

TECHNICAL ARTICLE

Application of Fecal Steroid Techniques to the Reproductive Endocrinology of Female Verreaux's Sifaka (*Propithecus verreauxi*)

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Solid phase extraction, high performance liquid chromatography, and radioimmunoassay were used to test the validity of fecal steroid analysis for assessing ovarian function in sifaka (*Propithecus verreauxi*). Daily fecal samples were collected over a 4 month period from two cycling female sifaka, and single samples were collected from females during normal gestation and males while housed at the Duke University Primate Center. Tests of radioimmunoassay validity indicated that solid phase extraction and microradioimmunoassay techniques were reliable and accurate methods for quantifying ovarian steroids in sifaka feces. The progesterone (P₄) antibody specifically quantitated only P₄, while several estrogen metabolites made small contributions to immunoreactive measures of estradiol (E₂). A 1:10 dilution reduced these contributions to 3–15% of the estimated E₂ concentration. Although the spectral data suggested that E₂ was not the major metabolite present, it accounted for the majority of the immunoreactivity at normal assay dilutions. Fecal profiles of immunoreactive E₂ and P₄ in the conceptive female resembled serum profiles of other strepsirhines. E₂ and P₄ were elevated at the end of the conceptive cycle and were more markedly increased in late pregnancy in the two pregnant females. Mating behavior and indices of sexual interest were observed in conjunction with E₂ peaks, although not all peaks were accompanied by observations of sexual behavior. © 1995 Wiley-Liss, Inc.

Key words: strepsirhine, sifaka, fecal steroids, ovarian cycles

INTRODUCTION

Verreaux's sifaka (*Propithecus verreauxi*) is a diurnal, seasonally reproducing strepsirhine primate inhabiting riverine and dry forests of south and southwest Madagascar [Pollock, 1979; Tattersall, 1982]. Social groups range in size from 2–13 individuals, in which female philopatry and social dominance appear to be the norm [Richard, 1987; Kubzdela et al., 1992; Young et al., 1990; but see Pereira et

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al., 1990]. Although *P. verreauxi* is one of the better known Malagasy lemurs [Jolly, 1966; Richard, 1974, 1977, 1992], its reproductive behavior and mating system remain poorly documented. Previous studies suggest that sifaka have a 3 month breeding season [Jolly, 1966; Richard, 1974, 1977; Haring, 1988] during which behaviorally asynchronous females are receptive to males for up to 96 h once, and occasionally twice, per breeding season [Brockman, 1994].

Understanding the significance of this unique breeding system for individual fitness and the evolution and history of social groups requires examination of the endocrine events that mediate breeding activity. Acquiring such data for this rare and endangered species [Harcourt & Thornback, 1990] necessitates noninvasive methods of hormone analysis. Recent data suggest that fecal steroid analysis is a promising method for assessing ovarian function in both captive [Risler et al., 1987; Perez et al., 1988; Wasser et al., 1988; Shideler et al., 1994] and free-ranging primates [Wasser et al., 1988, 1991; Clarke et al., 1991; Strier & Ziegler, 1994]. This paper tests the validity of radioimmunoassay procedures in sifaka fecal extracts and examines profiles of ovarian steroids from two captive sifaka during the annual breeding season.

MATERIALS AND METHODS

Study Subjects

Fecal samples were obtained from sifaka housed at Duke University Primate Center (DUPC), (Durham, NC), the only breeding colony of this species in the United States. Animals were housed in male–female pairs and maintained and provisioned as described elsewhere [Haring, 1988]. Single samples were obtained following the normal mating season and prior to the birth season from four pairs of *P. verreauxi* and one pair of *P. tattersalli*. Daily serial samples were collected from two wildcaught female *P. verreauxi* (estimated 6 and 8 years of age) during a period (June–October) that local breeding histories suggested would extend from the pre- to postbreeding period. One female, Octavia (6109f), had given birth to three infants conceived at DUPC prior to 1990; the other female, Marcella (6110f), had never produced offspring.

Fecal Sample Collection and Preservation

Fecal samples (4–15 g) were collected in entirety immediately after voiding, packaged in Whirl-Pak bags, coded by ID number, time, and date, and frozen at -20°C . Samples were collected at the same time each morning (0800–1000) to minimize bias that might be introduced if fecal steroid excretion exhibited the diurnal variation reported for urinary steroids [Collins et al., 1979]. To assess diurnal variation in steroid excretion, multiple samples were collected on a small sample of days and analyzed for temporal variation in steroid content. Differences in voiding patterns between females resulted in fewer samples for one of the serially sampled females (Marcella: $n = 49$) than the other female (Octavia: $n = 96$) and some samples obtained outside of the standard time period. Urinary/fecal comparisons of excreted steroids were not feasible, as sifaka do not urinate per se. Rather, droplets of urine are deposited on vertical substrates during scent-marking activities and are thus unsuitable for collection.

