

Excretory Fate of Estradiol and Progesterone in the African Elephant (*Loxodonta africana*) and Patterns of Fecal Steroid Concentrations throughout the Estrous Cycle

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We developed and validated a noninvasive method to quantify fecal estrogens and progestins as a tool for monitoring long-term ovarian activity in free-ranging African elephants. The lag times between iv injection of [³H]estradiol and [¹⁴C]progesterone and peak excretion of radioactivity in urine and feces were approximately 4 hr and 48 hr, respectively. The majority of progesterone metabolites recovered was excreted in feces (55%) versus urine (45%), whereas comparatively little of the recovered estradiol metabolites were excreted in feces (5%) compared to urine (95%). Intrasample variation in fecal hormone concentrations was extremely high but could be substantially reduced by extracting well-mixed fecal powder from freeze-dried samples, taken from the central or premixed portion of the wet sample. This method resulted in a close correspondence between matched serum and fecal progestins (mean correlation = 0.81, range 0.61–0.94) collected from five nonpregnant adult females over a 7-month period. Fecal estrogen profiles were more ambiguous, tending to overlap with those of fecal progestins. We conclude that analyses of fecal progestins can provide an effective, noninvasive

means of characterizing ovarian activity in free-ranging African elephants. © 1996 Academic Press, Inc.

African elephants (*Loxodonta africana*) were heavily poached during the late 1970s and early 1980s, dramatically reducing numbers from 1.5 million to approximately 600,000 individuals between 1980 and 1986 (Ricutti, 1993). In response, the Convention on International Trade in Endangered Species listed the African elephant as an Appendix 1 species in 1989—the highest protection status given to an endangered species. This action virtually eliminated the ivory market, creating one of the most successful international bans on poaching in history. However, pressure still persists to downlist the African elephant and thus lift the ivory ban.

Evidence from Tanzania suggests that the long-term consequences of poaching reach far beyond that of slaughtering elephants (Poole, 1989; Balozzi, 1989;

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Barnes and Kapela, 1991). In Tanzanian national parks such as Mikumi and Ruaha, most of the adults over 25 years of age have been removed by poaching, causing considerable disruption of the elephant's matriarchal social structure and reproductive function. Almost no calves between the ages of 2 and 9 could be found in Ruaha in 1989, suggesting that an entire generation of elephants may have been lost to mortality and/or stress-induced reproductive suppression (Barnes and Kapela, 1991). Poole (1989) and Balozi (1989) reported delayed puberty by up to 5 years in heavily poached herds. Yet, the postpubertal period of adolescent sterility appears to have decreased; females are giving birth to their first offspring at a younger age, largely because older females are no longer present to compete for reproductive opportunities (Barnes and Kapela, 1991). These younger females produce small young, at the potential expense of offspring survivorship. Maternal survivorship may also be compromised owing to long-term negative consequences for maternal bone growth in young reproductive females (Chan *et al.*, 1982, 1987; Israel and Newell-Morris, 1995; Morris *et al.*, 1995). These problems are compounded by the tendency for female mammals to rapidly cycle and conceive again after losing offspring during early lactation (Altmann *et al.*, 1978; Fedigan and Rose, 1995). In fact, many infants can be seen on the ground in elephant herds previously subjected to heavy poaching. This gives the false impression that the population is rebounding and, therefore, that the ivory ban should be lifted to allow culling as a means of population control.

Physiological documentation of the above reproductive impacts could help clarify some of the perceived, long-lasting consequences of elephant poaching. Such information would simultaneously illuminate some of the profound but obscure risks that could result from resumption of the ivory trade. Heavily poached groups can be readily discerned from less intensively poached groups by the former's truncated age distribution (few adult females over 25 years of age and an absence of juveniles; Barnes and Kapela, 1991). The objective of the present study was to develop a non-invasive method for evaluating physiological differences in long-term reproductive function between poached and unpoached African elephant herds. Urine from free-ranging elephants is too difficult, if not dangerous, to collect. We, therefore, validated a

technique to quantify estradiol and progesterone metabolites (estrogens and progestins, respectively) excreted in more easily collected feces.

