Excretion Patterns of Fecal Progestagens, Androgen and Estrogens During Pregnancy, Parturition and Postpartum in Okapi (Okapia johnstoni)

Satoshi KUSUDA1), Koki MORIKAKU2), Ken-ichi KAWADA3), Kenji ISHIWADA4)# and Osamu DOI3)

1)Laboratory of Animal Reproduction, United Graduate School of Agricultural Science, Gifu University, Gifu 501-1193, 2)Preservation and Research Center, City of Yokohama, Kanagawa 241-0804, 3)Laboratory of Animal Reproduction, Faculty of Applied Biological Sciences, Gifu University, Gifu 501-1193 and 4)Yokohama Zoological Gardens Zoorasia, Kanagawa 241-0001, Japan

#Present: Kanazawa Zoological Gardens of Yokohama, Kanagawa 236-0042, Japan

Abstract. The aim of the present study was to establish a simple method to monitor ovarian activity and non-invasively diagnose pregnancy in okapi (Okapia johnstoni). The feces of a female okapi were collected daily or every 3 days for 28 months. Steroids in lyophilized feces were extracted with 80% methanol, and the fecal levels of immunoreactive progestagens (progesterone and pregnanediol-glucuronide), androgen (testosterone), and estrogens (estradiol-17β and estrone) were determined by enzyme immunoassays with commercially available antisera. Using the progesterone profiles, the durations of the luteal phase, follicular phase, and estrous cycle were determined to be 11.1 ± 0.4, 5.3 ± 0.6, and 16.5 ± 0.7 days (n=22), respectively. Fecal levels of immunoreactive progesterone, pregnanediol glucuronide, and testosterone gradually increased from early pregnancy and peaked several months before parturition. More pregnanediol glucuronide was excreted in feces than progesterone during late pregnancy, but not during the estrous cycle. Although the fecal concentrations of immunoreactive estradiol-17β and estrone change a little throughout pregnancy and non-pregnancy, they rose sharply and temporarily on the day following parturition. The present study indicates that fecal assays with commercial antisera for progesterone and pregnanediol glucuronide are useful for evaluating luteal activity and diagnosing pregnancy and indicates that estrogens might have some role as a trigger of parturition.

Key words: Estrous cycle, Fecal hormone, Okapi (Okapia johnstoni), Pregnancy, Progesterone

Okapi (Okapia johnstoni), a member of the family Giraffidae, was first described scientifically in 1901 [1]. An estimated 6,200–10,650 individuals of this species live in the northern and eastern Democratic Republic of the Congo [2]. Possible habitats are extremely limited to areas of dense rainforest. According to the 2006 IUCN Red List of Threatened Species [3], okapi is classified as Low Risk/Near Threatened (LR/nt) and is not in immediate danger of extinction, but they are considered vulnerable because of deforestation, their delicate resistance to disease [4], and civil strife in the Democratic Republic of the Congo (DRC) [5].

In captivity, okapi were exhibited for the first time at the Antwerp Zoo in Belgium in 1919 [2].
Currently, the captive population numbers a total of 144 animals at 40 zoos in America, Europe, and Japan [6, 7] and 15 animals at the Epulu station of the Okapi Wildlife Reserve in the Ituri tropical rainforest, which is in the northeastern area of the DRC. At least two hundred and forty have been born in captivity since 1970 [7, 8], and captive breeding is successful. In Japan, Yokohama Zoological Gardens Zoorasia was the first zoo to exhibit female and male okapis in 1997, and they have produced a total of 2 offspring, one in 2000 [9] and another in 2003. As of February 2006, a total of 7 okapi are being kept and exhibited at Yokohama Zoological Gardens Zoorasia (5 okapi) and Tokyo Ueno Zoological Gardens (2 okapi) [7].

Endocrinological observations on reproductive physiology in female okapi have mainly been reported by the groups of Loskutoff [10-13] and Schwarzenberger [14–17]. Both groups have attempted to monitor luteal activity and diagnose pregnancy by assays of hormone metabolites in excreta. The estrous cycle of the okapi was determined to be approximately 15 days based on changes in the concentrations of pregnanediol glucuronide (PdG) in urine [11, 13] and feces [14]. Urinary levels of estrogens have been reported to increase sharply 1–2 days after complete luteal regression [12]. A peak consistent with estradiol-17β (E2) elution patterns has also been observed by high performance liquid chromatography in okapi urine collected during estrus [10]. However, the excretion patterns of fecal estrogens have not been clarified.

