

## Faecal Progesterone Assay and Its Use in Comparing Reproductive Status in Four Groups of Captive Asian Elephants

D. M. Naveen J. Dissanayake<sup>1,2,3,\*</sup>, Charles Santiapillai<sup>3</sup>, Dennis L. Schmitt<sup>4,5</sup>, Ashoka Dangolla<sup>6</sup> and H. P. Sonali D. Palliyage<sup>3,7</sup>

<sup>1</sup>Centre for Mountain Ecosystem Studies, Kunming Institute of Botany, Chinese Academy of Sciences, Heilongtan, Kunming, Yunnan, China

<sup>2</sup>University of Chinese Academy of Sciences, Beijing, China

<sup>3</sup>Postgraduate Institute of Science, University of Peradeniya, Peradeniya, Sri Lanka

<sup>4</sup>Darr College of Agriculture, Missouri State University, Springfield, Missouri, USA

<sup>5</sup>Ringling Bros. Center for Elephant Conservation, Polk City, Florida, USA

<sup>6</sup>Faculty of Veterinary Medicine & Animal Science, University of Peradeniya, Peradeniya, Sri Lanka

<sup>7</sup>Department of Animal Production and Health, North-Western Province, Kurunegala, Sri Lanka

\*Corresponding author's e-mail: naveendissanayake@yahoo.com

**Abstract.** We tested and validated a non-invasive reproductive monitoring technique using blood and faecal samples from captive elephants in Sri Lanka (n = 24) and USA (n = 18). Enzyme-linked immunosorbent assay (ELISA) was performed to determine the reproductive status utilizing progesterone antibodies. Correlation between faecal and serum progesterone values were significant. Management of elephants as herds significantly favoured higher oestrous cyclicity than management as individuals. The reproductively active proportions of elephants among the tested groups in Sri Lanka and in USA were 85% and 81% respectively. Therefore, the country elephants were living in, did not make a significant difference in reproductive status.

### Introduction

In Sri Lanka, there were 532 captive Asian elephants (*Elephas maximus*) in 1970 and 214 in 1997, which dropped to around 112 in 2011 (Fernando *et al.* 2011). At present, there are around 100 privately owned captive elephants, while the captive population in Pinnawala Elephant Orphanage is around 90. The captive elephant in Sri Lanka is an iconic feature in religious-cultural parades such as the annual 'peraheras'. However, the privately owned captive elephant population in Sri Lanka is in decline. As wild elephant capture is barred via the Fauna & Flora Ordinance of Sri Lanka, if cultural activities are to continue to use elephants, the privately owned captive population needs to be maintained by captive breeding.

Globally, despite efforts to conserve wild Asian elephants, captive numbers have been declining over time (Sukumar 2003). Asian elephants cannot

be traded internationally since they are listed in Appendix I of the Convention on International Trade of Endangered Species (CITES).

Few studies (Wijesinghe *et al.* 2002; de Silva *et al.* 2009) have assessed the breeding potential of privately owned captive female elephants in Sri Lanka. Reproductive monitoring is important for maintaining the numbers of captive elephants in Sri Lanka.

Captive Asian elephants have been successfully bred naturally and through artificial insemination (Brown *et al.* 2004a). Monitoring levels of reproductive hormones, primarily using progesterone, in captive female elephants plays a significant role in attempts towards captive breeding (Schmitt *et al.* 2001). Hormone levels in faeces have been found to be closely parallel to that of serum (de Silva *et al.* 2009; Freeman *et al.* 2010; Ghosal *et al.* 2010).

We examined the reproductive status of privately owned captive female Asian elephants, which were managed at the Millennium Elephant Foundation (MEF; keeping a total of 9 females) and the Pinnawala Elephant Orphanage (PEO; 50 females) in Sri Lanka, and those in the Ringling Bros. Center for Elephant Conservation (CEC; 18 females) and the Blue Unit of Ringling Bros. Circus (BU; 4 females) in Florida, USA. Females below 8 years of age were not included in the study. The MEF and CEC elephants were managed as non-herds (no sexually mature animals together) and those in PEO and BU were managed as herds (always more than one sexually mature female together).

Our objectives were to validate the existing faecal progesterone extraction method for female Asian elephants and to compare reproductive activity among herd and non-herd captive groups.

