Assessment of estrus cyclicity in the Asian elephant (*Elephas maximus*) by measurement of fecal progesterone metabolite 5α-P-3OH, using a non-invasive assay

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Reproductive management of the Asian elephant (*Elephas maximus*) is important for its conservation. To monitor its estrous cyclicity, we earlier used an indirect ELISA to show that levels of fecal progesterone (P₄)-metabolite (allopregnanolone: 5α-P-3OH) in semi-captive females sampled randomly positively correlated with serum P₄ levels [12]. In this longitudinal study (51 weeks), we measured levels of fecal 5α-P-3OH and serum P₄ in seven semi-captive female elephants. Females exhibited three types of hormonal profiles. Four females showed cyclical patterns of fecal 5α-P-3OH and serum P₄ typical of normal estrous cycles, two showed acyclic pattern while one showed high values indicative of a pregnant animal. Values for anestrous or follicular phases were <0.3 μg g⁻¹ (5α-P-3OH) and <0.3 ng mL⁻¹ (P₄); for luteal phase 0.32–11.09 μg g⁻¹ (5α-P-3OH) and 0.32–1.48 ng mL⁻¹ (P₄); for pregnancy 1.41–7.38 μg g⁻¹ (5α-P-3OH) and 0.39–1.6 ng mL⁻¹ (P₄). A positive correlation (t = 8.8, p < 0.01, n = 321) between levels of fecal 5α-P-3OH and serum P₄ was observed. A random sample of 30 free-ranging female elephants showed fecal 5α-P-3OH values of 0.06–23.4 μg g⁻¹, indicating them to be in different stages of estrous cyclicity. This study is the first to assess the reproductive phases of female Asian elephants based on the correlative-patterns of both the fecal 5α-P-3OH and serum P₄ values over multiple estrous cycles. This has a potential application in the reproductive management and conservation of Asian elephants.

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1. Introduction

A self-sustainable captive population is believed to be important for the conservation of the Asian elephant (*Elephas maximus*), listed as “Endangered” in the IUCN Red List of Mammals ([IUCN Red List of Threatened Species. Version 2011.1](https://www.iucnredlist.org/species/3623/11151803)). Although significant information is available on the reproductive biology of captive Asian elephants through monitoring of serum P₄ [1,7,33], their reproductive cyclicity has not been tracked so far through the use of a non-invasive method. Moreover, the reproductive physiology of free-ranging female Asian elephants is virtually unknown. For conservation of both captive and free-ranging populations of elephants, the assessment of female reproductive endocrine status would be invaluable. Conventionally, an animal’s reproductive cyclicity is assessed by monitoring profiles of circulating reproductive hormones [3,4,41] a procedure that is difficult or impossible in the case of large, endangered mammals such as elephants. Recently, non-invasive methods to measure fecal hormonal metabolites in elephants and other mammals, have gained considerable importance, primarily because of the relative ease of fecal sample collection of wild animals in their natural habitat. These have been successfully employed in lion-tailed macaque (*Macaca silenus*) [14], meerkat (*Suricata suricatta*) [45], Asiatic lion (*Panthera leo persica*) [34], bighorn sheep (*Ovis canadensis*) [22], okapi (*Okapia johnstoni*) [25], white rhinoceros (*Ceratotherium simum simum*) [21,26] and Asian one-horned rhinoceros (*Rhinoceros unicornis*) [24].

Studies have shown that progesterone (P₄) metabolites belonging to the 5α-pregnane family are predominant in blood circulation for both Asian elephants and African savanna elephants (*Loxodonta africana*) [13]. Further, the measurement of allopregnanolone (5α-P-3OH) in fecal samples is useful in determining the reproductive state of female African elephants [10]. To assess the estrous cyclicity of Asian elephants, kept in zoos, serum profiles of reproductive hormones such as P₄, oestrogen, luteinizing hormone, and follicle-stimulating hormone [1,3,4,15,16], as well as measurements of urinary P₄ metabolite i.e., 5β-pregnatriol [18] have been reported.
However, due to practical, legal and ethical considerations, it is difficult to collect blood and/or urine samples of semi-captive or free-ranging populations of elephants. To overcome this problem, a non-invasive method to measure the levels of fecal 5α-P-3OH would be useful.

