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# Estimation of faecal estrogen and progesterone profile at estrous and post estrous period irrespective of mating in lioness

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**Abstract**
Repeated collection of blood samples for hormonal evaluations is necessary for successful investigation of reproductive-endocrine relationships. However, repeated blood sampling is not possible in captive animals like lion; therefore non-invasive faecal steroid metabolite evaluations have mainly been used to study female reproduction and provide information regarding the oestrous behaviour, mating behaviour, pregnancy, abortion and seasonality. Six adult lioness of different ages were selected from Nandankanan Biological Park, Bhubaneswar for the study. Faecal samples were collected within 12 hrs after defecation in the morning. The sex steroid hormones were extracted from wet faeces and analysed by ELISA. The overall value of estrogen and progesterone (ng/g) were 1.22 ± 0.24 and 58.22 ±1.02 respectively during breeding season. The overall mean values of estrogen (ng/g) were 1.24± 0.02 and 1.03 ± 0.002 in estrus and post-estrus respectively. Similarly, the faecal progesterone concentrations (ng/g) were recorded to be 32.08 ± 0.92 and 62.92± 0.28 for corresponding estrus period. Faecal estrogen values during estrus were found to be significantly higher than the post estrous period. Similarly post estrus values of progesterone were significantly higher as compared to follicular phase of estrous cycle. Non-invasive hormonal assay from faecal material could be made from captive wild animals where other conventional method might incur risk to life.

**Keywords:** Estrogen, progesterone, estrus, ELISA

## Introduction
The Lion *(Panthera Leo)* is the fearest and most magnanimous of the four footed beasts in the world. The lion's name derives from the Latin *Leo* and the generic component of its scientific designation, *Panthera*¸ is presumed to derive from Greek *pan* (all) and *ther* (beast). The name came into English through the classical languages, but *panther*, is probably of East Asian origin meaning "*the yellowish animal*". The lion is a mammal and second largest in the family Felidae, being slightly smaller than the tiger (*Pantheratigris*). The lion is an iconic species, and its conservation is involved in attempts to prevent the animal from becoming extinct and preserving its natural habitat.

Lions are unusual amongst cats in displaying a striking sexual dimorphism due to the availability of a large number of females in grouped social structures allowing larger males to be able to monopolize breeding. There are significant differences in the size of both within and between lion populations. The avg. body wt. of adult lion is 189 kg and lioness as on avg. of 126 kg. Lions have two pairs of mammary glands (rarely three), one inguinal & one abdominal.

Male lions become sexually mature at around 26 months old, but unlikely to breed before the age of four or five years, primarily due to a lack of opportunity until they are large enough at around this age to take over a pride and therefore its breeding rights. Females generally conceived for the first time at 32 – 33 months with most lionesses having given birth by the time they are four years of age. Females can breed until they are of 15-years old, but reproduction usually starts to decline at 11 years of age. Lionesses are annually polyestrous, estrus lasting 4-7 days with intervals between periods of a few days up to more than a year. They have a post-partum estrus but do not conceive if the litter survives. If the litter is lost a new one may be produced within four months.

There is little data available on the reproductive endocrinology of lions, primarily because of the difficulty in obtaining frequent blood samples.

An alternative method for assessing endocrine activity in felid species is the analysis of steroid hormone metabolites in the faeces. Faecal hormone analysis has been used successfully in a variety of felid species associated with successful reproduction and as a tool to examine the efficacy of hormone therapies.

It was suggested that the onset of estrus is indicated by faecal estrogen concentrations being elevated above baseline for at least 2 days with no increase in faecal progestagen (Graham *et al*. 2006) [1]. Estrogens are the end products of steroid metabolism and the compounds are found to be similar in plasma and faeces (Mostl *et al*. 1984) [2]. It was reported that progestin concentrations in nondomestic felids during pregnant and non-pregnant luteal phases are quantitatively similar, just as in the domestic cat and it is technically possible to diagnose pregnancy in nondomestic felids based on faecal progestins that remain elevated past the normal length of a non-pregnant luteal phase (Brown *et al*. 2001) [3]. Faecal progesterone metabolite analysis has been successfully used for monitoring corpus luteum function and pregnancy, abortion, puberty and seasonality in felids (Czekala *et al*. 1994) [4]. Faecal progesterone metabolites were used to successful monitoring of reproductive treatment therapies in wild as well as captive animals (Kirkpatrick *et al*. 1995) [5]. Faecal steroid analysis was commonly used as non-invasive method and accepted as a diagnostic tool and as a means to study the fundamental reproductive endocrinology in farm, wild and zoo animals (Schwarzenberger, 1996) [6].