## Materials and methods

Fresh faeces and venous blood from 14 female elephants in CEC and 4 females from BU who were 8–62 years old, were collected weekly during December 2008 – May 2009. Faecal samples were collected 48 hours after blood was drawn. Weekly fresh faecal samples from 21 females from PEO and 3 from MEF who were 9–65 years old, were collected from November 2009 to December 2010.

The faecal samples from USA were frozen (-20°C) within 1–2 hours of collection and stored until processed at Darr College of Agriculture at Missouri State University. Faecal samples from Sri Lankan elephants were processed at the Department of Zoology, University of Peradeniya. Blood samples were processed at the Darr College of Agriculture, Missouri, USA.

The reliability of the faecal assays could be affected if any step is performed inaccurately or due to contamination with urine (Hunt & Wasser 2003). We took precautions not to sample urine contaminated faeces by collecting samples from the top-most bolus of a dung pile, observing the periphery for any urine contamination, breaking open the top-most bolus and collecting the sample

from the interior, and not from the periphery of the bolus.

Faecal extractions and dilutions were prepared as described by Brown *et al.* (2004b) and Freeman *et al.* (2010). Standards and controls for the ELISA were prepared using progesterone standard (Fisher PGC8 0051, Seimens Medical Solutions Diagnostics®, Los Angeles, CA, USA) and assay buffer solution (Brown *et al.* 2004b). We prepared serial dilutions of standard progesterone for 100, 50, 12.5, 6.25, 3.125 and 0 pg/0.05 ml using assay buffer as the diluent and the concentrations 70 pg/0.05 ml and 30 pg/0.05 ml as upper and the lower controls of the assay respectively. Progesterone-3CMO-horseradish peroxidase (HRP) (Coralie Munro, University of California, Davis CA, USA) was used as the conjugate enzyme.

The 96-well microtiter plates (Nunc Maxi Sorp®, flat wells; Thermo Scientific, Waltham, MA, USA), previously coated with progesterone antibodies, were used to perform the assays (Brown *et al.* 2004b). The wells were read using a BIO-RAD® Model 550 Microplate Reader at 490 nm (measuring filter) with 570 nm (reference filter). Faecal progesterone values were plotted against the sampling dates for each female separately with serum progesterone, while correcting for autocorrelation due to repeated sampling.

The luteal phase of the oestrous cycle was determined by a significant increase in progesterone concentration, which persisted for at least 8 weeks. The follicular phase was determined by progesterone concentrations remaining low compared to the luteal phase. Pregnancy was identified by observing a persistent elevation in progesterone levels for at least 16 consecutive weeks. If, the progesterone concentrations remained low for more than a few months, the animal was identified as either non-cycling or lactating. Lactating females were later confirmed on their reproductive history.

The length of the oestrous cycle was determined by counting the number of weeks between the beginnings of two adjacent luteal phases in each

**Table 1.** Summary of the Linear Mixed Effects (LME) model showing the relationship between faecal and serum progesterone concentrations in 9 sampled captive female elephants in the USA.

	Value	Standard error	DF	t-value	p-value
(Intercept)	3.438	0.369	105	9.319	< 0.0001
Faecal	0.039	0.006	105	6.955	< 0.0001

progesterone profile. The mean and the standard deviation values of the two phases i.e. luteal and follicular, separately and of the whole cycle, were then calculated.

Correlation between serum and faecal progesterone was examined in 9 elephants with a Linear Mixed Effects model (LME) using the function ‘*lme*’ of package “nlme” (Pinheiro *et al.* 2017) in the statistical software R version 3.3.3 (R Core Team, 2017). We used faecal progesterone as fixed effects and individual elephants as random effects for the variation of serum progesterone. To examine the importance of managing female elephants as a herd versus non-herd, the Fisher’s exact test was done using the function ‘*fisher.test*’.

The number of reproductively active female elephants was determined by summing the number of normally cycling, pregnant, and lactating females in each facility. Non-cycling females were identified as reproductively inactive in each facility. We used the criteria in Brown *et al.* (2016) to evaluate the reproductive status of our sampled females. The country factor (Sri Lanka vs USA) was analysed using Fisher’s exact test.

## Results

We observed a significant correlation between faecal and serum progesterone ( $p < 0.001$ ) as per our LME analysis (Table 1). There was significant autocorrelation between repeated

measures. Therefore, an autoregressive correlation structure was added to account for autocorrelation. The likelihood ratio test, between the model with a correlation structure and without a correlation structure, was significant ( $p < 0.001$ ). Correlations between serum and faecal progesterone in different reproductive stages are shown in Figure 1.

