterone, and cortisol [26, 27]. The assay was incubated at 4°C for 3 h in a total volume of 500 μl. Standards (100 μl) and/or sample were incubated with assay buffer (200 μl), first antibody (1:100 000, 100 μl), and ^{125}I-P_4 tracer (20 000 cpm, 100 μl) for 2 h. Antibody-bound complexes were precipitated after a 1-h incubation with sheep anti-mouse gamma globulin (1:400, 1 ml in buffer containing 5% polyethylene glycol, 8000 M_r [Sigma Chemical Co. St. Louis, MO]) followed by centrifugation (30 min, 1500 × *g* at 4°C). The antibody typically bound 40–50% of the iodinated P_4 tracer with ~3% nonspecific binding. Assay sensitivity was 3 pg/ml.

E_2 and P_4 assays were validated for fecal extracts from the domestic cat, leopard cat, cheetah, clouded leopard, and snow leopard by demonstrating 1) parallelism between dilutions of pooled fecal extracts and the standard curve and 2) significant recovery of exogenous E_2 (5–240 pg) or P_4 (3.75–120 pg), respectively, added to domestic cat ($y = 0.95x + 1.11$, $r = 0.99$; $y = 1.05x + 0.81$, $r = 0.99$), leopard cat ($y = 1.08x - 1.13$, $r = 0.99$; $y = 0.97x - 1.79$, $r = 0.99$), cheetah ($y = 1.16x - 0.4$, $r = 0.99$; $y = 1.02x + 0.99$, $r = 0.99$), clouded leopard ($y = 1.08x + 0.99$, $r = 0.99$; $y = 0.96x + 1.00$, $r = 0.99$), or snow leopard ($y = 1.02x + 0.97$, r = 0.99 ; $y = 1.10x + 2.4$, $r = 0.99$) fecal extracts. Intra- and interassay coefficients of variation for both assays were < 10%. All fecal data are expressed on a per gram dry weight basis.

HPLC

The number and relative proportions of E_2 and P_4 metabolites in cat fecal extracts were determined by reverse-phase HPLC (Microsorb C-18 Column; Rainen Inc., Woburn, MA) using modifications of the methods of Monfort et al. [28]. Before HPLC, samples were passed through a C-18 matrix column (Spice Cartridge, Rainen, Inc.) and eluted with 5 ml of 80% methanol to remove contaminants (sample loss was ~10%) [29]. For separation of E_2 metabolites, fecal extracts were eluted with use of a gradient of 32–45% acetonitrile (ACN):water over 20 min, increasing to 60% over 40 min and then to 100% ACN over 20 min. P_4 metabolites were separated by use of a gradient of 20–32% over 15 min, increasing to 50% over 50 min and then to 100% over 55 min. One-milliliter fractions were collected over an 80- or 120-min period (1 ml/min flow rate) for estrogens and progestogens, respectively. Elution profiles of tritiated E_2, E_1, and estrone-sulfate were determined in separate runs using the estrogen gradient. Tritiated P_4, 17α-OH-P_4, pregnanediol, and pregnanediol-glucuronide profiles were determined by use of the progestogen gradient.

HPLC fractions of domestic and nondomestic cat fecal eluates were taken to dryness and reconstituted in assay buffer, and E_2 and P_4 immunoreactivity was quantified by the appropriate RIA.

Gas Chromatography/Mass Spectrometry (GC/MS)

Identification of specific E_2 metabolites in domestic cat feces has been accomplished by Shille et al. [18] by means of GC/MS analysis; therefore, a similar analysis was not conducted on samples from the E_2 radiolabel study. GC/MS was employed to identify P_4 metabolites from domestic cats administered [^{14}C]P_4. For this analysis, twenty fecal samples (~0.2 g each) were extracted and purified by HPLC, and the fractions containing the major metabolite peaks were analyzed by means of GC/MS as described by Shackleton [30].

Longitudinal Evaluations

For assessing ovarian activity in nondomestic felid species, fecal samples were collected 3–5 times weekly from a leopard cat, five cheetahs, five clouded leopards, and two snow leopards. These species were maintained under standard zoological conditions at the following institutions: leopard cat (International Wildlife Conservation Park/Bronx Zoo, Bronx, NY); cheetahs (Phoenix Zoo, Phoenix, AZ; Caldwell Zoo, Tyler, TX); clouded leopards (Conservation and Research Center, Front Royal, VA; Nashville Zoo, Nashville, TN); snow leopards (Oklahoma City Zoological Park, Oklahoma City, OK).