Pregnancy in okapi is detectable by transabdominal ultrasonography and measurements of changes in body weight [8, 9, 18]. Since mating occurs during pregnancy in some cases [1], routine hormone monitoring is more useful for determining the time of conception. Urinary PdG and 20α-dihydroprogesterone [11] and fecal PdG [14–16] have been reported to be excreted in significantly higher levels in pregnant versus non-fertile okapi. Thus, assays of these hormones have enabled pregnancy to be diagnosed non-invasively.

The aim of this study was to establish a simple method of assaying fecal hormone concentrations to monitor ovarian activity and diagnose pregnancy non-invasively. We focused on 1) evaluating a simple method of extracting steroids from feces and 2) establishing generally available methods utilizing enzyme immunoassay systems with commercially available antisera for progesterone (P4), PdG, testosterone (T), E2 and estrone (E1).

Materials and Methods

Animal and collection of samples

A female okapi, named Layla (International Studbook No. 468; born on: 22 July 1996 at the Dallas Zoo; Fig. 1), was used in this study. This female was temporarily kept in an approximately 1,000 m² outdoor area during the day and in 26.88 m² indoor chambers during its nocturnal period in the Asian Tropical Forest Zone (currently being kept and exhibited in the African Tropical Rain Forest Zone) at Yokohama Zoological Gardens ZOORASIA [9]. She gave birth normally (21 November 2000) after a gestation period of 428 days based on the day of the final mounting behavior by a male (20 September 1999). The animal was fed a daily diet of alfalfa hay, pellets, carrot, Komatsuna (Japanese mustard spinach), lettuce, and celery. Drinking water was freely available.

Fresh-looking feces (Fig. 1) were collected from the floor of the animal’s sleeping chamber by 0900 h daily or every 3 days from 8 October 1999 (18 days after observation of the final mounting behavior) to
EXCRETION OF FECAL STEROIDS IN PREGNANT OKAPI

4 February 2002. Feces were placed in a Ziploc® bag and stored at –20°C until assay.

**Extraction of steroids**

For the assay of progestagens (P₄ and PdG), androgen (T), and estrogens (E₂ and E₁), the steroids in the fecal samples were extracted using a methanol extraction method [19]. Briefly, one or two grain-shaped frozen feces were lyophilized for approximately 24 h and crushed using a hammer. A portion of the powder, 0.1 g was then extracted with 80% methanol in ultra pure water (5 ml) by vortex-mixing for 30 min. After centrifugation at 2,500 rpm for 10 min, the supernatant (methanol fraction) was removed into a clean tube and diluted at a ratio of 1:20 for the P₄, PdG and T assays and 1:4 for the E₂ and E₁ assays using assay buffer (0.04 M disodium hydrogen phosphate dihydrate buffer containing 0.1% bovine serum albumin and 0.05 M sodium chloride).

**Enzyme immunoassays**

Fecal concentrations of immunoreactive P₄ (iP₄), PdG (iPdG), T (iT), E₂ (iE₂), and E₁ (iE₁) were determined using enzyme immunoassays, as described previously [19]. For the P₄ assay, a P₄ standard (Wako Pure Chemical, Osaka, Japan), P₄-3-CMO-HRP (dilution of 1:500,000, FKA301; Cosmo Bio, Tokyo, Japan), and P₄ antibody (1:250,000, LC-28; Teikoku Hormone, Kanagawa, Japan) were used. The main cross-reactivity of the antibody was 100% for P₄ and 62.2% for 5α-pregnanedione. For the PdG assay, a PdG standard (Sigma), PdG-HRP (1:100,000, FKA333; Cosmo Bio), and PdG antibody (1:800,000, FKA334-E; Cosmo Bio) were used. The main cross-reactivity of the antibody was 100% for PdG and pregnanediol and 16.0% for 20α-hydroxyprogesterone. For the T assay, a T standard (Wako Pure Chemical Industries), T-11α-succinate-HRP (1:2,000,000, FKA103; Cosmo Bio), and T antibody (1:1,000,000, ED-131; Teikoku Hormone) were used. The main cross-reactivity of the antibody was 100% for T and 41.3% for 5α-dihydrotestosterone. For the E₂ assay, an E₂ standard (Wako), E₂-3-CPE-HRP (1:80,000, FKA235; Cosmo Bio), and E₂ antibody (1:2,000,000, QF-121; Teikoku Hormone) were used. The main cross-reactivity of the antibody was 100% for E₂ and 8.0% for E₁-3-sulfate. For the E₁ assay, an E₁ standard (Sigma), E₁-3-CME-HRP (1:2,000,000, FKA233; Cosmo Bio), and E₁ antibody (1:2,000,000, FKA234-E; Cosmo Bio) were used. The main cross-reactivity of the antibody was 100% for E₁, 40% for E₁-3-sulfate, and 15% for E₁-3-glucuronide. Serial dilutions of fecal extracts from samples demonstrated parallelism with the standard curves in P₄, PdG, T, E₂ and E₁ assays.

