Non-Invasive Endocrine Profiling of Captive Asian Elephants (Elephas *maximus*) in Two South Indian Zoos

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Abstract:

Asian elephant (*Elephas maximus*) is an endangered species (IUCN 2009). It is crucial to breed elephants in captivity so that captive stocks do not need to be supplemented by removing animals from the wild. Unfortunately, captive breeding programs across the globe met with limited success and therefore more effort is needed to improve breeding in captivity. Endocrine profiling can help us understand the physiology of the captive elephants and help us direct our efforts to improve breeding in captivity. Non-invasive fecal sampling has been carried out to assess ovarian cyclicity of six female captive elephants at Nehru zoological park, Hyderabad and Mysore zoological park. Four of them showed ovarian cyclicity. Two male elephants were also monitored for assessment reproductive status and three musth sessions were recorded in one of the elephants over a period of one year. Cortisol and Testosterone concentrations were also measured during the musth period to understand the dynamics of cortisol and testosterone during Musth. No significant correlation was found between cortisol and testosterone during musth but elevated cortisol levels were observed during the period of musth. This study will help in building better breeding strategy and in turn improve breeding in captivity.

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P.Vivekananda Reddy

Introduction:

Asian Elephant (*Elephas maximus*) is listed under endangered species (IUCN 2011) with both *ex situ* and *in situ* populations decreasing at an alarming rate. Its survival is under threat because of continuous poaching, loss of habitat and corridor due to rapid fragmentation of the original habitat. The captive Asian elephants constitute 22–30% of the Asian elephants (Lair et al., 1997; Sukumar et al., 2003) and are indispensable workforce for forest departments, tourism and other religious purposes. The captive elephants are managed by traditional knowledge and skills of *mahouts*. Over the years, the quality of *mahouts* available has declined due to the lack of comparable economic benefits and improper welfare measures owing to the dwindling importance of captive elephants (MoEF 2004, Vanitha *et al.,* 2009), which in turn affects the management of captive elephants (Vanitha *et.al.,* 2009). Unfortunately, captive breeding in these populations has not been successful, thus making it difficult to keep the captive populations alive and self sustaining (Rees, 2003). So, captive populations are regularly supplemented with elephants caught from wild, adding further pressure on struggling wild population. Keeping this in mind, we need to understand the importance of assisted reproductive technologies in breeding of captive elephants (Sukumar *et al.*, 2006). In spite of continuous efforts by conservation authorities, captive breeding in elephants has met with limited success. Less than 20% of Asian and 10% of African elephants of reproductive age have given birth to offspring in captivity (Asian Elephant Studbook, 2000, 2001). This is mainly attributed to ovarian inactivity and acyclicity in captive female elephants (Keay *et al.,* 2006). The knowledge of status of ovarian activity can help us to make the breeding program more effective. The main reasons behind ovarian acyclicity are thought to be chronic stress, reproductive pathologies, neoplasias etc (Millspaugh *et al.,* 2004).

In general, stress is usually regarded as any condition that threatens the homeostasis of the animal. Although no single biochemical assay can really assess stress, the

measurement of hormones like ACTH, glucocorticoids, catecholamines, prolactin, etc., can help us understand the physiological status of the animal (Mostl *et al.,* 2002). These hormones are released in stressful conditions to help the organism defend itself against the stressor. Although short term release of these hormones is known to increase the fitness via energy mobilization but long periods of cortisol is known to decrease fitness by reproductive failure, immuno-suppression and muscular atrophy and behavioral stereotypes. After cortisol is released in the cortex of the adrenal glands, about 90% of the cortisol is bound to corticoid binding protein (CBG), 6% to albumin and about 4% of the cortisol is free and available for target cells. Since cortisol is lyophylic and smaller in size it can diffuse passively across cells and thus is available in all bodily fluids (Bayazit *et al.,* 2009)

Although how stress affects reproduction is not clearly understood it is proposed that increased activation of hypothalamo-pitutary-adreno axis results in decreased Luteinizing Hormone (LH) secretion thus affecting reproduction. So measurement of cortisol can be potential indicator of stress (Wingfield *et al.,* 2002). Various studies have measured cortisol from blood, saliva, urine and feces and used it as indicator of stress (Bayazit *et al.,* 2009, Menargues *et al.,* 2008). Measurement of cortisol from blood can provide a snapshot of the physiological status of the elephant. But regular sampling can be difficult as the animal has to be trained and must be physically restrained. Because of the stress of physical restraint the cortisol levels can raise and thus provide a false picture of the underlying physiology. Measurement of hormone activity from urine and faeces can provide an alternate non- invasive measure and also provide a 'pooled' measure of the gonadal activity. This also minimizes the effect of pulsatile and diurnal variations of many circulating hormones. The ease of collecting fecal samples compared to urine and saliva has also popularized the technique (Mostl *et al.,* 2002).

