Successful Artificial Insemination of an Asian Elephant at the National Zoological Park

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For decades, attempts to breed elephants using artificial insemination (AI) have failed despite considerable efforts and the use of various approaches. However, recent advances in equipment technology and endocrine-monitoring techniques have resulted in 12 elephants conceiving by AI within a 4-year period (1998–2002). The successful AI technique employs a unique endoscope-guided catheter and transrectal ultrasound to deliver semen into the anterior vagina or cervix, and uses the ''double LH surge'' (i.e., identifying the anovulatory LH (anLH) surge that predictably occurs 3 weeks before the ovulatory LH (ovLH) surge to time insemination. This study describes the 6-year collaboration between the National Zoological Park (NZP) and the Institute for Zoo Biology and Wildlife Research (IZW), Berlin, Germany, that led to the refinement of this AI technique and subsequent production of an Asian elephant calf. The NZP female was the first elephant to be inseminated using the new AI approach, and was the fifth to conceive. A total of six AI trials were conducted beginning in 1995, and

conception occurred in 2000. Semen was collected by manual rectal stimulation from several bulls in North America. Sperm quality among the bulls was variable and was thus a limiting factor for AI. For the successful AI, semen quality was good to excellent (75–90% motile sperm), and sperm was deposited into the anterior vagina on the day before and the day of the ovLH surge. Based on transrectal ultrasound, ovulation occurred the day after the ovLH surge. Pregnancy was monitored by serum and urinary progestagen, and serum prolactin analyses in samples collected weekly. Fetal development was assessed at 12, 20, and 28 weeks of gestation using transrectal ultrasound. Elevated testosterone measured in the maternal circulation after 36 weeks of gestation reliably predicted the calf was a male. Parturition was induced by administration of 40 IU oxytocin 3 days after serum progestagens dropped to undetectable baseline levels. We conclude that AI has potential as a supplement to natural breeding, and will be invaluable for improving the genetic management of elephants, provided that problems associated with inadequate numbers of trained personnel and semen donors are resolved. Zoo Biol 23:45–63, 2004.   © 2004 Wiley-Liss, Inc.

INTRODUCTION

The populations of Asian elephants (*Elephas maximus*) are in decline throughout the subcontinent. The International Union for the Conservation of Nature (IUCN) estimates that there now are only 38,000–51,000 Asian elephants remaining, and most populations are highly fragmented due to human encroachment and conversion of land to agriculture [Lair, 1997]. Domesticated elephants, which currently number as many as 16,000, were once an integral part of Asian culture but they now struggle to survive in a changing economy [Lair, 1997]. Nearly all ex situ Asian elephant populations, including domesticated elephants, are threatened and not self-sustaining because reproductive rates are very low [Lair, 1997; Wiese, 2000]. The preservation of elephants both in situ and ex situ will require better means of protecting habitats, improving captive breeding, and maintaining adequate genetic diversity.

Asian elephants in North America are particularly vulnerable because more than half of the females are over 30 years of age and are considered post-reproductive. Without continued importation or an increase in fecundity several times the historical rate, this population is predicted to become demographically extinct within 50 years [Wiese, 2000]. The challenges facing elephant managers are significant. While reproduction through natural mating is often desired, it is not always possible. In North America, there are few mature bulls available for breeding, and transporting animals between facilities is expensive, stressful, and carries the risk of disease transmission. Even after the elephants are introduced to each other, they may exhibit a lack of sexual interest [Schmidt, 1993]. Older females often develop uterine fibroids, ovarian cysts, and unexplained ovarian quiescence that may prevent conception [Brown, 1999, 2000; Hildebrandt et al., 2000a]. Furthermore, it appears that many adult bulls are experiencing fertility problems associated with poor sperm quality and lack of libido [Hildebrandt et al., 2000b] (D. Schmitt and T. Hildebrandt, unpublished results). Alternatively, the use of techniques such as artificial insemination (AI) would enable females that do not have ready access to a male

to reproduce, and offer managers more flexibility in making genetic management decisions.

Over the past two decades, numerous attempts to use AI in elephants have failed. The main problems were related to improper placement of semen, and timing of insemination relative to ovulation. In the early 1990s, a unique insemination method was developed that relied on endoscopy and transrectal ultrasound to guide a semen catheter through the distal vagina and into the cervix of the elephant [Hildebrandt and Schnorrenberg, 1996; Hildebrandt et al., 1999]. The subsequent discovery of the "double LH surge" in the mid 1990s permitted researchers to time the insemination to coincide with ovulation by identifying the anovulatory LH (anLH) surge, which predictably occurs 3 weeks before the ovulatory LH (ovLH) surge [Kapustin et al., 1996; Brown et al., 1999]. This work describes the 6-year collaboration between the National Zoological Park (NZP) and the Institute for Zoo Biology and Wildlife Research (IZW) that led to the refinement of these techniques and the eventual successful AI of an Asian elephant at the NZP.

MATERIALS AND METHODS

Animal History and Husbandry

This project was approved by the NZP IACUC. The dam was an Asian elephant (Shanthi, SB165) that was wild-born in Sri Lanka in 1976 and orphaned at only a few months of age. She was hand-reared at the Pinnawala Elephant Orphanage and sent to the NZP in 1977, where she was housed with two other Asian elephant females and an African female (the latter died in June 2000). The elephants were housed together in an indoor/outdoor enclosure with free access to the outside yard at all times, except when temperatures dropped below 5°C. There are no bulls at the NZP. The animals were fed a pelleted vitamin-mineral-protein supplement three times daily, and grass hay and water were provided ad libitum. All of the elephants were managed in a free-contact system (i.e., direct interaction between keepers and elephants in the enclosure) and were accustomed to routine blood sampling. Blood was collected from an ear vein without sedation, allowed to clot for at least 1 hr, and centrifuged for recovery of serum. Serum was stored at –20°C until hormonal analyses were performed. Blood samples have been collected weekly since 1988 for progestagen analysis to monitor estrous cyclicity. Daily progestagen and LH monitoring during the follicular phase began in January 1995 and, with the exception of two cycles in 1997, was conducted for every cycle through conception in 2000. The female was considered a good candidate for AI because she was very tractable and was considered to be fertile, having given birth to a healthy calf in 1993. That birth had been traumatic, however, and she had a retained placenta that required oxytocin and estradiol cypionate treatment over a period of several weeks [Murray et al., 1996]. Once all remnants of placenta were removed, she recovered quickly and clinical problems were resolved.

