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Is the yak (*Poephagus grunniens* L.) really a seasonal breeder?

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Abstract

Yaks are considered to be seasonally polyestrous and breeding occurs from July to November. Here we show that some yaks in peak non-breeding season do exhibit cyclic luteal activity without exhibiting any behavioral signs around expected estrus. A total of eight non-lactating yaks were selected from the Yak Farm belonging to National Research Centre on Yak for various sets of experiments. The animals were maintained as per semi range system of management. They were allowed to graze during daytime and fed concentrate mixture @2 kg/animal/day as per standard farm practices of the center. Blood samples were collected on alternate days for 30 days by jugular venipuncture from the yaks during peak breeding season (July to November) and from the same yaks in non-breeding season (February to March). The plasma samples were analysed for progesterone and estradiol-17β by RIA and EIA procedures, respectively. During breeding season, the mean plasma progesterone at estrus was basal (≤0.2 ng/ml) and there after started to increase and reached a peak at days 15–16 of the cycle and then declined rapidly to basal levels at estrus. Mean plasma estradiol-17β concentrations were high at estrus and then declined to basal level at days 2–3 of the cycle. The mean plasma estradiol-17β concentrations stayed low for the remaining part of the estrous cycle except for a small elevation, which was exhibited between days 6 and 12 of the cycle.

During non-breeding season, in 50% of the animal studied, plasma progesterone and estradiol-17β levels were at basal level. However, in the remaining animals distinct luteal activity was recorded in terms of plasma progesterone profile. High concentrations of progesterone were maintained in one animal, which suggested existence of cystic corpus luteum. The trend of progesterone and estradiol-17β profiles in other three animals indicated that they could be cycling although they did not exhibit estrus symptoms.

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It was concluded from the present study that with improved nutritional support some yaks exhibit estrous cyclicity even during the non-breeding period of the year.

Keywords: Yak; Seasonal; Progesterone; Estradiol-17β

1. Introduction

Yak, the multipurpose bovid, which provides milk, meat, wool and much needed pack on the precipitous slopes, belongs to the sub family Bovine of family Bovidae. Their natural habitat spreads from 3000 to 6000 m above m.s.l. in the middle and inner Himalayas. The average temperature in natural habitat in summer varies from 4 °C to 6 °C while in the winter; minimum temperature even reaches −40 °C to −50 °C. Yak milk and meat are the main source of protein requirement of the isolated highlanders who have little access to modern life. Yak milk is used for preparation of different milk products like churpi (concentrated sour wet cheese), butter, ghee, toffee and salted tea for their own consumption as well as for religious offering and earning other livelihood commodities such as rice, maize, salt, etc. from the lower altitude habitats through barter system. The total world yak population is about 15 millions and are found in China, Mongolia, Kazakhstan, Tajikistan, Tuva, Buryat, Russia, Pakistan, India, Nepal and Bhutan. Drastic decline in yak population all over the world has become a cause of concern to the development authorities and to animal scientists because this animal largely caters to the needs of the highlanders whose, economy largely depends on yak husbandry besides sheep and goat rearing.

All available literatures in this species indicate that yaks are seasonally polyestrous and breeding occurs from July to November [1–6]. Although some information is available on endocrine profile during breeding season [6,7], no such information is available during non-breeding season on this species. Hence, we decided to determine the profiles of plasma progesterone and estradiol-17β during non-breeding season and to compare them with the profiles in breeding season.

2. Materials and methods

2.1. Experimental animals and blood sampling

A total of eight non-lactating yaks were selected from the Yak Farm (2730 m above m.s.l.) belonging to National Research Centre on Yak for various sets of experiments. The yaks were 4–5 years of age with body weights ranging from 250 to 300 kg. The animals were maintained as per semi range system of management. They were allowed to graze during daytime and fed concentrate mixture comprising of maize grain, wheat bran, mustard cake, mineral mixture and salt @2 kg/animal/day as per standard farm practices of the center. During breeding season, the average daily temperature during the season ranged from 11 °C to 19.77 °C and the pasture was at its best in terms of quality and quantity. However, as the temperature declined after November, the nutritive value of grasses declined due to seeding and subsequent wilting which also reduced its availability to the

yaks. The yaks were closely monitored for the onset of estrus (heat) by observing behavioral signs and bull (teaser) parading at 06:00, 12:00, 18:00 and 24:00 h for 30 min and further confirmed by observing uterine tone on rectal palpation. Blood samples were collected in EDTA coated tubes by jugular venipuncture from the yaks during peak breeding season (July–November) and from the same yaks in non-breeding season (February–March, when average daily temperature ranged from 1.25 °C to 10.75 °C) on alternate days for 30 days. The samples (5 ml) were immediately cooled in ice and centrifuged free of blood cells at 4 °C as soon as possible. The plasma thus obtained was stored frozen at −20 °C until hormone analysis.