Behavioral Observations

Each pair was observed daily for behavioral indices of estrus (attempted or completed genital inspections, mounts, and copulations) during the morning sample collection period. These notes were supplemented by opportunistic observations made by keepers over the course of the day. Although these records may represent

underestimates of behavior, the strictly delimited nature of sexual behavior in this species (0.5–96 h [Brockman, 1994]) and the excellent monitoring conditions at DUPC suggest that these observations provide a useful guide to the actual timing of sexual behavior.

Fecal Steroid Extraction

A 0.1 g sample was homogenized in 2 ml methanol:acetone (8:2, v/v) and filtered with a 0.2 mm G Prep PTFE centrifuge filter (Gelman Sciences, Ann Arbor, MI). The filtrate was solid-phased extracted on small columns of reversed-phased octadecylsilane (C18) bonded to silica (Baxter/Burdick and Jackson, Muskegon, MI), using the method of Shackleton and Whitney [1980]. The sample was diluted 1:2 with water and then layered onto a column primed according to manufacturer's directions. The column was washed with 5 ml water, and steroids were eluted with 2 ml methanol (88–91% of E_2 and P_4 were eluted in the first 2 ml). Steroid recovery was monitored by adding radiolabeled tracer (1,500 cpm $^{125}I-E_2$ plus 1,500 cpm $^{125}I-P_4$) to each sample. In these initial investigations, recovery of the combined steroid tracer was used to estimate average steroid recovery; subsequent experiments have shown nearly identical recoveries for E_2 ($71 \pm 0.8\%$; $n = 20$) and P_4 ($74 \pm 0.6\%$; $n = 20$).

Hydrolysis of Steroid Glucuronides

In investigations of steroid glucuronides, fecal extraction was carried out using the solid phase method for separation of conjugated and unconjugated steroids described in Belanger et al. [1990]. Recovery experiments using unlabeled E_2 -3-glucuronide as tracer indicated that 95.4% of conjugated steroid was recovered in the conjugated phase and 4.6% in the free phase. The conjugated fraction was evaporated under nitrogen and reconstituted in 5 ml of 4 nM phosphate buffer, pH 6.8, containing 500 Fishman units beta-Glucuronidase (Sigma, St. Louis, Mo.). Following a 24 h incubation at 37°C, the released steroids were extracted on C18 columns using the procedures for unconjugated steroids described above. Duplicate samples of E_2 -3-glucuronide (1,000 pg/ml) were carried through these incubations to estimate the completeness of hydrolysis. The concentration of the E_2 glucuronide control carried through the same procedures was equimolar with the concentration added, indicating that the hydrolysis of simple glucuronide conjugates was complete. Aliquots of the unconjugated fraction were subjected to the same procedures to control for procedural losses. Each fraction and control was then analyzed for E_2 and P_4 by radioimmunoassay and by combined high performance liquid chromatography and radioimmunoassay.

Radioimmunoassay (RIA)

Estradiol. E_2 radioimmunoassay was carried out using microassay procedures developed by Worthman et al. [1990]. Validations were performed using reagents from both the E_2 -Quant-In Vitro ^{125}I RIA Diagnostic Test kit (Leeco Diagnostics Inc., Southfield, MI) and the Pantex Direct ^{125}I Estradiol 174M kit (Santa Monica, CA). Working buffer for the E_2 assay was 0.1% gelatin phosphate buffered saline, pH 7.4. An aliquot of fecal extract was evaporated under nitrogen and reconstituted in the working buffer at a 1:10 dilution. In the Leeco version, ^{125}I E_2 tracer (50 μ l) and 100 μ l of antiserum diluted 1:4 in working buffer were added to aliquots (200 μ l) of the standards (diluted 1:5 to give concentrations of 1–400 pg/ml), samples, and controls (diluted 1:5). Each was vortexed and incubated overnight at room temperature. The following morning, 500 μ l PEG second antibody, diluted 1:2, was added, and the incubates were vortexed, incubated an additional

20 min at room temperature, and centrifuged at 1,500g for 1 h at 4°C. In the Pantex version, 20 µl tracer and 500 µl of antiserum, diluted 1:8, were added to 50 µl of standards, samples, and controls diluted 1:10. After overnight incubation at room temperature, 500 µl PEG second antibody, diluted 1:4, was added, and the incubates were vortexed, incubated 1 h at room temperature, and centrifuged at 1,500g for 1 h at room temperature. The supernatants were decanted, and the radioactivity of the precipitate was determined by 10 min counts in a Packard RIASTAR gamma counter (Packard, Downer's Grove, IL) with RIASMART and Expert QC software.