## Material and Methods

### Source of animals and collection of faecal sample

Healthy Breeding lioness of different agewere selected from Nandankanan Biological Park, Bhubaneswar for the study. Six adult lioness were included in the present investigation out of them four adult lines are considered as per the requirement (Table 1). The average body weight of each lioness is 110 Kg to 140 Kg. Faecal samples from each of the lioness were collected 3 times during estrus (early, mid, late) and also after mating i.e. early mating, expected mid pregnancy and late pregnancy from November 2016 to May 2017. These faecal samples were collected (Figure 1) from individual animal in sterilized containers during morning between 7.30 A.M. to 9.30 A.M. in the feeding enclosure. The collected samples were stored at -35 °C until extraction.

**Table 1:** Description about the lions

| Name of Lions | Date of birth | Sex | Age (in years) | Male encounter | Female encounter |
|---|---|---|---|---|---|
| Rukmani | 26-05-2010 | Female | 7 | Yes | - |
| Radha | 26-05-2010 | Female | 7 | Yes | - |
| Lioness of cell 5(African lioness) | Not recorded | Female | 5-6 yr (Approx) | No | - |
| Enclosure(29/B) African liones | Not recorded | Female | 5-6 yr (Approx.) | No | - |

**Fig 1:** Collection of faecal sample of lion

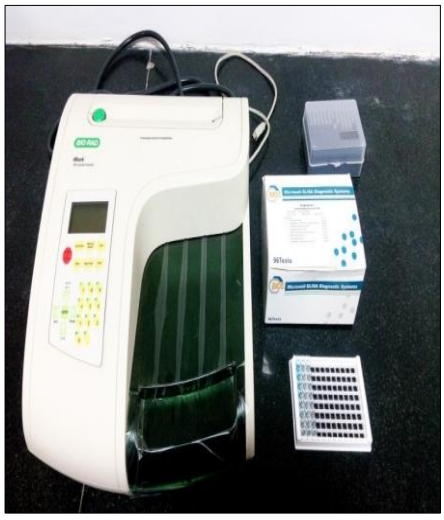

**Fig 2:** ELISA reader for hormone estimation

### Extraction of faecal samples

Before extracting, the faecal samples were brought to room temperature (20 $^0$C-27 $^0$C). Then 0.5g wet faeces was weighed and taken in a clean, sterilized test tube. After that 5 ml of 80% methanol were added to each test tube containing the faeces and kept for 10 minutes. Finally the mixture was mixed in a vortex mixture for 5 minutes. Samples were then centrifuged at 2500 rpm for 10 min and 1 ml of the supernatant was removed and stored in a 2ml polypropylene tube at -20 °C until hormone assays were performed.

### Estimation of sex steroid hormones

Estimations of sex steroid hormones such as estrogen, progesterone were done quantitatively by direct immuno-enzymatic technique using Multi Label Plate Reader (Figure 2).

### Progesterone

Progesterone concentration from fecal samples were assayed for quantitative estimation by direct immuno-enzymatic technique using progesterone ELISA kit in ELISA reader.

### Principle

It is based on competitive enzyme immuno assay. Anti-progesterone antibodies are immobilized on micro well plates. Progesterone in the sample competes with HRP labeled progesterone for binding to the immobilized antibody. For this the essential reagents required include antibody, enzyme antigen conjugate and fecal extraction containing the native antigen, a competitive reaction results between the native antigen and the enzyme antigen conjugate for a limited number of antibody binding sites.

A simultaneous reaction between the biotin attached to the antibody and the streptavidin immobilized on the micro well occurs. This effects the separation of the antibody bound fraction after decantation or aspiration. The enzyme activity in the antibody bound fraction was inversely proportional to the native antigen concentration. By utilizing several serum references of known antigen concentration, a dose response curve was generated from which the antigen concentration of an unknown sample could be ascertained.

**Estrogen**

The estrogen concentration was estimated by immune enzymatic method using ELISA Kit in Multi Label Plate reader.

**Principle**

The essential reagents required for an enzyme immunoassay include antibody, enzyme-antigen conjugate and native antigen. Upon mixing the biotinylated antibody with a serum containing the antigen, a reaction results between the antigen and the antibody. After a short incubation, the enzyme conjugate was added (This delayed addition permits an increase in sensitivity for low concentration samples). Upon the addition of enzyme conjugate, competition reaction results between the enzyme analog and the antigen in the sample for a limited number of antibody binding sites (not consumed in the first incubation).