The sire, Calvin (SB147), was captive-born at the Calgary Zoo in 1986. His sire and dam also were orphans at Pinnawala. Calvin was obtained in 1989 by the African Lion Safari (Cambridge, Canada), where he resided with two male and eight female Asian elephants. He sired his first calf in 1998, as well as five more before the successful birth of the present AI calf (Kandula). Calvin was managed in free-contact

and trained for semen collection. He was moved to the Hanover Zoo, Germany, in March 2000, and has successfully bred there.

Male Reproductive Evaluation and Semen Collection

Semen for the first four AI attempts (October 1995, April 1996, November 1996, and March 1997) was collected from an Asian elephant bull at the Dickerson Park Zoo, Springfield, MO (Onyx, SB104; DOB 1962). For the fifth AI (April 1999), Onyx and two bulls at the African Lion Safari (Calvin and Rex, SB263; DOB 1968) were utilized. For the last AI (February 2000), semen was collected from Calvin and bulls at the Fort Worth Zoo, Fort Worth, Texas (Groucho, SB203; DOB 1971), and the Ringling Brothers Center for Elephant Conservation, Polk City, FL (Romeo, SB343; DOB 1993). Onyx, Calvin, and Groucho were proven breeders.

Semen collections were scheduled every other day (first four AIs) or every day (last two), for a total of three inseminations per trial. Semen was collected by manual rectal stimulation [Schmitt and Hildebrandt, 1998] from bulls in restricted contact (i.e., keepers handled the bulls from outside the enclosure) in an elephant restraint device, except for Calvin, who was collected in free-contact from a paddock area of the elephant barn. Before the semen was collected, fecal material was removed by manual cleaning and irrigation with lukewarm water (1–2-cm-diameter hose, 5–15 l/min water flow rate). Penile erection was induced by rectal message of the pelvic portion of the urethra, near the seminal colliculus. Ejaculation was stimulated by massaging the region over the ampulla of the ductus deferens. The semen was collected in 2–4 fractions to avoid potential urine contamination. Collection devices (a plastic palpation sleeve (i.e., a condom) and a plastic bag that funneled the semen into a 50-ml tube at the end of a pole) were placed over or near (respectively) the end of the penis to collect semen, and were changed between fractions. Immediately after collection, each fraction was assessed for volume and sperm motility. Seminal fractions of similar motility were pooled, and a 100-μl aliquot of sperm suspension was fixed in 1 ml 0.3% glutaraldehyde/PBS and shipped with the semen for later sperm concentration and morphological analyses [Howard et al., 1990]. The remaining semen was diluted 1:1 in Ham's F10 medium (Irvine Scientific, Irvine, CA) with Pen-Strep, glutamine, pyruvate, and 5% fetal calf serum, and centrifuged at 400 g for 10 min. It was then extended 1:1 at 35–37°C in 1) TEST without glycerol (first four AIs) or 2) Berlin-cryomedium (BC), a basic solution of balanced buffered electrolytes and non-electrolytes (e.g., disaccharides) with 15.6% (V/V) egg yolk and 6.25% (V/V) DMSO. To avoid osmotic shock, the extenders were added slowly to the semen ($\sim 1/10$ volume every 30 sec) and then cooled at 4°C in a portable semen cooling chamber (Equitainer; Hamilton-Thorne, Beverly, MA) for shipment to the NZP. At least 30 min before insemination, the extended semen was warmed in a water bath to 37°C and assessed for sperm motility. Whole semen from each bull was screened for tuberculosis and found to be negative by polymerase chain reaction (PCR) for the *M. tuberculosis* complex, and by culture for tuberculosis organisms (National Veterinary Services Laboratory, U.S. Department of Agriculture, Ames, IA). The semen also tested negative for elephant-specific herpes virus (L. Richman, Johns Hopkins University, Baltimore, MD).
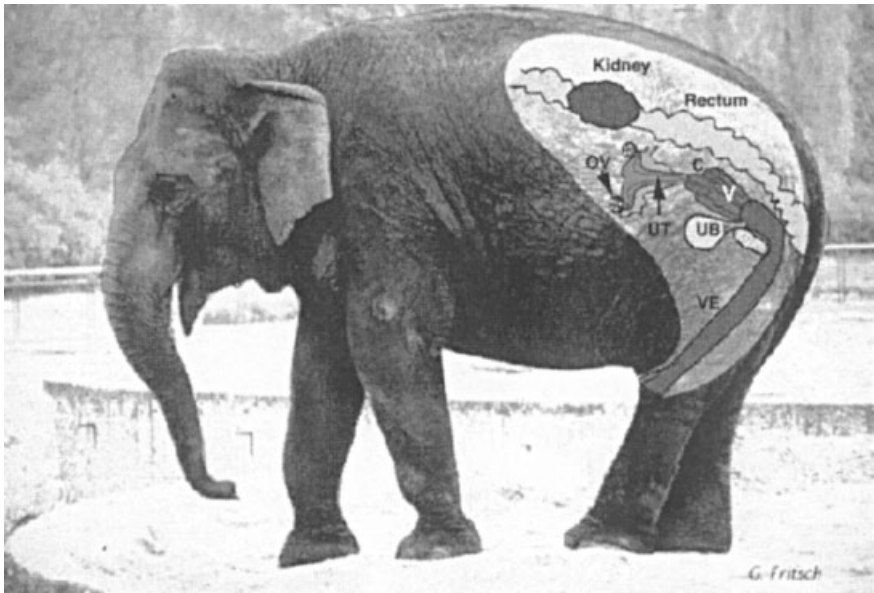
Fig. 1. Schematic diagram of the female African elephant urogenital tract. OV, ovaries; UT, uterus bicornis; C, cervix; V, vagina; VE, vestibule; UB, urinary bladder.

Transrectal Ultrasound of the Female Reproductive Tract

A schematic of the female elephant reproductive tract is shown in Fig. 1. Before each AI series, the entire urogenital tract was scanned using a real-time, B-mode ultrasound system (Ultraschallkopftraeger; A. Schnorrenberg Chirurgieme-chanik; Woltersdorf, Germany) to rule out the possibility of reproductive pathologies that might have compromised the AI [Hildebrandt et al., 1997, 2000a]. The examination was conducted in free contact, without the use of sedatives, while the female was in a standing position. Feces were removed manually and the rectum was irrigated with lukewarm water. A 3.5 MHz convex transducer with ultrasound gel for coupling was used to visualize the caudal region of the genital tract (vestibule, urethra, vagina, urinary bladder, cervix, and caudal corpus uteri) and a 5.0–7.5 MHz transducer attached to a specially designed 450-mm extension was used for the cranial region (cranial corpus uteri, uterine horns, ovaries, and surrounding tissues).

