

# Estrogen and LH Dynamics During the Follicular Phase of the Estrous Cycle in the Asian Elephant

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Pituitary and corpus luteum hormone patterns throughout the elephant estrous cycle have been well characterized. By contrast, analysis of follicular maturation by measurement of circulating estrogens has been uninformative. This study tested the ability of a urinary estradiol-3-glucuronide radioimmunoassay to noninvasively assess follicular development during the nonluteal phase of the elephant estrous cycle, and to determine the relationship between estrogen production and the “double LH surge.” Daily urine and serum samples were collected throughout seven estrous cycles from three Asian elephants, and urine was collected from an additional three females, for a total of 13 cycles. Serum was analyzed for luteinizing hormone (LH), and urine was analyzed for estrogens and progestins. Elephants exhibited a typical LH pattern, with an anovulatory LH (anLH) surge occurring approximately 21 days before the ovulatory LH (ovLH) surge. The urinary estrogen pattern indicated the presence of two follicular waves during the nonluteal phase. The first wave (anovulatory) began 5 days before the anLH surge and reached a maximum concentration the day before the peak. Thereafter, urinary estrogens declined to baseline for 2 weeks before increasing again to peak concentrations on the day of the ovLH surge. Urinary progestins were baseline throughout most of the follicular phase, increasing 2–3 days before the ovLH surge and continuing into the luteal phase. These results support

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previous ultrasound observations that two waves of follicular growth occur during the nonluteal phase of the elephant estrous cycle. Each wave is associated with an increase in estrogen production that stimulates an LH surge. Thus, in contrast to serum analyses, urinary estrogen monitoring appears to be a reliable method for characterizing follicular activity in the elephant. *Zoo Biol* 22:443–454, 2003. © 2003 Wiley-Liss, Inc.

**Key words:** elephant; urinary hormones; estrogen; luteinizing hormone; progestins; follicular development

## INTRODUCTION

Asian elephants in captivity are not self-sustaining, and their numbers are decreasing at such a rate that Wiese [2000] predicted the North American population will become extinct within 50 years. To manage this species better, a more complete understanding of its reproductive physiology is needed. Current findings suggest that the elephant exhibits some unusual, if not unique, reproductive characteristics. The female has the longest estrous cycle of any land mammal studied to date: 13–17 weeks in duration, with a 4–6-week follicular phase and an 8–10-week luteal phase (first reports, Asian [Hess et al., 1983] and African [Plotka et al., 1988]). It is well recognized that ovarian cyclicity is best characterized in elephants (as in other spontaneous ovulators) by monitoring corpus luteum activity. However, in contrast to most mammals, the major circulating luteal steroid in the elephant is not progesterone, but reduced pregnanes: 5 $\alpha$ -pregnane-3,20-dione (5 $\alpha$ DHP) and 5 $\alpha$ -pregnane-3-ol-20 one (5 $\alpha$ -P-3-OH) [Heistermann et al., 1997; Hodges et al., 1997; Schwarzenberger et al., 1997; Hodges, 1998]. Another unique endocrine feature of the elephant is the “double luteinizing hormone (LH) surge,” wherein two LH surges occur during the follicular phase of the cycle [Kapustin et al., 1996; Brown et al., 1999]. The first surge is anovulatory (anLH) and occurs 2–3 weeks after circulating progestins decline to baseline, whereas the second surge occurs ~3 weeks later and does induce ovulation (ovLH). In addition to serum progestins and LH, other hormones (FSH, prolactin, and inhibin) have been measured in the serum of Asian elephants [Brown et al., 1991, 1999], providing reference data for assessing both corpus luteum and pituitary function. In contrast, there has been no success in establishing a reliable endocrine monitor for evaluating follicular activity. The original speculation that presumed changes in estrogen status reflect successive 3-week follicular waves [Plotka et al., 1988] has not been confirmed by actual measurements of circulating estradiol. Rather, concentrations are low and typically exhibit no clear cyclic pattern [Hodges, 1998; Niemuller et al., 1999; Brown, 2000].

Because of inconclusive estrogen data, it was not known whether the extended nonluteal period in the elephant constituted a true “follicular phase,” or only a single wave of development occurred just before ovulation. This was recently clarified by an ultrasound study that identified two successive follicular waves, each about 3 weeks in duration, during the now-confirmed follicular phase of the cycle [Hermes et al., 2000]. The two waves are morphologically distinct, however, and only the second results in dominant follicle selection and ovulation. This study was conducted

to determine whether 1) measuring estrogens in urine samples collected daily throughout the follicular phase of the elephant estrous cycle would reflect these two waves of follicle development, and 2) estrogen excretory patterns differed between the ovLH and anLH surges.