A simultaneous reaction between the biotin attached to the antibody and the streptavidin immobilized on the micro well occurs. This effects the separation of the antibody bound fraction after decantation or aspiration. The enzyme acitivity in the antibody bound fraction was inversely proportional to the native antigen concentration. By utilizing several diffent serum references of known antigen concentration, a dose response curve generated from which the antigen concentration of an unknown was ascertained.

**Analysis of Data**

The data obtained in the experiment were analyzed by adopting the standard statistical procedures (Snedecor and Cochran, 1989) [7]. Duncan multiple comparison test was utilized to determine the significant differences among the individual species and stages of estrus cycle. The SPSS 22 statistical software programs were used for the variance analysis and Duncan multiple comparison tests, respectively (Gürbüz *et al*. 2003) [8].

**Results**

**Faecal estrogen and progesterone concentration in adult captive lioness**

The mean faecal estrogen concentration (ng/g) of the lioness were found to be 1.28±0.02, 1.27±0.06, 1.12± 0.02 and 1.20±0.04 respectively (Table 2) for Rukmani, Radha, African lion(29/B) and African lion(safari) (Figure 3) with an overall average of 1.22± 0.24. The corresponding concentrations (ng/g) of progesterone were estimated to be 61.95± 0.49, 62.61± 0.69, 52.09± 0.30, 56.25 ± 0.58 with an overall mean of 58.22±1.02 (Figure 4).

With respect tofaecal estrogen concentration no such significant difference ($p$>0.05) was found among Rukmani, Radha and African lion (Safari). However, the estrogen concentration of Rukmani and Radha differed significantly ($p$<0.05) from that of African lion (29/B). On the contraryestrogen concentration of both the African lion did not reveal any significant difference.

The faecal progesterone concentration of Rukmani and Radha did not display any significant difference ($p$>0.05) but a statistical significant difference ($p$<0.05) value wasobserved between both the African lions (Table 2).

**Table 2:** Faecal Estrogen and Progesterone concentration (ng/g) of four Lioness in breeding season from November 2016 to June 2017

| Name of lioness | Estrogen | Progesterone |
|---|---|---|
| Rukmani | $1.28^{b}$±0.02 (5) | $61.95^{a}$± 0.49 (5) |
| Radha | $1.27^{b}$±0.06 (5) | $62.61^{a}$± 0.69 (5) |
| African lion(29/B) | $1.12^{a}$± 0.02 (5) | $52.09^{b}$± 0.30 (5) |
| African lion(safari) | $1.20^{ab}$±0.04 (5) | $56.25^{c}$± 0.58 (5) |
| Overall mean value | 1.22± 0.24 (5) | 58.22±1.02 (5) |
| 'p' value | 0.05 | 0.05 |

Means bearing same superscript in a column do not differ significantly (*$p$<0.05)

Figures in parenthesis indicate the number of faecal samples.