### *Reproductive status in Sri Lanka*

Out of the 24 elephants, 14 were cycling (13/21 from PEO and 1/3 from MEF) and 3 were non-cycling (1 from PEO and 2 from MEF). None from MEF were pregnant or lactating while 2 were lactating and 5 were pregnant in PEO (Table 2). Therefore, 21 females were reproductively active (Fig. 3). The three non-cycling females were 42, 32, and 20 years old. The length of the oestrous cycle in cycling females was  $14.2 \pm 1.4$  weeks ( $n = 255$ ). The length of follicular and luteal phases were  $4.5 \pm 1.3$  weeks ( $n = 109$ ) and  $10.0 \pm 1.4$  weeks ( $n = 146$ ) respectively.

### *Reproductive status in the USA*

Out of 18 elephants, 10 were cycling (6/14 from CEC and 4/4 from BU). Four from CEC were non-cycling. In CEC, 3 were lactating and one was pregnant while none were lactating or pregnant in BU (Table 2). The non-cycling elephants in CEC were 58, 57, 63 and 47 years old. Ten from CEC and all 4 from BU were reproductively active (Fig. 3). The length of the

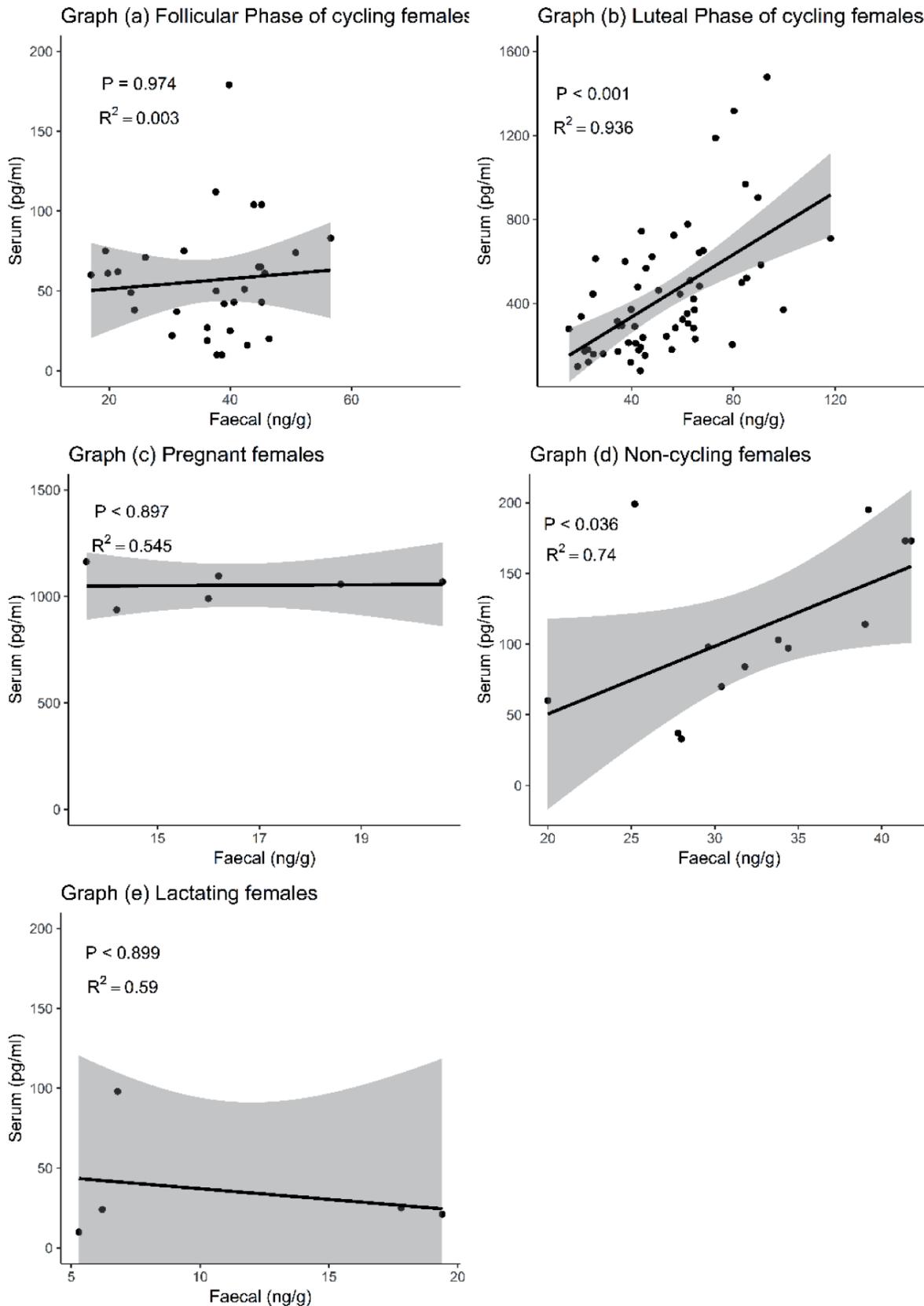
**Table 2.** Location and reproductive status of captive Asian elephants. CY = cycling, PG = pregnant, LA = lactating, NC = non-cycling.