We earlier successfully developed an indirect, enzyme-linked immunosorbent assay to measure fecal 5α-P-3OH and demonstrated a significant positive correlation between the levels of fecal 5α-P-3OH and serum P₄ in semi-captive female Asian elephants [12]. In the earlier study, we analyzed a relatively small sample set (n = 38) and performed coarse temporal analysis (with samples collected once in 15 or 30 days) that did not assess phase distinctions (follicular or luteal) in the estrous cycle of females [12]. Because female Asian elephants have an estrous cycle (12–16 weeks long) consisting of a 4–6 weeks long follicular phase (ovulatory phase) and a 8–10 weeks long luteal phase (non-ovulatory phase) [2,15] and because it is necessary to clearly distinguish these two stages of the estrous cycle, repeated sampling at close time intervals (1 week) over several cycles are required.

Another factor that has to be considered while assessing the reproductive status of semi-captive and free-ranging females is the possible effect of dietary composition on steroid hormone metabolite excretory patterns. Of relevance, here, is the observation that various ecological/environmental and in situ conditions, including resource availability and quality of forage, influence reproductive performance and mating strategies of animals. This is reported for the African elephant [42], sifaka (Propithecus verreauxi) [9] long-tailed macaques (Macaca fascicularis) [8] and meerkat [45]. This influence could possibly be pronounced in semi-captive forest-camp elephants that are fed daily with a supplementary diet of sugar cane, rice and millets which are quite different from the fodder species consumed by elephants in the wild [28]. It is therefore unclear whether or not such differences in feed-consumption, qualitatively or quantitatively, influence the fecal steroid metabolite (5α-P-3OH) excretory pattern. Reports indicate that increased dietary fibre has a negative effect on P₄ metabolite excretion in primates [38,39]. It is thus necessary to examine the fecal P₄ metabolite (5α-P-3OH) excretory pattern in free-ranging versus semi-captive female elephants to look for possible dietary influence.

We thus carried out this study with the following major aims: to examine the correlation of fecal 5α-P-3OH and serum P₄ profiles in simultaneously sampled semi-captive elephants over several expected phases of their estrous cycle; to measure fecal 5α-P-3OH content in random (opportunistic) individual samples of free-ranging females in order to assess their reproductive status and to determine whether or not feed-consumption has any influence on measured fecal 5α-P-3OH in free-ranging versus semi-captive elephants.

2. Materials and methods

2.1. Study area and animals

Sampling of semi-captive animals was carried out on seven female and one male Asian elephant (Table S1). Four of these females and the single male elephant were from the forest camp at Mudumalai Wildlife Sanctuary (MWLS), while the remaining three females belonged to the forest camp of the adjoining Bandipur National Park (BNP) in the southern Indian states of Tamil Nadu and Karnataka, respectively. The two camps are located c. 20 km apart in tropical dry forests with similar vegetation, rainfall and other environmental conditions. Elephants in these camps were captured from the wild by the forest department and were maintained under semi-captive conditions for a period of at least 10 years. This enabled the animals to become habituated and acclimatized to a captive environment. In the sanctuary, animals were used for logging operations (in the past) and/or engaged for eco-tourism [30,43]. The age of individual animals was estimated based on their height measurements, as described by Sukumar et al. [17,30,32] and records maintained at MWLS and BNP (Table S1). At both camps, elephants were maintained as mixed groups of adult and sub-adult females, calves, and males of different age groups. Elephants were brought to the camps once/twice a day, early in the morning and/or late in the afternoon for providing them supplementary feed and were then released back to the forest at night for foraging. We observed that these females in the forest camps had access to bulls in the wild as well as in captivity [35].

Random fecal sampling of the free-ranging females was carried out in the tropical dry forests of MWLS, covering an area of 321 km² with an average elephant density of about two individuals/km² [27,36]. The study area is a part of the Nilgiri Biosphere Reserve as well as Project Elephant Range No. 7 which holds the single largest population of the Asian elephant globally [28,37].