2.2. Plasma progesterone estimation

Progesterone was estimated by a simple, direct radioimmunoassay procedure [8]. Radioimmunoassay of progesterone estimation was carried out in plasma using 20 μl of plasma. The sensitivity of the assay for progesterone by direct plasma estimation was 4 pg/tube, which corresponds to 0.2 ng/ml, the 50% binding limit being 70 pg/tube. The intra and inter-assay coefficients of variation for progesterone were 8.4 and 12.0%, respectively. The progesterone antiserum (anti-progesterone-11α-hemisuccinate-BSA) cross-reacted with 4-pregnane-3,20-dione, 11α-hydroxyprogesterone and corticosterone to the extent of 100, 110 and 0.2%, respectively. The cross reactivity of the antiserum was <0.01% with cortisone and hydrocortisone and <0.001% with estradiol-17β, estriol and testosterone.

2.3. Estradiol-17β estimation

A highly sensitive heterologous enzymeimmunoassay procedure for plasma estradiol-17β estimation using the second antibody coating technique was developed for the first time in yak. The procedure employed 1 ml of plasma sample, which was extracted and reconstituted in buffer, an antiserum against estradiol-17β and estradiol-17β-horse radish peroxidase as the enzyme conjugate. Details of the procedure are as follows.

2.3.1. Preparation of affinity purified goat lgG antirabbit lgG

About 40 ml plasma from a goat immunized against rabbit lgG was mixed with rabbit lgG agarose and was loaded onto a small column (column size: 1.5 cm × 5.0 cm). The non-specific proteins were eluted with PBS (pH 7.2). The bound proteins were then eluted with 15 ml of 0.1 M glycine-HCl, pH 2.0. All the steps were performed at room temperature. The eluted fractions (3 ml each) were collected in vials containing 0.2 ml of 1 M Tris–HCl, pH 8.0. The eluted lgG was dialyzed overnight against PBS and the protein content determined by measuring the absorbance spectrophotometrically at 260 and 280 nm, and extrapolated from a normograph.

2.3.2. Preparation of enzyme label

Horse-radish peroxidase (Serva, Germany) was used for coupling to estradiol-17β-glucuronide using the mixed anhydride method [9] with modification. Briefly, the coupling procedure was carried out as follows. Solution A was prepared by freeze-drying a mixture of 2 mg of estradiol-17β-glucuronide dissolved in 50 μl of methanol and 20 μl of 1N HCl

at 50 μm for 2 h. After dissolving the residue in 500 μl of *N*,*N*-di methyl-formamide (Merck, Germany), 6.25 μl of 4-methylmorpholine (Merck, Germany) was added and the solution cooled to −15 °C.

Solution B was prepared by slowly adding 375 μl of dimethyl formamide to 500 μl of horse-radish peroxidase (4.75 mg/ml of water) and the solution cooled to 0 °C.

The coupling reaction was performed by first adding 6.25 μl of isobutyl chloroformate (Sigma, USA) to solution A with continuous stirring for 3 min at −15 °C and the pH of the resulting mixture was immediately corrected to pH 8.0 with 1N NaOH. The mixture was initially incubated for 1 h at −15 °C followed by 2 h at 0 °C. After the addition of 10 mg of $NaHCO_3$, the product was dialysed overnight and further purified by gel chromatography (Sephadex G-25 fine; column: 1.6 cm × 50 cm). The enzyme label so obtained was immediately tested for its titer and stored at −60 °C.

2.4. Benzene extraction of plasma samples

The plasma samples were extracted with benzene as described below:

(1) Yak plasma samples (1 ml) were taken in duplicate in 15 mm × 125 mm glass tubes.
(2) Benzene (6 ml) was then added to each tube containing the plasma sample. The tubes were then vortexed for 2 min and allowed to stay for 1 min. Five milliliter of the upper organic layer was pipetted out in another set of 12 mm × 100 mm glass tubes.
(3) The benzene was then evaporated to dryness in a hot air oven at 50 °C. The process takes nearly 2 days.
(4) The residue was dissolved in 300 μl EIA assay buffer and the tubes were vortexed for 30 s for complete solubilisation.