Progesterone. P₄ RIA was carried out using microassay procedures developed by Worthman and Stallings [unpublished], using reagents from the Pantex ¹²⁵I P₄ Kit for serum determinations. Working buffer was phosphate buffered saline, pH 7.4, with 0.1% bovine serum albumin and 0.1% sodium azide as preservative. An aliquot of fecal extract was evaporated under nitrogen and reconstituted 1:1 in working buffer. ¹²⁵I P₄ tracer (100 µl) and 100 µl of antiserum diluted 1:6 with working buffer were added to aliquots (100 µl) of the standards (diluted 1:5 to give concentrations of 0.04–16 ng/ml), samples, and controls (diluted 1:5) (Bio-Rad, ECS Division, Anaheim, CA). Each was vortexed and incubated overnight at room temperature. The following morning, second antibody (Pantex; 100 µl) was added, and the incubates were vortexed, incubated 1 additional hour at room temperature, and then centrifuged at 1,500g for 1 h at 22°C. Following decanting of the supernatants, radioactivity of the precipitate was determined by 10 min counts in a gamma counter.

Tests of RIA validity. Sensitivity was determined from the quantity of unlabeled hormone required to inhibit binding of tracer by an amount equal to two standard deviations below the mean binding observed in the absence of unlabeled hormone. The accuracy with which the radioimmunoassay assessed concentrations of fecal steroids was determined by the addition in duplicate of the steroid standards to aliquots of a fecal extract with low levels of the respective steroid. Precision was assessed by the tube error of duplicates and intra- and interassay coefficients of variation. Parallelism was assessed by serial dilutions of fecal extracts previously determined to contain high concentrations of E₂ or P₄ and comparison of the slope of the resultant regressions to the slope of the standard curve. Cross-reactivities were determined by the addition of estrogen metabolites to the standard reaction mixture at concentrations ranging from 2 pg/ml to 2 µg/ml; ED50s were calculated using the nonlinear curve-fitting program, ALLFIT, as described by Whitten et al. [1992].

High Performance Liquid Chromatographic Analysis (HPLC)

Apparatus. Liquid chromatography was carried out using an LC-250 binary pump, an LC-250 photodiode array UV detector, column oven, and an Epson Equity II computer, all from Perkin-Elmer (Norwalk, CT). The mobile phase was continuously degassed with a stream of helium. Samples were injected with a Rheodyne 7125 injector fitted with a 20 µl loop. A fraction collector (Gilson, Middletown, WI) was used for the timed collection of fractions of the eluent from the LC column. The column (25 cm × 4.6 mm I.D.) was Hypersil ODS, 5 µm, from Sigma-Aldrich (Aldrich, Milwaukee, WI). Components were identified by comparison of retention times to those of external standards (1–10 µg) run after each sample analysis. Steroid standards were prepared from aliquots of ethanolic stock solutions reconstituted in the mobile phase at concentrations of 50–500 µl/ml. Because the photodiode array detector allows collection of the absorption spectra of each detected peak, it was possible to carry out further verification of peak identity by

comparing the absorption spectra of the observed peaks to those of authentic standards. The predictable shifts in direction of spectra peaks induced by the position of substituents also aided in selection of potential candidates for peaks with retention times that did not correspond to standards, resulting in several cases in the matching of an unidentified peak with a new authentic standard that corresponded in both retention time and spectra.

Procedures. Gonadal and adrenal steroids were first separated in a general procedure for a range of common steroids using a reverse phase gradient technique [O'Hare et al., 1976]. The mobile phase was methanol-water, 50–100% in 30 min with a concave gradient of $y = -x^3$ and a flow rate of 1 ml/min at 45°C. UV detection was at 240 and 255 nm. Retention times, determined for ten steroid standards, were reproducible to within 1% CV over a 3 month period. Androgens and progestins were subsequently analyzed with a reverse-phase gradient technique for the detection of testicular steroids [O'Hare et al., 1976]. The mobile phase was acetonitrile-water, 50–80% in 12 min followed by 80–100% in 5 min with a concave gradient of $y = -x^3$ and a flow rate of 1 ml/min at 45°C. UV detection was at 240 and 280 nm. Retention times, determined for ten androgens and four progestins, were reproducible to within 1% CV over a 3 month period. Estrogens were subsequently analyzed using a reverse-phase isocratic technique that allows the simultaneous detection of the principle classical steroidal estrogens along with equine estrogens and plant estrogens [Setchell et al., 1987]. The mobile phase was 37% acetonitrile in 0.1 M ammonium acetate buffer, pH 4.6, with a flow rate of 1 ml/min for 23 min at 30°C. UV detection was at 280 nm and 260 nm. Retention times, determined for 15 estrogens and two isoflavonoids, were reproducible to within 1% CV over a 3 month period.