METHODS

Radiolabel Infusion Study

A radiolabel infusion study was conducted to determine (1) the degree to which estradiol and progesterone metabolites were excreted in feces and urine, (2) the lag between the appearance of estradiol and progesterone in blood and the time they were excreted, and (3) whether estrogens and progestins were evenly distributed within a single fecal sample.

A single adult female African elephant (Nancy, 38 years of age) housed at the National Zoological Park was injected (iv into an ear vein) with [³H]estradiol (E2; 1000 μ Ci, \sim 89 Ci/mmol; New England Nuclear, Boston, MA) and [¹⁴C]progesterone (P4; 100 μ Ci \sim 56 Ci/mmol; New England Nuclear) along with 250 mg of cold P4 (Sigma-Aldrich, Milwaukee, WI) at 09:00 hr. The cold P4 was intended for possible future GC mass spectrometry analyses. Cold E2 was not administered because characterization of E2 metabolites in feces is less complicated; unlike P4, E2 is at the end of the metabolic pathway and therefore is rarely metabolized beyond estrone in free or conjugated forms (Möstl *et al.*, 1987; Wasser *et al.*, 1994; Shideler *et al.*, 1993; Ziegler *et al.*, 1996).

The female was isolated in an indoor enclosure for the first 24 hr postinfusion and all urine was collected in a pan placed into the drain of the holding pen. After this period, radioactivity in the urine was assumed to have returned to background levels (Czekala *et al.*, 1992) and she was rejoined with the herd during the day. All fecal material also was collected beginning immediately after injection and continuing through 3 days postinfusion. The female was observed continuously during daytime hours and feces were collected immediately upon defecation. She was housed alone at night and fecal material was collected each morning following her release into the outside exhibit area.

Immediately upon defecation, 5-g aliquots of feces were removed from the outer and inner portions ($n =$

4 each) of each fecal sample. The entire sample was then thoroughly mixed and an additional 8 5-g subsamples were collected. A 0.5-g aliquot was removed from each of the 16 subsamples, across the 33 full samples, and counted for radioactivity using the method described by Wasser *et al.* (1994). Intrasample variation on the outside versus inside and well-mixed portions of the sample were compared by analysis of variance, using percentage CVs calculated for each of the 4, 4, and 8 subsamples, respectively, across all fecal samples. The remainder of the well-mixed subsamples was then pooled and lyophilized for 10 randomly selected samples. Dried samples were gently pulverized and the fecal powder was separated out from the hay. Several 0.2-g subsamples of well-mixed fecal powder were counted for radioactivity, %CVs were calculated as above and compared to the respective %CV of the original 8 well-mixed wet subsamples, using a paired, one-tailed Student's *t* test.

Blood and Fecal Sample Collection Analysis

Five nonpregnant adult female African elephants (Ivory, age 11; Tombi, age 16; Cita, age 25; Kubwa, age 17; and Sophie, age 26), housed at the Indianapolis Zoo, participated in this study. The elephants were housed in a large dirt enclosure and brought indoors at night. A diet of elephant pellets, hay, browse, and fresh fruits and vegetables was fed to the animals daily. No male elephants were housed at the facility.

Weekly blood samples (~5 ml) were collected in a heparinized tube from an ear vein of the unanesthetized elephants for 28 consecutive weeks. Blood samples were centrifuged within 30 min after collection and the serum was stored frozen (-20°). Fecal samples were collected ~48 hr after each blood withdrawal. Upon defecation, ~300 g of well-mixed feces were placed in a plastic zip-lock bag and stored frozen (-20°). All fecal samples were lyophilized and the fecal powder separated from the hay, as described above, and stored frozen until analysis.