The leopard cat, three of the cheetahs, two of the clouded leopards, and the two snow leopards were used in parallel

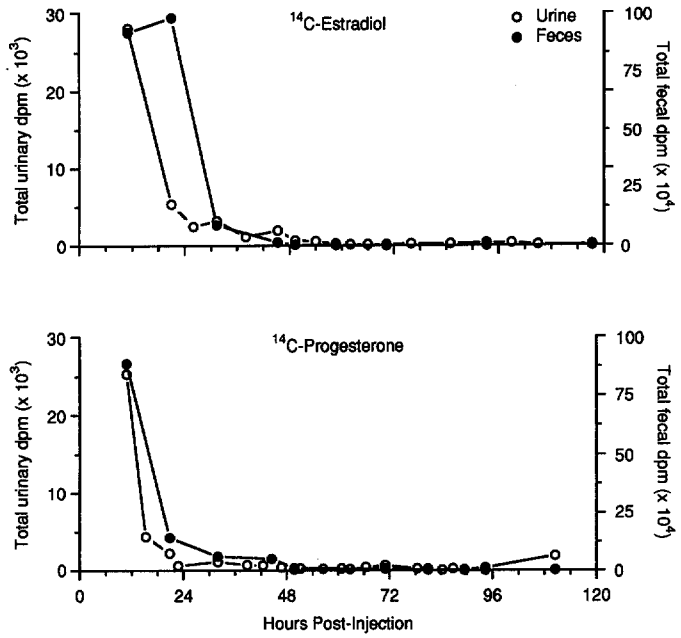

FIG. 1. Representative profiles of excretory time course for [^{14}C]E_2 and [^{14}C]P_4 after i.m. injection (3 μCi each) at Time 0 in the domestic cat.

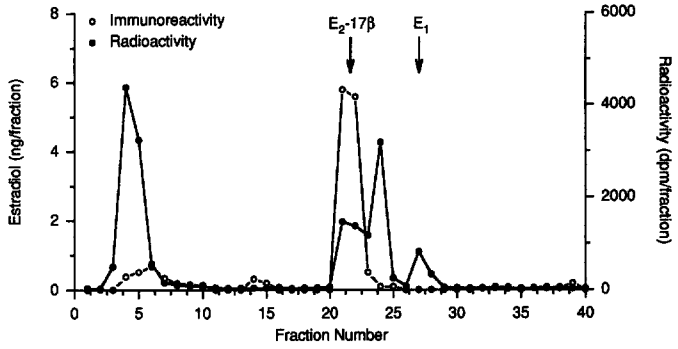

FIG. 2. HPLC separation of metabolized fecal estrogens after [^{14}C]E_2 injection in the domestic cat. Immunoreactivity of each fraction was determined by RIA. Retention times of radioactive and immunoreactive peaks were compared to [^{3}H]E_2-17β and [^{3}H]E_1 reference tracers.

gamete biology/assisted reproduction studies. All had been given i.m. injections of eCG and hCG to induce follicular development and ovulation, respectively [31]. After ovulation was confirmed by laparoscopic observation of ovarian corpora lutea, each female was artificially inseminated (AI) by transabdominal sperm deposition directly in utero [31, 32]. Pregnancies resulted in the leopard cat, one cheetah, and one clouded leopard; the remaining animals underwent a nonpregnant luteal phase (designated as pseudopregnancy). Longitudinal fecal collections also were conducted during pregnancies in one cheetah and two clouded leopards, and during pseudopregnancies in one cheetah and four clouded leopards after natural matings. Samples were collected for at least 95 days postbreeding or AI, or until 2 wk after parturition.

Statistical Analysis

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 the surge was determined by values that exceeded preceding values by 50%. Basal P_4 metabolite concentrations were calculated from values preceding the preovulatory E_2 surge; mean P_4 metabolite concentrations during pregnancy or pseudopregnancy contained values from the time of observed mating, estrus, or AI to parturition or the return of P_4 to baseline. Differences among species in baseline fecal E_2 or P_4 metabolite concentrations, preovulatory peak E_2 concentrations, or mean and peak (i.e., highest point) P_4 metabolite concentrations during pregnancy or pseudopregnancy were determined by a one-way analysis of variance followed by Duncan's New Multiple Range test. Because only one leopard cat was available for study, statistical comparisons with this species were not conducted. Mean data are ± SEM.

RESULTS

Radiolabel Studies

Total radioactivity recovered in urine and feces for both steroids was ~60%. After [^{14}C]E_2 was injected, 97.0 ± 0.6% and 3.0 ± 0.6% of the radioactivity (as a percentage of total radioactivity recovered) was excreted in feces and urine, respectively. For [^{14}C]P_4, feces and urine contained 96.7 ± 0.9% and 2.6 ± 1.2% of the recovered radioactivity, respectively. Virtually all (> 90%) of the radioactivity in urine was detected in the first sample collected at 9 or 11 h postinjection for E_2 and at 8, 12, or 13 h postinjection for P_4 (Fig. 1). Peak radioactivity in feces also occurred in the first sample collected at 11 or 21 h for E_2 and at 12, 24, or 50 h for P_4 (Fig. 1).