2.2. Sample collection

Fecal and blood samples were collected from four semi-captive female elephants at MWLS (Kamatchi, Bhma, Sumangala and Indra) (Table S1) at a frequency of once a week for a period of nearly one year (February 23, 2008 to February 14, 2009; 51 weeks). Similarly, control samples were also collected from a male elephant (Sujay) at MWLS for a period of 16 weeks (February 23, 2008 to June 28, 2008). At BNP (Table S1) three semi-captive females were sampled at weekly intervals over variable time periods (26 weeks: February 25, 2008 to August 31, 2008 for Chitra; 34 weeks: February 25, 2008 to October 25, 2008 for Diana; 35 weeks: February 25, 2008 to November 2, 2008 for Darisha). Animals were trained to lie down in a lateral recumbent position at the instruction of the mahout and blood samples (~5 mL) were drawn from ear vein using 10 mL vacutainer tubes by a qualified veterinarian (NK). Following the blood sampling, the animals were appropriately rewarded. Protocols for animal handling and blood sampling were laid down and approved by the Tamil Nadu and the Karnataka forest departments.

Blood samples were kept at room temperature for 6–7 h until the blood clotted and the serum was separated out after centrifugation for 10 min at 2000 rpm. The serum was frozen at −20 °C until further analysis in the laboratory at the Indian Institute of Science, where the assay for P₄ was carried out. Freshly dropped fecal samples were collected from each elephant within 1–2 h of blood collection. Approximately, 40 g of feces was removed from the centre of a bolus to avoid cross-contamination with urine or other fecal samples in the vicinity. Fecal samples were frozen in the field at −20 °C within 2–3 h of collection and taken to the laboratory in sealed boxes with ice packs. Serum samples were stored at −20 °C and the fecal samples at −70 °C until analysis. Due to logistical reasons, we could not sample blood and feces for the female Darisha (at BNP) for two consecutive weeks (16th and 17th week as indicated in Fig. 1D).

To collect fecal samples from free-ranging female elephants, we observed female-led family herds for a period of four months from May to August 2008 in MWLS and collected fresh fecal samples. Sampling was carried out only for adult female elephants (>20 years, using height as a proxy to determine the age of the individual) [31]. Each sampled female was photographed and characterized morphologically with features such as ear-fold, tail characteristics, pigmentation pattern were recorded and later verified with our field database. We collected 30 fecal samples from different females belonging to 17 different herds. Protocols employed for
Fig. 1. Correlation between the levels of serum progesterone (−−−) and fecal allopregnanolone (5α-P-3-OH) (−•−•) for four adult female elephants i.e., Indra (A), Sumangala (B), Diana (C) and Darisha (D). From each animal, the fecal sample was collected on the same day following the animal’s blood sampling. Blood and fecal sampling was carried out at weekly intervals for a period of 51 weeks for Indra (A) and Sumangala (B), for 34 weeks for Diana (C) and 35 weeks for Darisha (D). Plotted values are mean ± SEM, n = 4 assay replicates for each data point. Dotted line (-----) in Darisha’s profile (D) indicates the gap in sampling interval during 16th and 17th week. FP = Follicular phase; LP = Luteal phase; EC = Estrous cycle.
collection, storage and processing were as described and were similar to those of the semi-captive animals.

2.3. Measurement of fecal 5α-P-3OH through enzyme-linked immunosorbent assay

Steroid metabolites extracted from the fecal samples were analyzed according to a protocol as described in Ghosal et al. [12]. Samples were extracted with methanol and then the methanol phase was evaporated and reconstituted further with 1% (wt vol⁻¹) gelatin supplemented with Tris buffered saline containing 2% BSA (GTBS) and used for ELISA for estimating P₄ metabolite (allopregnanolone, 5α-P₃-3OH). Extraction efficiency, determined by monitoring the recovery of [³H] P₄ added to the samples of semi-captive females prior to the addition of the other reagents, was 80 ± 4.35% (mean ± SD, n = 80). Moreover, the extraction efficiency calculated for the fecal samples of free-ranging females (73 ± 11.0%, n = 30) was not significantly different from the coefficients calculated for the samples of semi-captive females.

For determining the concentration of 5α-P₃-3OH in the fecal samples of the females, by ELISA, we used rabbit polyclonal antibodies (1 μg/well) against allopregnanolone (Abcam Company Ltd., USA). This antibody was reported to have 100% reactivity to (5α-P₃-3OH) allopregnanolone (Makaira Ltd., UK) and less than 1% cross-reactivity with other progesterone metabolites. The sensitivity of the assay at 90% binding was 0.25 μM (i.e., 4 ng/well). The intra- and inter-assay coefficient of variations obtained by repeated measurements of the internal controls ranged between 12.8% and 16.0%, respectively.