## MATERIALS AND METHODS

### Animals

Information pertaining to the location, age, and number of cycles monitored for each elephant is given in Table 1. Daily urine and blood samples were collected from three Asian elephants, and daily urine was collected from three additional elephants, for a total of 13 estrous cycles. Blood was collected from the ear veins of unsedated animals, and urine was collected either free-catch or off the enclosure floor using a syringe immediately after the animal urinated. The blood was allowed to clot and the serum was separated by centrifugation. All samples were frozen after collection until hormonal analysis was performed.

### Urinary Creatinine

Urine was analyzed for creatinine (Cr) to account for individual fluid volume differences. Samples were diluted 1:100 with distilled water, and 100  $\mu$ l were analyzed in duplicate in 96-well flat bottom microtiter plates (Costar; Cambridge, MA). A 1:1 alkaline picrate reagent (100  $\mu$ l 0.04N picric acid:0.75N NaOH) was added to all samples and standards (Sigma, St. Louis, MO), and incubated for 15 min at room temperature. Absorption was measured at 490 nm in a microplate reader (E max; Molecular Dynamics, Sunnyvale, CA). Results were expressed as mass of steroid/mg Cr, and samples with values of <0.1 mg Cr/ml were discarded.

### LH

Serum LH was quantified by a  $^{125}$ I double-antibody radioimmunoassay (RIA) that utilized a monoclonal anti-bovine LH antiserum (518-B7), an ovine LH label (AFP8614B, NHPP-NIDDK), and ovine LH standards (NIH-LH-S25) in a phosphate-buffered saline (PBS)-based system (0.01 M  $\text{PO}_4$ , 0.9% NaCl, 2 mM EDTA, 0.01% thimerosal, pH 7.4) [Brown et al., 1999]. The assay was incubated at room temperature in a total volume of 500  $\mu$ l PBS plus 0.5% BSA (PBS-BSA).

**TABLE 1. Elephant demographics**

Name	Species	Location	Date of birth	Number of cycles	Collection dates
Taj <sup>a</sup>	Asian	Marine World Vallejo, Ca	1943	1	June 00–Sept 00
Tina <sup>a</sup>	Asian	Marine World Vallejo, Ca	1957	1	June 00–Sept 00
Shanthi <sup>a</sup>	Asian	National Zoo	1976	5	May 98–Jan 02
Mary <sup>b</sup>	Asian	San Diego Wild Animal Park	1964	3	July 01–June 02
Cha Cha <sup>b</sup>	Asian	San Diego Wild Animal Park	1966	2	Sept 01–June 02
Kitty <sup>b</sup>	Asian	Have Trunk Will Travel	1967	1	May 98–98

<sup>a</sup>Both urine and serum samples were collected.

<sup>b</sup>Only urine samples were collected.

Standards (100  $\mu$ l) and/or sample were incubated with PBS (200  $\mu$ l) and first antibody (1:750,000, 100  $\mu$ l) for 24 hr. Then  $^{125}$ I-LH (25,000 cpm, 100  $\mu$ l) was added, and the tubes were incubated for an additional 24 hr. Antibody-bound complexes were precipitated by centrifugation after a 1-hr incubation with goat anti-mouse gamma globulin (1:200, 1 ml in PBS containing 5% polyethylene glycol, Mr 800; Sigma Chemical Co., St. Louis, MO). The antibody typically bound 30% of the iodinated tracer with 8% nonspecific binding. Assay sensitivity was 0.3 ng/ml.

### Estradiol

A  $17\beta$ -estradiol RIA was modified by substituting estradiol-3-glucuronide (E2-3G) as standard in order to measure estradiol conjugates in elephant urine. Antibody crossreactivity using the modified assay was: 100% with E2-3G; 76% with estradiol- $17\beta$ ; 8.9% with estradiol- $17\alpha$ ; 7.2% with estrone; 2.8% with estrone-3-glucuronide; 1.7% with estradiol-17-glucuronide; 1.4% with estrone-3-sulfate; and <0.06% with estradiol-3-sulfate-17-glucuronide, testosterone, cortisol, and pregnenediol-3-glucuronide. For the assay, 20  $\mu$ l of unextracted urine were added to 500  $\mu$ l of 0.1M PBS containing 0.1% gel (PBS-gel) and vortexed for 10 sec. Then 100  $\mu$ l anti-estradiol (1:24,000; R2-P; ICN, Costa Mesa, CA), 100  $\mu$ l E2-3G standards (39–5,000 pg), and 100  $\mu$ l  $^3$ H-estradiol- $17\beta$  (10,000 cpm; NEN Life Science Products, Boston, MA) were incubated overnight at 4°C. Unbound steroid was removed by the addition of 250  $\mu$ l of Norit A charcoal/Dextran T-70 suspension. After 30 min at 4°C, the charcoal and unbound steroid were removed by centrifugation at 500 g for 15 min. The aqueous phase was decanted directly into scintillation vials, and 5 ml of liquid scintillation fluid (Ultima Gold; Packard BioScience, Meriden, CT) were added. The assay was validated for elephant urine by demonstrating parallelism between dilutions of urine and the standard curve ( $r=0.983$ ). The recovery of E2-3G added to urine was 110% and 103% (625 pg and 312 pg added, respectively).