E_2 Metabolism

Metabolism of E_2 in the domestic cat has been described by Shille et al. [18, 19]; therefore, [^{14}C]E_2 data generated in our study were used primarily for among-species comparisons. Differential extraction of domestic cat feces with diethyl ether indicated that metabolized E_2 was excreted in nearly equal amounts as conjugated (aqueous phase, 43.6 ± 4.8%) and unconjugated (organic phase, 55.4 ± 4.4%) steroid forms. HPLC analysis confirmed that E_2 was excreted as both conjugated and unconjugated metabolites (Fig. 2). The unconjugated steroid peaks coeluted with [^{3}H]estradiol-17β (E_2-17β) and [^{3}H]E_1 reference tracers, respectively. RIA of HPLC fractions determined that E_2 immunoreactivity was associated primarily with unconjugated metabolized E_2 (82% of the total immunoreactivity), although some immunoreactivity was also associated with the

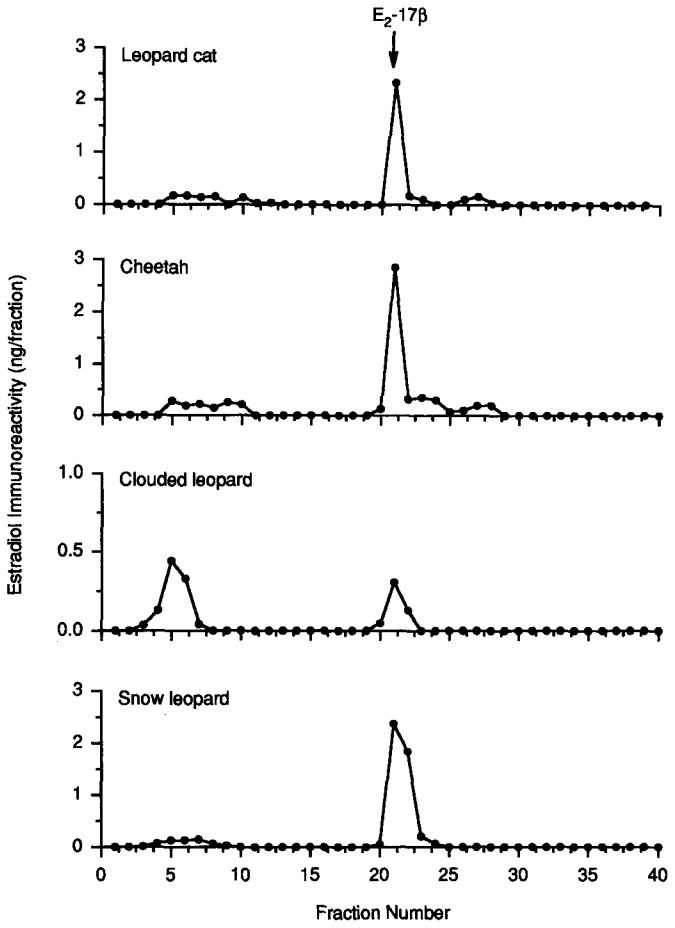

FIG. 3. Total immunoreactive estrogens in HPLC fractions of extracted fecal samples from the leopard cat, cheetah, clouded leopard, and snow leopard. Retention times of immunoreactive peaks were compared to the [^{3}H]E_2-17β reference tracer.

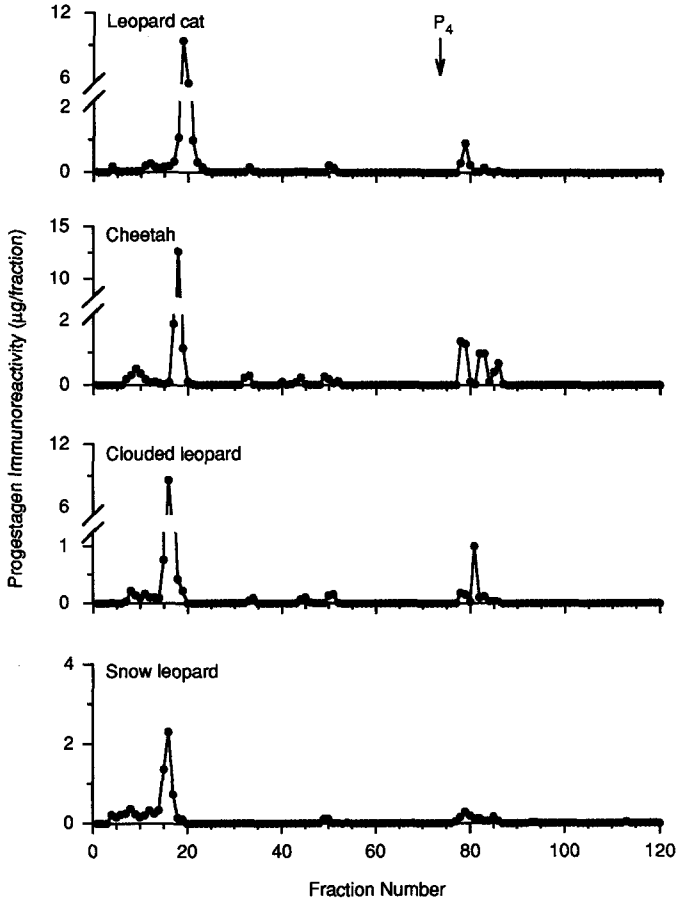

FIG. 5. Total immunoreactive progestogens in HPLC fractions of extracted fecal samples from the leopard cat, cheetah, clouded leopard, and snow leopard. Retention times of immunoreactive peaks were compared to the [^{3}H]P_4 reference tracer.

conjugated peak (18%) (Fig. 2). Similarly, RIA of fecal eluates from nondomestic species revealed that E_2 immunoreactivity coincided primarily (~85%) with unconjugated E_2. The only exception was in the clouded leopard, which produced considerable immunoreactivity associated with the presumably conjugated peak (60% of total immunoreactivity) at fractions 3–6 (Fig. 3).