Facility	Country	Managed as	CY	PG	LA	NC	Total
MEF	Sri Lanka	non-herd	1	0	0	2	3
PEO	Sri Lanka	herd	13	5	2	1	21
CEC	USA	non-herd	6	1	3	4	14
BU	USA	herd	4	0	0	0	4

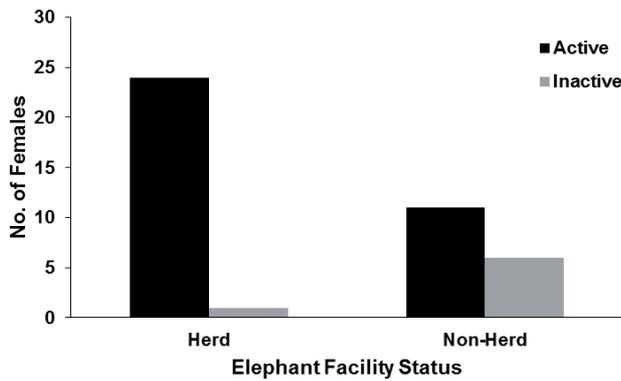
oestrous cycle was  $15.2 \pm 1.5$  weeks ( $n = 148$ ). The length of follicular and luteal phases were  $5.9 \pm 0.9$  weeks ( $n = 54$ ) and  $9.3 \pm 1.5$  weeks ( $n = 94$ ) respectively.

*Reproductively active and inactive females*

The proportion of individuals that were reproductively active was significantly higher



**Figure 1.** Correlation plots of faecal vs serum progesterone levels detected in the assays. The black line is the regression line and the grey area is the 95% confidence interval.



**Figure 2.** Reproductively active and inactive females in herd and non-herd facilities.

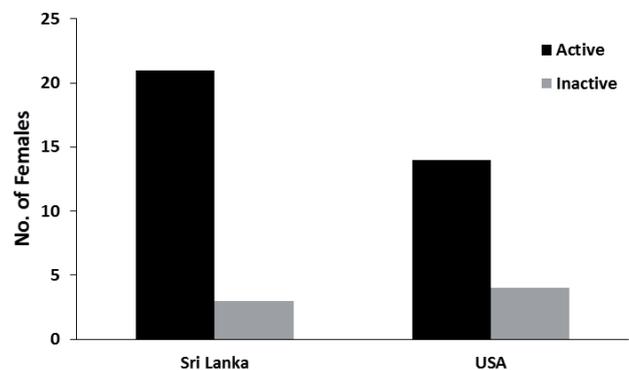
( $p = 0.008$ ) among elephants managed as a herd (24/25) compared to those managed as a non-herd (11/17) (Fig. 2). The proportion of reproductively active animals (Fig. 3) was not significantly different between Sri Lanka and USA ( $p = 0.403$ ).

## Discussion

We observed a significant correlation between serum and faecal progesterone levels, confirming that previously reported (de Silva *et al.* 2009; Ghosal *et al.* 2010). We assessed more females and used simpler methods of hormone extraction, compared to previous reports. We found that the correlation was high in the luteal phase of cycling females and non-cycling females but was not significant in the follicular phase of cycling, pregnant, and lactating females. Therefore, it is the luteal phase where progesterone levels elevate that could be detected by our simple hormone extraction method. Despite the fact that pregnant cows have high serum progesterone levels (Brown 2006), our sample size of lactating females may not have been adequate to draw conclusions. In non-cycling and lactating cows, the serum progesterone is known to be low (Brown 2006), and may have been undetectable with our methodology. Therefore, our method is unable to detect all the reproductive phases in female Asian elephants, but captures the most important, luteal phase. The value of this method, to monitor reproductive status in wild elephants has also been reported from South Africa (Freeman *et al.* 2007) and India (Ghosal *et al.* 2010). Our method is satisfactory to identify

whether a given female is regularly cycling and to recognise when she approaches oestrus. The length of the reproductive cycle and follicular and luteal phases reported herein are similar to published values (Hess *et al.* 1983; Plotka *et al.* 1988; Hodges 1998).