### Progestins

Progestin concentrations were determined by RIA using a progesterone monoclonal antibody (Quidel Clone 425, San Diego, CA), 100  $\mu$ l  $^3$ H-progesterone (NEN Life Science Products, Boston, MA) and progesterone standards (7.8–1,000 pg) in a PBS-gel buffer system. Neat urine (10  $\mu$ l) or standards were incubated with 100  $\mu$ l antibody (1:32,000) and 10,000 cpm overnight at 4°C. Unbound steroid was removed by incubation with 250  $\mu$ l of Norit A charcoal/Dextran T-70 suspension for 30 min at 4°C and centrifugation at 500 g for 15 min. The aqueous phase was decanted directly into scintillation vials, and 5 ml of liquid scintillation fluid were added. The antibody crossreacts 100% with progesterone; 96% with  $5\alpha$ -pregnane- $3\beta$ -ol-20-one; 36% with  $5\alpha$ -pregnane- $3\alpha$ -ol-20-one; 15% with  $5\beta$ -pregnane- $3\beta$ -ol-one; 15% with  $17\beta$ -hydroxyprogesterone; 13% with pregnenolone; 7% with  $5\beta$ -pregnane- $3\alpha$ -ol-20-on; 5% with  $5\beta$ -pregnane- $3\alpha,17\alpha$ -diol,  $20\alpha$ -one; and <1% with pregnenediol-3-glucuronide, androstenedione, testosterone, estrone, estradiol, estriol, 21-hydroxyprogesterone,  $20\alpha$ -hydroxyprogesterone, and cortisol. The assay was validated for elephant urine by demonstrating parallelism between dilutions of urine and the standard curve ( $r=0.99$ ). The recovery of progesterone added to urine was 98% and 79% (250 pg and 125 pg added, respectively).

Coefficients of variation were <15% for interassay and <10% for intrassay variation for all assays.

### High-Pressure Liquid Chromatography (HPLC)

The number and relative proportions of estrogen and progesterin metabolites in elephant urine were determined by reverse-phase HPLC using an octadecylsilane column (RP C-18 column; Beckman, Allendale, NJ). Urine was analyzed neat for conjugated and free metabolites, and after enzyme hydrolysis to determine the proportion and type of steroid conjugates. For hydrolysis, 100  $\mu$ l of urine were combined with 20  $\mu$ l glucuronidase aryl sulfatase (Boehringer Mannheim Corp., Indianapolis, IN) in 400  $\mu$ l of acetate buffer (0.1 M, pH 5.0). Following incubation at 37°C overnight, the samples were extracted with 5 ml anhydrous diethyl ether. The supernatant was decanted, dried, and reconstituted in 20  $\mu$ l of methanol. Then 20  $\mu$ l of unextracted, undiluted urine (i.e., neat) or hydrolyzed methanol extract were applied to the HPLC with <sup>3</sup>H-estradiol or <sup>3</sup>H-progesterone (3,000 cpm; New England Nuclear, Boston, MA) as co-chromatographic markers. Samples were eluted with methanol:water (3:2, pH 3.0 for estrogens; 3:1, pH 5.35 for progestins), and 1-ml fractions were collected over 90 min (estrogens) or 60 min (progestins) (1 ml/min flow rate). All HPLC fractions were taken to dryness and reconstituted in PBS-gel, and metabolite immunoreactivity was quantified by RIA.

### STATISTICAL ANALYSIS

From three of the six elephants, samples were collected throughout several successive cycles, so those hormone profiles were combined for each individual and the average values were used for analysis. For females from which both urine and serum were collected ( $n=3$  females, 7 cycles), LH and estrogen data were aligned to the day of the ovLH peak (day 0). Temporal changes in urinary estrogens and serum LH were analyzed using factorial analysis of variance (ANOVA), and post hoc differences were identified using Scheffe's F-test. Mean baseline urinary estrogen concentrations were calculated from samples collected 30–40 days before the ovLH peak. For urine-only profiles ( $n=6$  females, 13 cycles), estrogen and progesterin data were aligned to the day of the second estrogen peak (day 0). All analyses were conducted using Statview 5.0.1 (SAS Institute, Inc.)

### RESULTS

Estrogen immunoreactivity in unextracted urine separated by HPLC was associated with four polar peaks that eluted at fractions 7, 12, 15, and 22. The first peak is unidentified, but may be an estradiol di conjugate, given its polar elution pattern. The second and third peaks are similar in size and correspond to the elution time of E2-3-glucuronide and tri-hydroxy E2. The fourth and largest peak is consistent with the elution time of E2-3-sulfate. After hydrolysis and HPLC, the estrogen conjugate immunoactivity was absent and the hydrolyzed estrogen eluted with the fraction containing estradiol-17 $\beta$  radioactivity. Thus, the polar immunoreactive peaks all appear to be some form of estradiol conjugate. For the progesterin analysis, HPLC separation of unhydrolyzed urine identified one minor peak that eluted 11 fractions before the progesterone tracer, and two major immunoreactive peaks that eluted 8 fractions before and 2 fractions after the progesterone tracer. These metabolites were not further characterized.