P_4 Metabolism

According to differential extraction with diethyl ether, most P_4 metabolites were excreted as conjugated (aqueous

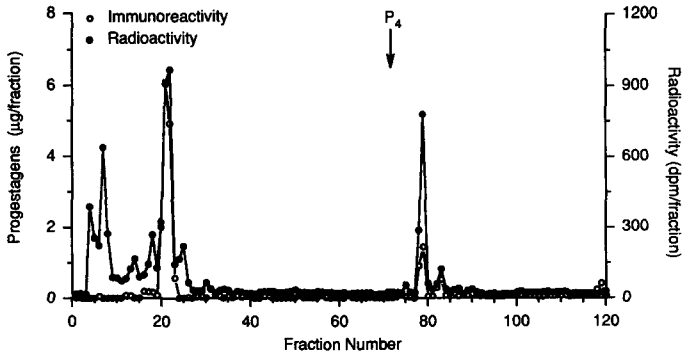

FIG. 4. HPLC separation of metabolized fecal progestogens after [^{14}C]P_4 injection in the domestic cat. Immunoreactivity of each fraction was determined by RIA. Retention times of the radioactive and immunoreactive peaks were compared to the [^{3}H]P_4 reference tracer.

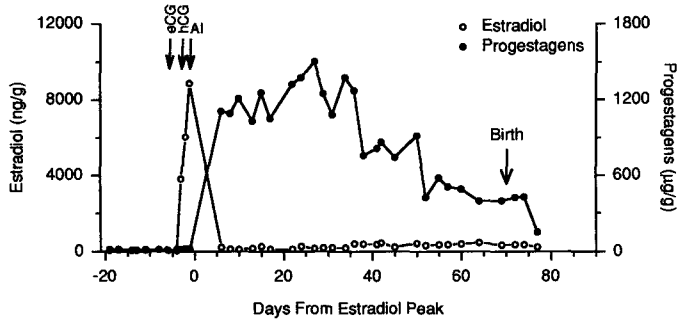

FIG. 6. Fecal estradiol and progestogen metabolite profiles during pregnancy in a leopard cat subjected to gonadotropin treatment and AI. All data were aligned to the estradiol peak (Day 0). The female produced two kittens.

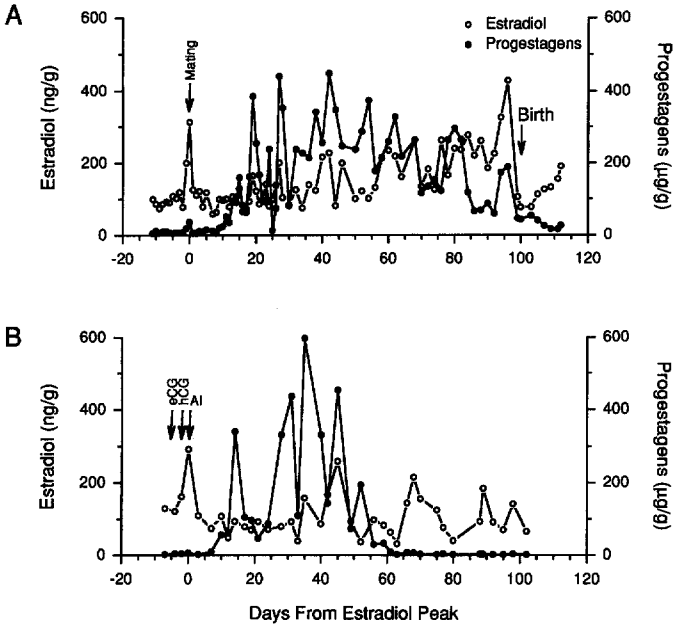

FIG. 7. Representative fecal estradiol and progestogen metabolite profiles during a pregnancy resulting from natural breeding (**A**) and pseudopregnancy resulting from AI (**B**) in the cheetah. All data were aligned to the estradiol peak (Day 0). The pregnant female produced two cubs.

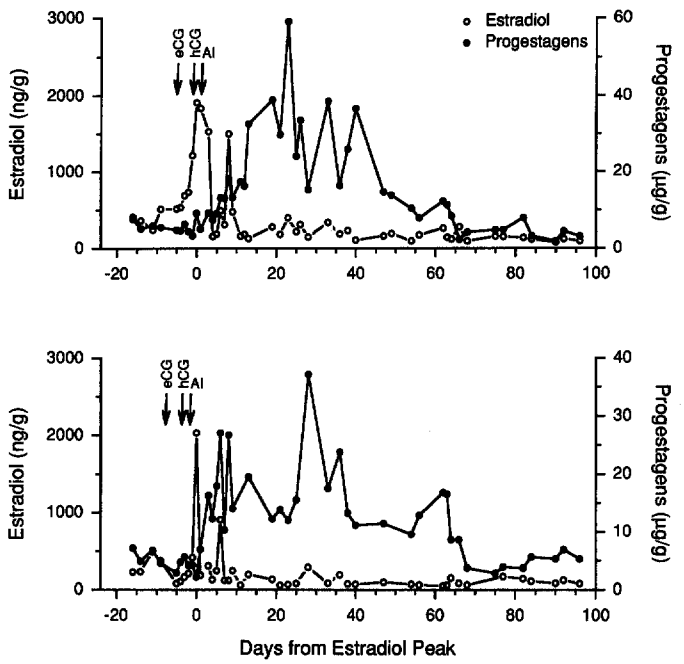

FIG. 9. Fecal estradiol and progestogen metabolite profiles during two pseudopregnancies resulting from ovulation induction/AI in the snow leopard. All data were aligned to the estradiol peak (Day 0).