In PEO, the females were kept as a herd during daytime, and that could be a reason for the high birth rate and high number of reproductively active females. It is clear that herd living favours being reproductively active also when the BU elephants are considered, because all four were active, and they too were managed as a herd. The privately owned females in Sri Lanka are managed as non-herd animals, and they have never been pregnant. Privately owned female elephants in Sri Lanka are mostly owned by individuals. The owners do not invest in breeding, possibly because it reduces their earning capacity until calves are weaned. Captive elephant populations in general have not been self-sustaining due to problems related to captive breeding (Sukumar 2003). Most captive elephants are managed as non-herds and therefore social interactions are minimal. In such situations, even if there is a successful birth, a cow might reject or kill the calf due to inexperience because, this highly social animal learns most of the postnatal behaviour from other elephants (Sukumar 2003). Therefore, our results indicate the importance of managing captive elephants in herds for keeping them reproductively active. In North America, monitoring of reproductive hormones is done regularly, resulting in many successful attempts of captive breeding (Brown *et al.* 2016). If captive breeding is to be promoted in Sri Lanka



**Figure 3.** Reproductively active and inactive females in Sri Lanka and USA.

using privately owned elephants, hormone level monitoring in faeces can be used.

According to Brown *et al.* (2004c), the continuous cycling of females, which are not bred, may have a negative cumulative effect on reproductive health. Further, the reproductive potential may be affected due to heavy workload (Sukumar 2003) and lack of breeding (Hermes *et al.* 2004). Additionally, Hermes *et al.* (2004), state that reproductive disorders may be prevented either by natural breeding or by assisted reproduction (*viz.* AI). Most Sri Lankan privately owned captive females have not been bred early in their lives and therefore, they could be acyclic or their breeding capacity may have been negatively affected. Thus, before the privately owned captive numbers decline to non-recoverable levels, breeding efforts should be initiated. If adequate numbers of births among captive elephants are to be maintained in Sri Lanka among privately owned females, it is essential to monitor their reproductive status while implementing a sound breeding policy.

In our study, there was a 65 years old female (Anusha from PEO) who exhibited normal reproductive cycles, which suggests that there is no reproductive senescence in some female Asian elephants. There were 4 females aged above 50 in CEC (Jewel, 57; Susan, 57; Lutzi, 59; Mysore, 63), who have never been managed in a herd and were not cycling. Anusha is managed in the herd in PEO in which favourable social exposure for breeding is present, which could be the reason for her to be cycling. Lack of reproductive senescence in elderly female elephants has been previously reported by Brown *et al.* (2016). Therefore, non-herd management could possibly be a reason for captive females to be acyclic or show irregular cycling patterns.

Our results suggest that the role of environmental conditions in maintaining cyclicity in female elephants is minimal, since a substantial number of females in the USA (not an elephant range country), were reproductively active. This is in agreement with Freeman *et al.* (2009) who show that travelling among facilities with different environmental conditions had no significant

effect on reproductive health in elephants. Faecal sampling is convenient, non-invasive and does not require assistance of professional elephant handlers. Therefore, it may be applied to wild females also, if they could be tracked to collect serial samples.

### Acknowledgements

We thank Feld Entertainment Inc. for funding this research and the staff of Ringling Bros. Center for Elephant Conservation and their travelling Blue Unit circus for the assistance provided. We also thank University of Peradeniya, Sri Lanka and Dr. Chandana Rajapakse of Pinnawala Elephant Orphanage, Sri Lanka for kind cooperation during the study period. Inputs from Dr. Elizabeth Freeman and Crystal Eng are highly appreciated in developing the methods in this study.