2.4. Measurement of serum progesterone through radioimmunoassay

Serum P₄ analysis was carried out following the protocol as described in our earlier study [12]. Briefly, P₄ hormone was extracted from the serum samples with diethyl ether, then the aqueous phase was frozen using liquid nitrogen, ether was evaporated and reconstituted in 1% (wt vol⁻¹) gelatin supplemented with phosphate buffered saline (GPBS). Radio-immunoassays were carried out in duplicates using P₄ (4-Pregnene-3, 20-dione, P₄; Sigma, USA) as standard, [³H]-P₄ (96.6 Ci/mmol; Perkin-Elmer, Singapore) as a radioactive label and anti-P₄ antibody (Niswender Co., USA) as the antisera in [12]. Sensitivity of the assay at 90% binding was 7.8 pg 100 μL⁻¹. Inter- and intra-assay coefficient of variation was 22.8% and 15.8%, respectively, being generated by repeated measurements of internal standards that were included in every assay performed. The measured P₄ concentrations were finally expressed in nanograms (ng) per mL of the serum sample.

2.5. Statistical analyses

Linear mixed effect (LME) model approach in R version 2.7.1 [6], an extension of simple linear models, was applied to test the strength of the relationship between the fecal 5α-P₃-3OH and serum P₄ concentrations. The values of fecal 5α-P₃-3OH levels and serum P₄ concentrations were pooled across all the sampled individuals (both male and females) and then log transformed for incorporation in the LME model. Since the log transformed data followed a normal distribution, Pearson’s product moment correlation was carried out between the measured concentrations of fecal 5α-P₃-3OH and the fitted values of fecal 5α-P₃-3OH, as predicted by the LME model. All values reported here are expressed as mean ± SD of four assay replicates for a particular female and also as mean ± SD of the pooled values across the females; in the figures, the values are plotted as mean ± SEM of the assay replicates.

3. Results

3.1. Profiles of fecal 5α-P₃-3OH and serum P₄ in semi-captive female elephants

We observed three patterns of hormonal profiles, the profiles characteristically observed during the estrous cycle, the non-cycling phase, and the pregnant stage (Figs. 1 and 2).

3.1.1. Profiles indicating estrous cyclicity

Of the seven female elephants sampled at both MWLS and BNP, four females (Indra, Sumangala, Diana and Darisha; Table S1) showed regular cycles in hormonal patterns throughout the sampling period (Fig. 1A–D) as monitored through the measurement of both fecal 5α-P₃-3OH and serum P₄ levels.

The patterns of both fecal 5α-P₃-3OH and serum P₄ in these four female elephants indicated that they were experiencing a typical estrous cycle with follicular and luteal phases. We assigned the follicular phase of the estrous cycle to an animal when the values of both fecal 5α-P₃-3OH (<0.3 μg g⁻¹) and serum P₄ (<0.3 ng mL⁻¹) were low for a time period >3 weeks. The luteal phase was assigned when values were higher in fecal 5α-P₃-3OH (>0.3 μg g⁻¹) and serum P₄ (>0.3 ng mL⁻¹) over a period >5 weeks (Fig. 1). The lowest values of fecal 5α-P₃-3OH and serum P₄ measured for the four cycling females were 0.04 ± 0.02 μg g⁻¹ (Indra) and 0.1 ± 0.01 ng mL⁻¹ (Sumangala), respectively, while the highest corresponding values were 11.09 ± 0.72 μg g⁻¹ (Sumangala) and 1.48 ± 0.25 ng mL⁻¹ (Diana). Indra and Sumangala showed similar patterns of fecal 5α-P₃-3OH concentration in the range 0.04 ± 0.02–11.09 ± 0.72 μg g⁻¹ and of serum P₄ in the range 0.10 ± 0.01–1.34 ± 0.51 ng mL⁻¹ (Fig. 1A and B). However, for Diana and Darisha the lowest value of fecal 5α-P₃-3OH was marginally higher than the other two females (Indra and Sumangala), ranging from 0.12 ± 0.01–6.91 ± 0.73 μg g⁻¹ while serum P₄ concentration fluctuated between 0.09 ± 0.01–1.48 ± 0.25 ng mL⁻¹ (Fig. 1C and D).

