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—Original Article—

Fecal Progestagens to Detect and Monitor Pregnancy in Captive Female Cheetahs (*Acinonyx jubatus*)

Itsuki ADACHI[1)], Satoshi KUSUDA[2)], Hitomi KAWAI[2)], Megumi OHAZAMA[3)], Atsushi TANIGUCHI[4)], Natsuko KONDO[4)], Masato YOSHIHARA[4)], Ryuta OKUDA[5)], Tatsuya ISHIKAWA[5)], Iwai KANDA[6)] and Osamu DOI[2)]

[1)]The Graduate School of Agricultural Science, Gifu University, Gifu 501-1193, [2)]Faculty of Applied Biological Sciences, Gifu University, Gifu 501-1193, [3)]Himeji Central Park, Himeji 679-2121, [4)]Tama Zoological Park, Tokyo 191-0042, [5)]Fuji Safari Park, Shizuoka 410-1231 and [6)]Kyushu Natural Zoological Park, Oita 872-0722, Japan

Abstract. The purposes of the present study were to establish a noninvasive monitoring assay of fecal progestagen measurement to detect pregnancy and to identify the components of fecal progestagens in early, middle and late pregnancy in cheetahs. Feces were collected from 7 female cheetahs and analyzed from 30 days before the last copulation to parturition in 9 pregnancies. Blood was collected from one cheetah. Fecal progestagen and serum progesterone concentrations were determined by enzyme immunoassay (EIA). The profiles of the fecal progestagen concentrations were similar to the serum progesterone profile. Fecal progestagen and serum progesterone concentrations remained at the baseline until copulation. In the mean fecal progestagen profile during pregnancy (92.8 ± 0.4 days; from the last copulation to parturition), the concentrations increased 3–4 days after the last copulation and remained high until parturition. To investigate changes in the components of progestagen metabolites in the tripartite periods of gestation, fecal progestagens were analyzed by HPLC-EIA. Marked immunoreactive peaks consistent with 5α-pregnan-3α/β-ol-20-one and 5α-pregnan-3,20-dione and small peaks consistent with 5β-pregnan-3α/β-ol-20-one were detected. There were no distinct difference in the components of progestagens among the first, second and third trimesters of pregnancy. The hormone assay, as an indicator of fecal 5α-reduced pregnanes, is useful for detecting pregnancy and monitoring pregnant luteal activity in cheetahs.

Key words: Cheetah, Fecal hormone, Progesterone, Steroid metabolites

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Captive breeding and management are important for the *ex-situ* conservation of cheetahs (*Acinonyx jubatus*); however, breeding cheetahs is difficult in captivity. The causes of this difficulty may be attributed to basic reproductive abilities, such as the high rate of morphologically abnormal sperm [1, 2], high cub mortality [2] and reproductive suppression occurring among female cheetahs housed together or in close proximity [3, 4]. Recent studies have also suggested that appropriate husbandry and management based on the singularity lifestyle of free-ranging cheetahs are effective for captive breeding [5]. In Japanese zoos, breeding loans, such as exchanging or moving female cheetahs between institutes, have enabled successful breeding. These trials might have caused changes in individual relations among females and reproductive suppression.

While the breeding opportunities have increased in captive cheetah breeding programs, the high infant mortality needs to be improved. It is therefore necessary to detect pregnancy reliably and to provide appropriate management before and after parturition. About 30% of the cheetah cubs born in captivity die before six months of age [2], and more than 80% of the loss occurs during the first month [5]. For these reasons, reliable pregnancy determination is one of the most important tools to prepare for parturition and for the unlikely event of hand rearing.

For endocrine monitoring in pregnant animals, repeated blood sampling for assessing circulating hormones and ultrasound examination cause stress to potentially both the mother and fetus. Moreover, these procedures are impractical with intractable nondomestic felids, including cheetahs. Fecal steroid metabolite analysis to monitor reproductive endocrinology is noninvasive and has been widely used for zoo and wildlife species [6, 7]. However, information about the types of fecal hormone metabolites among the reproductive stages is limited. It has been reported that in the Eurasian lynx (*Lynx lynx*), the excretion pattern of fecal progestagen metabolites is different among pregnancy stages [8]. Understanding the excretion pattern, such as the components of fecal hormone metabolites, among reproductive stages is important for validating the assay method used for monitoring the stages.