The profile of urinary estrogens in relation to serum LH and urinary progestins in three cycling female Asian elephants is shown in Figure 1. Two LH surges occurred  $\sim 21$  days apart, and concentrations were markedly higher for 1 day only. The mean anLH surge concentration was about two-thirds the magnitude of the ovLH surge, but concentrations were not significantly different ( $62.9 \pm 18.5$  ng/ml;  $92.7 \pm 22.9$  ng/ml, respectively) ( $P > 0.05$ ). Estrogen concentrations averaged  $15.6 \pm 5.8$  ng/mg Cr at baseline (30–40 days before the ovLH peak), and increased significantly beginning about 5 days before each LH surge ( $P < 0.05$ ). Mean peak estrogen concentrations were observed 1 day before the anLH surge ( $133.5 \pm 23.1$  ng/mg Cr) and one day before the ovLH surge ( $83.0 \pm 16.7$  ng/mg Cr). Peak estrogen values were not significantly different ( $P > 0.05$ ). Urinary progestins were low throughout the follicular phase until about 2–3 days before the ovLH surge, at which time the concentrations increased and then remained elevated throughout the luteal phase.

Urinary estrogen and progestin profiles, including those of the three additional elephants from which only urine samples were collected ( $n=6$  females), are shown in Figure 2. Again, urinary estrogen concentrations did not differ between the first ( $102.2 \pm 12.7$  ng/mg Cr) and second ( $111.6 \pm 19.4$  ng/mg Cr) peaks, which were associated with the anLH and ovLH surges ( $P > 0.05$ ). With all six individuals included, there was more variation in the days between the two estrogen peaks (16–22 days; mean =  $19.5 \pm 0.9$  days). Because the data were aligned to the second (preovulatory) estrogen peak, the first surge appears broader than that observed in individual females. In cycles in which urine was collected after ovulation ( $n=12$ ), an increase in urinary estrogens also was observed during the luteal phase ( $n=10$ ) (Fig. 3).

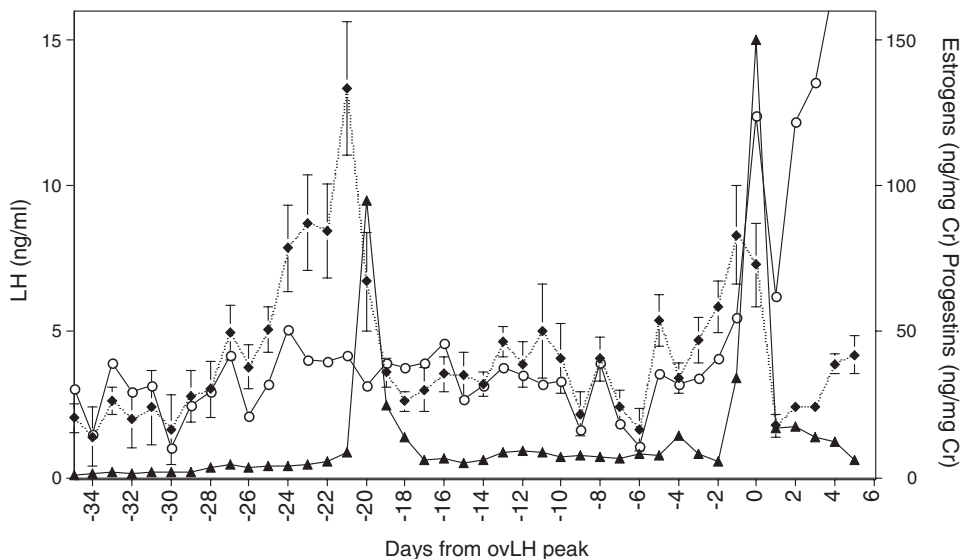


Fig. 1. Urinary estrogens (solid diamonds: mean  $\pm$  SEM) and progestins (open circles: mean) aligned to the ovulatory serum LH peak (solid triangles: mean).