The female elephants usually show estrous cycles of 13-17 weeks duration with 4-6 weeks follicular phase and 8-10 week luteal phase. The circulating progesterone metabolized into metabolites belonging to the family of 5α pregnanes like 5α-pregnane-3, 20-dione and 5α-pregnane-3-ol-20-one (Wasser *et al.,* 1996). It has been measured

that approximately 55% of these metabolites are excreted in the faeces and also correspond to serum analyses with a 2-day lag time. So monitoring of 5α-pregnanes can help us understand the cyclicity status of the females. A unique phenomenon observed in elephant among other mammals is the occurrence of 2 LH surge (i.e. anovulatory and ovulatory LH surges) with in duration of 20 days during follicular phase (Brown *et al.,* 1999). Recent studies have shown that anovulatory LH surge determines the timing of the ovulatory LH surge marking the appropriate time for release of the egg. Variation in duration and timing of the cycle is also shown to be present because of varying climatic conditions. Hot, humid climate is proposed to alter activity of HPA or HPO axes during early follicular phase resulting in lower GnRH levels and thus delayed anovulatory LH surge (Tilbrooke *et al.,* 2000, Thitaram *et al.,* 2007).

Various studies have attempted to understand the physiological and behavioral differences between cycling and acycling elephants (Freeman *et al.,* 2004). Ovarian activity is thought to be mediated by social influences. The non- cycling elephants are found be usually dominant in the hierarchy (Freeman *et al.,* 2010). The patterns of thyroid hormones (Thyroid stimulating hormone, TSH; free and total Thyroxin; T4 and T3) and Cortisol are found to be similar between cycling and acycling elephants. Their endocrine profiles varied only in patterns of FSH, prolactin and estradiol. African elephants show elevated prolactin levels during the follicular phase which is not observed in Asian elephants (Brown *et al.,* 2004).

However, successful non-invasive assessment of ovarian cyclicity can be done by measurement of concentration of Progesterone metabolites (5ß-preganes, 5α-pregnanes, etc) in fecal matter (Wasser *et al.,* 1996). In this study, ovarian activity was assessed based on monitoring the concentrations of Progesterone metabolites in the fecal samples. Knowing the status of the ovarian activity will help us in directing our efforts in captive breeding and reproductive health.

A free ranging bull matures at the age of 15yrs. After which it departs from its maternal group in search of new groups and resources *(Poole et al.,* 1987; 1989b*)*. Occasionally, Male elephants enter in to a sexually active mode known as Musth. Although timing of musth is not the only breeding season but this sessions are usually accompanied with

high testosterone levels, heightened aggression and predominate association with females, temporal gland secretion and continuous urine dribbling (Poole *et al.,* 1987*)*. The duration of musth and the level of sexual activity are shown to be dependent on the availability of females and many other factors *(*Rasmussen *et al., 2005)*. Musth is believed to an alternative reproductive strategy given that 20-25% of the reproductive success in elephants is attributed to sexually active non- musth bulls *(*Rasmussen *et al., 2005)*. During Musth, the male spends most of its time searching for a femate and less time in feeding resulting in significant weight loss (Poole *et al., 1982)*. These conditions are believed to cause stress to the animal but however the association between glucocorticoids and musth is not clearly understood (Ganswindt *et al.*, 2003).

Musth in free ranging bulls starts from the age of 25-30 whereas it is shown to begin at around 13yrs of age, in captivity (Rasmussen *et al*., 2008). However factors contributing to such early musth sessions in young bulls are not clearly understood. But it is proposed that early access to females and lack of competition may be the causes. Introduction of older males is known to suppress musth and aggressiveness in these younger males in captivity (Bradshaw *et al.,* 2005). During musth, management of such bulls in facilities like zoos is a problematic. So such males are restricted with chains and are kept isolated all throughout the musth period which is a huge waste of reproductive resources. Understanding the changes involved during musth session and prediction of musth in advance could immensely help in management of these bulls and breeding in captive conditions. In this study, three musth sessions were observed and effort has been made to understand the dynamics of Cortisol and Testosterone during musth.

Materials and Methods

Sample Collection

Fecal samples were collected for once in 3 days from the elephants in Nehru zoological park and Mysore Zoological Park in the morning at around 7am and are stored at -30^{o}C within an hour of sample collection. To avoid sampling error, the feces was well mixed (with gloves) before collecting ~50g in a zip lock bag. All necessary precautions to avoid contamination are taken. Details of elephants under study are briefed in Table 1.

Table 1. Details of name, age, sex and physical health of the study animals in Mysore and Hyderabad zoological parks.

S.No	Name	ID	Sex	Age	Physical rating	Duration of sample collection
1	Jamuna	NZP_01	Female	38Y	3	July 2010 to Dec 2011
2	Asha	NZP_03	Female	39Y	3	July 2010 to Dec 2011
3	Vanaja	NZP_04	Female	30Y	3	July 2010 to Dec 2011
4	Airavati	MZP_03	Female	8Y	4	Oct 2011 to Dec 2011
5	Gajalakshmi	MZP_04	Female	32Y	5	Oct 2011 to Dec 2011
6	Padmavati	MZP_05	Female	57Y	4	Oct 2011 to Dec 2011
7	Abhi	MZP_07	Male	7Y	4	Oct 2011 to Dec 2011
8	Rama	MZP_08	Male	17Y	2	Oct 2011 to Dec 2011
9	Vijay	NZP_05	Male	28Y	3	July 2010 to Dec 2011

The physical rating of the animal was given on the scale of 1 to 5(worst =1 and best= 5) based on the rating used and described by Foley et.al (2000):

1. Animal emaciated, as exhibited by clearly protruding bone structures around face, ribs, ileum and pelvis

2. Ribs no longer visible, but depression around wing of ileum and lumbar region clearly apparent, highly concave skin on pelvis area

3. Depression around wing of ilium and lumbar depression clearly visible, with skin on pelvic area shallowly concave

4. Lumbar depression flat or broadly con- vex and wing of ilium barely visible

5. Scapular and pelvic bones not visible

Extraction of Steroid metabolites:

The fecal samples were extracted according to the procedure described in Gandswindt *et.al*., (2005) with some modifications. The sample stored at -30^{o}C is thawed and dried at 70^{o}C overnight. The dried sample were pulverized and sieved before collecting 0.2g of fecal sample in 15ml Tarson tube for extraction. 5ml of methanol was added to the sample and vortexed for 20mins. After this, samples were centrifuged at 4000rpm followed by collection of supernatant in 5ml plastic cryovials which are stored at -30^{o}C and later used for quantification.