2.5. Estimation of recovery percentage

Fixed amounts of estradiol-17β viz. 12.5, 25, 50 and 100 pg were added in 1 ml yak charcoal-dextran treated plasma in quadruplicate. Four tubes containing only 1 ml charcoal dextran treated yak plasma were also run as blank tubes. The hormone was extracted with benzene and then reconstituted in EIA assay buffer by the procedure as described above. Then 50 μl of the extracted sample was taken and the amount of hormone estimated by EIA procedure, which is described in detail in the next section. The recovery percentage was then calculated by the following formula:

$$\frac{\text{concentration of hormone determined by EIA/well} \times 7.2}{\text{amount of hormone added per tube}} \times 100$$

The recovery percentage obtained in the study is given in Table 1.

2.6. EIA procedure

First coating: The first coating was performed by adding 0.63 μg of goat lgG dissolved in 100 μl of Coating buffer (pH 9.6) per well of the microtiterplate (Greiner Labortechnik, Germany). The plates were then incubated overnight at 4 °C.

Table 1
Recovery of exogenous estradiol-17β after extraction

Sl. no.	Estradiol-17β (pg/tube) added ($n = 4$)	Estradiol-17β recovered (pg)	Percentage recovery
1.	12.5	11.23 ± 0.41	89.9
2.	25	19.3 ± 0.36	77.2
3.	50	42.6 ± 0.23	85.2
4.	100	93.3 ± 0.76	93.3

Second coating: For blocking the remaining binding sites, 300 μl of EIA assay buffer (pH 7.2) containing 1% BSA was added to all the wells and the plates were incubated for 40–50 min at room temperature under constant shaking.

Washing: The coated plates washed twice with 350 μl of the washing solution (0.05% Tween-20) per well using an automated microtitre plate washer (Model: EL 50 8MS, USA).

Assay protocol: Duplicates of 50 μl of reconstituted buffer containing plasma extract or estradiol-17β standards ranging from 0.2 to 100 pg/50 μl prepared in EIA assay buffer (pH 7.2) were pipetted into respective wells along with 100 μl of diluted enzyme conjugate (1:100,000 in EIA assay buffer, pH 7.2) with the aid of a dilutor dispenser. Then, 100 μl of antiserum (obtained from Institut Fuer Physiologie, Freising-Weihenstephan, Germany, diluted 1:100,000 in EIA assay buffer, pH 7.2) was added to all the wells except the blank. The plates were incubated overnight at 4 °C after 30 min of constant agitation in the dark.

Substrate reaction: The microtiterplates were washed four times, as before, with the washing solution (0.50% Tween 20) and then incubated with 150 μl of the *Substrate solution* for 40 min in dark at room temperature. The reaction was stopped by adding 50 μl of 4 N H_2SO_4 to each well and the yellow colour obtained thereafter was measured at 450 nm with an eight-channel automatic microtiterplate reader (Microscan, India).

2.7. Assessment of estradiol-17β enzymeimmunoassay

Sensitivity: The sensitivity of the assay in extracted plasma was 0.2 pg/50 μl/well, which corresponds to 1.45 pg/ml of plasma.

Precision: The intra- and interassay coefficients of variation of the assay were 7.4 and 8.3%, respectively.

Specificity: The cross reactivity of the antiserum has previously been reported [10]. The antiserum exhibited cross reactivity with Estrone and Estradiol-17α to the extent of 0.7% and 0.9%, respectively. Cross-reactivities with estriol, trenbolone, testosterone, zeranol and DES were <0.25%.

2.8. Statistical analysis

Means and standard errors of means for the two hormones were calculated for each sequential blood sample. The data for hormonal concentrations were analyzed by an

ANOVA for repeated measures technique with a post test for linear trend to compare hormonal changes during different days of estrous cycle, across time using the Graphpad InStat software.