Serum progesterone and estradiol were quantified using the method described by Brown and Lenhardt (1995) employing solid-phase ¹²⁵I radioimmunoassay kits for P4 and E2, respectively (Coat-a-Count, Diagnostic Products Corp., Los Angeles, CA). The P4 antisera cross-reacts 100% with progesterone, 43.8% with 5 α -pregnane-3 β -ol-20-one, 31% with 5 β -pregnane-3 α -

ol-20-one, 23.3% with 5 β -pregnane-3 β -ol-20-one, 16% with 5 α -pregnane-3 α -ol-20-one, 3.5% with 5 β -pregnane-3 β , 20 α -diol, 2% with 20 α -dihydroprogesterone, 1.3% with 5 β -pregnane-3,20-dione, and <1% with 5 β -pregnane-3 α ,17 α -diol, 5 α -pregnane-3 α ,20 α -diol, 5 α -pregnane-3 β ,20 α -diol,17 α -hydroxyprogesterone, and 5 α -pregnane-3 α , 17,20 α -triol (Wasser *et al.*, 1994; kit literature). The E2 antisera crossreacts 100% with estradiol, 10% with estrone, 4.4% with *d*-equilenin, 1.8% with estrone- β -D-glucuronide, and <1% with all other steroids measured (kit literature). Feces were extracted using the method described by Wasser *et al.*, (1994) and measured for estrogens and progestins using methods described by Risler *et al.*, (1987) and Wasser *et al.* (1994), respectively. Briefly, 0.18–0.2 g lyophilized fecal powder was boiled (20 min) in 10 ml of 90% ethanol:distilled water. The supernatant was recovered following centrifugation (500g for 15 min). The pellet was resuspended in 5 ml 90% ethanol, vortexed (1 min), and recentrifuged. Both ethanol supernatants were combined, dried completely, and redissolved in 1 ml methanol. Samples were diluted (1:20 for estrogens; 1:200 for progestins) in assay buffer (0.01 M PO₄, 0.14 M NaCl, 0.5% BSA, 0.01% sodium azide) before RIA analysis. Duplicate extracted fecal samples also were subjected to ether:water (10 ml ether:1 ml water) extraction after drying to determine the percentage of conjugated steroids in the samples.

The 17 β -estradiol antibody used to analyze fecal estrogens cross-reacted 100% with 17 β -estradiol, 77% with 6-keto-estradiol, 2% with estrone and 17 α -hydroxyprogesterone, 0.8% with estriol and ethinlyestradiol, 0.6% with 5-andostene-3 β ,17 β -diol, <0.01% with cortisol and cholesterol, and 0.00% with testosterone (Risler *et al.*, 1987). The monoclonal P4 antibody used to analyze fecal progestins cross-reacted 100% with P4, 96% with 5 α -pregnane-3 β -ol-20-one, 36% with 5 α -pregnane-3 α -ol-20-one, 15% with 5 β -pregnane-3 β -ol-20-one, 15% with 17 β -hydroxyprogesterone, 13% with pregnenolone, 7% with 5 β -pregnane-3 α -ol-20-one, 5% with 5 β -pregnane-3 α -ol-17 α -diol, and < 1% with pregnanediol-3-glucuronide, androstenedione, testosterone, E2, estrone, estriol, 21-hydroxyprogesterone, 20 α -hydroxyprogesterone, and cortisol (Grieger *et al.*, 1990; Wasser *et al.*, 1994).

The slope of the curve showing percentage of binding of pooled, serially diluted fecal samples for both

estrogens and progestins, was not significantly different ($P > 0.05$) from that of its respective standard curve, demonstrating parallelism. Fifty percent binding occurred at a sample dilution of 1:20 for estrogens and 1:200 for progestins. Recovery of exogenous estradiol (5 to 240 pg) and progesterone (3.75 to 120 ng) added to extracted fecal samples resulted in a net recovery of 98% ($y = 1.0x + 5.87$; $r = 0.99$) and 96% ($y = 1.1x + 4.29$; $r = 0.99$), respectively.

RESULTS

Radiolabel Infusion Study

Peak radioactivity in the urine occurred within 4.5 hr postinfusion for both estrogens and progestins (Fig. 1). By contrast, peak metabolite excretion in feces occurred 48–50 hr postinfusion (Fig. 2). Fifty-five percent of the metabolized [^{14}C]progesterone recovered was excreted in feces compared to 45% in urine. By contrast, only 5% of the metabolized [^3H]estradiol recovered was excreted in feces compared to 95% in urine.

The subsampling variance from the same wet fecal sample was highest on the outside (mean %CV \pm SEM = 29 ± 6) compared to the inside (mean %CV \pm SEM = 20 ± 4) of the sample, although these differences were not significant. Mixing the wet sample did not further reduce the variation (mean %CV \pm SEM = 19 ± 4).