The objectives of our study were to establish a noninvasive monitoring assay of fecal progestagen measurement to detect pregnancy and to identify the components of fecal progestagens in early, middle and late pregnancy in cheetahs.

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Correspondence: S Kusuda (e-mail: kusuda@gifu-u.ac.jp)

Materials and Methods

Animals and sample collection

Fecal samples were collected from 7 female cheetahs (Nos. 1–7) kept at Himeji Central Park (HCP), Tama Zoological Park (TZP),

Table 1. The seven female cheetahs investigated in this study

Animal	Name	Birth date	Facility	Sample collection
No. 1	Nancy	Apr 19, 1998	HCP	Blood, feces
No. 2	Kikyou	Apr 23, 2003	TZP	Feces
No. 3	Sumire	Jun 5, 2003	FSP	Feces
No. 4	Toria	Dec 18, 2002	FSP	Feces
No. 5	Pranky	Jan 2003 [1)]	FSP	Feces
No. 6	Ruby	Nov 21, 2005	FSP	Feces
No. 7	Ten	Aug 1, 2003	KNZP	Feces

[1)] Day of birth was unknown. HCP: Himeji Central Park. TZP: Tama Zoological Park. FSP: Fuji Safari Park. KNZP: Kyushu Natural Zoological Park.

Table 2. Gestation details of the female cheetahs used in this study

Animal	Last copulation	Parturition	Gestation (days) [1)]	Fetal number
No. 1	Jul 7, 2006	Oct 9, 2006	94	4
	Jan 27, 2008	Apr 30, 2008	94	3
No. 2	Nov 2, 2007	Mar 5, 2008	94	4
	Jun 21, 2009	Sep 22, 2009	93	3
No. 3	Oct 6, 2008	Jan 6, 2009	92	4
No. 4	Dec 6, 2007	Feb 5, 2008	91	5
No. 5	Apr 29, 2008	Aug 2, 2008	95 [2)]	3
No. 6	Dec 19, 2008	Feb 20, 2009	93	4
No. 7	Apr 21, 2008	Jul 23, 2008	93	7

[1)] From the day of last copulation to parturition. [2)] Cesarean section 95 days after last copulation.

Fuji Safari Park (FSP) and Kyushu Natural Zoological Park (KNZP) (Table 1). Feces were collected in the morning one to seven times a week from 2006 to 2009. Fecal samples were stored at –30 C immediately after collection. Fecal progestagen concentrations were measured from 30 days before the last copulation to parturition in 9 pregnancies during the fecal collection period.

Blood was collected every one or two weeks from cheetah No.1 from 2005 to 2006 (Table 1). The animal was transferred to a squeeze cage and manually restrained, and then blood was collected from a lateral caudal vein. Serum was separated from whole blood and stored at –30 C until assay. Serum progesterone concentrations were measured using 10 samples (–26, –19, –12, –4, 0, 16, 32, 46, 62 and 73 days from last copulation).

Fecal and serum hormone analyses

The fecal extraction procedure was described in a previous report [7]. Briefly, frozen feces were lyophilized for approximately 36 h. A portion of the fecal powder, 0.1 g, was then extracted with 5 ml of 80% methanol by vortex-mixing for 30 min. After centrifugation at 2,500 rpm for 10 min, the supernatant methanol fraction was diluted at a ratio of 1:40 for the assay with assay buffer. Serum progesterone was extracted twice with diethyl ether, and the extracts were diluted at a ratio of 1:4 with assay buffer. Fecal and serum assay samples were determined by enzyme immunoassay (EIA) with progesterone antiserum (LC-28; Aska Pharma Medical, Kanagawa, Japan), as previously reported [7]. The main cross-reactivity of this antiserum was 100% for progesterone, 28.7% for 5α-pregnan-3β-ol-20-one, 18.3% for 5α-pregnan-3α-ol-20-one, 16.7% for 5α-pregnan-3,20-dione, 4.3% for 5β-pregnan-3β-ol-20-one and 2.1% for 5β-pregnan-3α-ol-20-one. The intra- and interassay coefficients of variation were 5.0 and 14.5%, respectively. Serial dilution curves of the fecal extracts demonstrated parallelism to the standard curve for progesterone (data not shown).

High-performance liquid chromatography (HPLC)

To investigate changes in the components of progestagen metabolites throughout gestation, fecal progestagens were analyzed with HPLC-EIA using each pooled feces during the tripartite periods of gestation (1–15, 30–60 and 75–90 days from last copulation) in female cheetahs. HPLC separation of progestagens was performed by a modified version of the procedure described in a previous report [9].