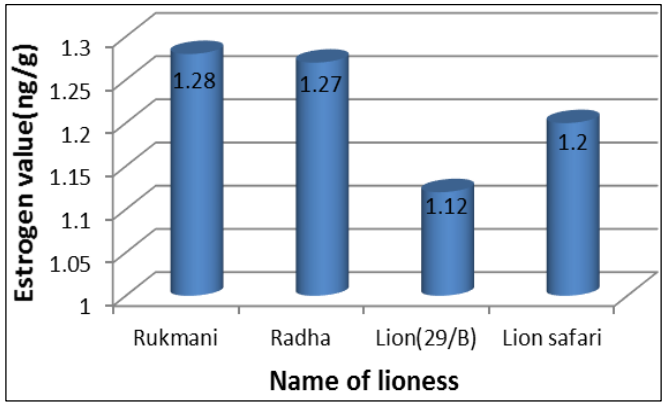


**Fig 3:** Mean faecal estrogen concentration (ng/g) of captive lionesses during breeding season

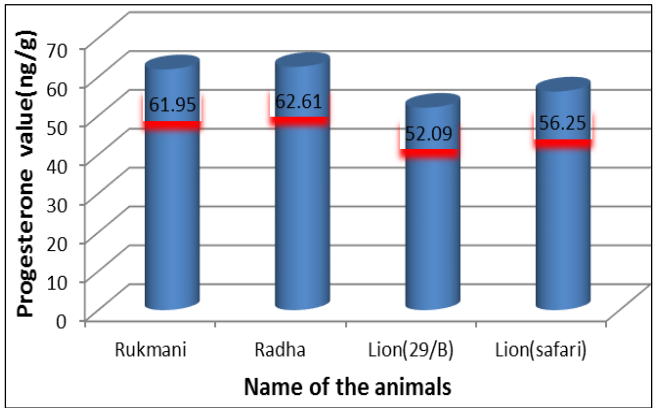


**Fig 4:** Mean faecal progesterone concentration (ng/g) of captive lionesses during breeding season

**Faecal estrogen and progesterone concentration at estrous and post estrous period**

Faecal estrogen and progesterone concentrations (ng/g) of the captive lioness Rukmani and Radha at estrous and post estrous period were estimated. It was found that estrogen concentration (ng/g) at estrous and post estrous was 1.24± 0.02 and 1.03 ± 0.002 (Figure 5) respectively (Table 3). The corresponding progesterone values (ng/g) were measured (Table 3) to be 32.08 ± 0.92 and62.92± 0.28 (Figure 6). A statistical significant difference ($p$<0.05) of faecal estrogen and progesterone concentration between estrous and post estrous period was observed. Post estrous progesterone concentration was found significantly ($p$<0.05) higher than the concentration at estrous.

Faecal estrogen concentrations at estrous and post estrous period ofRadha and Rukmani were determined (Table 4). Estrogen concentration (ng/g) at estrous was found to be 1.28±0.03 and 1.22±0.02 respectively (Table 4) which differed significantly ($p$<0.05). The corresponding estrogen value (ng/g) at post estrous phase was measured (Figure 7) to be 1.03±0.02 and 1.03±0.007 (Table 4), which differed significantly ($p$<0.05).

Faecal Progesterone concentration (ng/g) of Radha (34.21± 2.10) was significantly higher ($p$<0.05) than that of Rukmani (31.15±0.64) at estrous (Table 5). Similarly post estrous Progesterone concentration differed significantly ($p$<0.05) between Radha (63.21±0.89) and Rukmani (62.63±0.90) (Figure 8).