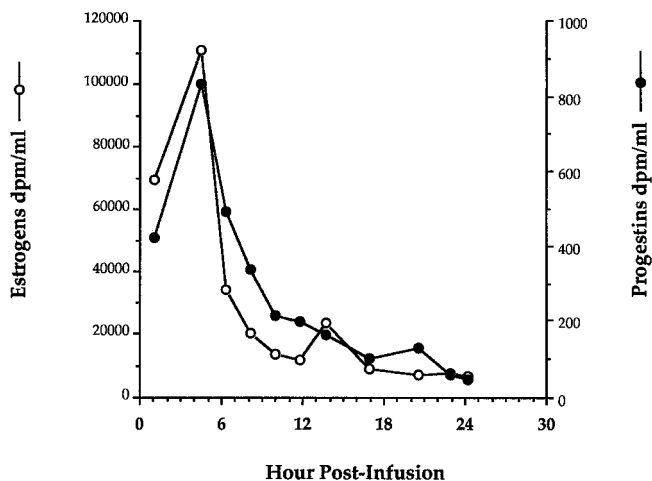


FIG. 1. Excretion time course of radiolabeled estrogens and progestins in urine of a female African elephant following iv infusion.

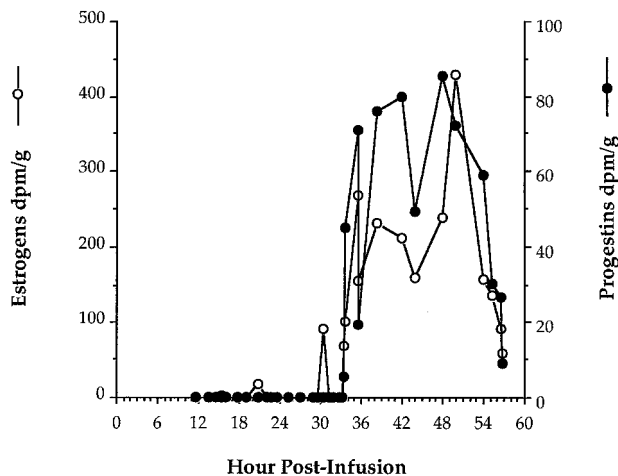


FIG. 2. Excretion time course of radiolabeled estrogens and progestins in feces of a female African elephant following iv infusion.

However, a significant reduction in subsample variance occurred when using well-mixed, dried fecal powder taken from premixed wet samples (mean %CV \pm SEM = 8 ± 1 ; $P < 0.05$).

Extraction recoveries of the metabolized [^3H]estradiol and [^{14}C]progesterone from feces were 75 and 86%, respectively. Ether:water extraction studies revealed that 80% of each steroid was excreted as free steroid and 20% as conjugates in feces.

Longitudinal Endocrine Assessments

Close correspondence was found between serum progesterone and fecal progestins (lagged by 2 days) for all cycling females (Figs. 3a–3d; mean correlation coefficient = 0.81, range 0.61–0.92, Table 1). One female (Sophie) was acyclic, with serum progesterone levels continuously below the detection limit of the assay (Fig. 3e). Stress may be a factor in Sophie's otherwise idiopathic infertility. She is the oldest female in the group (age 26 years), is extremely responsive to keeper commands, and ceased cycling within the

TABLE 1

Correlation Coefficients between Serum Progesterone and Fecal Progestins Collected 2 Days Later for Cycling Females

Animal	<i>r</i> value	<i>P</i> value
Ivory	0.92	0.0001
Kubwa	0.77	0.0001
Cita	0.94	0.0001
Tombi	0.61	0.0016