Immunoreactivity of steroid metabolites. Following separation of steroid metabolites by chromatography, the eluted fractions were further tested for their immunoreactivity with the steroid antisera used in RIA. The chromatographic eluent was collected in 0.3 min fractions, and the fractions were reconstituted in assay buffer and subjected to RIA.

RESULTS

Steroid Recovery and Radioimmunoassay Validity

Steroid recovery averaged $69.0 \pm 0.8\%$ overall, with recoveries from the homogenization, filtration, and solid phase column stages of the extraction procedure being $94.2 \pm 0.6\%$, $91.4 \pm 1.3\%$, and $82.7 \pm 1.8\%$ ($n = 5$), respectively. Table I shows that the RIA procedures provided precise and accurate estimates of fecal E_2 and P_4 , with evidence of parallelism for both fecal E_2 and P_4 . Extraction solvents carried through the extraction procedure and reconstituted in assay buffer gave readings of zero in both radioimmunoassays, as did phytoestrogens (coumestrol, genistein) at concentrations as high as $1 \mu\text{g/ml}$. Reagents from the two E_2 kits yielded similar estimates of accuracy and reliability, and comparisons of E_2 concentrations in extracts of sifaka samples measured in the two assays did not differ significantly over a thirtyfold range of values (paired $t = 0.983$, $P = 0.332$, $n = 36$).

Comparison of Free and Conjugated Steroids

Only a small fraction of the E_2 (4%) and P_4 (8%) was present in the glucuronide fraction following separation and hydrolysis. RIA of the hydrolyzed glucuronide and free fractions following separation by HPLC suggested that a larger portion (40%) of E_2 was conjugated than was indicated by radioimmunoassay prior to chromatography; the discrepancy is likely due to the fact that other estrogen me-

TABLE I. Accuracy and Precision of Fecal Steroid Radioimmunoassays in *Propithecus verreauxi*

Steroid	Sensitivity ^a	Accuracy (n)	Paral- lelism	Tube error (n)	Intraassay CV (%) ^b		Interassay CV (%) ^c	
					Low (n)	High (n)	Low (n)	High (n)
Estradiol								
Leeco	0.36 pg/ml	113 ± 6%; y = 1.01x - 0.03; r = 0.999 (7); P < 0.001	Yes	1.9 (19)	16.5 (8)	3.8 (4)	8.1 (9)	15.6 (9)
Pantex	0.36 pg/ml	121 ± 15%; y = 0.90x + 6.46; r = 0.99 (7); P < 0.001	Yes	1.6 (20)	5.8 (6)		2.7 (3)	17.3 (10)
Proges- terone								
0.006 ng/ml	117 ± 9%; y = 1.02x + 0.10; r = 0.981 (8); P < 0.001	Yes	1.1 (18)	6.6 (8)	7.4 (8)	8.7 (6)	20.4 (3)	

^aDefined as the quantity of unlabeled hormone required to inhibit binding of tracer by an amount equal to two standard deviations below the mean binding in the absence of unlabeled hormone.

^bWithin assay coefficient of variation of fecal sample pools.

^cBetween assay coefficient of variation of serum controls.

tabolites, present primarily in unconjugated form, are also detected by the E₂ antisera (see below). These determinations do not preclude the possibility of greater representation by steroid sulfates or more complex glucuronides but suggest that the unconjugated state is likely to be the predominant form of both E₂ and P₄ in sifaka feces.