The mean fecal 5α-P₃-3OH concentration of these four cycling females during the follicular phase was 0.16 ± 0.06 μg g⁻¹ (n = 55, 10 phases across four females) and serum P₄ level was 0.18 ± 0.05 ng mL⁻¹ (n = 57, 10 phases across four females) (Table 1A and B). Similarly, during the luteal phase the mean concentrations of fecal 5α-P₃-3OH and serum P₄ were 1.97 ± 2.05 μg g⁻¹ (n = 118, 11 phases across 4 females) and 0.61 ± 0.25 ng mL⁻¹ (n = 116, 11 phases across 4 females), respectively (Table 1A and B). The period during which the values of both fecal 5α-P₃-3OH and serum P₄ were lower lasted for 4–7 weeks for all the four females. However, the four females exhibited higher levels of both fecal 5α-P₃-3OH and serum P₄ for a period of 10–12 weeks. One such peak and trough cycle of both fecal 5α-P₃-3OH and serum P₄ lasted for approximately 15–18 weeks, in the case of the four females (Fig. 1). On the whole seven estrous cycles (complete peak-trough cycle) were monitored across 4 females. In five of the seven cycles monitored, a defined luteal-phase rise as determined by fecal 5α-P₃-3OH analysis (>0.3 μg g⁻¹) occurred on the same day as that of serum P₄ (>0.3 ng mL⁻¹).

3.1.2. Profile indicating pregnancy status

The levels of both fecal 5α-P₃-3OH and serum P₄ measured for the female Chaitra from BNP (Table S1) remained relatively high throughout the sampling period of 26 weeks (Fig. 2A). The range and mean value of fecal 5α-P₃-3OH was 1.41–7.38 μg g⁻¹ and 3.29 ± 1.79 μg g⁻¹ (n = 27), respectively (Table 1A). The range and mean value of serum P₄ concentration was 0.39 to 1.6 ng mL⁻¹ and 0.69 ± 0.28 ng mL⁻¹ (n = 27), respectively (Table 1B).
Pregnancy status of this female was evident by the birth of a male calf (arrow, Fig. 2).

3.1.3. Profiles indicating non-cyclicity

Two female elephants (Kamatchi and Bhama, Table S1) showed a ‘flat-line pattern’ of profiles for levels of both fecal 5α-P-3OH (<0.3 μg g\(^{-1}\)) and serum P₄ (<0.3 ng mL\(^{-1}\)) (Fig. 2B and C) during the sampling period of 51 weeks, thereby indicating them to be non-cycling or being anestrous. The above values were similar to those (fecal 5α-P-3OH: <0.3 μg g\(^{-1}\) and serum P₄: <0.3 ng mL\(^{-1}\); Fig. 2D) seen typically in a male elephant (Sujay, Table S1), used as a negative control.

3.2. Association between fecal 5α-P-3OH and serum P₄ levels in semi-captive females

Linear mixed effect (LME) analysis showed a strong positive association between the fecal 5α-P-3OH and the circulating P₄ concentrations across all the elephants (\(r = 0.85, p < 0.0001\)). To further validate the results of the LME analysis, Pearson’s product moment correlation was carried out between the measured levels of fecal 5α-P-3OH concentrations and the fitted values for the same as predicted by the LME model. This showed a significant positive correlation (Pearson’s \(r = 0.85, p < 0.0001, n = 321\)) between the measured concentrations of fecal 5α-P-3OH and the fitted values of fecal 5α-P-3OH, which the LME model predicted.

To depict the degree of overlap of hormone values across different reproductive stages of animals, the hormonal profiles (5α-P-3OH & P₄) of three individuals (Sumangala, Kamatchi and Chaitra, Table S1) of different reproductive states (cycling, non-cycling and pregnant, respectively) were plotted (Fig. 3). The plot indicated a considerable overlap of hormone values between follicular phase of estrous cycle and anestrous phase and also between the luteal phase of the estrous cycle and the pregnancy period. Besides, a comparison made on the observed values of fecal 5α-P-3OH and serum P₄ determined with those published for Asian and African elephants [1, 10, 13, 19, 20, 40, 42] indicated a greater difference in the content of fecal 5α-P-3OH and similar levels of serum P₄ (Table 1A and B).