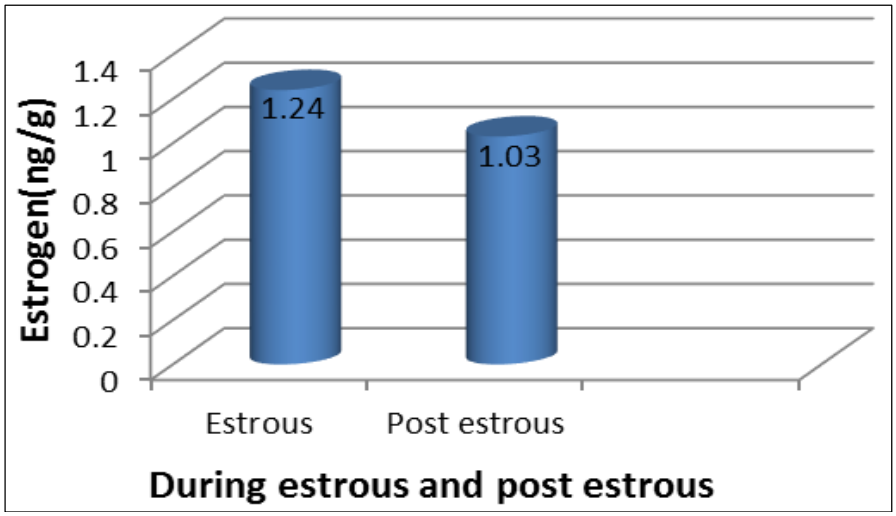


**Fig 5:** Mean faecal estrogen concentration (ng/g) of captive Lionesses at different phases of estrous cycle

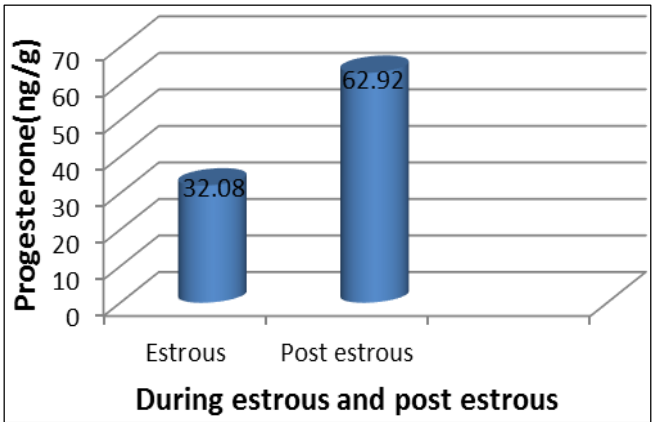


**Fig 6:** Mean faecal progesterone concentration (ng/g) of captive Lionesses at different phases of estrous cycle

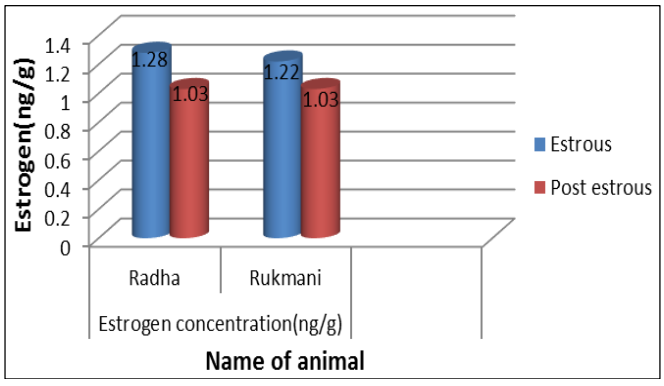


**Fig 7:** Mean faecal estrogen concentration (ng/g) of Radha and Rukmani at estrous and post estrous period

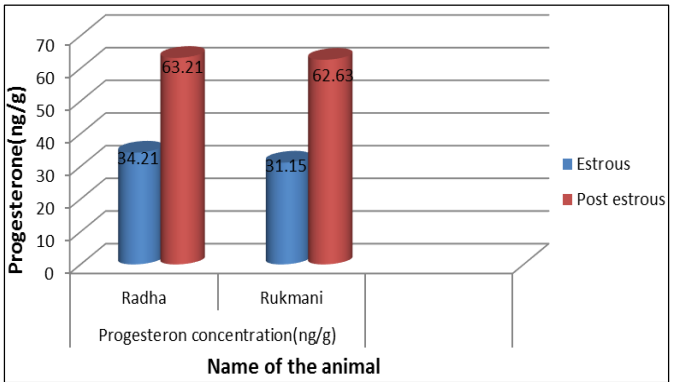


**Fig 8:** Mean faecal progesterone concentration (ng/g) of Radha and Rukmani at estrous and post estrous period

**Table 3:** Faecal Estrogen and Progesterone concentration (ng/g) (Mean ± SE) of captive Lioness at estrous and post estrous period

| Stages of estrous cycle | Estrogen | Progesterone |
|---|---|---|
| Estrous | $1.24^{a}$± 0.02 (5) | $32.08^{a}$ ± 0.92 (5) |
| Post Estrous | $1.03^{b}$ ± 0.002 (5) | $62.92^{b}$± 0.28 (5) |

Means bearing same superscript in a column do not differ significantly $p$<0.05
Figures in parenthesis indicate the number of faecal samples.

**Table 4:** Faecal Estrogen concentration (ng/g) (Mean±SE) in Radha and Rukmani at estrous and post estrous period

| Stages of estrous cycle | Radha | Rukmani |
|---|---|---|
| Estrous | $1.28^{a}$±0.03 (5) | $1.22^{b}$±0.02 (5) |
| Post Estrous | $1.03^{A}$±0.02 (5) | $1.03^{B}$±0.007 (5) |

Means bearing same superscript in a column do not differ significantly $p$<0.05
Figures in parenthesis indicate the number of faecal samples.

**Table 5:** Faecal Progesterone concentration (ng/g) (Mean± SE) in Radha and Rukmani at estrous and post estrous period

| Stages of estrous cycle | Radha | Rukmani |
|---|---|---|
| Estrous | $34.21^{a}$±2.10 (5) | $31.15^{b}$±0.64 (5) |
| Post Estrous | $63.21^{A}$±0.89 (5) | $62.63^{B}$±0.90 (5) |

Means bearing same superscript in a column do not differ significantly $p<0.05$

Figures in parentheses indicate the number of faecal samples.

**Faecal progesterone concentration of lioness during pregnancy**

It was observed that during pregnancy (30-60 days) the mean progesterone concentration (ng/g) inRadhaand Rukmani was 122.87±1.14and 118.67±0.62 respectively (Table 06) which differed significantly ($p<0.05$). Progesterone value had almost increased double fold than the value recorded during non-pregnancy period for the same hormone was observed.