3. Results

3.1. Breeding season

3.1.1. Estrus behavior

The yak cows were closely observed for different behavioral changes during estrus and were also subjected to rectal palpation for the changes in the genital organs at estrus. The duration of estrus as judged from the existence of behavioural symptoms varied from 9 to 23 h. The common signs of estrus and their frequency of occurrence in yak were being followed and mounted by male yaks (100%), standing to be mounted (100%), frequent urination (75%), raising of tail (83.33%), swelling of vulva (66.67%), congestion of vulvar mucous membrane (75%), restlessness and alertness (33.33%) and loss of appetite (16.67%). Bellowing was not observed during estrus. Estrual discharge by yaks was very less.

3.1.2. Plasma progesterone profile

The mean ± S.E.M. levels of plasma progesterone during the different stages of estrous cycle are presented graphically in Fig. 1. The estrous cycle length was calculated on the

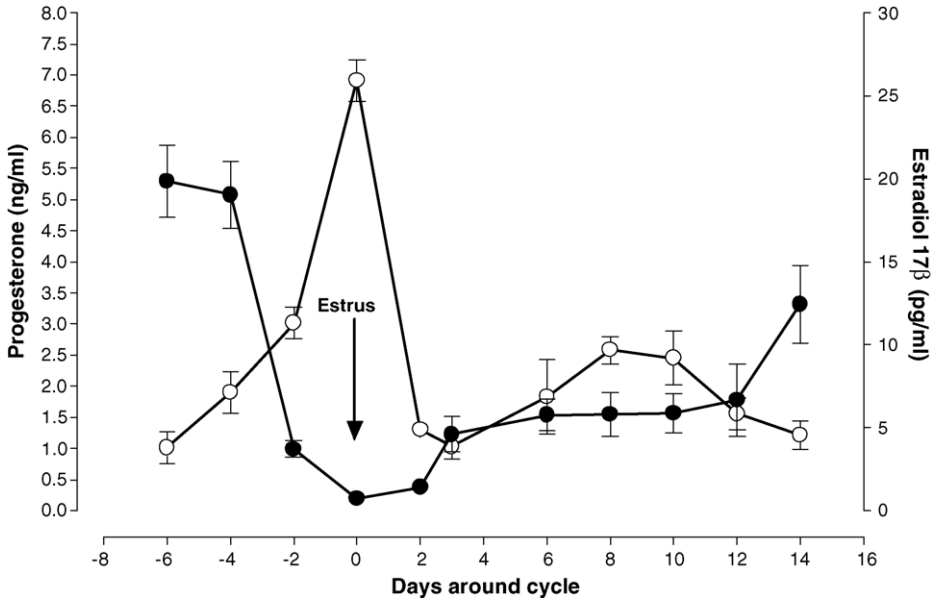

Fig. 1. Plasma progesterone and estradiol-17β profile (mean ± S.E.M.) during estrous cycle in yak ($n = 8$). Progesterone (●) and estradiol-17β (○).

basis of plasma progesterone profile and it varied between 20 and 22 days in individual yaks with a mean of 21.3 ± 0.6 days. On account of variation in estrous cycle length, the plasma hormone profiles have been adjusted in relation to day of estrus. The differences of mean plasma progesterone concentration during different days of the estrous cycle were found to be statistically significant ($p < 0.01$). The plasma progesterone concentration at estrus was basal (≤0.2 ng/ml) and thereafter started to increase and reached a peak at days 15–16 of the cycle, thereafter declined rapidly to basal levels at estrus (Fig. 1). In the early luteal phase (0–6 days), mid luteal phase (7–12 days) and late luteal phase (13–19 days) of the estrous cycle, the mean plasma levels of progesterone were 0.84 ± 0.32, 1.63 ± 0.07 and 3.67 ± 0.99 ng/ml, respectively.

3.1.3. Estradiol-17β profile

The mean ± S.E.M. levels of plasma estradiol-17β profile during the different stages of estrous cycle are presented graphically in Fig. 1. The differences of mean plasma estradiol-17β concentrations during different days of the estrous cycle were found to be statistically significant ($p < 0.01$). Mean plasma estradiol-17β concentrations increased during late luteal phase to peak concentrations at estrus. The hormone concentrations then declined sharply to basal level at days 2–3 of the cycle. The mean hormone concentrations stayed low except for a small elevation between days 6 and 12 of the cycle. In the early luteal phase (0–6 days) period, mid luteal phase (7–12 days) period and late luteal phase (13–19 days) of the estrous cycle, the mean plasma levels of estradiol-17β were 10.39 ± 2.52, 8.26 ± 1.2 and 6.7 ± 1.69 pg/ml, respectively.