phase, 77.8 ± 1.3%) rather than unconjugated (organic phase, 22.2 ± 1.3%) steroid forms. HPLC analyses detected several polar (presumably conjugate) radioactive peaks at fractions 4–8 and 17–25 (27 and 50% of the total radioactivity, respectively) and two less polar peaks at fractions 78–79 (20%) and 82–83 (3%) (Fig. 4). None of these peaks coeluted with the [^{3}H]P_4 tracer (fractions 73–74). The presumably conjugated metabolites were not enzyme-hydrolyzable and could not be identified by GC/MS. The unconjugated steroid peaks contained 5β-pregnane-3α-ol-one (22.3% of the unconjugated steroid radioactivity), 5α-pregnane-3α-ol-one (13.9%),

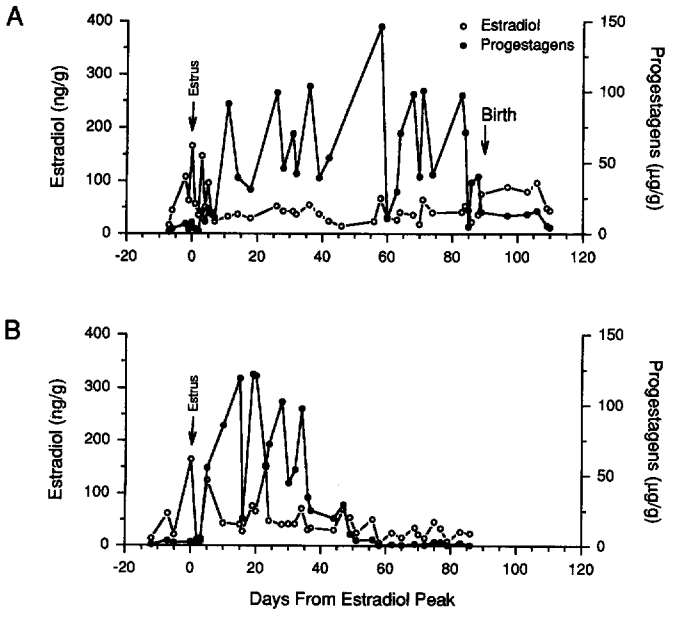

FIG. 8. Representative fecal estradiol and progestogen metabolite profiles during a pregnancy (**A**) and pseudopregnancy (**B**) resulting from natural matings in the clouded leopard. All data were aligned to the estradiol peak (Day 0). The pregnant female produced four cubs.

TABLE 1. Fecal E_2 concentrations associated with pregnancy and pseudopregnancy.

Species	No.	E_2 metabolite concentrations (ng/g dry fecal weight) Basal[a] (range)	Peak (range)
Leopard cat			
Pregnancy	1	313.7	9085
Cheetah			
Pregnancy	2	70.6 ± 15.9[b] (54.7–86.4)	289 ± 24[b] (266–313)
Pseudopregnancy	3	65.8 ± 5.9[b] (58.6–77.6)	527 ± 136[b] (312–799)
Clouded leopard			
Pregnancy	3	33.2 ± 12.6[c] (29.5–56.4)	170 ± 36[c] (98–212)
Pseudopregnancy	5	37.5 ± 15.4[c] (28.2–58.1)	185 ± 37[c] (103–250)
Snow leopard			
Pseudopregnancy	2	225.1 ± 35.1[d] (190.2–258.8)	1969 ± 61[d] (1908–2031)

[a]Calculated from values not associated with pre-ovulatory E_2 surge.
[b,c,d]Within column values for fecal E_2 metabolite concentrations with different superscripts were different ($p < 0.05$). Statistical analyses were not conducted on the single leopard cat.

TABLE 2. Fecal P_4 metabolite concentrations during pregnancy and pseudopregnancy.