HPLC and Immunoreactivity of Fecal Steroids

Because the rarity of this species in captivity precluded metabolic studies, the fecal metabolites of E₂ and P₄ and their immunoreactivity were investigated using HPLC. Peaks corresponding to the retention times of 17OHP and 20αOHP, as well as P₄, were detected using the adrenal (data not shown) and androgen/progestin programs (Fig. 1). Spectral estimates of concentration indicated that the metabolite present in highest concentration corresponded to the retention time of 20αOHP. Nevertheless, only a single immunoreactive peak, corresponding to the retention time of authentic P₄, was detected by the P₄ antibody. Spectral peaks corresponding to the retention times of E₂ and several other estrogens and estrogen metabolites were detected by the adrenal (data not shown) and estrogen programs (see Fig. 2). The metabolite present in highest concentration corresponded to the retention time of 4-hydroxyE₂. Although the spectral data suggested that E₂ was not the major metabolite present, it accounted for the overwhelming majority of the immunoreactivity (see Table II) at normal assay dilutions. When extracts were assayed undiluted, however, the immunoreactive contributions of other estrogen metabolites, primarily the catechol estrogen 2-hydroxyE₁, increased to as much as 48% of the estimated dose. Comparison of the Leeco and Pantex antisera indicated nearly identical patterns of immunoreactivity.

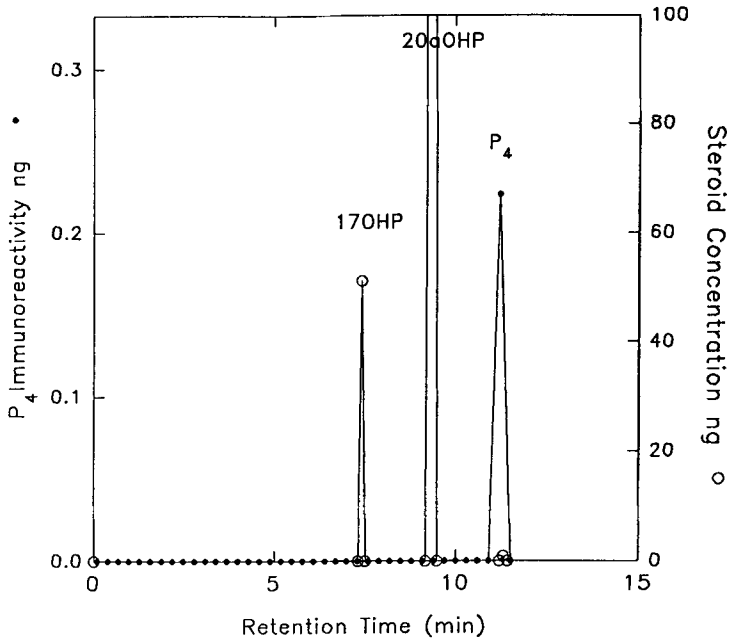


Fig. 1. Comparison of progesterone immunoreactivity (closed circles) and concentrations (open circles) of chromatographic peaks corresponding in retention time and absorption spectra to progesterone standards. Fractions were separated on the androgen/progestin HPLC program and collected at 0.3 min intervals. Immunoreactivity represents the nanogram/fraction estimated by P_4 RIA. Progesterone concentrations were estimated by comparing height of absorptive peaks to a linear curve fit to serial dilutions of progesterone standards ($r = 0.99$). 17OHP, 17-hydroxyprogesterone; 20 α OHP, 20 α -hydroxyprogesterone; P_4 , progesterone.

Diurnal Variability in Steroid Concentrations

The influence of time of fecal collection on steroid concentration was examined in seven sets of samples in which collections were made twice in the same day. Concentrations of E_2 and P_4 in the later samples averaged $104.3 \pm 26.8\%$ (E_2 : 25.9–208.3%) and $124 \pm 35.5\%$ (P_4 : 27.3–301.8%) of morning values, suggesting no consistent effect of collection time, although variation was considerable (E_2 : CV = 67.9; P_4 : CV = 76.0).

Relation of Fecal Steroid Levels to Reproductive State

Table III compares E_2 and P_4 concentrations in fecal extracts of male and female sifaka in several reproductive states. Concentrations of both steroids were low in males and females outside the breeding season. E_2 and P_4 concentrations were markedly elevated in two females sampled in the last trimester of pregnancy. Moreover, both steroids were twice as high in the sample taken from one female within 1 week of parturition than in the sample taken approximately 6 weeks before parturition.