**Data analysis**

All fecal data are expressed on a per gram dry feces basis. Data are presented as the mean ± standard error of the mean (SEM). Analyses were conducted using Student’s t-test. One estrous cycle is the period from the first significant rise in fecal iP₄ level of one cycle to the rise in the following cycle. The day of sampling before the first significant increase in fecal iP₄ and iPdG levels was defined as day 0.

**Results**

**Excretion patterns of fecal steroids during pregnancy and parturition**

The changes in the fecal concentrations of the progestagens, androgen and estrogens from early pregnancy to recurrence of the estrous cycle postpartum are shown in Fig. 2. At 18 days after observation of final mounting behavior, the fecal iP₄ and iPdG levels were already about 10 times the postpartum basal levels. Fecal iP₄, iPdG, and iT levels gradually increased during the greater part of the pregnancy, and at 1–3 months before parturition, they reached approximately 50, 90, and 10 times the basal levels, respectively. The day after parturition, the iP₄, iPdG, and iT values drastically decreased. Immunoreactive P₄ values were slightly higher than iPdG values during the first half of the pregnancy, whereas about twice as much iPdG as iP₄ was extracted during the second half of the pregnancy.

More iE₂ than iE₁ was excreted throughout the pregnancy. Fecal iE₂ values exhibited a slight increase 20–70 days before parturition. Fecal iE₂ and iE₁ values sharply and temporarily increased on the day following parturition.

**Excretion patterns of fecal steroids during estrous cycles postpartum**

The changes in the fecal concentrations of the progestagens, androgen, and estrogens during non-cycling and cycling after parturition are shown in
The fecal progestagen levels exhibited a temporal increase 58 days after parturition and cyclic patterns from 70 days after parturition. Fecal iP4 values were slightly higher than iPdG values during estrous cycles but not during pregnancy. The fecal profiles for androgen and the estrogens did not indicate cyclic changes like those of the progestagens.

Averaged changes in the fecal concentrations of the progestagens during 22 estrous cycles are shown in Fig. 4. The length of the luteal phase, as indicated by increased iP4 and iPdG levels, was 11.1 ± 0.4 days (n=22). The length of the follicular phase, as indicated by a non-fluctuating baseline level, was 5.3 ± 0.6 days (n=22). Therefore, the estrous cycle, the total length of the luteal and follicular
Fig. 3. Changes in the fecal concentrations of progestagens, androgen and estrogens during postpartum estrous cycles in the female okapi.

Fig. 4. Averaged excretion patterns of fecal immunoreactive progesterone (○) and pregnanediol glucuronide (●) from 22 estrous cycles of a female okapi. Day 0 is the day of sampling before the first apparent increase in the levels of these hormones. Estrous cycle (±SEM): 16.5 ± 0.7 days. Luteal phase (LP): 11.1 ± 0.4 days. Follicular phase (FP): 5.3 ± 0.6 days.
phases, was 16.5 ± 0.7 days (n=22).

Discussion

This study examined the overall patterns of fecal excretion of progestagens, androgen, and estrogens during pregnancy and the estrous cycle in an okapi.