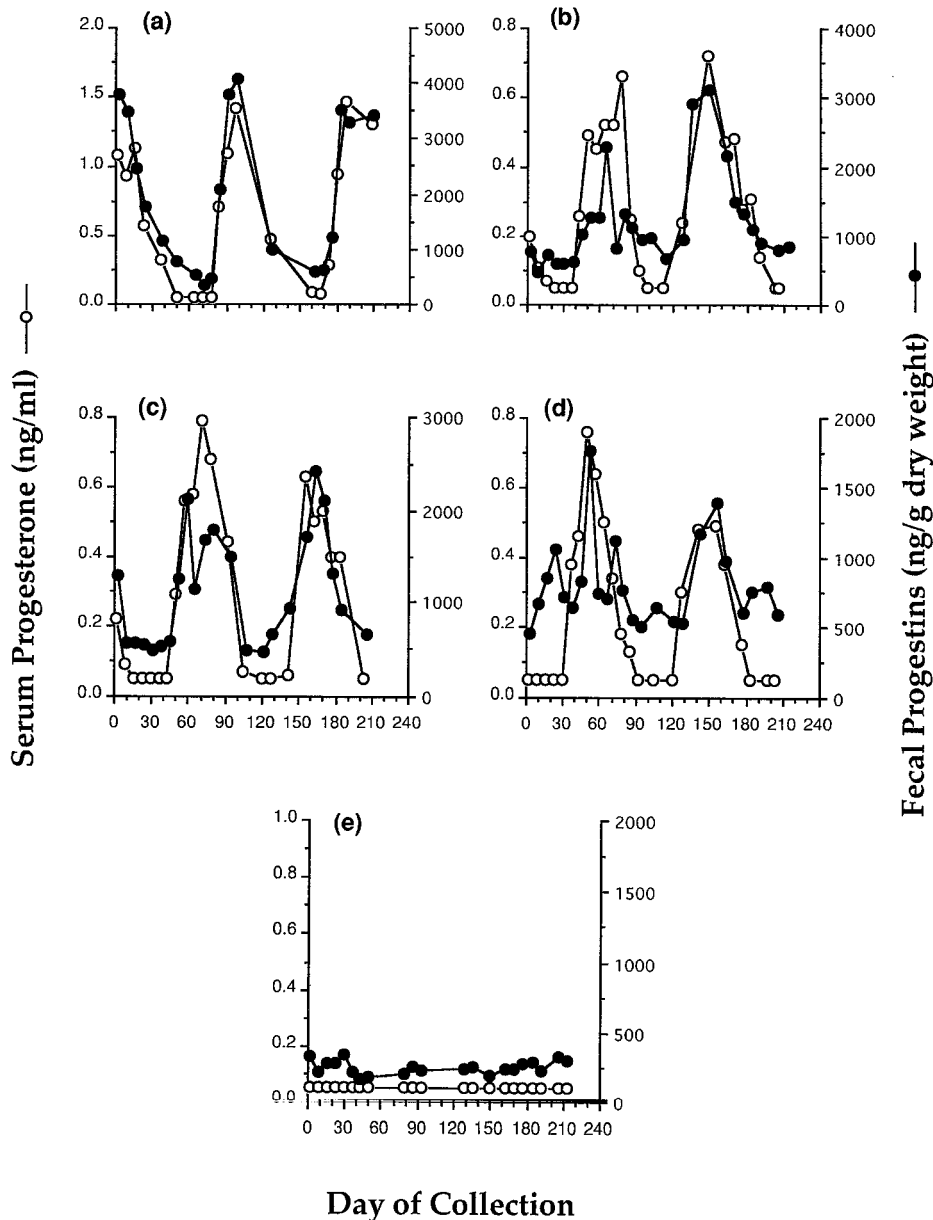


FIG. 3. Correspondence between serum progesterone and fecal progestins collected 2 days later from (a–d) four cycling and (e) one acyclic adult female African elephants.

same month that the youngest female in the group (Ivory) first began cycling. Rectal ultrasound of Sophie revealed normal but completely inactive ovaries and uterus (T. Hildebrandt, personal communication).

Serum estradiol levels (not shown) were low, often undetectable, and inconsistent across all females, as previously reported (Plotka *et al.*, 1988; Hess *et al.*, 1983; but see Taya *et al.*, 1991). By contrast, fecal estrogen concentrations were within the measurable

range, but tended to track fecal P4 for the cycling females (Figs. 4a–4d).