Species	No.	Duration (d)[a] (Range)	P_4 metabolite concentrations (μg/g dry fecal weight) Basal[b] (Range)	Mean (Range)	Peak[c] (Range)
Leopard cat					
Pregnancy	1	70	16.2	872.2	1503
Cheetah					
Pregnancy	2	94 ± 3[d] (91–97)	4.6 ± 1.1 (3.5–5.7)	157 ± 46[d] (111–203)	465 ± 17[d] (448–482)
Pseudopregnancy	2	53 ± 2[e] (51–60)	5.0 ± 0.7 (3.8–6.2)	243 ± 96[d] (113–430)	908 ± 433[d] (363–1364)
Clouded leopard					
Pregnancy	3	90 ± 2[d] (86–93)	4.7 ± 0.9 (2.9–6.2)	99 ± 31[d] (73–141)	193 ± 78[e] (87–345)
Pseudopregnancy	5	48 ± 2[e] (43–51)	4.9 ± 0.7 (2.5–6.6)	119 ± 31[d] (66–227)	265 ± 82[e] (122–585)
Snow leopard					
Pseudopregnancy	2	69 ± 2[f] (67–71)	5.1 ± 0.3 (4.8–5.4)	21 ± 4[e] (17–24)	48 ± 11[f] (37–59)

[a]Calculated from time of observed mating, estrus or AI to parturition or return of P_4 to baseline concentrations.
[b]Calculated from values preceding mating or AI.
[c]Determined as the highest pregnancy or pseudopregnancy value.
[d,e,f]Within column values for fecal P_4 metabolite concentrations with different superscripts are different ($p < 0.05$). Statistical analyses were not conducted on the single leopard cat.

5β-pregnane-3β-ol-one (61.8%), 5α-pregnane-3β-ol-one (3.2%), and 5α-pregnane-3α,20α-diol (3.3%). Co-chromatographic HPLC profiles of extracted fecal samples from the domestic cat revealed that P_4 immunoreactivity coincided with a major conjugated peak (fractions 20–23) and the unconjugated metabolized peaks (Fig. 4). Nonimmunoreactive, radioactive peaks in fractions 3–20 were found to contain lignins and equol by GC/MS. P_4 immunoreactivity in leopard cat, cheetah, clouded leopard, and snow leopard fecal eluates purified by HPLC was similarly associated with presumably conjugated and unconjugated metabolite peaks (Fig. 5). There were no consistent differences between the proportions of conjugated versus unconjugated P_4 metabolite immunoreactivity during early or late pregnancy or pseudopregnancy across species (data not shown).

Extraction Tests

Compared to 100% ethanol, ethanol containing 10% water increased extraction efficiency of the metabolized (from the radiolabel study) [^{14}C]E_2 (74.7 ± 3.3% versus 90.1 ± 0.8%) and [^{14}C]P_4 (56.9 ± 0.7% versus 87.2 ± 1.4%), respectively. Extraction efficiency of labeled ([^{3}H]E_2 and [^{14}C]P_4) or unlabeled (E_2 or P_4) steroids added to fecal samples before extraction exceeded 90%. However, other variations of the extraction procedure (see *Materials and Methods*) failed to enhance efficiency further.

Studies conducted to examine whether fecal material could be analyzed wet strongly suggested that the most accurate results were obtained when samples were dried, pulverized, and well mixed before analysis. Correlation coefficients for fecal E_2 and P_4 concentrations, respectively, between matched wet and dry samples were variable for both the cheetah ($r = 0.79 \pm 0.13$, range = 0.38 − 0.91; $r = 0.80 \pm 0.08$, range = 0.59 − 0.89) and clouded leopard ($r = 0.39 \pm 0.09$, range = 0.22 − 0.54; $r = 0.27 \pm 0.16$, range = 0.04 − 0.59). Furthermore, when individual fecal samples were divided into sections before analysis, the variability in steroid distribution across each sample was considerable. Average coefficients of variation of E_2 and P_4 metabolite concentrations calculated from sectioned fecal samples averaged 27.1 ± 15.7% (range 9.9–68.1%) and 23.0 ± 3.4% (range, 6.6–41.6%), respectively, for undried sectioned samples and 15.6 ± 5.2% (range, 7.1–41.6%) and 21.2 ± 5.8% (range, 8.2–48.4%), respectively, for dried sectioned samples. The greatest coefficients of variation were associated with samples containing large proportions of hair residue. Fecal water content varied within individuals and, to some extent, among species. The percentage of water in cheetah, clouded leopard, and snow leopard feces was similar ($p > 0.05$), averaging 64% (range, 41–81%), whereas that in leopard cat feces was higher ($p < 0.05$; average 83%; range, 74–91%). The higher water content of leopard cat feces was due to defecation by that animal in its water dish.

Longitudinal Profiles

Figures 6–9 represent typical fecal steroid metabolite profiles generated in the nondomestic felid species. Fecal E_2 and P_4 metabolite profiles during pregnancy in the leop-

ard cat after exogenous gonadotropin therapy and AI are depicted in Figure 6. Fecal E_2 concentrations increased ~30-fold over baseline within 3 days of eCG injection, peaked the day before AI, and then declined to baseline 5 days after hCG injection. E_2 concentrations remained basal throughout pregnancy. Fecal P_4 metabolite concentrations were at nadir before AI (16 μg/g), increased within 5 days after AI, and were highest (~100-fold increase) between Days 5 and 35 of pregnancy (~1500 μg/g). Although elevated throughout gestation, P_4 concentrations gradually declined after midgestation, reaching baseline within ~2 wk postpartum.

