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Ovarian follicular dynamics, hormonal profiles and ovulation time in Mithun cows (*Bos frontalis*)

Vikram Ramesh[1] | **Laishram S. Devi**[2] | **Vivek Joshi**[3] | **Moonmoon Mech**[1] | **Kobu Khate**[2] | **Meraj H. Khan**[1]

[1]Animal Reproduction and Gynecology, Indian Council of Agricultural Research – National Research Centre on Mithun, Medziphema, Nagaland, India

[2]Livestock Production Management, Indian Council of Agricultural Research – National Research Centre on Mithun, Medziphema, Nagaland, India

[3]Veterinary Medicine, Indian Council of Agricultural Research – National Research Centre on Mithun, Medziphema, Nagaland, India

Correspondence
Vikram Ramesh, Animal Physiology and Reproduction Laboratory, ICAR-National Research Centre on Mithun, Medziphema, Dimapur, Nagaland 797 106, India.
Email: vikram.r@icar.gov.in; vikki955@gmail.com

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Abstract

The study aimed to evaluate follicular dynamics and concentrations of estradiol-17β (E2), progesterone (P4), follicle stimulating hormone (FSH) and luteinizing hormone (LH) during the oestrous cycle and to determine ovulation time in Mithun cows. Ovaries of experimental cows (n = 7) were examined daily by transrectal-ultrasonography for three consecutive oestrous cycles (n = 21). The characteristics of follicular waves, dominant follicle, largest subordinate follicle and corpus luteum and ovulation time were evaluated. The plasma samples were analysed throughout the interovulatory interval to determine the differences in the hormonal profiles (E2, P4, FSH and LH) between different follicular wave cycles. Out of eighteen oestrous cycles analysed, three-wave follicular cycles were maximum (n = 12: 66.66%) followed by two (n = 4: 22.22%) and four waves (n = 2: 11.11%). The two and three waves were statistically compared, and no significant ($p > .05$) differences were observed in day of wave emergence, number of follicles (≥3 mm) recruited, maximum diameter of the ovulatory dominant follicle, growth rates of ovulatory and anovulatory dominant follicles and maximum diameter of corpus luteum. The diameter of dominant follicles was significantly ($p < .05$) greater than subordinate follicles in both ovulatory and anovulatory waves. No significant differences were observed in peak concentrations of estradiol-17β and follicle stimulating hormone between ovulatory and anovulatory waves in all wave cycles. A preovulatory luteinizing hormone surge was observed a day before ovulation in all wave cycles. Progesterone concentrations were lower than 0.5 ng/ml during oestrus and increased sharply to the maximum levels of ≥3.8 ng/ml in all wave cycles. Ovulation time (mean ± SEM), irrespective of follicular waves was 10.5 ± 0.64 h after the end of oestrus. It was concluded that Mithun cows have a preponderance of three follicular waves with little difference between the two- and three-follicular waves and ovulation occurred 10.5 h after the end of oestrus.