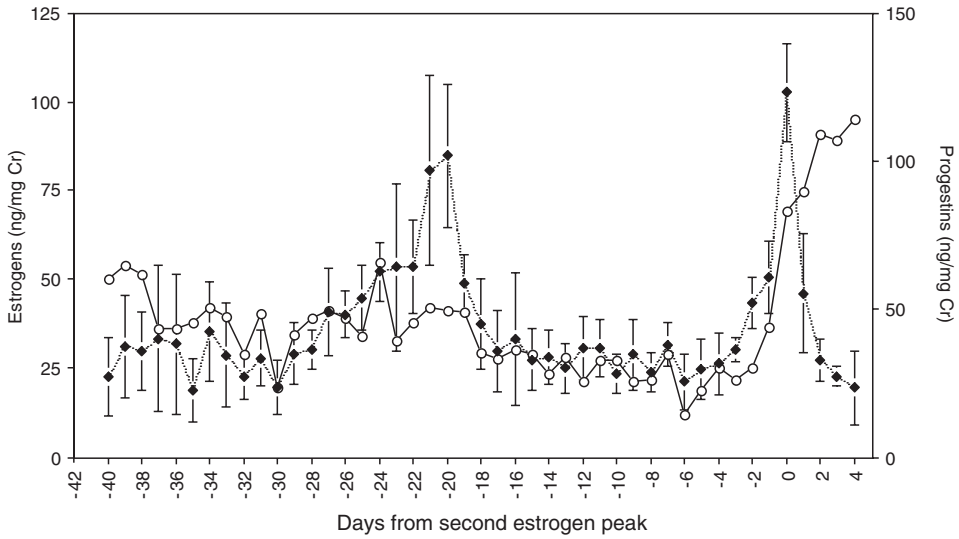


Fig. 2. Urinary estrogens (solid diamonds: mean  $\pm$  SEM) and progestins (open circle: mean) aligned to the second estrogen peak (n=6).

**DISCUSSION**

This study demonstrates for the first time the existence of two waves of estrogen excretion during the follicular phase of the elephant estrous cycle, each of which stimulates an LH surge. The findings are consistent with ovarian ultrasound data obtained in a previous study of African elephants [Hermes et al., 2000]. In that study it was observed that two follicular waves occurred with a 3-week periodicity in

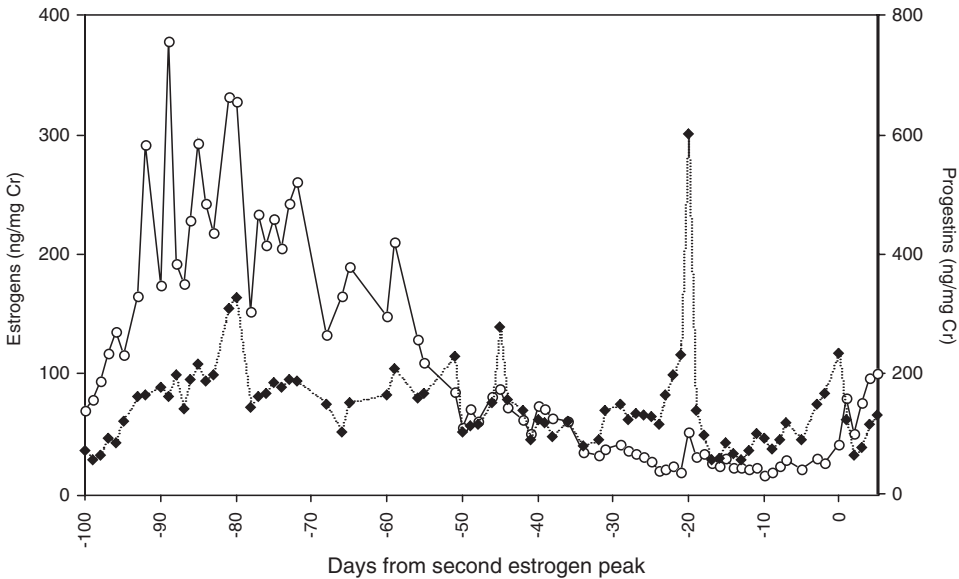


Fig. 3. Urinary estrogens (solid diamonds) and progestins (open circles) from one elephant (Mary, San Diego Wild Animal Park) evaluated over a 110-day period.



conjunction with the double LH surge, and that the two follicular waves were morphologically distinct. The first wave was characterized by multiple (two to four) follicles ranging from 10 to 19 mm in diameter that developed within the parenchyma of both ovaries. None of these follicles became dominant. After the anLH surge, these structures appeared to collapse and slowly luteinize, although they remained steroidogenically inactive. During the second wave, only one antral follicle developed, beginning 5–7 days before the ovLH surge. It grew to ~20 mm in diameter and changed from a round to an oval shape just before ovulation ~24 hr after the LH peak. Thus, the developmental pattern of these two follicular waves by ultrasound imaging is consistent with the temporal urinary estrogen profile observed in the present study.

It is notable that such a clear pattern emerges using a urinary estrogen conjugate assay when earlier attempts to assess estrogen dynamics using serum analyses failed to show any relationship with elephant reproductive status [for reviews see Hodges, 1998; Brown, 2000]. One possible reason for the inconclusive serum results is that the free steroid is quickly metabolized to estradiol and estrone conjugates in the bloodstream [Czekala et al., 1992]. Thus, techniques to measure circulating estradiol would have excluded the identification of a conjugated form due to the extraction process. We also found that excreted estrogens were conjugated, (primarily estradiol conjugates) and thus a broad-spectrum antibody was required to detect the metabolized forms. In addition, in order for changes to be detectable, the two estrogen waves must be sampled daily, and few studies have collected samples with this frequency. The only clear evidence of an elevation in serum estradiol before the ovulatory increase in luteal progesterone was reported by Taya et al. [1991]. However, in that study peak concentrations were low (<7 pg/ml), and the first surge was missed because the samples were collected weekly. In two intensive studies of the double LH surge, Kapustin et al. [1996] and Brown et al. [1999] found that on average estradiol concentrations tended to be higher the day before each LH surge. However, in both of those studies the estradiol concentrations varied considerably and the profiles were not consistent across individuals.