Prior to HPLC, feces were lyophilized and pulverized, and 2.0 g of fecal powder was extracted with 10 ml of 80% methanol. The 5 ml supernatant was added to 35 ml assay buffer, and the total volume was passed through a Sep-Pak C-18 column (Sep-Pak plus C-18 Environmental Cartridges; Waters Milford, MA, USA). Progestagens were eluted with 5 ml absolute methanol. To separate fecal progestagens, a 20 μl extract sample was injected onto the HPLC with a reverse-phase Nova-Pak C-18 column (3.9 × 30.0 mm; Waters, Milford, MA, USA). For the HPLC, an isocratic solvent of acetonitrile (ACN)/water (H_2O) (40/60, v/v) was used at a flow rate of 1.0 ml/min, and 120 fractions (1 ml each) were collected. Each fraction was extracted twice with diethyl ether and determined by EIA for progesterone. The immunoreactive fraction numbers were compared with reference tracers, and fecal progestagens were iden-

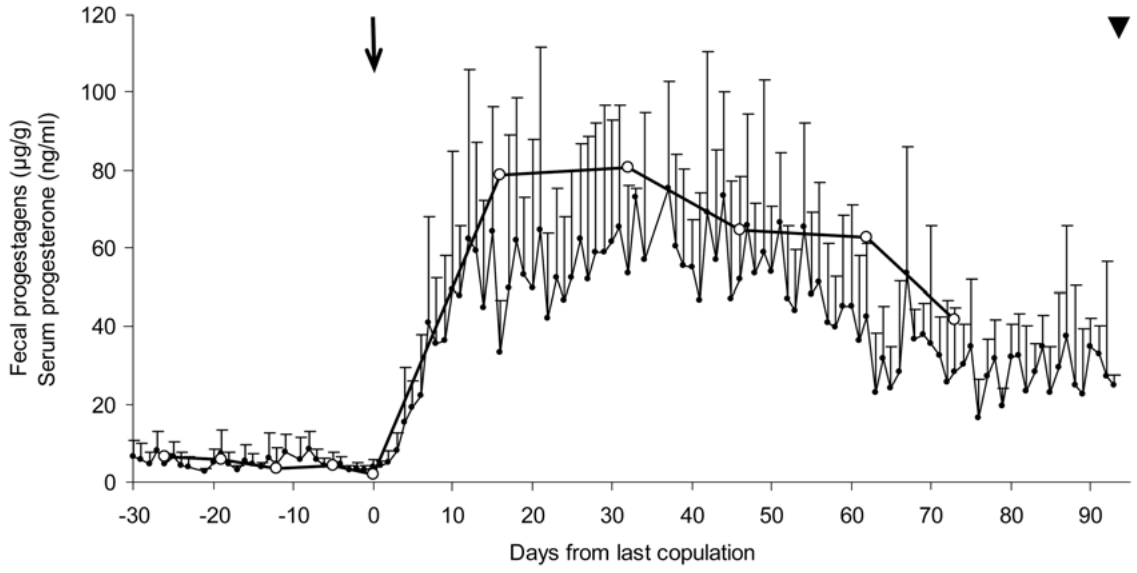

Fig. 1. Profiles of fecal progestagen and serum progesterone concentrations from 30 days before last copulation to parturition in cheetahs. Fecal progestagens (closed circle) are indicated as the mean (± SEM) profile of the concentration in 8 pregnancies from 6 cheetahs. Serum progesterone (open circle) is indicated as the profile of the concentrations in cheetah No. 1. The arrow indicates the day of last copulation. The arrowhead indicates the day of parturition.

tified. Progesterone, 5α-pregnan-3,20-dione and 5α/β-pregnan-3α/β-ol-20-one were used as reference tracers. Each serial dilution curve of these pregnanes demonstrated parallelism to the standard curve for progesterone (data not shown).

Data analysis

All fecal data are expressed as per gram of dry feces. Data are presented as the mean ± standard error of the mean, SEM. A relationship between serum progesterone and fecal progestagen concentrations was evaluated by a correlation coefficient. The correlation coefficient between serum progesterone concentrations in cheetah No. 1 and mean fecal progestagen concentrations in 8 pregnancies from 6 cheetahs was calculated between blood on that day and feces the following day because of the lag time for blood-circulating progesterone to be defecated into feces (within 1-2 days for the domestic cat [10]).