Fetal development was evaluated by transrectal ultrasound conducted at 12, 20, and 28 weeks of gestation. Scans were conducted with the female in both standing and lateral recumbent positions without sedation. The ultrasound at 12 weeks was conducted using the B-mode ultrasound system described above. The examinations at 20 and 28 weeks utilized a scanning system (hand-held, multi-frequency 4–7 MHz convex transducer) capable of 3-D imaging (Kretz Technik; Zipf, Austria).

AI

The female was unsedated and standing, and an aseptic technique was employed throughout the procedure. The catheter equipment was soaked in
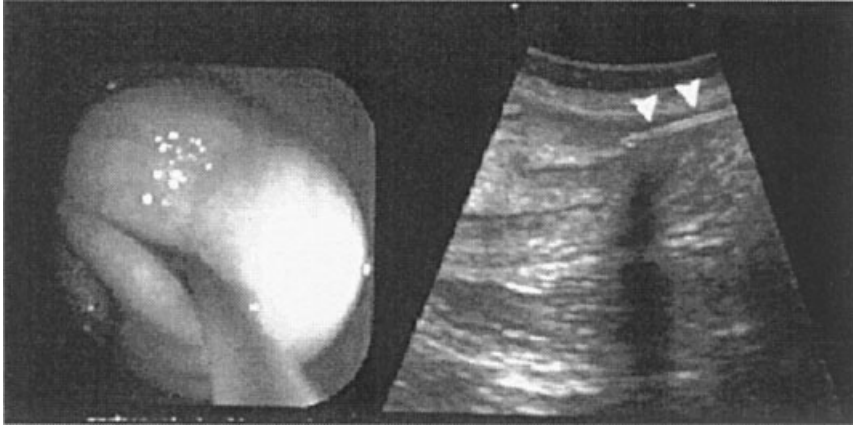
Fig. 2. Endoscopical image of the vaginal os and 2-mm-diameter insemination catheter during an AI (left panel). Sonogram (3.5 MHz convex transducer) of the insemination catheter (white arrowheads) placed at the beginning of the portio vaginalis (right panel).

Nolvasan for at least 30 min before use. A custom-made balloon catheter (2.5 cm diameter × 140 cm length) was lubricated with nonspermacidal sterile gel (Priority Care STER-I-GEL®; NLS Animal Health, Owings's Mill, MD) and inserted into the vestibule to slightly distend the reproductive tract for optimal visualization and placement of a flexible 2.5-m video chip endoscope (EPM-1000; Pentax, Inc., Hamburg, Germany) containing a disposable insemination catheter (3 mm diameter, 300 cm length) in the working channel. Both endoscopic and ultrasonographic visualization were used to guide the semen catheter into the distal vagina or cervix (Fig. 2). Semen deposition was visualized ultrasonographically to verify placement. Two to three inseminations (once per day) per series were conducted depending upon the availability of good-quality semen.

Hormone Analysis

LH (AFP8614B; NHPP-NIDDK) and prolactin (NIDDK-oPRL-I-2) were iodinated using chloramine-T [Brown et al., 1999]. Serum LH was quantified by a ^{125}I double-antibody radioimmunoassay (RIA) that utilized a monoclonal anti-bovine LH antiserum (518-B_7), an ovine LH label, and NIH-LH-S18 standards [Brown et al., 1999]. Serum prolactin was measured using a heterologous ^{125}I double-antibody RIA that employed an anti-human prolactin antiserum (NIDDK-anti-hPRL-3), an ovine prolactin label, and standards (NIDDK-oPRL-I-2) [Brown and Lehnhardt, 1995, 1997]. Assay sensitivities (based on 90% of maximum binding) were 0.039 and 0.156 ng/ml, respectively.

Serum progesterone was measured by a solid-phase ^{125}I RIA (Diagnostic Products Corp., Los Angeles, CA) [Brown and Lehnhardt, 1995]. Assay sensitivity was 0.05 ng/ml. Increases in serum progestagens were considered indicative of a luteal phase if concentrations exceeded 0.05 ng/ml for more than 2 consecutive weeks [Brown and Lehnhardt, 1997]. Estrous cycle length was calculated as the number of days from the first increase in serum progestagens until the next rise. The luteal phase included those days from the initial rise in progestagens until concentrations

returned to baseline (defined as concentrations <0.08 ng/ml for at least 5 consecutive days, excluding single point increases).

Fetal sex was determined by measuring the concentration of maternal circulating testosterone in samples collected from conception through 65 weeks of gestation, as described by Duer et al. [2002] (Total Testosterone; Diagnostic Products Corp., Los Angeles, CA). The assay was modified by increasing the amount of serum analyzed (200 μl), increasing the incubation time to 4 hr, and adding two additional low standards to the standard curve. The assay sensitivity, based on 200 μl serum, was 20 pg/ml.

For comparative purposes, serum LH and serum and urinary progestagens were also analyzed by enzyme immunoassay (EIA). The LH EIA utilized the same monoclonal antibody as in the RIA (518-B_7), a biotin-conjugated ovine LH label, and bovine LH (NIH-LH-B10; AFP-5551B) standards [Graham et al., 2002]. The LH label was prepared using an EZ-Link™ Sulfo-NHS-LC-Biotinylation kit (catalog #21430; Pierce, Rockford, IL). The assay sensitivity was 0.038 ng/ml. The progestagen EIA [Munro and Stabenfeldt, 1984] utilized a monoclonal progesterone antibody (1:10,000; Quidel clone #425), horseradish peroxidase-conjugated progesterone label (1:40,000; C. Munro, University of California–Davis), and progesterone standards (catalog #P0130; Sigma Chemical Co., St. Louis, MO). This antibody crossreacts with a variety of reduced pregnanes in serum and excreta in a wide range of species, including elephants. Serum and urine samples were diluted 1:4–1:16 in assay buffer (0.1 M PO_4, 0.14 M NaCl, 0.1% BSA, pH 7.0) before progestagen analysis. The assay sensitivity was 0.016 ng/ml. The EIAs were validated for elephant serum (LH and progestagens) and urine (progestagens) by demonstrating 1) parallelism between serially diluted samples and the respective standard curves, and 2) $>90\%$ recovery of added standard hormone to pooled samples.

All of the serum and urine samples were analyzed unextracted. Urinary progestagen data were indexed by creatinine concentration. The intra- and interassay coefficients of variation for all assays were <10 and $<15\%$, respectively. Hormone and estrous cycle data are presented as means $\pm$ SEM.

RESULTS

AI

Ultrasound examinations of the reproductive tract, conducted before each AI trial, indicated the presence of multiple cysts in the endometrium. These cysts were located around the placental scar from the previous pregnancy, and were limited in number at the first AI. However, cysts were more prevalent and generalized throughout the uterine body and both horns at subsequent ultrasound examinations. Anechogenic mucus was observed in the vagina and cervix, often characterized as a discontinuous white line. The uterus was more fluid-filled and the endometrium was echogenic.