Quantification of Steroid metabolites using indirect competitive ELISA

ELISA Protocol for quantifying any steroid metabolite:

1. The NUNC 96 well maxisorp high binding plate is coated with 50ul of diluted antibody (antibody specific to that steroid metabolite) in each well and is kept at 4^{o}C overnight in an airtight humid container. Antibody dilutions are made with Coating buffer (0.05M Sodium bicarbonate buffer at pH 9.6).

2. On the following day the plate is washed with Wash buffer (0.15M NaCl, 0.05% Tween 20) for 4 times and is dried on a paper towel. Meanwhile 50µl of each diluted samples and standards are mixed with 50µl conjugated HRP (specific to that steroid). All the sample dilutions and standard dilutions are made with EIA buffer (i.e. 0.1 M Phosphate Buffered saline containing 0.1% BSA at pH 7.0).

3. After the plate is dried, these mixed samples are added to the wells. All the samples were added within 10mins to reduce intra assay variation. After addition of samples, the plate is incubated at 37^{o}C for two hours. After incubation the plate is washed again and any unbound antigens in the samples or conjugated HRP will be washed away.

4. After Incubation for 2hrs, 50µl of TMB/H_2O_2 (substrate to HRP) is added. Activity of HRP on the TMB substrate results in formation of blue color. The plate is incubated at room temperature until an absorbance value of 0.8 to 1.0 is reached. Then 50ul of 1N Hydrochloric acid (HCl) is added to each well to stop the activity of HRP. Addition of HCl results in yellow color in wells and accordingly absorbance is measured at 450nm Multiscan IT spectrophotometer.

5. Based on the absorbance values obtained from the known concentrations a standard curve is plotted. Based on Beer lamberts law, this standard curve is used to obtain the concentration of the unknown samples.

The standard ELISA protocol validated for steroid metabolite estimation by Graham *et al.* (2001) is used to quantify progesterone metabolite (P4), Cortisol and testosterone from the extracted samples. The Progesterone (CL425-mono clonal), Cortisol (R4866) and Testosterone (R156/7) antibodies and conjugated HRP's for ELISA are commercially available and are obtained from Dr. Caroline Munro, University of California Davis.

Standardization of the protocol:

Based on standardization curve, checker board ELISA and parallelism curves obtained, the dilutions of antibody, conjugated HRP and fecal extracts are given in Table 2. The sensitivity of cortisol assay is 0.97pg/well at 90% binding. The sensitivity of progesterone metabolite (P_4) is 0.19pg/well at 90% binding. The sensitivity of Testosterone metabolite is 1.1pg/well at 90% binding.

Table 2. Details of dilution of antibody, HRP and sample extracts used in ELISA

For quantifying	**Progesterone**	**Cortisol**	**Testosterone**
Antibody Dilution	1: 6000	1: 9000	1: 10000
Conjugated HRP dilution	1: 100000	1: 250000	1: 200000
Sample dilution	1: 20	1: 2	1:8

Data Analysis:

After obtaining all the absorbance reading from the spectrophotometer, the standard curve is fitted on excel using semi log plot. Later concentration of steroid metabolites was calculated based on the equation of the best fit line. Regression values for all the standard curves is always greater than 0.95. In case of lower regression values, the plate is repeated to obtain better results. The Intra-assay variation and Inter-assay variation are 4% and 11% respectively.

RESULTS

Sample collection and extraction

A total of 1200 samples were collected from six females and two males held captive at Nehru zoological park, Hyderabad and Mysore zoological park, Mysore over a period of four to 12 months. Of which 808 samples were extracted for fecal progesterone, testosterone and cortisol analysis using ELISA developed in-house. The details of results of various assays are given below.

Assessment of ovarian cyclicity using progesterone metabolites

For monitoring ovarian cyclicity, a total of 426 fecal extracts from six females were used for fecal progesterone assays. Due to non-availability permission the sample duration ranged from 4 month to 12 months resulting changes in duration of monitoring individuals. Fecal samples from female Asha held in captive at Hyderabad could be collected over a period of 12 months where as females (Gajalakshmi, Iravathi, and Padmvati) could be collected for four months.

The fecal progesterone values ranged from a minimum of 5 ng to a maximum of 1200 ng / gm, where as non-cycling females it ranged from 3 - 250 ng/g. Of the six females monitored 4 females (Asha, Gajalaksmi, Iravati and Vanaja) showed a clear ovarian cyclicity which is evident from in the fecal progesterone values. Of the three females monitored in Mysore Zoo, two of them were cycling together. A female elephant named Asha showed two clear cycles of luteal phase followed by follicular phase (Figure 5.1). Similarly Gajalakshmi, Iravati, and Vanaja have also showed one cycle each (Figure 5.2 A, B, C and D). The average length of luteal phase of these females was 14 weeks and it ranged from 10 to 19 weeks. The mean fecal progesterone value during the peak of the luteal was 1050 ng/g and it ranged from 1000 to 1200 ng/g. The base line progesteone value of cycling females ranged 47 to 130 ng/g while the non-cycling females' value ranged from 3 to 140 ng/g. Base line fecal progesterone values did vary significantly between cycling and non-cycling female during the study period.