Figure 7 depicts fecal steroid profiles in one cheetah during a natural pregnancy (Fig. 7A) and in another during a pseudopregnancy after unsuccessful AI (Fig. 7B). Approximate 3-fold increases in fecal E_2 concentrations were observed during estrus or as a result of exogenous gonadotropin therapy compared to baseline values. E_2 concentrations were highest in the periovulatory interval (in both females) and immediately before parturition (in the pregnant female). P_4 metabolite concentrations rose within 2 wk of mating or AI. During gestation, P_4 metabolite concentrations peaked at ~Day 30, then gradually declined to approach nadir by parturition (97 days after mating). In contrast, P_4 metabolite concentrations during the sterile luteal phase declined to baseline by Day 60.

Representative fecal E_2 and P_4 metabolite profiles during a natural pregnancy and pseudopregnancy in the clouded leopard are presented in Figure 8. Approximate 5-fold increases in E_2 were observed during estrus compared to baseline. Again, after estrus, E_2 excretion returned to baseline whereas P_4 metabolite concentrations increased markedly, falling to baseline coincident with parturition (Fig. 8A) or termination of the sterile luteal phase (Fig. 8B).

Fecal steroid metabolite profiles for two snow leopard pseudopregnancies after gonadotropin treatment and AI are provided in Figure 9. Fecal E_2 concentrations increased approximately 9-fold over baseline within 6 days after eCG administration and were inexplicably followed by a second E_2 excretion surge approximately 1 wk later. Increases in P_4 metabolite excretion were observed within 6–7 days of hCG treatment and remained elevated for at least 60 days after the preovulatory E_2 surge.

Although only a single leopard cat was examined, overall mean, basal, and peak E_2 and P_4 metabolite concentrations appeared much greater in that species than in the cheetah, clouded leopard, and snow leopard (Tables 1 and 2). Among the latter three species, basal and estrual E_2 concentrations were lowest in the clouded leopard, intermediate in the cheetah, and highest in the snow leopard ($p < 0.05$). In contrast, basal P_4 metabolite concentrations were similar ($p > 0.05$) among the cheetah, clouded leopard, and snow leopard. During pregnancy or pseudopregnancy, overall mean P_4 metabolite concentrations were similar between the cheetah and clouded leopard ($p > 0.05$), but several-fold higher ($p < 0.05$) than that observed in the snow leopard. Peak concentrations during pregnancy or pseudopregnancy were highest in the cheetah, intermediate in the clouded leopard, and lowest in the snow leopard ($p < 0.05$).

There were no differences ($p > 0.05$) in mean or peak P_4 metabolite concentrations between pregnancy versus pseudopregnancy for cheetahs and clouded leopards (the only species with available data) (Table 2). In contrast, the duration of the nonpregnant luteal phase was only about half that observed for pregnancy ($p < 0.05$). Duration of pregnancy was similar ($p > 0.05$) for the cheetah and clouded leopard, as was the duration of pseudopregnancy. In contrast, pseudopregnancy in the gonadotropin-treated snow leopards was ~20 days longer ($p < 0.05$).

DISCUSSION

Attempts to facilitate or improve reproductive efficiency in the management of rare nondomestic felids often fails, in part, because necessary basic reproductive/endocrine information is unavailable. Thus, our first objective was to develop a noninvasive method for the longitudinal monitoring of ovarian activity in nontractable felid species. This was accomplished by determining the excretory fate of E_2 and P_4 after radiolabeled steroids were injected into the domestic cat. We confirmed the earlier findings of Shille et al. [18, 19] that E_2 is excreted in this species almost exclusively in feces as a non-enzyme-hydrolyzable conjugate (reported to be estradiol sulfate [18]) and unconjugated estrogens (estradiol and E_1 [18]). By means of an RIA specific for E_2, similar immunoreactive profiles of HPLC-separated fecal extracts were measured in four representative nondomestic species. One unexpected observation was the significant immunoreactivity associated with a polar, presumably conjugated, metabolite in the clouded leopard. The nature of this compound is unknown, but it is not estrone-sulfate because the cross-reactivity of that conjugate with our E_2 antibody is < 1%. Rather, it may be non-enzyme-hydrolyzable estradiol-sulfate as reported by Shille [18], which is present in high concentrations relative to E_2, since that compound does cross-react with our antibody. To completely resolve this question would require conducting an E_2 radiolabel infusion study in the clouded leopard. Nevertheless, from a comparative perspective, it is clear that E_2 is a common excretory product of estrogen metabolism among the different felid genera, and standard E_2 immunoassays are appropriate for assessing ovarian follicular activity in these species, either during natural estrus or after gonadotropin therapy.

Like E_2, most excreted P_4 radioactivity in the domestic cat was associated with feces rather than urine. However, in contrast to E_2, none of the [^{14}C]P_4 was excreted in its native form. This result is consistent with a recent preliminary report by Möstl et al. [33] showing that immunoreactive substances in domestic cat fecal eluates purified by HPLC were not P_4. By use of GC/MS analysis, unconjugated ra-