Results

Endocrine monitoring

Eight pregnancies from 6 cheetahs were spontaneous deliveries. One cheetah (No. 5) underwent a caesarean section 95 days after the last copulation. The mean gestation period from the last copulation to parturition was 92.8 ± 0.4 days (8 pregnancies from 6 females, except in the case of caesarean section in No. 5). The mean profile of fecal progestagen concentrations from 30 days before the last copulation to parturition in 9 pregnancies from 7 cheetahs is shown in Fig. 1. The progesterone concentrations in serum samples collected from cheetah No. 1 ranged from 19.23 to 80.66 ng/ml. There was a visual trend for the fecal progestagen profiles to be similar to the serum profile; however, it was difficult to show a reliable statistical correlation between serum progesterone and fecal progestagen concentrations (there were few matching samples between blood and feces collected on the same day, between blood on that day and feces the following day or between blood on that day and feces 2 days later).

Fecal progestagen concentrations remained at the baseline (average 5.3 μg/g) until copulation and then increased 3–4 days after the last copulation. Fecal progestagen concentrations during pregnancy reached a plateau at the peak level approximately 15 days after the last copulation, remained high (approximately 40–70 μg/g) for approximately 40 days and then gradually decreased until parturition.

Fecal steroid metabolites

The immunoreactive progesterone concentrations in HPLC fractions of extracted fecal samples in the three stages of gestation (first, second and third trimesters) from a female cheetah (No. 2) are shown in Fig. 2. The marked immunoreactive peaks, Nos. 70–72, 75–77 and 90–92, were consistent with 5α-pregnan-3β-ol-20-one, 5α-pregnan-3α-ol-20-one and 5α-pregnan-3,20-dione reference tracers, respectively. Small immunoreactive peaks were consistent with 5β-pregnan-3α-ol-20-one (No. 59–60) and 5β-pregnan-3β-ol-20-one (No. 63–64) tracers. Immunoreactive peak No. 47–48, consisting of progesterone, was very small. There were no distinct differences in the components of progestagens among the first, second and third trimesters of pregnancy; however, progesterone immunoreactivity fell in the third trimester of pregnancy. There was also no difference in metabolites among individuals (data not shown).

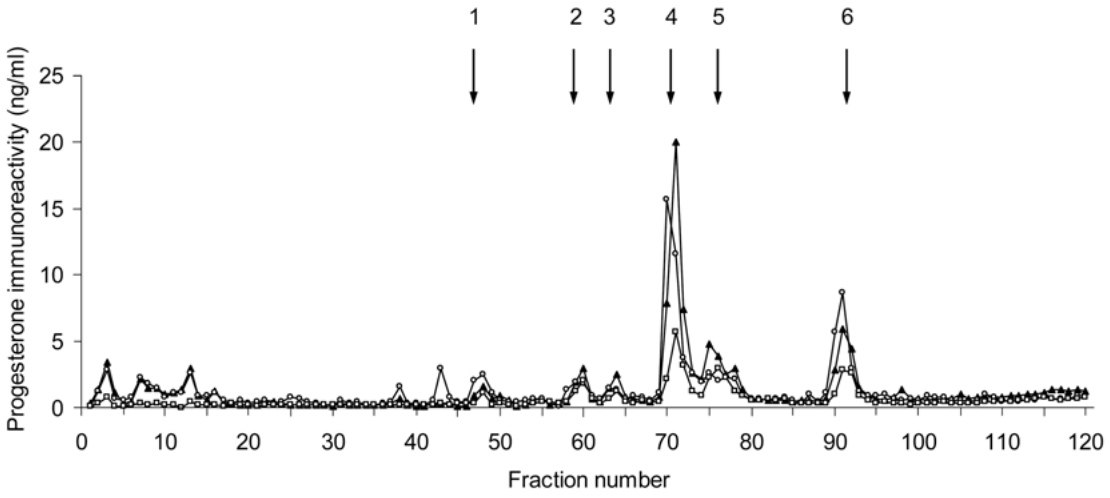

Fig. 2. Progesterone immunoreactivity in HPLC fractions of extracted fecal samples during early (○), mid- (▲) and late (□) pregnancy in a pregnant cheetah (No. 2). Arrows indicate the reference tracer as follows: 1, progesterone; 2, 5β-pregnan-3α-ol-20-one; 3, 5β-pregnan-3β-ol-20-one; 4, 5α-pregnan-3β-ol-20-one; 5, 5α-pregnan-3α-ol-20-one; and 6, 5α-pregnan-3,20-dione.