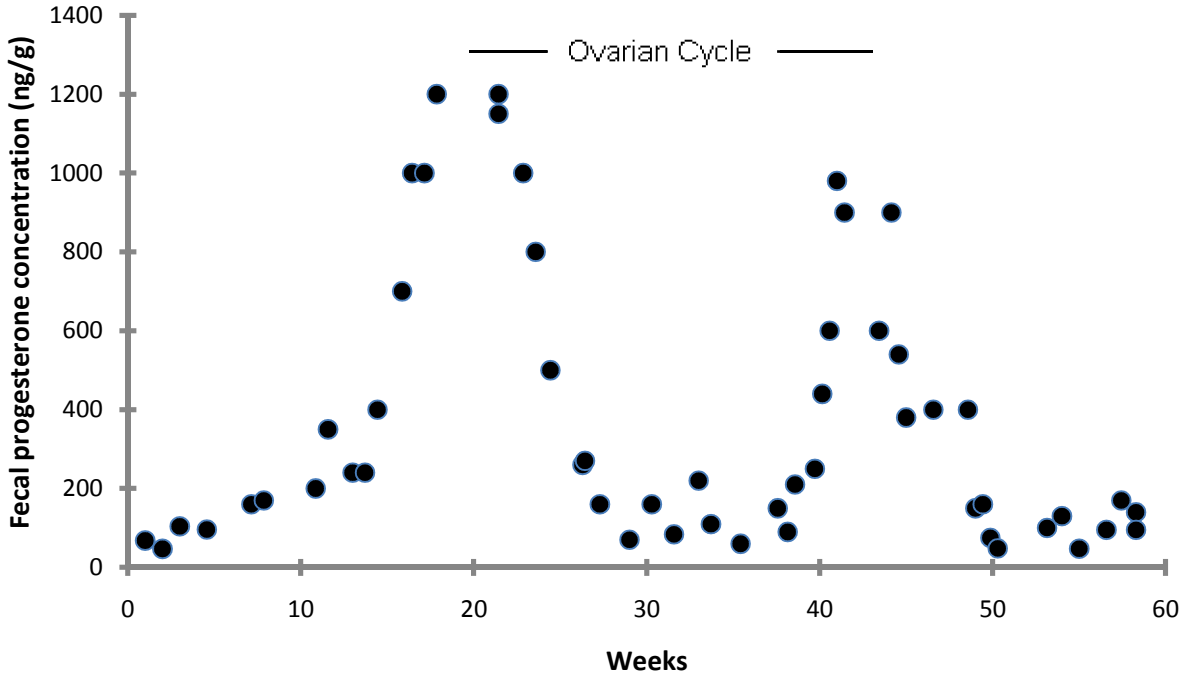

Figure 5.1 Weekly fecal progesterone concentrations in female elephant Asha

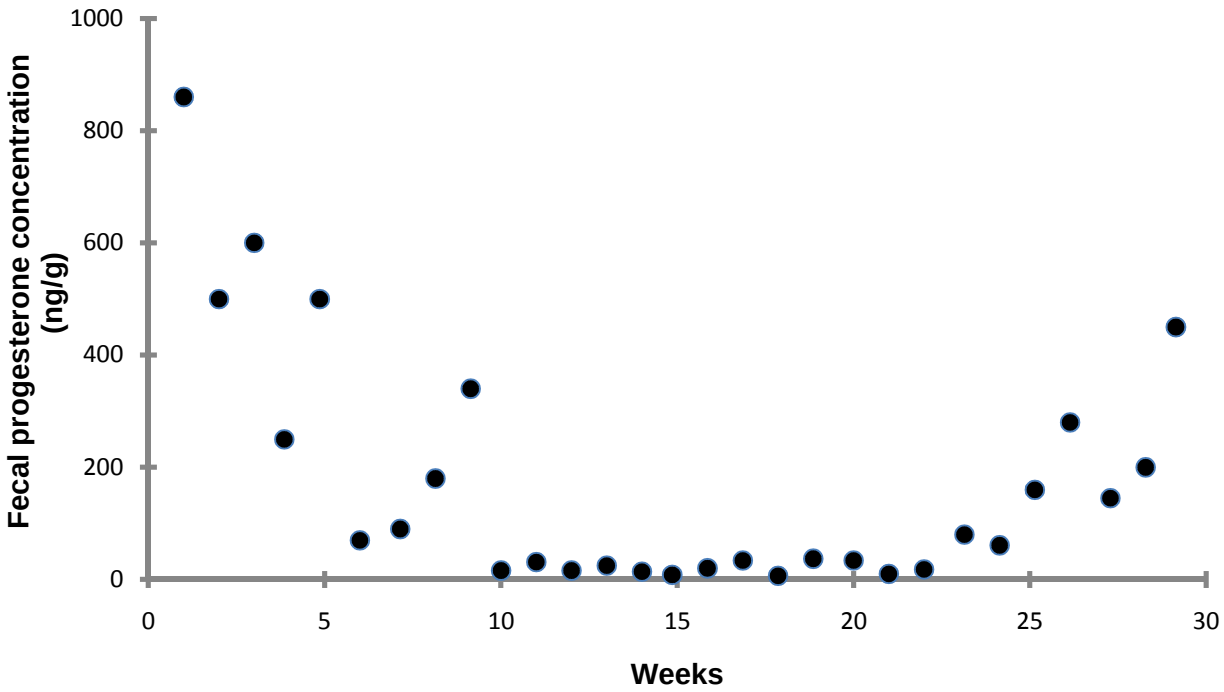