The lack of a reliable serum indicator of follicular activity led to the investigation of urinary estrogen analysis as a possible alternative, the biological relevance of which was based on radiolabel infusion studies that showed circulating estrogen conjugates are excreted primarily (95%) in the urine of Asian [Czekala et al., 1992] and African [Wasser et al., 1996] elephants. However, urinary estrogen data have also been inconsistent, likely because of sampling frequency limitations. For example, Fieß et al. [1999] evaluated total estrone and estradiol immunoactivity in hydrolyzed urine samples collected weekly and found no cyclic pattern or pronounced increase associated with mating. Analysis of biweekly urinary total estrogens over five cycles in an Asian elephant revealed that concentrations were elevated more during the luteal phase, indicating a 15–16-week cyclic pattern [Mainka and Lothrop, 1990]. In the present study, an increase in urinary estrogen conjugates also was observed during the luteal phase, suggesting the corpus luteum may be a source of estrogens in the Asian elephant, as it is in primates [Pryce et al., 1995]. However, because of the daily sampling interval, our study also identified the waves of estrogen production associated with follicular development during the nonluteal phase. Interestingly, an early report by Ramsay et al. [1981] concluded that the Asian elephant estrous cycle was 3 weeks in duration (18–26 days) based on a



short-term (8.5 weeks) study in which daily urine samples were hydrolyzed and analyzed for estrone and estradiol concentrations. Although their interpretation of the entire cycle length was incorrect, and they lacked progesterone data by which to align the estrogen surges with ovulation, the 3-week pattern they observed is consistent with our findings.

One objective of this study was to determine whether the estrogen milieu differed between the anLH and ovLH surges, and whether it reflected the differences observed in developmental patterns between the two follicular waves, or could explain why one LH surge is ovulatory and the other is not. In monovular species, such as the elephant, dominant follicle selection occurs as a multistep process that begins after the corpus luteum block is eliminated [Fortune et al., 2001; Ginther et al., 2001]. Increased FSH concentrations stimulate follicular development, including activating granulosa cell aromatase and estrogen production. Elevated peripheral estrogens and inhibin [Brown et al., 1999] then suppress FSH so that circulating concentrations fall below that needed to stimulate maturation of less developed follicles. Changes in components of the intrafollicular insulin-like growth factor (IGF) system also permit the future dominant follicle to secrete more estradiol and elicit an ovulatory LH surge [Fortune et al., 2001]. For unknown reasons, none of the follicles that develop during the first wave in the elephant achieve this maturation capability. It is possible that the extended FSH secretory pattern observed during the early follicular phase of the cycle results in concentrations that are too high to facilitate dominant follicle selection [Brown, 2000]. Only later, when FSH concentrations decline toward baseline, does a second wave result in ovulation. The follicular estrogens likely facilitate the FSH decline, although there were no clear quantitative or qualitative differences in the temporal estrogen pattern that would explain why one wave is ovulatory and the other is not. Estrogens are known to stimulate prolactin secretion, and in the African elephant prolactin concentrations are elevated during the follicular phase [Bechert et al., 1999]. However, a similar follicular increase in prolactin secretion has not been identified in the Asian elephant [Carden et al., 1998; Brown et al., 1999], and even in the African elephant there is no indication that concentrations are notably different between the early (i.e., associated with the anLH surge) and late (ovLH surge) follicular phase. Besides FSH, the only other notable difference between the two waves is the rise in progestins that precedes the ovLH surge, but not the anLH surge. These progestins may be of follicular origin because they increase before ovulation, which occurs about 24 hr after the LH surge [Hermes et al., 2000; Brown et al., 2002]. In an ultrasound study of the African elephant [Hermes et al., 2000], it was reported that multiple follicles from the first wave appear to collapse and then slowly luteinize. At 1–5 days before the ovLH surge, accessory corpora lutea become visible and may be the remnant, luteinized structures from the nonovulatory wave [Hermes et al., 2000]. Thus, these structures might also be a source of elevated circulating progesterones observed 2–3 days before the ovLH surge.