dioactive metabolites in our study were identified as pregnenolone epimers, several of which cross-reacted with our P_4 antibody [26, 27]. Wasser et al. [27] also reported the presence of pregnenolone epimers in baboon fecal extracts after P_4 radiolabel infusion. However, these unconjugated steroid metabolites constituted only a small proportion of the metabolized P_4 in the cat. In contrast, > 70% of the P_4 radioactivity was associated with conjugated steroids that were not enzyme-hydrolyzable. This was surprising considering that the majority of fecal P_4 metabolites in other species are excreted in the unconjugated form (primates [15, 27, 34], rhinoceros [9]), presumably the result of bacterial hydrolysis in the gut [35, 36]. We were unsuccessful in identifying this conjugated material by GC/MS analysis. The metabolized material coeluted with [^{3}H]pregnanediol-glucuronide, and quantification of progestogen excretory patterns has been accomplished in other species by means of pregnanediol-glucuronide immunoassays [8, 10, 11, 14, 15]; however, cats rarely produce glucuronides as major by-products of steroid metabolism [37]. Furthermore, our antibody does not cross-react with that conjugate. However, regardless of our inability to make a specific identification, our P_4 RIA was able to quantify this metabolite. Additionally, on the basis of comparison to other preliminary reports [20–22, 33], we appear to be measuring considerably more progestogen activity (circa, 10- to 100-fold higher concentrations). These differences may be due to extraction method and the fact that our data are expressed on a dry weight basis. But they are also likely to be related to the higher cross-reactivity of our antibody with the predominant P_4 metabolites in cat feces. Finding similar immunoreactive profiles of HPLC-separated fecal extracts between the domestic cat and the nondomestic counterparts further suggests that P_4 metabolism is relatively conserved among felid species and that this assay can be used confidently across species to evaluate luteal function.

Our second major objective was to simplify the extraction method originally developed by Wasser et al. [16] for analyzing excreted steroid metabolites in nonhuman primates. By omitting the dichloromethane extraction step (for removing lipids), we substantially reduced sample preparation time while eliminating the need for toxic chemicals. Boiling in ethanol containing 10% water also enhanced extraction efficiency by ~20 and 50% for [^{14}C]E_2 and [^{14}C]P_4, respectively, compared to boiling in 100% ethanol. This improvement probably occurred because cat feces contain a high proportion of conjugated steroids that are soluble in aqueous solutions. Increasing the water percentage to 20–50% was counter-productive, however, resulting in prolonged drying times and no improvement in extraction efficiency for either steroid. We also attempted to shorten processing time further by eliminating the drying and pulverizing steps that originally were intended to adjust for differences in water content [17]. Although correlations were found between matched wet and dry samples for both E_2 and P_4 metabolites, data were variable and apparently influenced by the amount and distribution of hair throughout the sample. This variation was even more evident when individual samples were divided into sections before analysis, where up to 8-fold differences were observed across sections. For E_2, this variation could mean the difference between characterizing a sample as baseline or estrual. Thus, results suggest that fecal steroids are not evenly distributed and that, if assayed wet, samples must be mixed well before extraction [13, 17]. In our laboratory, the convenience and ease of handling, storing, and processing dried fecal material presently outweighs the disadvantage of added drying time. Furthermore, expressing samples per gram dry weight may still be important, especially when diets are variable [25].

Our final objective was to demonstrate that there was a biological relationship between changes in fecal steroid metabolite concentrations and physiological factors known to affect ovarian activity. In this regard, there were several lines of positive evidence. First, results of longitudinal steroid analyses clearly indicated that significant increases in fecal E_2 concentrations were associated with behavioral estrus or eCG-stimulated ovarian follicular development. Second, mating activity and/or hCG administration also was followed by distinct increases in fecal P_4 metabolite concentrations indicative of ovulation and luteinization. Finally, the excreted steroid metabolite profiles measured here were typical of temporal patterns measured in the peripheral circulation of similarly treated felids (domestic cat [38–40], lion [41], snow leopard [42]). There was considerable day-to-day variability in concentrations of fecal E_2 and P_4 metabolites, however, suggesting that samples should be collected frequently (at least 3 times weekly).

In sum, the purpose of this initial study was to demonstrate a relationship between fecal steroid concentrations and biological activity, not to definitively characterize the reproductive cycle of each species. In that context, we did make several important observations. First, longitudinal monitoring of fecal E_2 and P_4 metabolite excretion provided an accurate index and a safe approach for assessing ovarian function in felids. Second, there was considerable variation in overall fecal E_2 and P_4 metabolite concentrations among species, suggesting that although steroid metabolism may be conserved among species, the absolute production of steroids from the different ovarian compartments may be species-specific. It was also clear that the technology appeared to have cross-species application without the need for major adjustments. Thus, fecal steroid analyses should prove invaluable for studying the basic reproductive biology of endangered felids, including 1) characterizing the estrous cycle, 2) determining the prevalence of induced versus spontaneous ovulation, and 3) examining the influence of season as well as a whole host of environmental/management factors on reproductive efficiency. Because the duration of pseudopregnancy appears to be about

half that of pregnancy, it may also be possible to use fecal P_4 metabolite analyses as an index of pregnancy after mid-gestation. Finally, although assisted reproduction is being used with some success in propagating endangered felids, pregnancy rates continue to be low [5, 31, 32, 43–45]. Perhaps one of the greatest uses of steroid metabolite monitoring will be in assessing the cause(s) of poor fertility after AI, in vitro fertilization, or embryo transfer, eventually allowing these tools to better contribute to species conservation.

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