Assay of the fecal concentrations of progestagens is helpful for evaluating ovarian function and diagnosing pregnancy in a variety of domestic and non-domestic mammals [reviewed in 20, 21]. In okapi, monitoring of the estrous cycle, pregnancy, parturition, and postpartum has been successful using the PdG assay with feces [14–16]. However, there are few reports on the use of the P4 assay in okapi. We established enzyme immunoassays using commercial P4 and PdG antisera and validated them by actually monitoring estrous cycles and pregnancy using okapi feces. There is considerable variation in the fecal levels of P4 metabolites among animal species, suggesting that the metabolism of steroids in the liver may be species-specific [21]. In our study, the levels of iP4 slightly differed from those of iPdG between late pregnancy and estrous cycles. This result suggests that the metabolism of P4 in the liver differs with reproductive stage in female okapi.

The normal estrous cycle of okapi is approximately 14.5 days in length, with the follicular phase and apparent luteal phase reported to last 7.9 and 6.7 days, respectively, in one study [11] and 8.3 and 6.9 days, respectively in another study [13] based on changes in urinary PdG. However, the true length of the follicular phase is considered to be shorter since urinary PdG levels during the follicular phase were at the lower limit of detection [13, 22]. The levels of all fecal PdG detectable with our assays were determinable to the follicular phase (5.3 days) in the okapi.

It has been reported that the serum T levels of Asian elephants (Elephas maximus) show a similar increase as serum P4 during the luteal phase of estrous cycles, and thus the corpus luteum of ovaries may produce T [23]. Androgens are also intermediates in the biosynthesis of estrogens. It has been reported that the fecal concentrations of androgen (17-oxo-androstanes) are an indicator of the follicular phase in female Indian rhinoceroses [24] and Malayan sun bears [25]. However, fecal androgen during the non-gestation period in the okapi was not useful as an index of the luteal or follicular phase. In contrast, more fecal androgen was excreted during pregnancy than during the non-gestation period in our study, suggesting that the pregnant corpus luteum or placenta of the okapi may produce androgen.

It has been reported that most of the tritiated E2 injected into the jugular vein of a female okapi is excreted via feces [10]. Although fecal immunoreactive estrogens were detectable in our study, the longitudinal results of monitoring did not show changes associated with follicular activity or estrus. In some species, estrogens are also produced in the placenta during pregnancy. The successful detection of pregnancy by assaying fecal estrogens has been reported in various ungulates, such as buffalo (Syncerus caffer), Grevy’s zebra (Equus grevyi), caribou (Rangifer tarandus), sable antelope (Hippotragus niger) and bison (Bison bison) [21]. Pregnancy in okapi could not be diagnosed using our assay of fecal iE2 and iE1. It has been reported that a variety of contaminants with estrogenic immunoreactivity are present in okapi urine [10]. When assaying fecal estrogens, a pretreatment such as purification of fecal steroid extracts may be required.

The fecal iE2 and iE1 levels increased sharply 1 day postpartum. However, the day of their peak could not be identified since fecal samples were not collected on the day of parturition and the 2nd and 3rd days postpartum. Given the report that tritiated E2 infused via the jugular vein was detected in feces at 72 h post-injection [10], circulating estrogens probably take 3 days to be excreted in feces. It seems that the circulating levels of estrogens reached a peak within the period from 3 days prepartum to the day of parturition. This is almost the same observation as those made in cattle, which reach a plasma E1 level peak in the last 4 days of pregnancy [26], and sheep, which reach a plasma E1 level peak at parturition [27]. It has been suggested that the extra estrogen produced at parturition acts to contract the uterine muscle [28]. Our results suggest that estrogens may have some role as a trigger of parturition in okapi as well as domestic ungulates.

In conclusion, the methanol extraction method for fecal steroids is simple and useful, and enzyme immunoassays with commercially available antisera are informative for the understanding estrous cycle, pregnancy, and parturition in okapi.
Fecal hormonal analyses are a reliable alternative to serum and urinary analyses in female okapi. The estrous cycle, pregnancy, and parturition are characterized by increased fecal concentrations of progestagens, progestagens and androgen, and estrogens, respectively. Using the fecal P₄ assay, the ovarian activity of all captive okapis should be assessed non-invasively to further advance the captive propagation program.

References


Acknowledgements

We gratefully acknowledge the help of the staff of Yokohama Zoological Gardens Zoorasia. This work was supported by the Preservation and Research Center, City of Yokohama which provided numerous disposable items for our experiments.