What remains to be determined is whether the follicular dynamics associated with two developmental waves in the Asian elephant are similar to those described ultrasonographically for the African elephant [Hermes et al., 2000]. Further studies are also needed to determine whether urinary estrogen profiles are similar between Asian and African elephants, especially given the observed species differences in LH secretion (surge concentrations are generally higher in Asian elephants) [Brown

et al., 1999]. Understanding the function of the first follicular wave and anLH surge continues to be of considerable interest. It may be part of a sophisticated mating strategy to ensure successful reproduction. Elephants have the longest interbirth interval of any land mammal, with the time between two fertile cycles ranging from 3 to 7 years. Estrus is a rare event, so females would benefit by evolving a mechanism to advertise impending fertility and attract bulls. Elephants have been observed in “false behavioral estrus” several weeks before conceptive mating occurs. It has also been shown that female Asian elephants excrete a urinary pheromone, (*Z*)-7-dodecenyl acetate (*Z*7-12:Ac), that stimulates male breeding behavior [Dehnhardt et al., 2001; Rasmussen, 2001]. After luteal progestins decline to baseline, *Z*7-12:Ac becomes detectable in the urine, increasing in a linear fashion throughout the follicular phase. This pattern suggests that follicles from both waves are capable of mediating this pheromonal signal. However, *Z*7-12:Ac concentrations are highest just before ovulation; thus, production is greatest during the second wave, in conjunction with dominant follicle selection. Considering the long distances bulls travel in search of estrous females in the wild, this strategy would be highly conducive to survival of the species.

In conclusion, it is somewhat ironic that if they are left undisturbed, Asian and African elephants reproduce well in the wild, but essentially all populations living ex situ (i.e., in zoos, circuses, working or tourist camps, and breeding centers) face possible “extinction” because of historically poor reproductive performance. Although some problems are logistical (i.e., limited breeding opportunities), others appear to have a physiological basis (e.g., ovarian acyclicity, and reproductive tract pathologies). Through advances in endocrine monitoring and ultrasonographic imaging, we are beginning to understand these phenomena, as well as the complex mechanisms that control normative reproductive function in the elephant. Several unique reproductive features have been discovered, including those related to luteal steroidogenic function, follicular development, and pituitary gonadotropin secretion. These findings are of interest from a scholarly perspective, but they are also useful for developing assisted-reproduction techniques, such as artificial insemination [Hildebrandt et al., 1999; Brown et al., 2002]. The use of the present noninvasive method for tracking follicular activity, in combination with other endocrine and ultrasonographic evaluations, could benefit the ex situ conservation of elephants by elucidating how pathologies impact ovarian cyclicity, and assessing the efficacy of mitigating treatments. Given the prediction that elephants in captivity face extinction within only a few decades, it is even more imperative to use all available tools to further study their reproductive biology and develop effective management strategies.

## CONCLUSIONS

1. Estrogens are excreted in waves during the follicular phase of the Asian elephant estrous cycle.
2. The two waves precede each of the LH surges that occur during the follicular phase of the cycle, and are consistent with ultrasound data obtained in studies of the African elephant.