Discussion

We validated that fecal progestagen measurement is effective for detection of pregnancy in cheetahs. Furthermore, the present study revealed that the components of fecal progestagens showed no distinct difference throughout pregnancy and among cheetahs.

In the profiles of fecal progestagens of the female cheetahs, progestagen remained at the baseline until copulation and markedly elevated only after copulation. This result supports previous reports that cheetahs are almost an induced ovulator [3, 11]. Fecal progestagen values elevated 3–4 days after copulation. In a cheetah, 2 distinct corpus lutea have been confirmed by laparoscopic examination 5 days after mating [1].

The 92.8 ± 0.4 day- pregnancy in our study was an average period, with previous studies reporting pregnancy periods of 89 days [12], 94.2 ± 0.5 days [3], 85–95 days [13] and 90–96 days [5]. A nonpregnant luteal phase was not observed throughout the present study period. It has been reported that the duration of fecal progestagen elevation in the mated nonpregnant luteal phase is 51.2 ± 3.5 days and that the duration is similar to that in the nonmated luteal phase after spontaneous ovulation, which occurs infrequently [3]. In cases of abortions, the progestagen level immediately returned to baseline [13, 14]. The combination of our results and these previous reports indicates that successful pregnancy can be distinguished from nonpregnant luteal phase/abortion by fecal progestagen measurement, which enables detection of pregnancy after approximately 50 days from the last copulation.

As a result of qualitative analysis of fecal progestagen metabolites in the pregnant cheetahs, several progestagen metabolites were detected. Fecal progestagens of the female cheetahs included 5α/β-pregnan-3α/β-ol-20-one and 5α-pregnan-3,20-dione, and nonmetabolized progesterone was barely present. A previous study in domestic cats described that progesterone was excreted primarily as conjugated metabolites and unconjugated pregnanolone epimers (primarily 5β-pregnan-3α/β-ol-one) and that progesterone is not detected [15]. Fecal progesterone metabolites in the female Eurasian lynx also contained 5α-pregnan-3β-ol-20-one and 5α-pregnan-3,20-dione [8]. Progesterone has not been usually detected in the feces of wild felid species, such as the tiger (*Panthera tigris*), lion (*P. leo*) [16], leopard cat (*Felis bengalensis*), clouded leopard (*Neofelis nebulosa*) and snow leopard (*Uncia uncia*) [15]. Although these felid species, including cheetahs, excrete similar pregnanes, the primarily metabolite might be different among species. Generally, the excretion of steroid hormone metabolites varies considerably among species, even closely related species [6]. In our study, it was unknown if the detected pregnanes were the major fecal metabolites in female cheetah feces because the progesterone antiserum used in our study probably does not uniformly cross-react with all pregnanes; however, our results indicate that circulating progesterone in pregnant cheetahs is almost entirely metabolized and excreted as reduced pregnanes, including 5α-reduced pregnanolone (5α-pregnan-3α/β-ol-20-one) and pregnanedione (5α-pregnan-3,20-dione). Comprehensive assay of these pregnanes in feces is useful for monitoring pregnancy/pregnant luteal activity in cheetahs.

In domestic cats, the corpora lutea produce progesterone for approximately 40–50 days of gestation; however, the amounts produced after 40 days are minimal, and the subsequent pregnancy is maintained by placental progesterone [17]. A previous report indicated that fecal progesterone decreased from 60–70 days of pregnancy in cheetahs, and it has been speculated that luteoplacental shift of the progesterone secretor might occur after 60 days of pregnancy [13]. In our study, the types of fecal progestagens showed no distinct difference among early (1–15 days), mid- (30–60 days) and late pregnancy (75–90 days) and among individuals. These results indicate that our assay method is effective for moni-

toring progestagen profiles during pregnancy.

In conclusion, the fecal progestagen assay, as an indicator of 5α-reduced pregnanes, is effective for detecting pregnancy and monitoring pregnant luteal activity in cheetahs. Continuous noninvasive monitoring of reproductive endocrinology is important for reliable management and care through pregnancy and parturition.

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