DISCUSSION

The results of the present study demonstrate that nearly all estrogens (95% of that recovered) are ex-

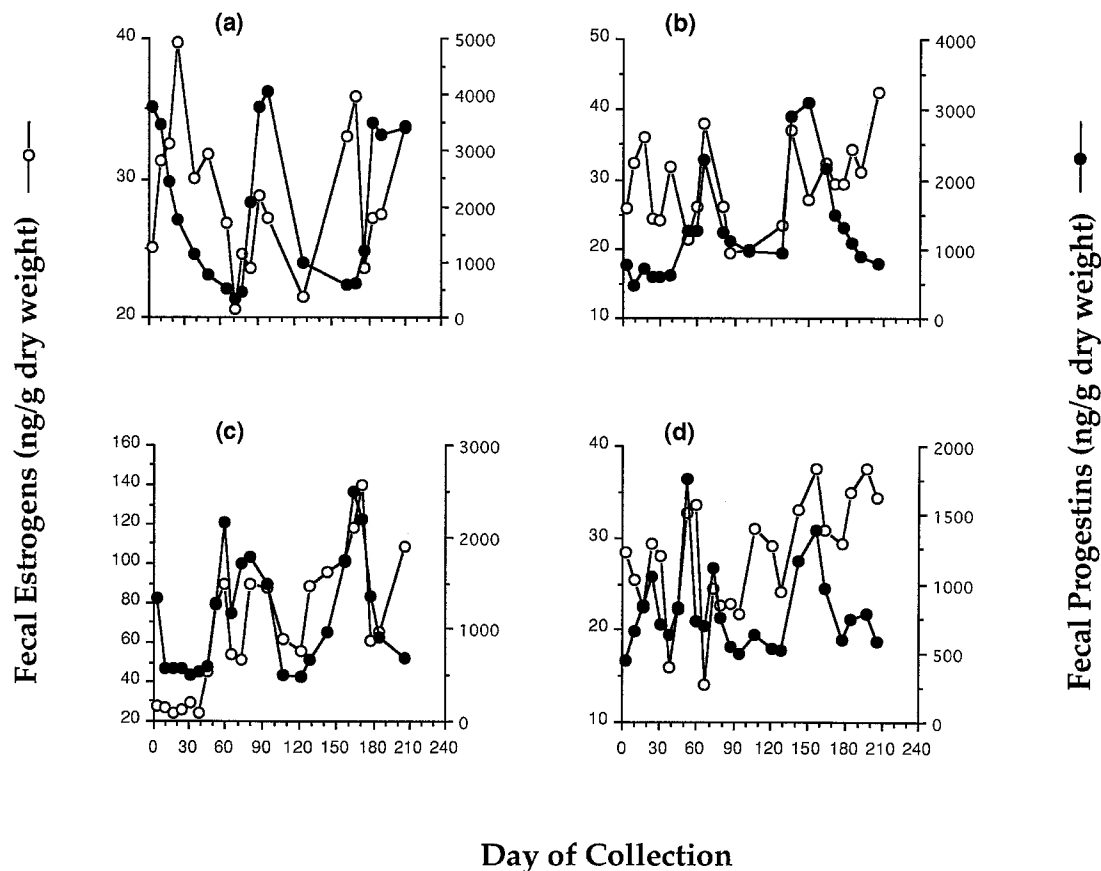


FIG. 4. Correspondence between fecal progesterins and estrogens in (a-d) four cycling adult female African elephants.

creted in urine, whereas the majority of progesterins (55% of that recovered) are excreted in feces in the African elephant. Urinary excretion is relatively rapid, peaking within 4.5 hr after secretion in blood. Czekala *et al.* (1992) reported a similar time lag of 2 hr for estradiol metabolite excretion in the urine of an Asian elephant. By contrast, we found fecal excretion to be much slower in onset and of longer duration, peaking at around 48 hr for both estrogens and progesterins. Extraction recovery studies of the radiolabeled estradiol and progesterone metabolites excreted in feces revealed high efficiency of our extraction method for these metabolites. At least 80% of both fecal estrogens and fecal progesterins were excreted in free versus conjugated forms. The predominant progesterone metabolites measured in elephant feces are probably epimers of 5α -reduced progesterins. Hodges *et al.* (1994) identified the predominant progesterins secreted by the corpus luteum to be 5α -pregnane- 3α -ol-20-one and 5α -pregnane- $3,20$ -dione. 5α -pregnane- 3β -ol-20-one