KEYWORDS
corpus luteum, estradiol-17β, follicular waves, ultrasonography

1 | INTRODUCTION

Mithun, a unique bioresource, provides livelihood security and plays a key role in the socio-economic and cultural life of the tribal people of Northeastern India; however, the International Union for Conservation of Nature grouped Mithun under vulnerable species of mammals in India (IUCN, 2017). In recent days, the focus has been shifted to adapting a semi-intensive Mithun rearing system for better management. For implementation and improving assisted reproductive technology (ART) procedures, understanding basic ovarian physiology is essential. Ovarian follicular dynamics and their relationship with hormones have been studied in detail in cattle (Chasombat et al., 2014; Ginther et al., 1989; Kastelic et al., 1990) and compared between *B. taurus* and *B. indicus* breeds (M. F. Martínez, G. A. Bo, M. Caccia, R. J. Mapletoft, unpublised data). These studies have helped in better understanding of ovarian physiology and documented that follicular growth occurs in a wave-like fashion during the oestrous cycle in *B. indicus* (Viana et al., 2000) and *B. taurus* (Ginther et al., 1989; Kastelic et al., 1990). In Mithun cows, earlier studies have documented that expression of behavioural oestrus is silent and it is positively correlated with the maximum peak concentration of estradiol 17β (E2) and total oestrogen during the peri-oestrous period (Dhali et al., 2006a; Mondal et al., 2006a). Furthermore, their studies observed that the E2: progesterone (P4) ratio significantly affects the expression of behavioural oestrus in Mithun cows. The E2, P4, follicle stimulating hormone (FSH) and luteinizing hormone (LH) were profiled during the oestrous cycle in Mithun cows (Dhali, Mishra, Mech, Karunakaran, & Rajkhowa, 2005; Dhali, Mishra, Karunakaran, Mech, & Rajkhowa, 2006b). Yet, earlier studies have investigated only hormonal profiles, and there are no ultrasonographic studies to date that can precisely document follicular and luteal dynamics in correlation with hormonal profiles (E2, P4, LH and FSH) throughout the oestrous cycle in Mithun cows. Further, ovulation prediction relative to the onset of oestrus has been done via behavioural signs of oestrus and rectal palpation in Mithun cows (Mondal et al., 2006b). However, the use of ultrasonography can precisely determine the ovulation time which is crucial for successful artificial insemination. Therefore, the objective of this study was to evaluate follicular dynamics and concentrations of hormones (E2, P4, FSH and LH) during the oestrous cycle and to determine ovulation time relative to the oestrus in Mithun cows.

2 | MATERIAL AND METHODs

2.1 | Experimental animals

The study was conducted at the Institute Mithun farm, Indian Council of Agricultural Research (ICAR)-National Research Centre on Mithun cows during the rainy season (April to June; temperature-humidity index: 69.5 ± 1.16) (mean ± SEM). The Mithun cows (n = 7; age = 5–7 years), multiparous, non-lactating, body condition score (3.5 on a 0–5 point scale) and mean ± SEM body weight of 448.93 ± 45 kg were used in the study. All Mithun cows were subjected to the clinical examination of the genital tract, and only those cycling with no reproductive abnormalities were included. The experimental animals were maintained under a semi-intensive system and the same managerial conditions. Mithun cows were fed according to ICAR (ICAR, 2013) feeding standards with a dry matter intake of 2.5% of body weight. Cows received 10 kg [paddy straw (50%) + Napier and Congo signal grasses (50%)], 2 kg concentrate fortified with the mineral mixture and salt. The crude protein (%) and crude fibre (%) were in the range of 8%–9% and 28%–30%, respectively. Animals were offered ad libitum drinking water. The outlines of the study are described in Figure 1.

2.2 | Oestrous synchronization and oestrous detection

The ovaries of experimental Mithun cows were examined by transrectal ultrasonography (Honda, HS-2100 V, Honda Electronics Co., Ltd) equipped with a 7.5 MHz probe to determine the presence of a corpus luteum (CL). If a CL was present, the cows were synchronized with prostaglandin F2α analogue (Cloprostenol, 500 μg, IM, Estrumate™, Vet Pharma, Friesoythe GmbH) and animals were evaluated twice a day after 48–72 h by parading teaser bull and rectal palpation for the onset of oestrus. The experiment started on the day of ovulation that is the disappearance of the dominant follicle as determined by transrectal ultrasonography and designated as day 0 for this experiment. The length of the oestrous cycle was determined as the interval between the two ovulations (interovulatory interval; IOI). The Mithun cows were evaluated for follicular dynamics during the three consecutive oestrous cycles, a total of 21 oestrous cycles were monitored. The three oestrous cycles where ovulation did not occur after the end of oestrus were excluded from data analysis.