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## REFERENCES

- Bechert US, Swanson L, Wasser SK, Hess DL, Stormshak F. 1999. Serum prolactin concentrations in the captive female African elephant (*Loxodonta africana*): potential effects of season and steroid hormone interactions. *Gen Comp Endocrinol* 114:269–78.
- Brown JL. 2000. Reproductive endocrine monitoring of elephants: an essential tool for assisting captive management. *Zoo Biol* 19:347–68.
- Brown JL, Citino SB, Bush M, Lehnhardt J, Phillips LG. 1991. Cyclic patterns of luteinizing hormone, follicle-stimulating hormone, inhibin, and progesterone in the Asian elephant (*Elephas maximus*). *J Zoo Wildl Med* 22:49–57.
- Brown JL, Schmitt DL, Bellem A, Graham LH, Lehnhardt J. 1999. Hormone secretion in the Asian elephant (*Elephas maximus*): characterization of ovulatory and anovulatory LH surges. *Biol Reprod* 61:1294–9.
- Brown JL, Goeritz F, Hermes R, Pratt-Hawkes N, Galloway M, Hildebrandt TB. 2002. Artificial insemination in the Asian elephant. *Biol Reprod (Suppl)* 66:259.
- Carden M, Schmitt D, Tomasi T, Bradford J, Moll D, Brown JL. 1998. Utility of serum progesterone and prolactin analysis for assessing reproductive status in the Asian elephant (*Elephas maximus*). *Anim Reprod Sci* 53:133–42.
- Czekala NM, Roocroft A, Bates M, Allen J, Lasley BL. 1992. Estrogen metabolism in the Asian elephant (*Elephas maximus*). *Zoo Biol* 11:75–80.
- Dehnhardt M, Heistermann M, Göritz F, Hermes R, Hildebrandt TB, Haber H. 2001. Demonstration of 2-unsaturated C19-steroids in the urine of female Asian elephants, *Elephas maximus*, and their dependence on ovarian activity. *Reproduction* 121:475–84.
- Fieß M, Heistermann M, Hodges JK. 1999. Patterns of urinary and faecal steroid excretion during the ovarian cycle and pregnancy in the African elephant (*Loxodonta africana*). *Gen Comp Endocrinol* 115:76–89.
- Fortune JE, Rivera GM, Evans ACO, Turzillo AM. 2001. Differentiation of dominant versus subordinate follicles in cattle. *Biol Reprod* 65:648–54.
- Ginther OJ, Beg MA, Bergfelt DR, Donadeu FX, Kok K. 2001. Follicle selection in monovular species. *Biol Reprod* 65:638–47.
- Heistermann M, Trohorsch B, Hodges JK. 1997. Assessment of ovarian function in the African elephant (*Loxodonta africana*) by measurement of 5 $\alpha$ -reduced progesterone metabolites in serum and urine. *Zoo Biol* 16:273–84.
- Hermes R, Olson D, Göritz F, Brown JL, Schmitt DL, Hagan D, Peterson JS, Fritsch G, Hildebrandt TB. 2000. Ultrasonography of the sexual cycle in female African elephants (*Loxodonta africana*). *Zoo Biol* 19:369–82.
- Hess DL, Schmidt AM, Schmidt MJ. 1983. Reproductive cycle of the Asian elephant (*Elephas maximus*) in captivity. *Biol Reprod* 28:767–73.
- Hildebrandt TB, Göritz F, Hermes R, Schmitt DL, Brown JL, Schwammer H, Loskutoff N, Pratt NC, Lehnhardt JL, Montali RJ, Olson D. 1999. Artificial insemination of African (*Loxodonta africana*) and Asian (*Elephas maximus*) elephants. In: Proceedings of the American Association of Zoo Veterinarians Annual Conference. p 83–6.
- Hodges JK. 1998. Endocrinology of the ovarian cycle and pregnancy in the Asian (*Elephas maximus*) and African (*Loxodonta africana*) elephant. *Anim Reprod Sci* 53:3–18.
- Hodges JK, Heistermann M, Beard A, van Aarde RJ. 1997. Concentrations of progesterone and the 5 $\alpha$ -reduced progestins, 5 $\alpha$ -pregnane-3,20-dione and 3 $\alpha$ -hydroxy-5 $\alpha$ -pregnan-20-one, in luteal tissue and circulating blood and their relationship to luteal function in the African elephant, *Loxodonta africana*. *Biol Reprod* 56:640–6.
- Kapustin N, Critser JK, Olson D, Malven PV. 1996. Nonluteal estrous cycles of 3-week duration are initiated by anovulatory luteinizing hormone peaks in African elephants. *Biol Reprod* 55:1147–54.
- Mainka SA, Lothrop CD. 1990. Reproductive and hormonal changes during the estrous cycle and pregnancy in Asian elephants (*Elephas maximus*). *Zoo Biol* 9:411–9.

- Niemuller C, Brown JL, Hodges JK. 1999. Reproduction in elephants. In: Knobil E, Neill J, editors. Encyclopedia of reproduction. Vol. I. New York: Academic Press. p 1018–29.
- Plotka ED, Seal US, Zarembka FR, Simmons LG, Teare A, Phillips LG, Hinshaw KC, Wood DG. 1988. Ovarian function in the elephant: luteinizing hormone and progesterone cycles in African and Asian elephants. *Biol Reprod* 38:309–14.
- Pryce CR, Schwarzenberger F, Dobeli M, Etter K. 1995. Comparative study of oestrogen excretion in female New World monkeys: an overview of non-invasive ovarian monitoring and a new application in evolutionary biology. *Folia Primatol* 64:107–23.
- Ramsay EC, Lasley BL, Stabenfeldt GH. 1981. Monitoring the estrous cycle of the Asian elephant (*Elephas maximus*), using urinary estrogens. *Am J Vet Res* 42:256–60.
- Rasmussen LEL. 2001. Source and cyclic release pattern of (*Z*)-7-Dodecanyl acetate, the pre-ovulatory pheromone of the female Asian elephant. *Chem Senses* 26:611–23.
- Schwarzenberger F, Strauss G, Hoppen H-O, Schaftenaar W, Dieleman SJ, Zenker W, Pagan O. 1997. Evaluation of progesterone and 20-oxo-progestagens in the plasma of Asian (*Elephas maximus*) and African (*Loxodonta africana*) elephants. *Zoo Biol* 16:403–13.
- Taya K, Komura H, Kondoh M, Otagawa Y, Nakada K, Watanabe G, Sasamoto S, Tanabe K, Saito K, Tajima H, Narushima E. 1991. Concentrations of progesterone, testosterone and estradiol-17 $\beta$  in the serum during the estrous cycle of Asian elephants (*Elephas maximus*). *Zoo Biol* 10:299–307.
- Wasser SL, Papageorge S, Foley C, Brown JL. 1996. Excretory fate of estradiol and progesterone in the African elephant (*Loxodonta africana*) and pattern of fecal steroid concentrations throughout the estrous cycle. *Gen Comp Endocrinol* 102:255–62.
- Wiese RJ. 2000. Asian elephants are not self-sustaining in North America. *Zoo Biol* 19:299–310.