For each trial, two to three inseminations were conducted around the time of expected ovulation. For the insemination on 12 February 1999, extended semen from Onyx and Calvin was pooled for insemination. Only Onyx (DPZ) and Calvin (ALS) produced semen of high enough quality for insemination. The characteristics of Onyx and Calvin's inseminated semen are depicted in Tables 1 and 2,

TABLE. 1. Ejaculate characteristics of an Asian elephant bull (Onyx) at the Dickerson Park Zoo used for artificial inseminations conducted in October 1995, April 1996, November 1996, March 1997 and April 1999

Parameter	Dates of artificial insemination									
	10/2/95	10/4/95	4/19/96	4/22/96	4/24/96	11/23/96	11/25/96	3/15/97	3/17/97	4/12/99
Sperm motility (% at collection)	80	85	95	80	75	95	60	70	90	80
Sperm motility status (at collection)[a]	2.5	3.0	3.5	3.5	3.0	3.0	2.5	3.0	3.5	2.5
Semen volume (ml)	27	17	17	47	12	20	7	13	12	48
Sperm concentration/ml ($\times 10^8$)	3.2	5.1	3.7	0.3	0.3	3.7	1.9	1.8	3.2	4.8
Post-collection insemination time (hr)	23	22	23	23	23	23	24	23	23	10
Sperm motility (% at insemination)	60	85	95	80	75	90	60	60	90	80
Sperm motility status (at insemination)[a]	2.5	3.0	3.5	3.5	3.0	3.5	2.5	3.0	3.5	2.5
Inseminant volume (ml)[b]	43	33	34	84	24	40	13	26	23	97
Total motile sperm inseminated ($\times 10^9$)	8.1	14.3	11.9	2.2	0.5	13.3	1.5	2.8	6.6	37.4
Sperm morphology (%)								NA	NA	
Normal	84	89	85	92	65	87	82	–	–	55
Abnormal acrosome	11	6	0	0	19	6	8	–	–	0
Bend midpiece w/ droplet	1	3	0	0	0	0	2	–	–	0
Bent midpiece w/o droplet	3	1	0	0	5	1	2	–	–	1
Proximal droplet	0	0	0	0	0	1	1	–	–	0
Distal droplet	0	0	0	0	0	0	1	–	–	3
Coiled tail	1	1	1	3	5	1	2	–	–	41
Bent tail	0	0	14	5	6	4	2	–	–	0
Day of ovLH surge	9/24/95			4/24/96			11/25/96	3/14/97		4/12/99
Days between anLH and ovLH surges	20			20			21	19		20

[a]Based on a scale of 1–5, where 1 = no forward progression, 5 = high progressive motility.
[b]Semen diluted 1:2 with extender.
NA, not available.

TABLE 2. Ejaculate characteristics of an Asian elephant bull (Calvin) at Lion Country Safari used for artificial inseminations conducted in April 1999 and February 2000

Parameter	Dates of artificial insemination				
	4/11/99	4/12/99	4/13/99	2/23/00	2/24/00
Sperm motility (% at collection)	20	85	85	80	95
Sperm motility status (at collection)[a]	1.0	1.0	1.0	3.0	3.5
Semen volume (ml)	12.0	5.5	17.3	51.5	5.5
Sperm concentration/ml ($\times 10^8$)	21.5	10.4	9.9	10.8	4.6
Post-collection insemination time (hr)	10	9	9	11	11
Sperm motility (% at insemination)	10	85	80	15	95
Sperm motility status (at insemination)[a]	1.0	1.0	1.0	3.5	4.0
Inseminant volume (ml)[b]	24	11	34.5	115	11
Total motile sperm inseminated ($\times 10^9$)	25.5	48.6	136.2	92.7	3.9
Sperm morphology (%)					
Normal	82	15	70	84	52
Abnormal midpiece	4	0	0	0	0
Coiled tail	1	0	0	1	0
Bend midpiece w/droplet	4	7	6	12	9
Bent midpiece w/o droplet	1	9	21	1	3
Proximal droplet	5	0	0	0	1
Distal droplet	2	10	3	2	35
Bent neck	1	0	0	0	0
Day of the ovLH surge		4/12/99		2/24/00	
Days between anLH and ovLH surges		20		20	

[a]Based on a scale of 1–5, where 1 = no forward progression, 5 = high progressive motility.
[b]Semen diluted 1:2 with extender.

respectively. Semen quality varied within and among bulls. For Onyx, sperm motility at collection ranged from 60% to 95%, with normal cell morphology ranging from 55% to 92%. Morphological abnormalities were associated with the acrosome or tail (coiled or bent tails). For Calvin, sperm motility ranged from 20% to 95%, with 52–84% normal sperm morphology. Most abnormalities were associated with the midpiece (with and without droplets). Timing of insemination for the first four AI trials took place about 24 hr after semen collection, whereas the latter two trials were performed within 12 hr of collection. In general, sperm motility and status of extended semen at insemination were similar to immediate postcollection values. There was evidence of head-to-head agglutination of spermatozoa (not quantified) in most of the ejaculates collected. Ejaculates collected from Rex, Romeo, and Groucho were not used for insemination because of low motility (<15%) (data not shown).

The timing of inseminations relative to the ovLH surge varied among AI trials (Tables 1 and 2). For the conceptive AI in 2000, AI was conducted on the day before and the day of the ovLH surge, with ovulation occurring the day after the surge (based on transrectal ultrasound). The first AI in October 1995 was conducted without use of the double LH surge, and was performed over a week late relative to the ovLH surge (evaluated retrospectively). Another AI in March 1997 was late when no semen was available from Onyx on the first scheduled day (13 March 1996). For all other AI attempts, inseminations were performed both before (1 or 2 days) and on the day of the ovLH surge.

Each insemination lasted between 0.5 hr and 2 hr, with subsequent trials requiring less time for proper catheter placement and semen deposition. After each procedure, the female was exposed to olfactory stimuli from the bull (e.g., urine and/or semen) placed on the ground. This exposure resulted in the observation of mild abdominal contractions and pelvic thrusting.

Hormone Profiles

Based on the female's historical serum progesterone profile, the estrous cycle averaged 107 ± 3 days with a 70 ± 3-day luteal phase and 37 ± 2-day follicular phase (n = 20 cycles). Beginning in January 1995, a total of 15 follicular phase LH profiles were characterized. Using the RIA, baseline LH concentrations were generally <1.0 ng/ml. Surge concentrations averaged 14.4 ± 1.7 ng/ml for the anLH surge, and 16.4 ± 1.6 ng/ml for the ovLH surge. The anLH surge occurred 19.7 ± 0.7 days after the drop in progestagens to baseline. The ovLH surge occurred 19.9 ± 0.2 days after the anLH surge, and 1–3 days after the rise in progestagens at the beginning of the subsequent luteal phase.