Fecal Steroid Profiles

Figure 3 depicts the E_2 and P_4 profiles of Octavia, who conceived during the sample period. The known range of gestation for this species, 154–198 days ($n = 12$ [Haring, 1988]), would place conception between August 21 and October 4. A marked elevation in fecal E_2 is seen during this period, followed by a more gradual

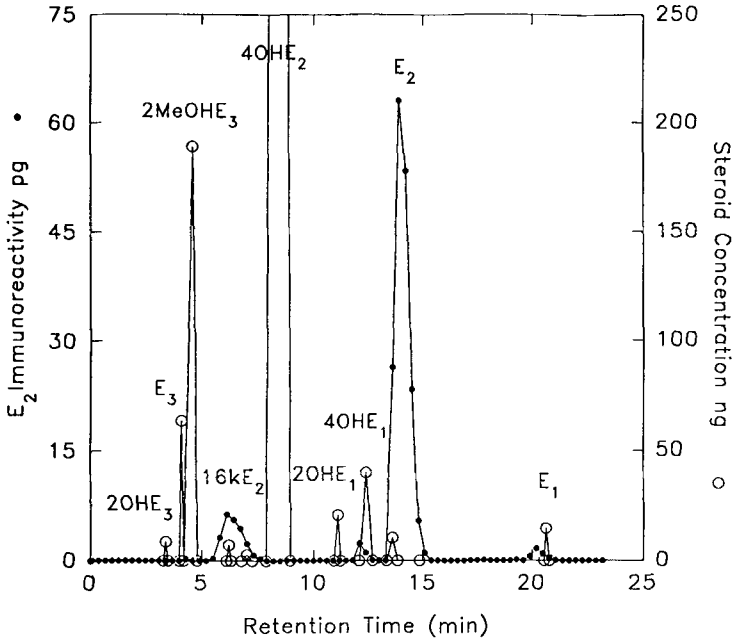


Fig. 2. Comparison of estradiol immunoreactivity (closed circles) and concentrations (open circles) of chromatographic peaks corresponding in retention time and absorption spectra to estrogen standards. Fractions were separated on the estrogen HPLC program and collected at 0.3 min intervals. Immunoreactivity represents the picogram/fraction estimated by E_2 RIA. Concentrations of estrogen metabolites were estimated by comparing area of absorptive peaks to a linear curve fit to serial dilutions of estrogen standards ($r = 0.99$). 2OHE₃, 2-hydroxyestriol; E₃, estriol; 2MeOHE₃, 2-methoxyestriol; 16kE₂, 16-keto-estradiol; 4OHE₂, hydroxyestradiol; 2OHE₁, 2-hydroxyestrone; 4OHE₁, 4-hydroxyestrone; E₂, estradiol; E₁, estrone.

rise in P_4 . A similar E_2 profile can be observed in late June–July. Baseline levels of E_2 are within the range of values observed in sexually inactive females (see Table III), while peak values are fifteenfold higher. Both of these E_2 elevations last 31–33 days and are characterized by a rapid fifteenfold elevation in E_2 lasting 5 days, followed 1–2 days later by a more modest, threefold elevation that declines gradually over the next 18–20 days. Elevations in P_4 follow the E_2 peak and last 25–27 days. An additional elevation in P_4 , accompanied by a modest rise in E_2 , can be observed at the onset of sampling in mid-June and may represent the latter portion of an additional “cycle.” Another P_4 elevation occurring during the first half of August that is not preceded by an E_2 elevation may represent a different endocrine response than the other putative cycles. In spite of the successful conception, no mounts or copulations were observed in this sifaka pair. However, the male attempted genital inspections near the time of the E_2 peak in this conceptive profile.

Figure 4 depicts the E_2 and P_4 profiles of Marcella, who did not conceive during the sampling period. A 5 day E_2 peak is coincident with copulations observed in September. Additional copulations occurred 28 days later, but hormonal data are lacking for this time period. Copulations were not observed during two E_2 elevations in late July to early August. In contrast to the conceptive profile of Octavia, Marcella’s P_4 levels were consistently low throughout the sample period.

TABLE II. Contributions of Estrogen Metabolites to Estradiol Immunoreactivity

Retention time (min)	Estrogen standard ^a	% Cross-reactivity ^b	% of immunoreactivity ^c	
			Octavia	Marcella
3.2	2-hydroxy-estriol	<<0.001		
4.2	Estriol	0.01		
4.6	2-methoxy-estriol	0.006		
6.1	16-keto-estradiol	0.12	0.1	11.4
6.3	16 α -hydroxyestrone	0.01		
8.4	4-hydroxyestradiol	0.05	2.4	
10.8	2-hydroxyesterone	0.004		
11.9	4-hydroxyestrone	0.02		1.7
13.7	Estradiol	100.00	97.5	85.2
20.6	Estrone	0.004		1.7

^aEstrogen standards corresponding to the retention times and absorption spectra of chromatographic and immunoreactive peaks.