may also have been present but not recognized by the antibody used in the Hodges *et al.* study. The above, combined with the high cross-reactivity of our P4 antibody for the 5α pregnaneolones (Wasser *et al.*, 1994), suggests the most likely metabolites in elephant feces to be 5α -pregnane- 3α -ol-20-one and 5α -pregnane- 3β -ol-20-one. The pregnanediones are not expected to be present in feces because this keto form cannot be conjugated, greatly reducing its likelihood of entering the GI tract via the bile duct (H. Meyer, personal communication).

Steroids were unevenly distributed in elephant feces. However, a homogenous subsample can still be acquired by removing ~ 200 g of the central, or a well-mixed, portion of the entire wet fecal sample upon collection, lyophilizing that portion, separating the dried fecal powder from the highly fibrous bulk material, and then analyzing a small aliquot (~ 0.2 g) of the well-mixed powder.

The utility of the above sampling method was

clearly demonstrated by the close correspondence we found between serum progesterone and fecal progestins collected ~48 hr later, both of which demonstrated 16-week cycles. Some variability was observed in fecal progestins compared to serum P4 (see especially Fig. 3d), probably owing to uneven hormone distribution in the large fecal mass. However, the variation was not great enough to obscure cyclicity in any of these females. Initial estimates of Asian (*Elephas maximus*) and African (*L. africana*) elephant estrous cycle lengths based on behavioral observations (Eisenberg *et al.*, 1971), vaginal cytology (Watson and D'Souza, 1975), and urinary estrogen excretion (Ramsey *et al.*, 1981) suggested the cycle was approximately 3 weeks in duration. However, more recent evaluation of circulating progesterone concentrations demonstrated that elephant estrous cycles actually were 13–16 weeks in length (Brannian *et al.*, 1981; Hess *et al.*, 1983; Plotka *et al.*, 1988; Brown *et al.*, 1991), consistent with our own findings. Combined, these studies suggest that fecal progestin measures show considerable promise for monitoring ovarian activity. Luteal phase fecal progestin concentrations ≥ 1500 ng/g appear to be indicative of luteal activity. However, a better indication of luteal function is probably a $>2\text{-}\sigma$ rise in progestin concentrations above baseline; this better accommodates between female variability and between laboratory antibody differences in progestin antibody affinities to elephant fecal progestin metabolites.

Fecal estradiol did not appear to be very useful for evaluating follicular activity in the elephant, in contrast to fecal progestins. No estrogen surge could be detected in feces (or serum), although a more sustained luteal rise in fecal estrogens was detected that paralleled fecal progestins (Figs. 4a–4d). This latter pattern could have biological significance, as it does for primates (e.g., Wasser, 1995). Moreover, failure to detect this luteal rise in serum E2 could reflect a problem in the serum rather than fecal measures (cf. Brown *et al.*, 1991; Taya *et al.*, 1991). Clearly, more data will be required to justify any conclusions about the significance of elephant fecal estrogen patterns.

Management and Conservation Implications

Easily collected, reliable noninvasive measures of reproductive cyclicity have a number of applications for conservation and management of African elephants. There is growing evidence that African el-

phants continue to experience severe behavioral, demographic, and reproductive problems as a long-term consequence of the social disruption resulting from heavy poaching (Poole, 1989; Balozi, 1989; Barnes and Kapela, 1991). These effects are presumably the result of stress associated with the disrupted social structure that occurs following removal of the majority of herd matriarchs over 25 years of age. It is essential that these long-term impacts be well quantified and considered in any decision to reopen the ivory trade, particularly given the associated high likelihood of renewed poaching (Ricutti, 1993). The present study suggests that fecal steroid monitoring, used in conjunction with behavioral and demographic observations, provides an effective tool for obtaining this information. These fecal steroid measures may also prove to be extremely valuable in helping to evaluate the effectiveness of remotely delivered contraceptives being proposed for use as an alternative to culling African elephant populations (e.g., Poole, 1993).

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