Figure 5.2A. Weekly fecal progesterone concentration in female elephant Vanaja

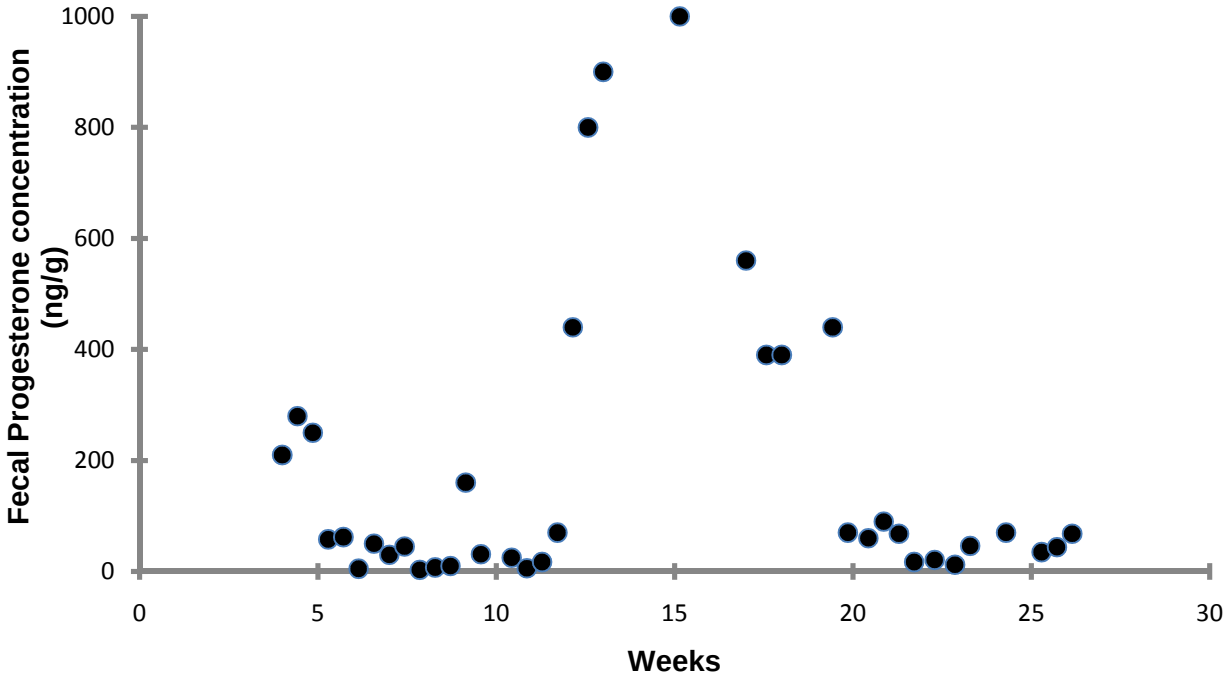

Figure 5.2B. Weekly fecal progesterone concentration in female elephant Gajalakshmi