3.3. Levels of fecal 5α-P-3OH in sampled free-ranging females

To estimate the concentration of fecal 5α-P-3OH in free-ranging elephants, we used our standard ELISA assay, developed with 0.1 μM to 1 mM of 5α-P-3OH, which had a linear range between 0.25 and 62.5 μM, with an EC₅₀ of 4.68 μM (Fig. S1). The one-time sampling of 30 free-ranging females is represented as a plot along with the standard curve for 5α-P-3OH (Fig. S1). The values of 5α-P-3OH showed a wide range across the females, from as low as 0.06 μg g\(^{-1}\) to as high as 23.4 μg g\(^{-1}\) of the fecal samples (Table S2). Animals could be categorized into three groups, namely, low (<0.3 μg g\(^{-1}\)) and medium (0.3–11 μg g\(^{-1}\)) categories which correspond to the levels of fecal 5α-P-3OH measured in the semi-captive females and high (>11 μg g\(^{-1}\)) values, greater than the levels measured for the semi-captive females. Of the 30 females analyzed, 76.6% and 10% of females belonged to medium and low categories, respectively; the rest 13.3% females showed high values of fecal 5α-P-3OH.

4. Discussion

The present study is the first to assess reproductive (estrus) cyclicity, monitored over a few possible estrous cycles, in semi-captive Asian elephants, through measurement of fecal 5α-P-3-OH (of the 5α-pregnane family). We have demonstrated a significant positive association between the measured levels of fecal 5α-P-3-OH and serum P₄ for a large sample size (\(n = 321\)). These findings are consistent with our earlier initial findings [12] that fecal 5α-P-3-OH can be used to assess the reproductive status of female Asian elephants.

Based on the profiles of fecal 5α-P-3-OH and serum P₄ concentrations of the seven females sampled, we observed that four females showed periodic cyclical changes of hormones-metabolites characteristic of normal estrous cycles (Fig. 1). This is consistent with the measurements made by other researchers of serum P₄ levels showing similar cyclical change during the follicular and luteal phases of the estrous cycle of Asian elephants [2, 15, 20]. In our study the duration of follicular (~6 weeks) and luteal (~11 weeks) phases of the reproductive cycle of about 17 weeks.

Table 1
Comparative overview of (A) fecal allopregnanolone (5α-P-3OH) levels in Asian elephants from our study and African elephants from published data; (B) serum progesterone levels in Asian elephants from our study and collated published data on both Asian and African elephants.

<table>
<thead>
<tr>
<th>Reproductive status</th>
<th>Fecal allopregnanolone (μg g(^{-1}))</th>
<th>Published data*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Our data Mean ± SD Range</td>
<td>Value/Range Reference</td>
</tr>
<tr>
<td>(A)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follicular</td>
<td>0.16 ± 0.05 0.04–0.3</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Luteal</td>
<td>1.97 ± 2.05 0.32–11.09</td>
<td>1.5–25</td>
</tr>
<tr>
<td>Non-cycling</td>
<td>0.10 ± 0.05 0.02–0.23</td>
<td>1–5</td>
</tr>
<tr>
<td>Pregnant</td>
<td>3.29 ± 1.79 1.41–7.38</td>
<td>10–20</td>
</tr>
<tr>
<td>(B)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follicular</td>
<td>0.18 ± 0.05 0.10–0.29</td>
<td>0.14 ± 0.01 [13]</td>
</tr>
<tr>
<td>Luteal</td>
<td>0.61 ± 0.25 0.32–1.48</td>
<td>0.71 ± 0.06 [13]</td>
</tr>
<tr>
<td>Non-cycling</td>
<td>0.14 ± 0.04 0.04–0.26</td>
<td>0.03–0.07 [1]</td>
</tr>
<tr>
<td>Pregnant</td>
<td>0.69 ± 0.28 0.39–1.6</td>
<td>0.67 ± 0.26 [19]</td>
</tr>
</tbody>
</table>

* With respect to African elephants.