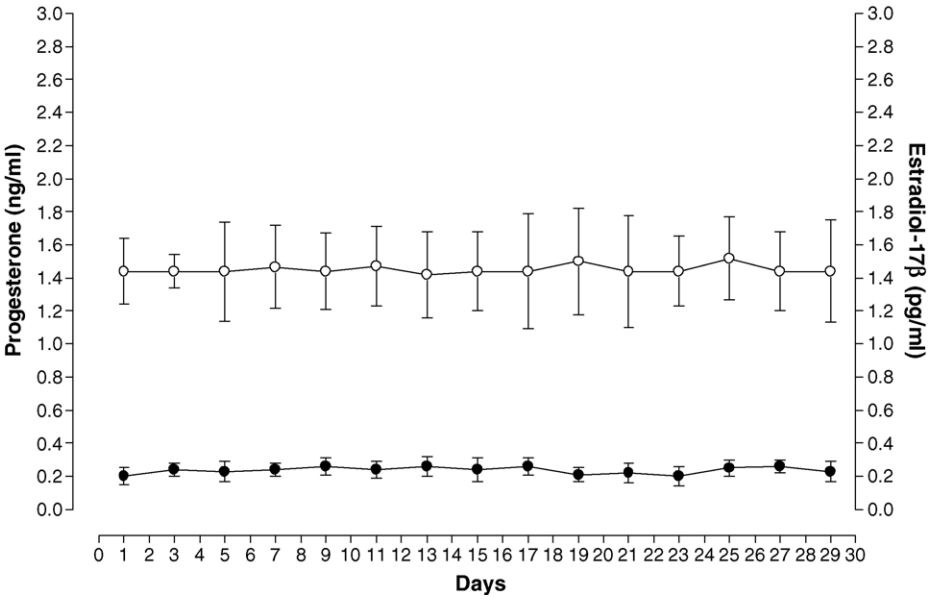

Fig. 2. Plasma progesterone and estradiol-17β profile (mean ± S.E.) during the non-breeding season in four non-cycling yaks. Progesterone (●) and estradiol-17β (○).