Hormone concentrations during pregnancy and the preceding luteal phase are presented in Fig. 3. For the conceptive cycle, LH concentrations were 13.9 ng/ml for the anLH and 19.9 ng/ml for the ovLH surge (Fig. 3, top panel). LH data generated using the EIA were similar to the RIA (r = 0.91; $P < 0.05$), except that overall concentrations were lower for the EIA because of standard differences. Peak LH concentrations using the EIA were 3.1 ng/ml for the anLH and 6.5 ng/ml for the ovLH surge.

After AI, progestagens increased to luteal-phase concentrations, followed by the gradual decline that normally marks the end of that period in a nonpregnant elephant. Progestagen concentrations declined by about half and then increased again, reaching concentrations that exceeded nonpregnant luteal phase levels by 12 weeks of gestation. The subsequent progestagen profile then followed a biphasic pattern. Based on the progesterone RIA, concentrations peaked ~20 weeks into gestation at 2.23 ng/ml, before declining to a transitory low of 0.51 ng/ml at 60 weeks (Fig. 3, top panel). Serum progestagens increased again through the end of gestation until concentrations decreased precipitously to undetectable levels during the final week (Figs. 3 and 4). The profile of serum progestagens using the more broad-spectrum CL425 antibody in the EIA paralleled that produced by the progesterone RIA (r = 0.95; $P < 0.05$) (Fig. 3, top panel), except that concentrations were about 10-fold higher in the EIA and were still detectable at parturition (Figs. 3 and 4). The urinary progestagen profile produced by EIA also mimicked that observed in circulation (r = 0.91 and 0.92 for RIA and EIA, respectively; $P < 0.05$), although the data were more variable (Figs. 3 (middle panel) and 4).

Serum prolactin secretion was stable throughout the estrous cycle, averaging 5.4 ± 0.6 ng/ml. Concentrations remained at baseline throughout the first 14 weeks of gestation and then increased markedly (Fig. 3, bottom panel). Serum testosterone in the maternal circulation was low (<20 pg/ml) for the first 30 weeks of gestation before increasing to a concentration of 0.94 ng/ml at 64 weeks, correctly indicating the calf was a bull (Fig. 5).
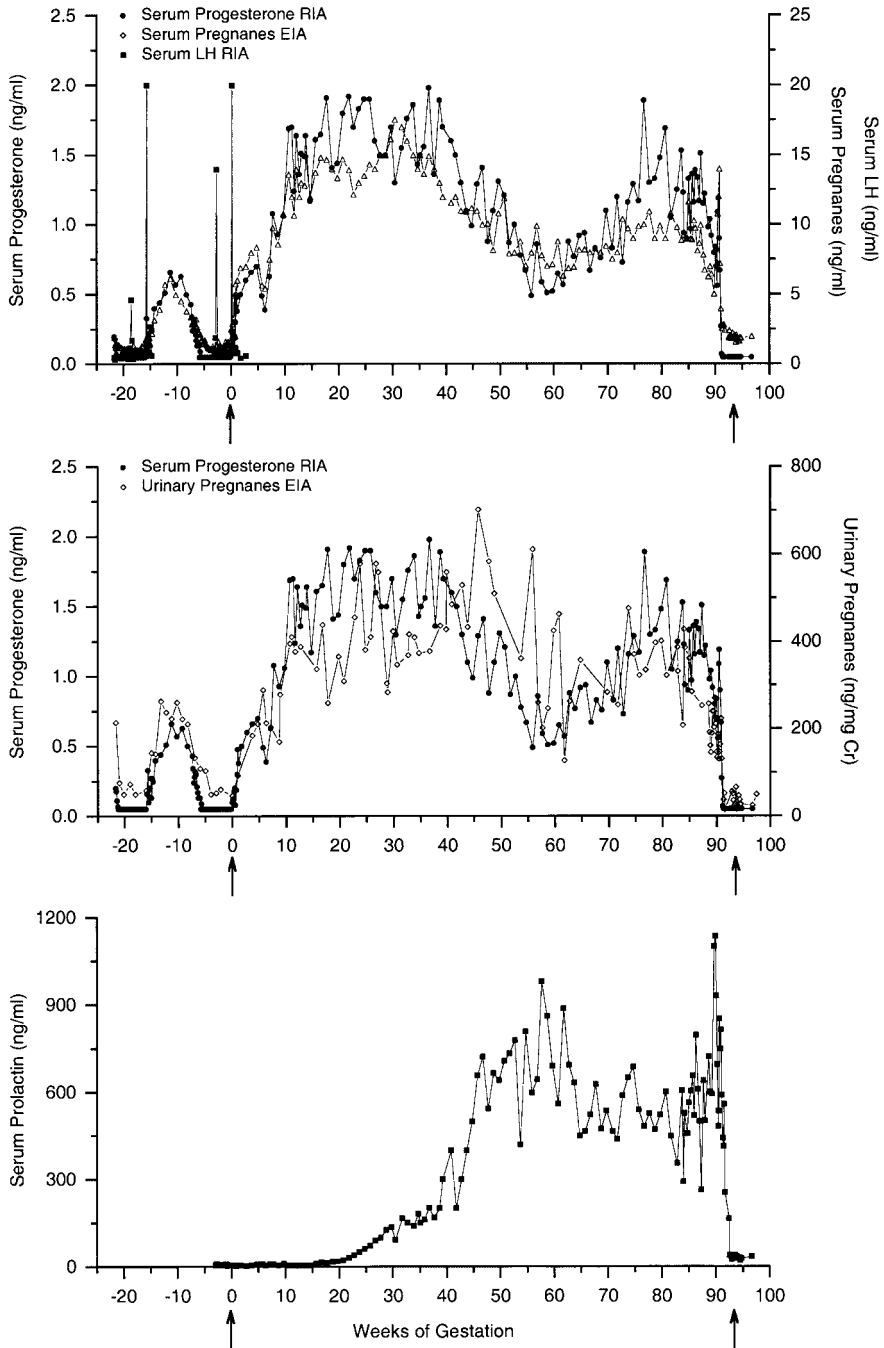
Fig. 3.   Profiles of serum LH determined by RIA, serum progestagens determined by commercial progesterone RIA (top and middle panels) and an EIA that uses CL425 antisera (top panel), urinary progestagens determined by EIA (middle panel), and serum prolactin determined by RIA (bottom panel) throughout a nonpregnant luteal phase and gestation in an Asian elephant that conceived by AI. Arrows indicate time of insemination and birth.
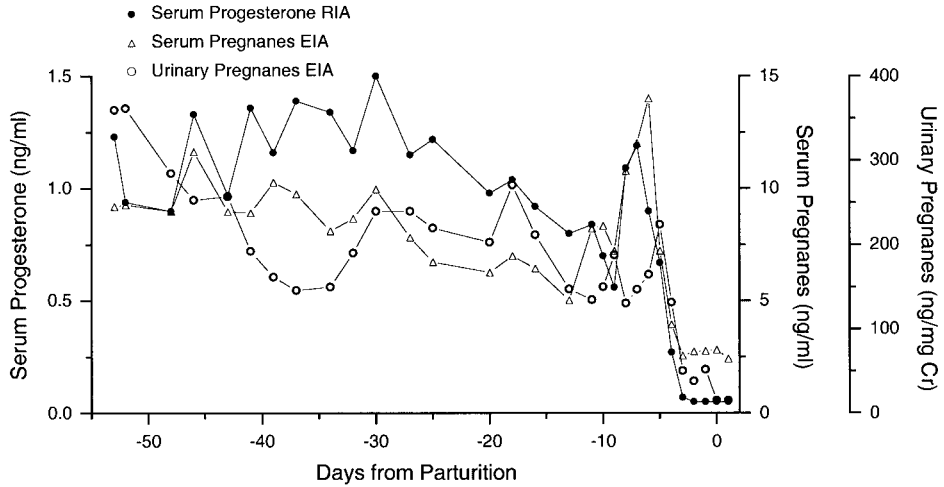
Fig. 4. Profiles of serum progestagens determined by commercial progesterone RIA and an EIA that uses CL425 antisera, and urinary progestagens determined by EIA during the last 55 days of gestation in an Asian elephant that conceived by AI.