^bRelative cross-reactivity of estrogen standards with Pantex E₂ antibody.

^cBased on RIA of chromatographic fractions of 200 μ l aliquots of extracts from fecal samples obtained at or near behavioral and hormonal estrus (Marcella: peak E₂ value; Octavia: 1 day prior to peak E₂).

TABLE III. Relation of Fecal Steroid Concentrations to Reproductive State

Reproductive state ^a	Species ^b	N ^c	Estradiol (ng/gm)	Progesterone (ng/gm)
Females				
Sexually inactive	Pv	2	4.5 \pm 0.7	83.4 \pm 23.4
	Pt	1	1.2	5.7
Pregnant	Pv	2	72.9 \pm 17.9	1121.1 \pm 480.4
Males				
Sexually inactive	Pv	4	7.4 \pm 2.3	27.8 \pm 6.5
	Pt	1	0.5	55.0

^aSexually inactive: sampled outside of normal breeding season and no mating activity observed; pregnant: samples collected 1–6 weeks prior to parturition.

^bPv, *Propithecus verreauxi*; Pt, *Propithecus tattersalli*.

^cNumber of individuals sampled.

DISCUSSION

These investigations show that solid phase extraction and microradioimmunoassay techniques provide accurate and reliable estimates of ovarian steroids in sifaka feces. These methods offer the advantages of rapid and efficient extraction and facilitate sample dilution to reduce the effects of cross-reacting metabolites and other constituents of fecal extracts that may interfere with RIA. Dilution of extracts prior to assay may be particularly important in the estimation of E₂ where cross-reacting metabolites can make major contributions to immunoreactivity. Absorption spectra and retention times suggest that catechol estrogens are the major immunoreactive components in undiluted extracts. As major metabolites of E₂ [Parvizi & Ellendorf, 1980; Hershcopf et al., 1986], these metabolites should mirror the excretion of E₂, and thus their contribution need not skew the estimation of E₂ secretion, but their instability at alkaline pH [Gelbke & Knuppen, 1972] may increase the variability of estimates. These problems can be avoided by a tenfold dilution of fecal extracts which minimizes metabolite contributions and reduces assay variability. With these precautions, immunoreactivities specifically measure P₄ and E₂ even though they are not the primary steroid metabolites found in

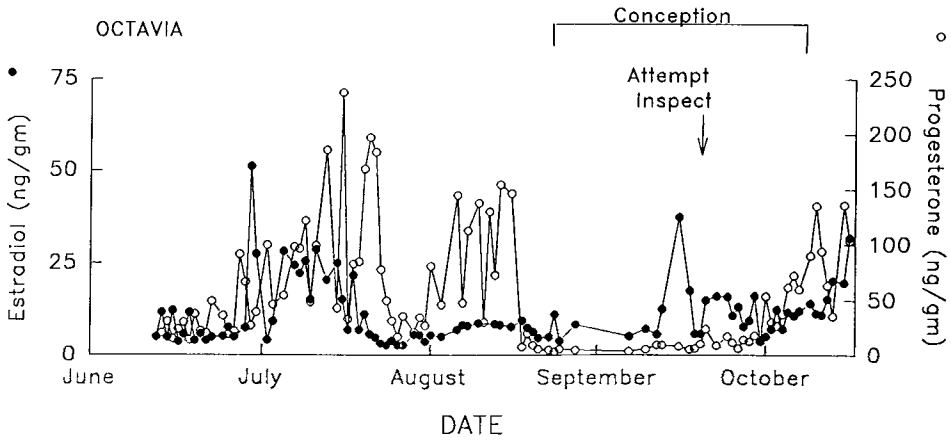


Fig. 3. Profile of E_2 and P_4 for the female Octavia. Arrows depict the timing of observed indices of estrus, and the bar indicates the period in which conception occurred, based on the date of birth and known gestation lengths.