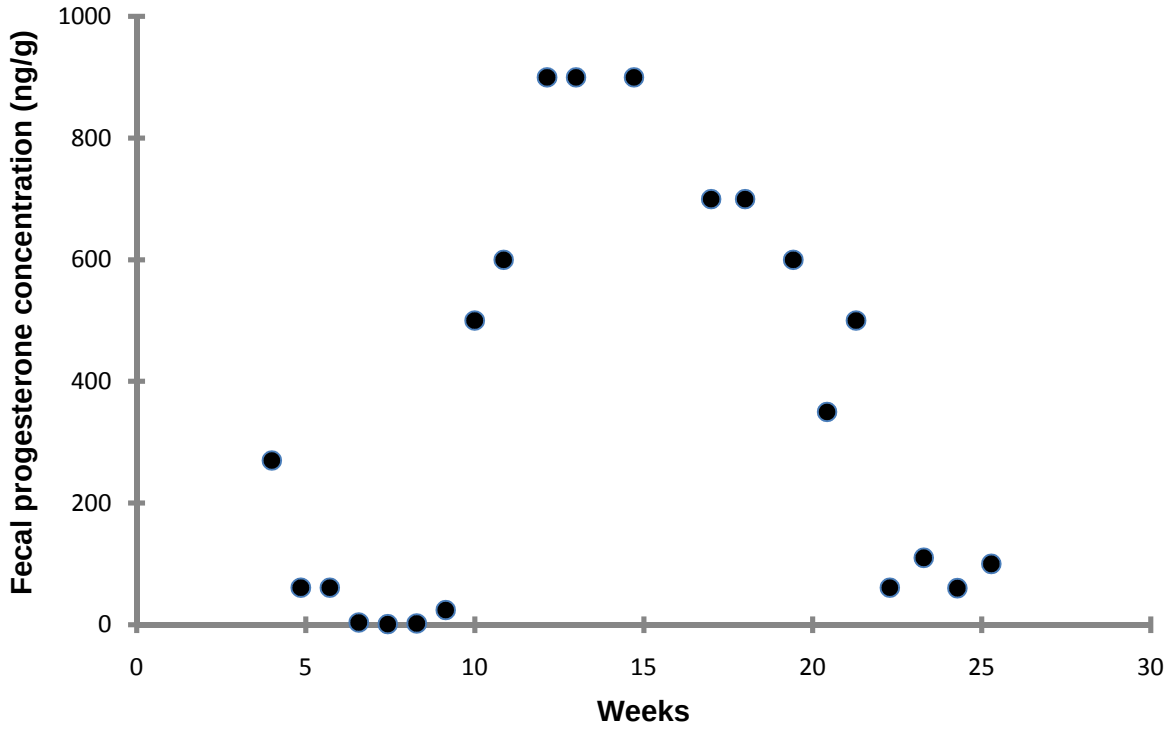

Figure 5.2C Weeekly fecal progesterone concentration in Airavati

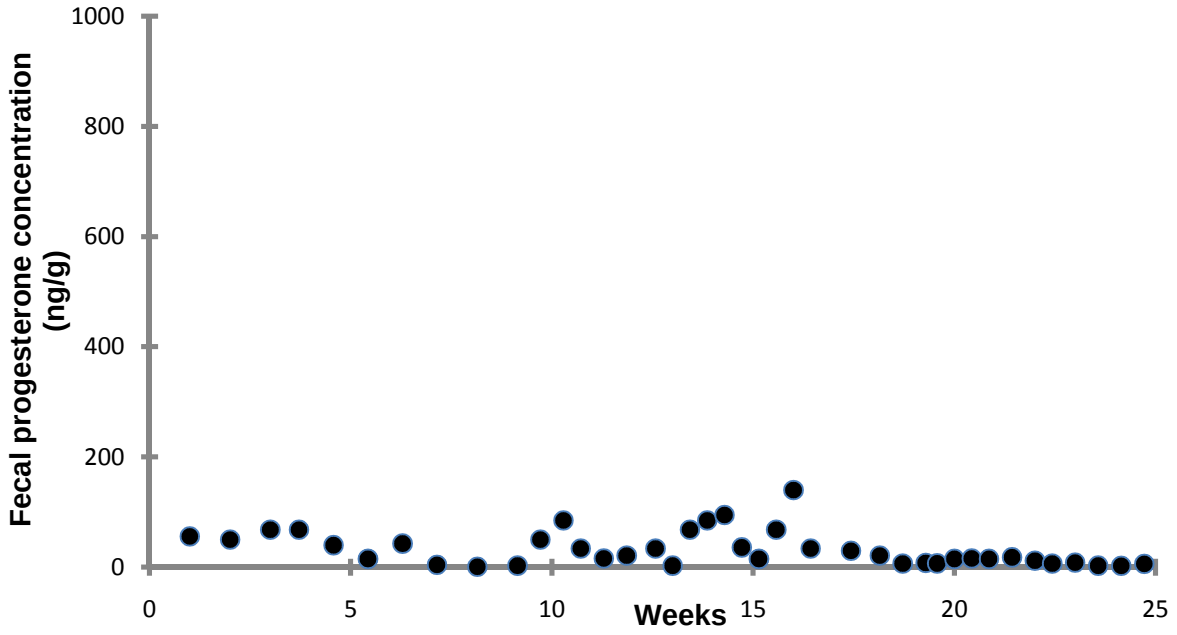

Figure 5.3A. Weekly fecal progesterone concentration in Jamuna

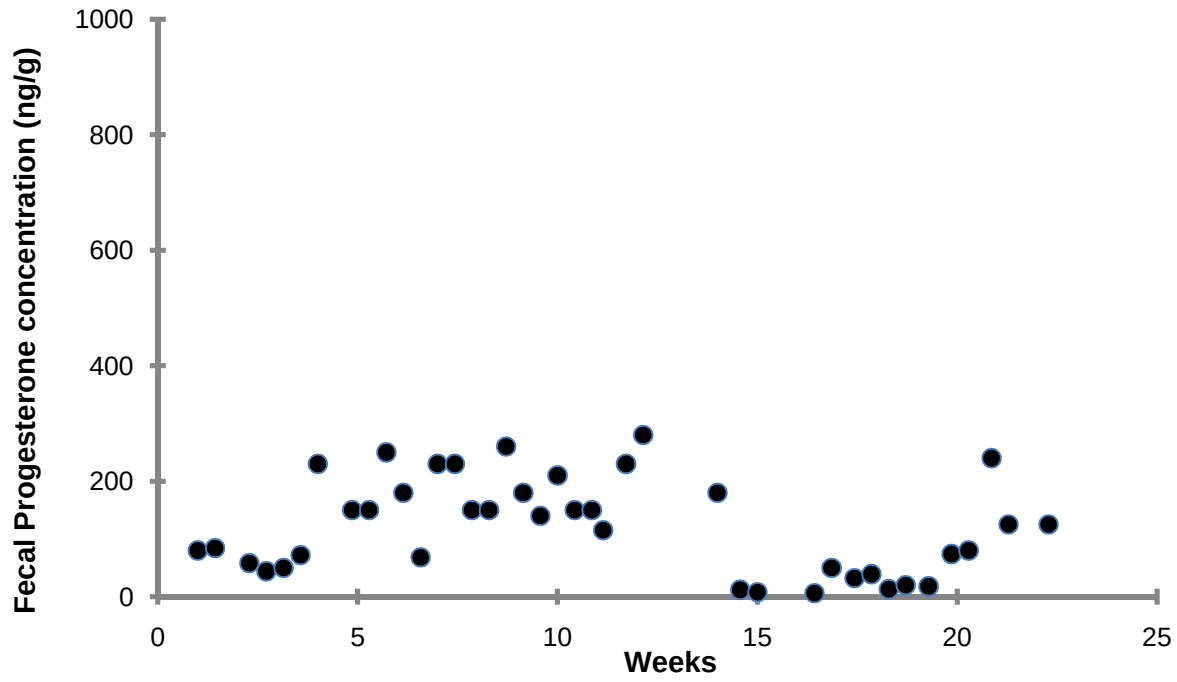

Figure 5.3B. Weekly fecal progesterone concentration in Padmavati

Fecal testosterone estimation:

A total of 400 fecal samples were collected from two males (one each from Mysore and Hyderabad zoos) and the samples were extracted for fecal testosterone analysis. The male, Vijay from Hyderabad zoo showed three clear peaks of testosterone during 12 months period (October 2010 to December 2011) while Rama from Mysore zoo did not show any clear peak of testosterone (Figure 5C) during the period. Testosterone concentrations ranged between 10ng/g and 100ng/g during non-musth period. During the musth period testosterone concentration elevated significantly and levels ranged between 150 and 450 ng/gm. Following testosterone peaks, the temporal gland secretion was observed