<table>
<thead>
<tr>
<th>Reproductive status</th>
<th>Serum Progesterone (ng mL(^{-1}))</th>
<th>Published data*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Our data Mean ± SD Range</td>
<td>Value/Range Reference</td>
</tr>
<tr>
<td>(A)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follicular</td>
<td>0.14 ± 0.01 0.10–0.29</td>
<td>0.05–0.15 [1]</td>
</tr>
<tr>
<td>Luteal</td>
<td>0.71 ± 0.06 0.32–1.48</td>
<td>0.3 – 1.4 [1,20]</td>
</tr>
<tr>
<td>Non-cycling</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Pregnant</td>
<td>0.67 ± 0.26 0.39–1.6</td>
<td>0.4–2.1 [1]</td>
</tr>
</tbody>
</table>

* With respect to Asian and African elephants.
was determined based on the patterns of both fecal 5α-P-3-OH and serum P₄. This too is consistent with earlier observations made on Asian elephants based on the pattern of serum P₄ [2,15,20], and on African elephants based on the hormonal profiles of both serum P₄ and fecal P₄ metabolites [10,16,40]. The variation (length and duration of estrous cycle) among the hormonal profiles of the cycling females (Fig. 1) can be attributed to individual animal variation, in terms of age group and past-fecundity status of females.
In the current study, the fecal 5α-P-3OH profiles showed a closer association with serum P₄ measurements in terms of timing of the luteal-phase rise (five of the seven estrous cycles in 4 elephants). Upon comparison of fecal 5α-P-3-OH profile of Asian elephants with the estrous cycle-associated profiles of fecal P₄ metabolites of African elephants [10], some similarities and differences were observed (Table 1A). First, the level of fecal 5α-P-3-OH observed during the follicular phase in Asian elephants was markedly lower (0.04–0.3 μg g⁻¹, Table 1A) than that measured in African elephants (1.5–2.5 μg g⁻¹, Table 1A). There was a distinct overlap in the range of the fecal P₄ metabolite values during the luteal phases of the estrous cycles, observed for both the Asian (0.32–11.09 μg g⁻¹) and African elephants (3–10 μg g⁻¹) but the minimal level observed in Asian elephants was about ten-fold lower than that reported for the African elephants (Table 1A). A striking observation made in this study is that the minimal levels of Asian elephants’ fecal 5α-P-3-OH were always several-fold lower than those reported for African elephants, regardless of the reproductive phase of the females studied (Table 1A). Overall, the range of the measured levels was dissimilar in the two elephant species compared (Table 1A). These apparent differences can be attributed to a difference in the assay procedure, the dissimilar patterns of steroid hormone metabolites’ excretions, and/or their compositional variations, and also may be due to the different habitats and foraging habits in the two elephant species studied. However, the similarities in the hormonal profiles can be due to the prevalence of comparable reproductive physiology of two closely-related species [28].

It was interesting to observe that, similar to earlier reports on the serum P₄ profile of Asian elephants during gestation [1,5,19], the magnitude of the fluctuating serum P₄ concentration measured in the female Chaitra was quite high (Fig. 2A and Table 1B). The pattern of fecal 5α-P-3-OH also closely resembled the profiles of 5α-P-3-OH, determined in African elephants (Table 1A). The observed magnitude of fluctuating levels of fecal 5α-P-3-OH and serum P₄ in this animal is puzzling. Whether or not factors such as pregnancy-associated ovarian activity, and the general well-being of the animal, social isolation (in captivity) and other ecological factors contribute to the observed hormone fluctuations are unclear. Incidentally, we had only one pregnant female in our sampling category and, hence, could not arrive at a firm conclusion. However, it would be interesting to investigate the profiles of more pregnant females.

The observation that two females (Fig. 2B and C) did not exhibit any cyclical changes in the levels of both fecal 5α-P-3-OH and serum P₄, throughout the entire sampling period (51 weeks) clearly indicate their non-estrus state. This is similar to the ‘flat-line’ profiles of serum P₄ for anestrous or non-cycling females recorded in western zoos [4] (Table 1A and B). The reproductive physiology of free-ranging female African savanna elephants shows a strong relationship with ecological conditions; thus with the onset of drought, and consequently insufficient quality of forage, females show depressed levels of ovarian activity [42]. However, of greater relevance in our study is the reproductive history of these two “flat-liner” semi-captive females who are unlikely to experience much dietary variation because of supplementary feed. Bhma had last conceived in the year 2003 at the age of ~ 56 years (Table S1). During this study (2008–2009), Bhma’s age was 62 years and thus she is likely to have stopped cycling given her old age. The other female Kamatchi was captured at the age of ~3 years and had lost her mother soon after. She had a history of at least two miscarriages prior to her first successful parturition at a very late age of 36 years (forest department reports at MWLS), while her third and last calf was born at the age of ~51 years (Table S1). Given the old age and history of the poor reproductive performance, these two females would have probably reached their anestrous state.