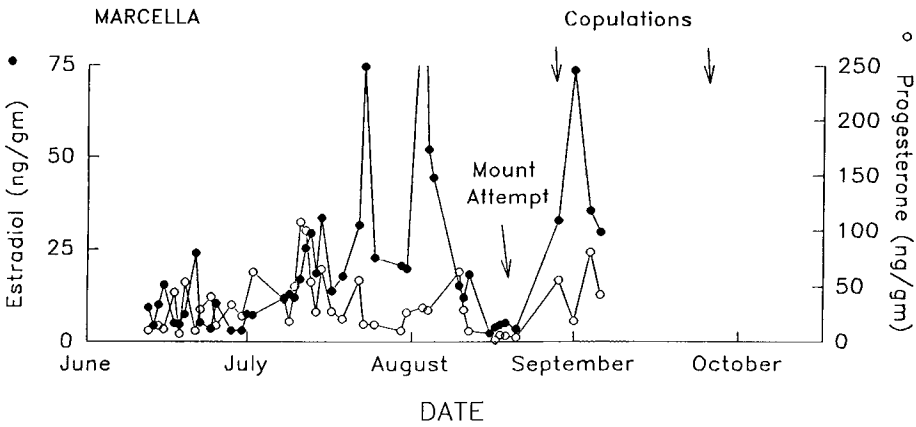


Fig. 4. Profile of E_2 and P_4 for the female Marcella.

sifaka feces. Tentative identifications of metabolites present in feces will facilitate the investigation of other steroids that may better reflect ovarian function and reproductive status.

In other investigations, the Whitten laboratory has shown that the same techniques can be accurately and reliably applied to the assay of gonadal steroids in baboons and rhesus macaques, producing steroid estimates and profiles that mirror serum steroids and reproductive state [Stavisky, 1994; Stavisky et al., 1991, 1994, submitted]. Although management concerns prevented serum comparisons in this study, substantial concordance was observed between the conceptive fecal profile (Fig. 3) and the available data on ovarian cyclicity and conception in other strepsirhine primates [Perry et al., 1992]. The durations of the sharp excursions in E_2 (5 days) and the prolonged elevations of P_4 (25–27 days) resemble the 5 day follicular phases and 24–28 day luteal phases reported for species of *Lemur* [Bogart

et al., 1977; Van Horn & Resko, 1977; Perry et al., 1992]. Although the fecal profiles show some day-to-day fluctuation in steroid levels, this variation is no different from that seen in daily urinary steroid profiles [Shideler & Lasley, 1982]. The rise in E_2 and continued elevations of P_4 , occurring at 30 days after the E_2 peak, resemble a gestational profile of *Lemur mongoz* at 30 (E_2) to 45 (P_4) days of gestation [Perry et al., 1992]. In contrast, the poor reproductive performance of Marcella suggests that her more erratic profile may be atypical, a hypothesis that we are testing in ongoing investigations of free-ranging sifaka.

The small number of cycles and animals examined here preclude any definitive conclusions about the endocrine bases of reproductive events in this species. Nevertheless, these data provide some insights into the reproductive biology of these rare and endangered primates. First, these endocrine data indicate that ovarian cyclicity may be more prolonged than indicated by the brief 3 month breeding season observed in free-ranging strepsirhines [Jolly, 1966; Richard, 1974; Koyama, 1988; Sauther, 1991]. Cyclic changes in E_2 and P_4 were observed in Octavia as early as June and as late as September, reflective of two ovarian cycles. Previous studies of captive and free-ranging strepsirhines suggest that cyclicity is prolonged when conception fails to occur [Perry et al., 1992; Brockman et al., 1987, 1994]. Secondly, these behavioral data accord with recent field observations indicating that some females may be receptive to males more than once during a breeding season [Brockman, 1994]. Marcella was observed mating twice, once in late August when E_2 was rising and then again 1 month later in late September. Last, although mating behavior and indices of sexual interest were observed in conjunction with E_2 peaks, not all peaks were accompanied by observations of sexual behavior. Although the latter may possibly reflect missed behavioral observations, similar hormone–sexual behavior disjunctions have been observed in free-ranging sifaka wherein females may sexually present to newly immigrated males coincident with diminished E_2 levels [Brockman, 1994]. Additional data will be needed to establish the significance of these observations, but they suggest that fecal steroid analysis may be a useful tool for exploring the relationship between ovarian cyclicity and mating behavior in captive and free-ranging sifaka.

CONCLUSIONS

1. Solid phase extraction and microradioimmunoassay techniques are reliable and accurate methods for quantifying ovarian steroids in sifaka feces.
2. The P_4 antibody specifically quantitated only P_4 while several estrogen metabolites made small contributions to immunoreactive measures of E_2 .
3. Sifaka fecal steroids are excreted predominantly in unconjugated form.
4. Fecal E_2 and P_4 profiles from a conceptive profile resembled serum profiles of other cycling and pregnant strepsirhines.
5. Ovarian cyclicity during the mating season may be more prolonged than indicated by the brief 3 month breeding season observed in free-ranging strepsirhines.
6. Mating behavior and indices of sexual interest were observed in conjunction with E_2 peaks, although not all peaks were accompanied by observations of sexual behavior.

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