in Vijay. Rama the other male did not show clear pattern in testosterone and temporal gland secretion. Duration of temporal gland secretion for Vijay ranged from 15 days to 45 days along with musth related behavior such as aggression, urine dribbling, etc., was observed.

Fecal cortisol estimation:

Fecal cortisol was estimated for both males (n=3) and females (n=3) using over 400 fecal samples to study physiological stress in these captive animals. Overall individual mean cortisol ranged from 2.4 to10 ng/g and the lowest was observed in Rama (male) and the highest in Asha (female). A weak negative correlation ($r = 0.22$, $n=22$) was observed between testosterone and cortisol during musth period in Vijay. However, overall cortisol concentrations were higher during musth period (Figure 5.3). No significant correlation was observed in the females.

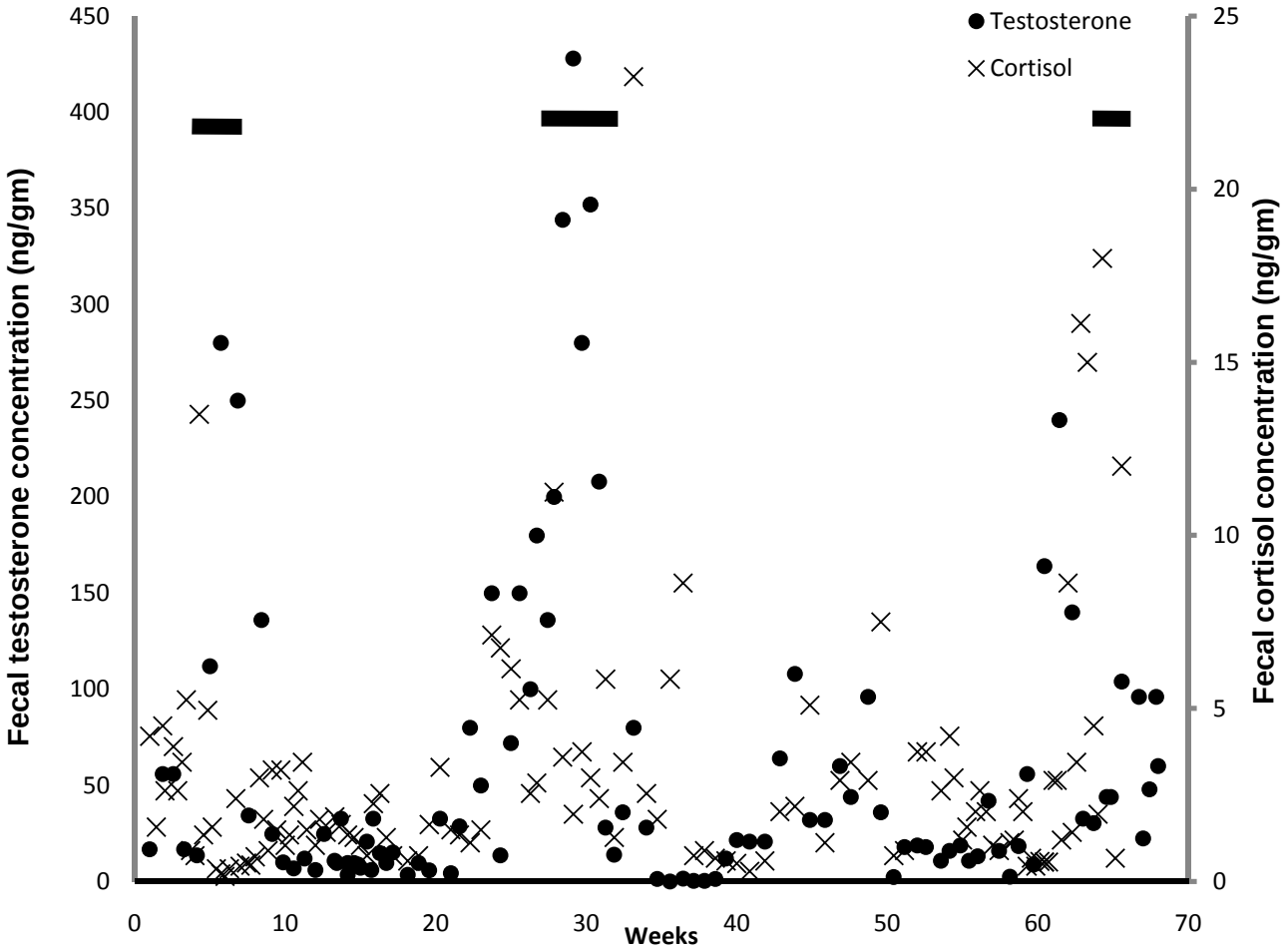

Figure 5.4. Fecal testosterone and cortisol profiles of the male elephant, Vijay. The dark bars (▬) indicate the periods of musth