Transrectal Ultrasound Assessments During Gestation

During gestation, the vagina filled with thick vaginal mucus and served as a mechanical and infectious protective barrier. Ultrasonographic images of the developing embryo/fetus are shown in Fig. 6. Shanthi was examined at 12 weeks of
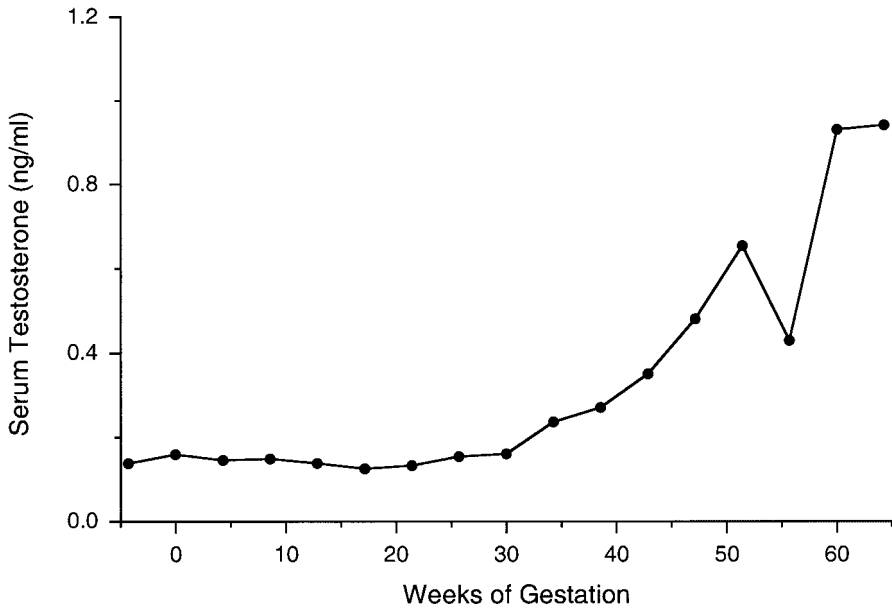
Fig. 5. Profile of serum testosterone determined by RIA during the first 65 weeks of gestation for fetal sex determination in an Asian elephant that conceived by AI.
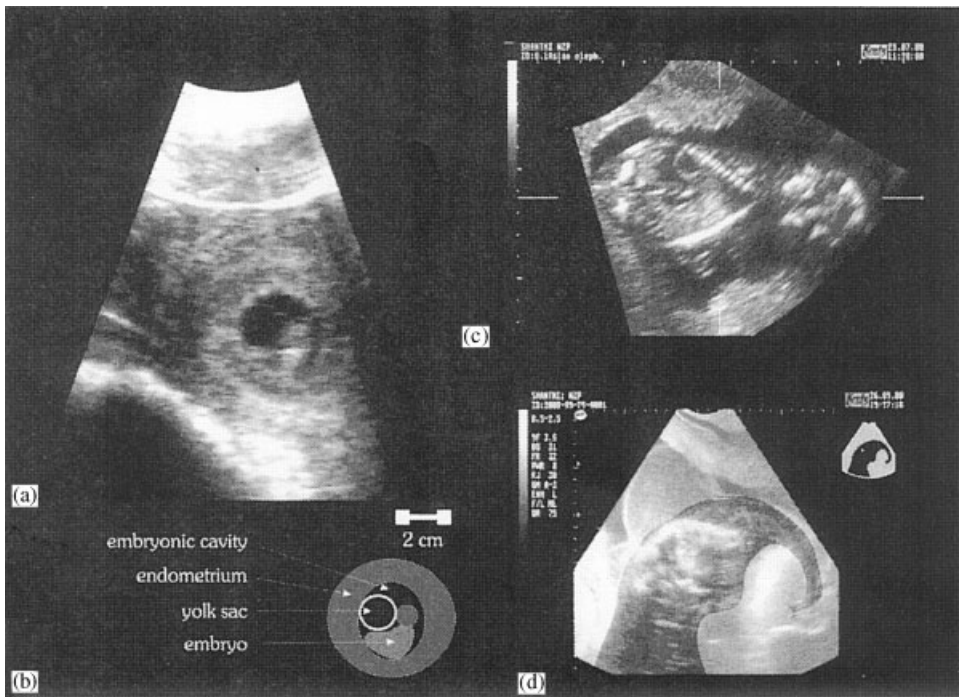
Fig. 6.   **A:** Sonogram (hand-held, 3.5 MHz convex transducer) of the embryo at 93 days of gestation. **B:** Schematic diagram of the anatomical relationships of the 12-week-old embryo. **C:** Sonogram (hand-held, multifrequency 4–7 MHz convex transducer) showing the median view of the fetus at 115 days of gestation. **D:** Sonogram (hand-held, multifrequency 4–7 MHz convex transducer) of the 215-day-old fetus showing the head region and developing trunk.

gestation and the embryo measured about 40 mm (Fig. 6a and b). At 20 weeks, the uterus was extensively vascularized and the fetus was estimated to be about 11.7 cm in length, with a head diameter of ~ 4 cm (Fig. 6c). Heart rate was estimated at 80 bpm. At 28 weeks, the fetus was estimated to be ~ 30 cm in length and the entire body could not be scanned in one field. Scans identified several structures, including the eye, brain cavity, backbone, foot, ribs, umbilical cord (with Doppler imaging of blood flow), and trunk (Fig. 6d). The right ovary (where ovulation occurred) measured 7 cm $\times$ 4 cm and contained a single corpus luteum (CL) 5.2 cm $\times$ 3.2 cm in size.