The levels of fecal 5α-P-3-OH and P₄ observed in the negative control male elephant examined for a duration of 16 weeks (Fig. 2D) were similar to those of non-cycling female elephants. These levels thus appear to be indicators of baseline values of serum P₄ and fecal 5α-P-3-OH in female Asian elephants; animals exhibiting higher than these values for a sustained period may indicate their varying degrees of ovarian activity.

The plot of the mean values of fecal 5α-P-3-OH and serum P₄ determined for three animals in different reproductive states i.e., cycling (Sumangala), non-cycling (Kamatchi) and pregnant (Chaitra), showed distinct overlap in the levels of the measured hormones (Fig. 3). The concentrations of fecal 5α-P-3-OH and serum P₄ during the follicular phase of the estrous cycle for Sumangala showed substantial overlap with the values measured during the non-cycling stages of Kamatchi (Fig. 3). Similarly, the levels of fecal 5α-P-3-OH and serum P₄ measured during the luteal phase of the estrous cycle for Sumangala showed considerable overlap with the values measured during the gestational phase of Chaitra (Fig. 3). This overlap of measured levels of hormones suggests the need for periodic sampling of a particular female on a finer temporal scale. Such data will help in assessing the accurate reproductive stage (i.e., cycling, non-cycling and pregnant periods) of female Asian elephants.

Our study showed a strong positive correlation between serum P₄ and fecal 5α-P-3-OH, thus providing an efficient tool to non-invasively assess the estrous state of semi-captive or free-ranging female elephants. Of significance here is the fecal 5α-P-3-OH measurements made on 30 different free-ranging females (Table S2), which showed varying concentrations of the metabolite across all females, thereby indicating that the sampled animals were at different reproductive phases of the estrous cycle (Table S2). Since 76.6% females exhibited high values of fecal 5α-P-3-OH, ranging from 0.3 to 11 μg g⁻¹ (medium category), they can be characterized to be either pregnant or belonging to the luteal phase of the estrous cycle. Only 10% of the females could be considered to be either in their follicular phase or non-cycling phase. Of interest, here, is the 13.3% females which showed a high value of fecal 5α-P-3-OH (>11 μg g⁻¹), not measured in the case of semi-captive females, indicating higher ovarian activity and perhaps higher fecundity in free-ranging females as compared to semi-captive ones. However, distinct characterization of the reproductive cycle in free-ranging elephants requires repeated sampling of the same individual at frequent time intervals, as there is considerable overlap in the values of fecal 5α-P-3-OH measured during different reproductive states (Fig. 3). This is only possible with free-ranging individuals that are monitored over long periods (>1 year), assisted by radio- or GPS (Global Positioning System) tracking system.
Because there was no statistically significant difference in the steroid extraction efficiency of the fecal samples between the free-ranging and the semi-captive females, it appears unlikely that diet can influence fecal steroid metabolite excretion patterns observed in female Asian elephants [38,39]. Wild populations of both Asian and African elephants experience various ecological and anthropogenic factors such as seasonal cycles, availability of forage, human-elephant conflict, and poaching for ivory [28,29,31,44], all of which could potentially affect reproductive cyclicity of female elephants [11,28,29,42]. In African elephants, studies demonstrated that disruption in the reproductive physiology of females in the PTR population could be disrupted/alterted as compared to female elephants in populations with a higher number of adult males [29, C. Arivazhagan and R. Sukumar, unpublished]. The non-invasive method of measuring fecal P4 metabolite, developed by us, can thus be useful to assess the reproductive state of free-ranging elephants under varying ecological conditions as well as population structures. This research was supported by the Ministry of Environment and Forests, Govt. of India, New Delhi. We thank the forest departments of Karnataka and Tamil Nadu states for providing permission to conduct research. We also acknowledge K. Bomman for his help and support in the field and Dr. Kavita Iywaran for her help in the statistical analyses of the data and M.S. Padmavathi in the preparation of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygenen.2011.10.004.

References


T.N.C. Vidya, R. Sukumar, Social organization of the Asian elephant (Elephas maximus) in southern India inferred from microsatellite DNA, J. Ethol. 23 (2005) 201–205.