### References

- Brown JL (2006) Reproductive endocrinology. In: *Biology, Medicine and Surgery of Elephants*. Fowler ME & Mikota SK (eds) Blackwell Publishing, Ames, IA. pp 377-388.
- Brown JL, Göritz F, Pratt-Hawkes et al. (2004a) Successful artificial insemination of an Asian elephant at the National Zoological Park. *Zoo Biology* **23**: 45-63.
- Brown JL, Olson D, Keele MN & Freeman EW (2004c) Survey of the reproductive cyclicity status of Asian and African elephants in North America. *Zoo Biology* **23**: 309-321.
- Brown JL, Paris S, Prado-Oviedo NA, Meehan CL, Hogan JN, Morfeld KA, & Carlstead K. (2016) Reproductive health assessment of female elephants in North American zoos and association of husbandry practices with reproductive dysfunction in African elephants (*Loxodonta africana*). *PloS ONE* **11**: e0145673.
- Brown JL Walker S & Steinman K (2004b) *Endocrine Manual for Reproductive Assessment of Domestic and Non-domestic Species*. Conservation & Research Center, Smithsonian's National Zoological Park, Front Royal, Virginia.

- Fernando P, Jayewardene J, Prasad T, Hendavitharana W & Pastorini J (2011) Current status of Asian elephants in Sri Lanka. *Gajah* **35**: 93-103.
- Freeman E, Abbondanza F, Meyer J, Schulte B & Brown J (2007) Use of a fecal field kit to monitor hormone activity in free-ranging elephants. In: *Proceedings of International Elephant Conservation & Research Symposium*. International Elephant Foundation, Orlando, FL.
- Freeman EW, Guagnano G, Olson D, Keele M & Brown JL (2009) Social factors influence ovarian acyclicity in captive African elephants (*Loxodonta africana*). *Zoo Biology* **28**: 1-15.
- Freeman EW, Abbondanza FN, Meyer JM, Schulte BA & Brown JL (2010) A simplified method for monitoring progestagens in African elephants under field conditions. *Methods in Ecology and Evolution* **1**: 86-91.
- Ghosal R, Sukumar R & Seshagiri PB (2010) Prediction of estrus cyclicity in Asian elephants (*Elephas maximus*) through estimation of fecal progesterone metabolite: Development of an enzyme-linked immuno-sorbent assay. *Theriogenology* **73**: 1051-1060.
- Hermes R, Hildebrandt TB & Goritz F (2004) Reproductive problems directly attributable to long-term captivity—asymmetric reproductive aging. *Animal Reprod. Science* **82-83**: 49-60.
- Hess D, Schmidt A & Schmidt M (1983) Reproductive cycle of the Asian elephant (*Elephas maximus*) in captivity. *Biology of Reproduction* **28**: 767-773.
- Hodges JK (1998) Endocrinology of the ovarian cycle and pregnancy in the Asian (*Elephas maximus*) and African (*Loxodonta africana*) elephant. *Animal Reproduction Science* **53**: 3-18.
- Hunt KE & Wasser SK (2003) Effect of long-term preservation methods on fecal glucocorticoid concentrations of grizzly bear and African elephant. *Physiological and Biochemical Zoology* **76**: 918-928.
- Pinheiro J, Bates D, DebRoy S, Sarkar D & R Core Team (2017) *nlme: Linear and Nonlinear Mixed Effects Models*. R Package Version 3.1-131.
- 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. *Biology of Reproduction* **38**: 309-314.
- R Core Team (2017) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.
- Schmitt DL, Hildebrandt TB, Hermes R & Goritz F (2001) Assisted reproductive technology in elephants. In: *Proceedings of 1st International Symposium on Assisted Reproductive Technology for the Conservation and Genetic Management of Wildlife*. Henry Doorly Zoo, Omaha, Nebraska. pp 2.
- de Silva SGNT, Fernando PRNK, Perera KA, Ariyaratne HBS, Pushpakumara PGA & Wijayagunawardane MPB (2009) Relationship between blood and faecal progesterone profiles in Sri Lankan elephant (*Elephas maximus maximus*): A non-invasive method for monitoring the oestrous cycle. In: *Proceedings of 61<sup>st</sup> Annual Convention of the Sri Lanka Veterinary Association*, Sri Lanka Veterinary Association. pp 34.
- Sukumar R (2003) *The Living Elephants: Evolutionary Ecology, Behavior and Conservation*. Oxford University Press, New York.
- Wijesinghe SK, Wijayagunawardane MPB, Perera KA & Dangolla A (2002) Cyclic changes in serum progesterone levels in the domesticated she elephant. In: *Proceedings of 54<sup>th</sup> Annual Convention and Scientific Sessions of the Sri Lanka Veterinary Association*. Plant Genetic Resource Centre, Gannoruwa, Peradeniya, Sri Lanka. pp 19.