Parturition

Progestagen concentrations dropped to baseline on 22 November 2001, and labor contractions began 2 days later on the morning of the 24th. Rectal palpation and transrectal ultrasound conducted mid-morning on the 24th determined that the cervix was dilated and one foot was in the birth canal. A mammary gland ultrasound also showed significantly more fluid in the milk channels; the ducts were larger and more defined than the previous day, and there was clearer definition of the glandular tissue. Milk stained both front legs and there was thick drainage from the left temporal gland. A mucus plug was passed 10 hr later, but then contractions ceased at 2300 hr. On the morning of the 25th, rectal palpation indicated that both feet were in

the birth canal, but contractions never resumed. Therefore, at 1250 hr on the 25th, oxytocin (40 IU, i.v.) was administered. Contractions began within 15 min of injection and birth occurred within 35 min. The male calf weighed 148 kg, was 1 m tall, and was delivered without incident in a head-first position. He walked within minutes and nursed within 2 hr of birth. The placenta was passed at 1500 hr on 26 November. Shanthi gained about 455 kg during the pregnancy (from ∼4,150 to 4,600 kg).

DISCUSSION

In October 1995, Shanthi became the first elephant to be inseminated using a new semen catheterization approach. After six attempts she became the second Asian and fifth elephant overall to conceive by AI. Therefore, although Shanthi was not the "first" to conceive, the initial trials conducted in this female were pivotal in the subsequent development of a reliable technique that has resulted in the successful insemination of at least a dozen Asian and African elephants to date.

For decades, the size and unique features of the elephant reproductive tract hampered efforts to develop assisted-reproduction techniques, such as AI. In accordance with its size, the elephant has the longest reproductive tract of any land mammal–approximately 2.5 m from vestibule to ovary [Balke et al., 1988]. The length and position of the urogenital canal (1.0–1.4 m) presents a challenge because it opens ventrally between the hind legs, runs vertically up toward the tail, and then curves cranially and horizontally above the bony pelvis. In addition, the vaginal opening is very small, especially in nulliparous cows (<1 cm in diameter), and although it dilates to ∼40 cm during parturition, it constricts back to nearly its original size after birth. During natural mating, the vestibule is the site of ejaculation; however, anecdotal evidence from early AI attempts suggested that this site was not suitable. Thus, a major challenge was to devise a way to deliver sperm deeper within the reproductive tract of the elephant. This was accomplished by incorporating a semen catheter into a flexible endoscope, passing it through a balloon guide catheter, and further directing it by the use of transrectal ultrasound. Using this approach, semen for AI can be deposited into the anterior vagina or cervix.

Another challenge was to time the insemination so that sperm were deposited around the time of ovulation. As with most mammals, the female is only fertile for 2–3 days at the end of the follicular phase. Given the nearly 4-month cycle length of the elephant, this period is difficult to predict. The first successful AI was accomplished in an Asian elephant at the Dickerson Park Zoo [Schmitt, 1998] using a combination of ovarian ultrasonographic examinations and the preovulatory increase in progestagens [Carden et al., 1998] to time the insemination. This worked well because the bull was maintained on-site. Another AI (Vienna, Austria) was also timed on the basis of daily ultrasound evaluations [Hildebrandt et al., 1999]. However, most facilities do not have ready access to a bull, or the ability to conduct daily ultrasound exams to monitor follicular growth and ovulation. Thus, when preparing for an AI, it is crucial to be able to reliably predict when the ovLH surge will occur. The discovery of the "double LH surge" in Shanthi was serendipitous. It was during the initial intensive characterization of the female's progestagen cycle

that a retrospective LH analysis in late 1995 revealed the 3-week timing between the anLH and ovLH surges. Around this time, a similar pattern was observed in the African elephant [Kapustin et al., 1996]. From repeated ultrasound examinations of African elephants, we now know that ovulation occurs ~ 24 hr after the ovLH surge [Hermes et al., 2000], a finding consistent with that observed in Shanthi. Based on this and other successful AIs, we conclude that conception occurs when inseminations are conducted within a day ($\pm$) of the ovLH surge.

A third challenge was obtaining fresh sperm samples ''on demand'' several days in a row. The major limitations of this procedure are the small number of adult bulls in the captive population, few of which are trained for semen collection, and the variable quality of semen. Another problem with the early AIs was the use of suboptimal semen extenders. For example, it was not until 1998 that the protective effect of DMSO on sperm membranes was discovered (Blottner and Schmitt, unpublished results). Now, at least for Asian elephants, the inclusion of 1–4% DMSO in the semen diluent is required to sustain sperm viability. Interestingly, however, it appears that DMSO is not needed to protect sperm membranes in African elephants (Schmitt and Loskutoff, unpublished results).

Postconception hormone monitoring identified transitory decreases in progestagen secretion at around 8 and 60 weeks, which appeared to signal shifts in luteal or placental steroidogenic activity. Based on histological examination [Smith and Buss, 1975] and analysis of luteal progestagens [Hodges et al., 1997] in other elephants, the CL of pregnancy is most steroidogenically active between 12 and 60 weeks of gestation. This period is characterized by progestagen concentrations that are higher, on average, than those during the nonpregnant luteal phase [Hess et al., 1983; Olsen et al., 1994; Brown and Lehnhardt, 1995]. The source of increased progestagens during the first half of gestation is unknown. Assays for eCG, hCG, and pregnancy-specific protein B have failed to detect a placental gonadotropin in Asian or African elephants [Brown, 2000]. However, an ultrasound study of wild African elephants (n = 66) identified new follicles and up to 10 fresh CL on both ovaries at 16–20 weeks postconception that persist for at least 50 weeks [Hildebrandt et al., 2000a]. These were smaller in size (≤ 2 cm diameter) than the CL derived from ovulation (generally 2–3 cm), and contained an internal fluid-filled cavity, which suggests that a pregnancy-specific factor may be produced. By contrast, only one CL, significantly larger than a nonpregnant CL, was observed at the 28-week ultrasound examination in Shanthi. Based on these observations, it would appear that higher gestational progestagens are due to multiple, smaller CLs in the African elephant vs. a single large CL in the Asian elephant. However, because this was the first time the ovary had been visualized in a pregnant Asian elephant, it is premature to speculate on such a species difference in gestational CL development or function.