Discussion:

This is the first report on non-invasive reproductive and stress monitoring in captive Asian elephants in Indian Zoos. About 800 fecal samples from six females and two males were used for estimating fecal progesterone, testosterone and cortisol metabolites using ELISA developed and standardized by LaCONES, CCMB. Of the six females, at least four of them showed reproductive cycle. Further, it has been demonstrated again that fecal hormone could be used effectively to monitor reproductive cycle and stress monitoring. Two thirds of the captive elephants under study are found to be cycling. A similar observation was found in captive African elephant in North American zoos (Brown *et al.,* 2004). Two out of six elephants are found to be non-cycling. One of the two non-cycling elephants, Jamuna, is of reproductive age (i.e. 38 years) and healthy. Whereas, the other non-cycling elephant, Padmavati (57 years old), is also in good health.

The length of observed reproductive cycle in the four cycling elephants ranged from 18 to 25 weeks with a mean of 20 weeks. Of the six female elephants, Gajalakshmi and Airavati showed a normal cycling with an average length of 18 weeks, consistent with published literature (Ghosal *et al.,* 2011, Brown *et al.,* 2004). The other cycling elephants showed longer estrus cycles with one of the cycles lasting up to 25 weeks. The longer estrus period can be because of difference in environmental conditions (Thitataram *et al.,* 2007), age and stress (Tilbrooke *et al.,* 2000). All the cycling females at Hyderabad zoo exhibited a longer estrus cycle compared to females of Mysore zoo which showed normal cycle length. The longer estrus period in elephants at Hyderabad zoo can be because of lack of social structure and poor conditions leading to stress (Bradshaw *et al*., 2005). These elephants at Hyderabad zoo are always kept bound with chains and are never allowed to interact with each other unlike the elephants at Mysore which are allowed to range and interact with other individuals during day. Apart from that, most of the females at Mysore zoological park are genetically related to each other so their group relatedness is very high compared to elephants at Hyderabad zoo. This high group relatedness can also be potential factor behind the normal cycling of

elephants at Mysore zoo (Gobush *et al*., 2008). However a longer study might further improve our understanding about the influence of this factor on reproductive cycle.

Of the two non-cycling elephants, Padmavati of age 57 might have entered post reproductive life as part of aging. She has given birth twice earlier at the age of 20 and 51 years which supports our reasoning that the elephant might have entered post reproductive stage as part of its life history. While the second one, Jamuna of age 37, is of reproductive age but is non-cycling which might be due to stress or any reproductive pathology (Millspaugh *et al.,* 2004). All the three elephants from this facility are showing either no cycles or abnormal cycles, which needs to be studied further investigated.

Three sessions of musth were observed during this study but the length of the musth varied significantly from each other and is not correlated with the amount of testosterone. The length of musth is shown to be dependent on environmental conditions, access to females, age of the individual etc. (Gangswindt et al., 2004). Factors like access to females and resources may not have changed significantly during this study; however, different environmental conditions could be the major factor behind this difference in lengths. Although testosterone and cortisol are not found to be significantly correlated but the amount of cortisol is higher during musth seasons.

Although the exact dynamics of cortisol and testosterone are not clearly understood, but one possible hypothesis can be that bulls tend to be more aggressive during Musth compared to non- musth periods, which makes it difficult for the mahout to control the elephant, so, they are always kept bound. Apart from that, Musth is usually accompanied with significant weight loss because of reluctance of animal to consume food. Normally an elephant spends 90% of the time in feeding. So, reluctance to feed can be stressful and can result in elevated cortisol. Gangswindt *et al.* (2003) did not find any significant rise in cortisol during musth in African elephants. So this may be case-dependent and factors contributing to the rise in cortisol may not directly relate to the musth. The elevation in cortisol can be because of the differences in management practices and may be dependent on individual differences. Further, a detailed study is required to understand the exact the causes of this elevated cortisol during musth.

This study once again highlights the importance of assessing ovarian cyclicity for an effective breeding program. The difference in average lengths of the cycles in individuals can be because of difference in group relatedness or because of difference in management of elephants in these two facilities. Further detailed investigation required to understand the impact of these factors on captive elephants. Non- cycling elephants can be suffering from reproductive pathologies or may have gone acyclic because of stress which needs to be further investigated in order to take essential steps to avoid such reproductive failures in future. Various studies have shown different patterns of cortisol during musth and the dynamics of cortisol and testosterone during musth are not clearly understood. The variation of cortisol can be case dependent and may depend on the outcome of the interaction between the musth related signs and environmental conditions. Further behavioral and endocrine monitoring can help us in understanding the dynamics between cortisol and testosterone better.

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