**Table 6:** Mean faecal progesterone concentration (ng/g) (Mean ± SE) during 30-60 days post mating of lioness Radha and Rukmani

| Name of lioness | During pregnancy (30-60days) | Inference |
|---|---|---|
| Radha (5) | $122.87^{a}$±1.14 | Cubbed on date 06-03-2017 |
| Rukmani (5) | $118.68^{a}$ ±0.62 | Suspected pregnancy |

Means bearing same superscript in a column do not differ significantly $p<0.05$.

Figures in parenthesis indicate the number of faecal samples.

## Discussion

**Faecal Estrogen and Progesterone concentration in different stages of Estrous cycle**

Various attempts to improve and facilitate a non-invasive method for the longitudinal monitoring of ovarian activity in non-tractable feline species have been attempted through the determination of execratory fate of estrogen and progesterone in the domestic cat. Estrogen is execrated in feline almost exclusively in faeces as a non-enzyme-hydrolisable conjugate (estradiol sulfate) and unconjugated estrogen (estradiol and estrogen) (Shille *et al*. 1990) [9].

From the present study it was clearly indicative of a rise of estrogen in estrous period which decreased significantly to a lower level at post estrous stage. During post estrus the progesterone concentration rose to a significantly higher level as compared to estrus stage of estrus cycle. During natural estrus lower progesterone concentration and rise in estrogen value favour LH release which facilitate both spontaneous and induced ovulation in certain animals like leopard (Schmidt *et al*. 1988) [10], snow leopard (Brown *et al*. 1995) [11], tiger (Graham *et al*. 2006) [1] and African lion (Briggs *et al*. 1990, Schramm *et al*. 1994) [12, 13].

The significant increase of estrogen during estrus in the present study partially supported the findings of early workers (Schmidt *et al*. 1979, Briggs *et al*. 1990, Graham *et al*. 1995, Umapathy *et al*. 2007) [14, 12, 15, 16] and they recorded 2-3 fold increase in estrogen value during estrus compared to post estrus value in tiger, clouded leopard and African lion. The differences in the actual concentration of hormonal metabolites within species might be influenced by antibody cross reactivity and among the felids might be due to species specific difference in steroid metabolism and excretion (Brown, 2006) [17]. In the present study the higher concentration of estrogen in estrus might be due to formation of multiple follicles and during the post estrus period, the dominance of estrogen is replaced by rise of progesterone as a result of luteal function.

Like estrogen, progesterone or its metabolites in the domestic cat are execrated mostly in the faeces rather than urine. However, in contrast to estrogen none of the progesterone is excreted in its native form (Brown *et al*. 1994) [18]. The progesterone value (ng/g) was the highest (62.92± 0.28) in post estrus sampling which significantly differed ($p<0.05$) from that of estrus (32.08 ± 0.92) period. In *Panthera* species estrogen is the prime inducer for sex arousal and motivates the female for courtship and coitus. The significant increase in estrogen level observed in present study endorsed the role of estrogen for sexual motivation and copulation (Seal *et al*., 1978, Graham *et al*. 2006) [19, 1].

The significant increases in progesterone in post estrus period observed in the present study substantiate its role for maintenance of pregnancy or luteal function following ovulation. The corpus luteum in case of feline species is the major source of progesterone hormone (Graham *et al.* 2006) [1]. The increase in the progesterone concentration during post estrus period might due to development of more numbers of corpus luteum as a result of multiple ovulations. The rise of progesterone in these species may be due to retention of corpus luteum as a result of actual pregnancy or may be due to pseudopregnancy (Putranto *et al.* 2007) [20]. In multiparous animal progesterone concentration in the blood is positively correlated with number of luteal tissue present on ovary.

Although rationality of comparison of progesterone value between pregnancy and non-pregnancy stages is superfluous but mean pregnancy progesterone value (ng/g) of Radha (122.87) and of Rukmani (118.67) increased nearly two fold than the non-pregnancy period suggesting high production of progesterone by the pregnant luteal tissue. In case of tiger luteal tissue is the main source of progesterone production which supported and sustained pregnancy (Umapathy *et al*. 2013) [21].

## Conclusion

Faecal estrogen values during estrus were found to be significantly higher than the post estrous period. Similarly post estrus values of progesterone were significantly higher as compared to follicular phase of estrous cycle.

Non-invasive hormonal assay from faecal material and urine sample could be made from captive wild animals where other conventional method might incur risk to life.

From the present study it could be implied that successful hormonal assay could be made from the wet faecal samples in lions and this technique could be useful for other endangered animals which need protection and conservation.

## Acknowledgement

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