A second transient progestagen decline at 60 weeks was clearly evident in Shanthi. Similar short-term reductions have been observed in other elephants, but they were not always as well defined [Olsen et al., 1994; Niemuller et al., 1997; Carden et al., 1998; Fiess et al., 1999] (Brown, unpublished results). In fact, during Shanthi's first pregnancy only a brief decline was observed at this stage [Brown and Lehnhardt, 1995]. Whether maintenance of luteal function after mid-gestation is related to ovarian and/or placental factors is not known. Given that prolactin is luteotropic in other mammals, the increase after 20–28 weeks could account for some of the progestagen activity observed later in pregnancy. The lack of a correlation

between circulating progestagen levels and luteal volume [de Villiers et al., 1989], and the positive relationship between fetal progestagen concentrations and gestational age [de Villiers et al., 1989] both suggest that the placenta may be a source of progestagens.

A notable finding was the good correspondence between a commercial progesterone RIA (which is specific for progesterone) and a broad-spectrum progestagen EIA, given that limited native progesterone is produced by the elephant. Rather, the major circulating luteal progestins in Asian and African elephants are 5α-pregnane-3,20-dione ($5\alpha DHP$) and 5α-pregnane-3-ol-20 one (5α-P-3-OH), with $5\alpha DHP$ predominating [Heistermann et al., 1997; Hodges et al., 1997; Schwarzenberger et al., 1997]. In the Asian elephant, 17α-hydroxyprogesterone (17α-OHP) is also present in the circulation, although it is not as abundant as $5\alpha DHP$ [Niemuller et al., 1993; Hodges, 1998]. Despite the higher immunoactivity exhibited by CL425 in the EIA, the two serum profiles were qualitatively similar throughout the luteal phase and pregnancy. The urinary pattern produced by the EIA similarly mimicked circulating profiles, although the data were somewhat more variable. In Europe, urinary monitoring of reproductive status in the elephant typically is conducted by analyzing progestagen metabolites (5α-P-3OH in African [Heistermann et al., 1997; Fiess et al., 1999] and pregnanetriol in Asian [Niemuller et al., 1993] elephants). Neither the EIA nor the RIA used in this study required hydrolysis or extraction of serum or urine, which differs somewhat from the European methods. In comparing the two systems, the commercial RIA was preferable to the EIAs for predicting parturition because the concentrations became undetectable in the days before birth. For assays using CL425, concentrations were always measurable, which made it more difficult to determine when absolute ''baseline'' had been reached.

In conclusion, the methodologies surely will continue to be refined, but it now appears that AI has the potential to be a viable supplement to natural breeding. Today, nearly all AIs are conducted using the double LH surge in conjunction with the nonsurgical IZW catheterization technique. However, surgical AI by vestibulotomy has also been performed successfully in at least two elephants, and thus is an option for animals that are not good candidates for the nonsurgical approach (Schmitt, unpublished results). These breakthroughs could not have come at a better time, given the low reproductive rates and age structure of the captive Asian and African elephant populations. At present, only $\sim 30\%$ of Asian elephant females are under 30 years of age [Keele, 1997]. The situation is somewhat better for African elephants: $\sim 70\%$ are between 10 and 30 years of age [Olson, 2000]. However, only a third of the females in this age group are managed by the SSP and thus likely to be included in any national breeding effort. In the wild, elephants can reproduce into their late 40s or even 50s. However, in captivity there is a higher incidence of stillbirths and dystocias in animals that experience their first conception after 30 years of age. In addition, older elephants develop uterine and ovarian pathologies at a much higher rate, especially if reproduction has not occurred for 10–15 years [Hildebrandt and Göritz, 1995; Hildebrandt et al., 2000a]. The cause of these pathologies, and their impact on fertility are unknown, although there is speculation that the continuous ovarian cyclicity of nonbred females may have a negative and cumulative effect on reproductive health. In the wild, most females are either pregnant or lactating, and they experience comparatively few reproductive cycles in their lifetime. Although Shanthi had produced a calf in 1992, her uterus contained

some cysts at the start of the project in 1995. These were initially found around an old placental scar, a finding consistent with that noted in free-ranging multiparous African elephants (Hildebrandt and Göritz, unpublished results). The progressive increase in the number of cysts in other uterine regions with each successive AI could be a remnant effect of the previous retained placenta [Murray et al., 1996]. Interestingly, these structures all but disappeared during gestation, which suggests that prolonged progestagen exposure and/or the absence of estrogens may be beneficial to urogenital tract integrity. Given these findings and the current demographic situation, the Elephant SSP/TAG now recommends that all elephants be bred at an early age and, whenever possible, females should produce several calves over their lifespan.

Still, despite the recent optimism about the use of AI to increase reproductive potential, there are a number of problems that must be overcome. First, there are not enough individuals trained in this technique. All AIs to date have been performed by only two groups (headed by T. Hildebrandt and F. Göritz, and D. Schmitt). Second, less than half of the facilities that express an interest in AI actually follow through on what is needed for preparation (i.e., training, resources, and commitment). Third, there are too few African and almost no Asian elephant bulls available for semen donation at this time. Of four bulls identified in 2002, only Calvin produced reliable semen, and he has since been sent to Germany. The other good sperm donor, Onyx, died recently. Clearly, for AI to be practical, methods for cryopreserving elephant sperm must be developed. This would provide a readily available source of semen and enable the establishment of a global genome resource bank that could include sperm samples from bulls living in range countries to ensure a genetically vigorous ex situ population. In addition, an ability to sex semen would permit the continuation of a skewed sex ratio in favor of females. Until these problems are solved, AI will continue to be a promising and highly sought after, but not quite practical, alternative to natural breeding.

CONCLUSIONS

1. AI has the potential to be an effective tool for improving the breeding management of elephants.

2. Before an AI is scheduled, females should be screened for reproductive fitness by progestagen monitoring to ensure regular ovarian cyclicity, and a reproductive tract ultrasound examination should be performed to ensure the absence of morphological abnormalities that could interfere with conception or pregnancy maintenance.

3. The best results are obtained when AI is coupled with the use of the double LH surge to time insemination.

4. Limiting factors to the successful use of AI in elephants include the lack of trained personnel and a reliable source of high-quality semen (fresh or frozen).

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