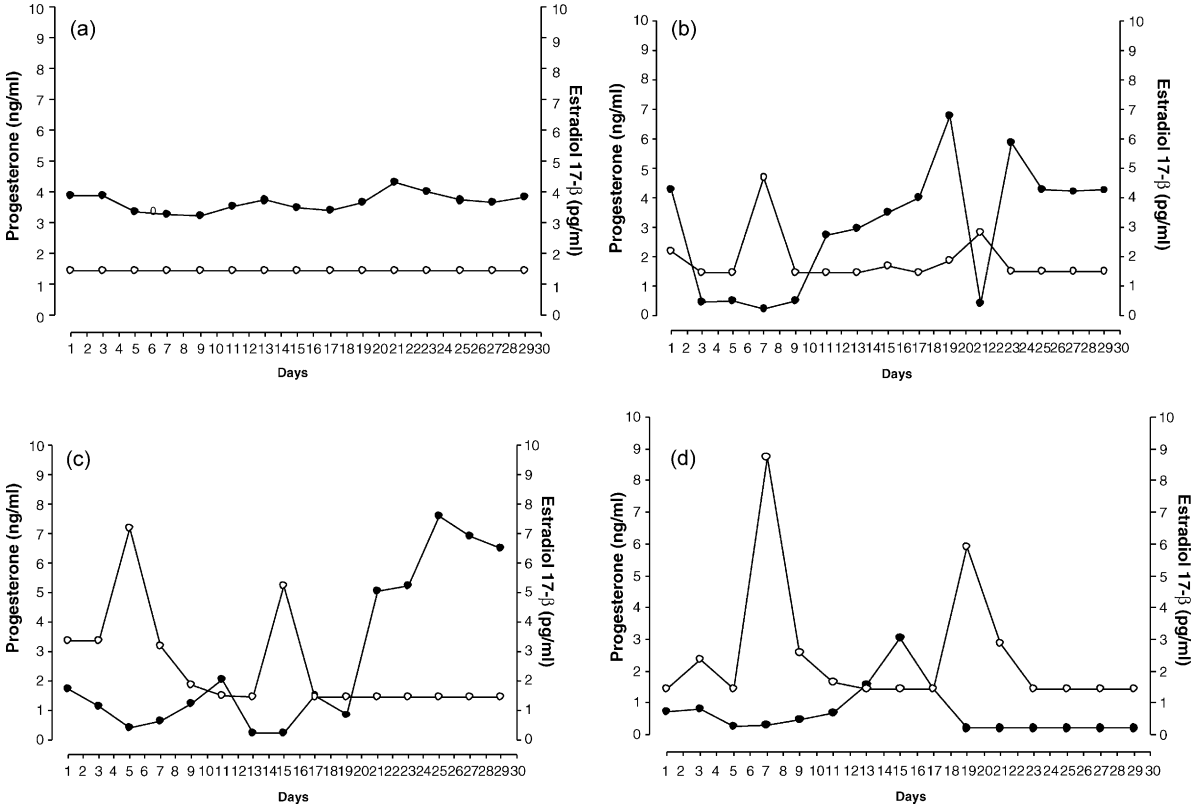

Fig. 3. Plasma progesterone and estradiol-17β profile for individual representative yaks during non-breeding season. Progesterone (●) and estradiol-17β (○). (a) Animal having cystic corpora lutea; (b–d) animals are cycling.

3.2. Non-breeding season

3.2.1. Estrus behavior

In the non-breeding season, no behavioral symptoms of estrus were observed in any yaks.

3.2.2. Plasma progesterone and estradiol-17β profile

The plasma progesterone and estradiol-17β profile during non-breeding season of eight animals studied have been depicted in Figs. 2 and 3. Plasma progesterone and estradiol-17β levels were very low as anticipated in 50% of the animals studied (Fig. 2). However, higher levels of progesterone were recorded in the remaining animals (Fig. 3). In one animal (Fig. 3a) high concentrations of progesterone were maintained throughout the sampling period while in the remaining three animals Fig. 3b–d some cyclic changes in plasma progesterone, and, elevations in estradiol-17β concentrations around expected estrus were observed.

4. Discussion

Although some behavioral signs of estrus were observed, these were not pronounced and hence could not be considered as reliable indicators of estrus.

In the present study, the plasma progesterone profile during different days of estrous cycle agrees well with Yu et al. [7]. However, Yu et al. [7] observed three peaks of estradiol-17β in plasma and milk on the day of estrus, and on days 5 and 14 of the estrous cycle in white yaks in China.

During non-breeding season, higher levels of progesterone were maintained at constant level in one animal (Fig. 3a) without any major fluctuation. It could be due to existence of cystic corpora lutea in this animal. However, the trend of progesterone and estradiol-17β profiles in other three animals (Fig. 3b–d) indicated that they were cycling although they did not exhibit any estrus signs.

Management system for yak follows, predominantly, a traditional pattern, which is dictated by climate and season. In general, the transhumance form of management predominates. During the winter season, when the land is covered with snow at high altitudes, the yak herdsmen migrate with their yaks to lower elevations (3000 m above m.s.l.) in search of pastures but the quality and quantity of grass available to the yaks is very poor. On account of poor nutrition during winter months, body condition of the yaks deteriorates and heavy loss of body weight occurs (15–25%) [5]. With the rise of temperature and humidity during summer months, the snow melts which eventually help in growth of grass in the pasture. During this time, the yak herdsmen along with their yaks migrate to higher elevations in search of lush green fodder. With better quality and quantity of pastures available during summer the yak females improve in body condition and gain weight and make up for their long period of feed deprivation and weight loss during the winter and start exhibiting estrous cyclicity. The environmental and nutritional stress in winter months as described above could probably be the main factors for the occurrence of seasonal anestrous during winter months as reported earlier [1–5]. However, in the present

study where the winter scarcity of feeding was taken care of to some extent by concentrate feeding, at least three animals were found to be cycling during the non-breeding season.

Behavioral symptoms of estrus are brought about by the effect of estradiol produced by growing follicles on central nervous system [11]. The absence of behavioral symptoms in some of the cycling animals during non-breeding season could be due to insufficient production of estradiol from the developing follicles possibly due to nutritional and environmental stresses [12,13]. The mean peak estradiol-17β levels obtained at estrus in yaks during breeding season (25.3 ± 0.3 pg/ml) were significantly higher ($p < 0.01$) than those recorded in the three yaks during non-breeding season (6.9 ± 0.4 pg/ml). Whatever may be the reason; our observation casts doubt on the ''seasonal breeding pattern'' of yak and we propose that this could be corrected by improved feeding management practices during winter months.

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