2.3 | Follicular dynamics and data processing

The real-time transrectal ultrasonography equipped with a 7.5 MHz probe (Honda, HS-2100V, Honda Electronics Co., Ltd) was employed to monitor the cycles of follicular waves daily from the day before ovulation. All examinations were conducted by the same person throughout the experiment. At the onset of each follicular wave, the ovarian antral follicles ≥3 mm diameter were identified and recorded as previously described by Pierson & Ginther (1987). The dominant follicle (DF) and subordinate follicle (SF) were defined as previously described (Viana et al., 2000). The interovulatory interval (IOI) was expressed as two, three or four follicular wave cycles based on graphical representation. The rate of growth and rate of regression of the DF and SF were calculated as previously described (Ginther et al., 1989). The linear regression was used to determine the growth and regression rates (mm/day) separately for each follicle and the mean growth or regression rate (slope) was calculated. For

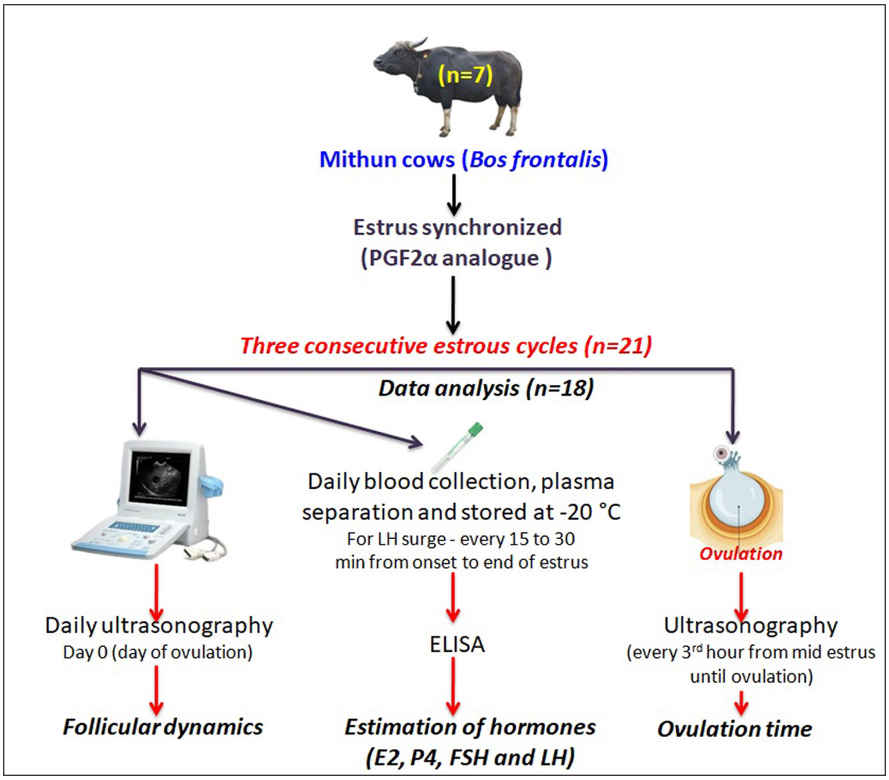

FIGURE 1 Flow diagram of the experimental design: Mithun cows ($n = 7$) were used in the study and oestrus synchronized using the PGF2α analogue (prostaglandin F2α analogue). Three consecutive oestrous cycles, a total of 21 oestrous cycles were evaluated for follicular dynamics, hormonal profiles (E2, P4, FSH and LH) and ovulation time. E2, estradiol-17β; P4, progesterone; FSH, follicle stimulating hormone; LH, luteinizing hormone

each follicular wave, the following parameters were evaluated: day of emergence, wavelength (days), number of recruited follicles, DF and SF diameter at emergence (mm), DF and SF linear growth and atresia rate (mm/day), DF and SF maximum diameter (mm), day at DF and SF maximum diameter, DF and SF lifespan/persistence (days) and onset of DF and SF atresia (day). The corpus hemorrhagicum (CH) and corpus luteum (CL) development were monitored as previously described (Barkawi et al., 2009). For each IOI following CL characteristics were evaluated: CH diameter (mm), growing phase and regression phase (days), growth rate and regression rate (mm/day), CL maximum diameter (mm), day at CL maximum diameter, CL lifespan/ persistence (days) and onset of CL atresia (day).

2.4 | Blood collection and estimation of hormones

The plasma samples were analysed throughout the interovulatory interval (IOI) to determine the differences in the hormonal profiles between different follicular wave cycles. Daily blood samples were collected for three consecutive oestrous cycles via jugular venipuncture in 5 ml heparinized vacutainer tubes and kept at 4°C immediately after collection. The plasma was separated by centrifugation at 2000 ***g*** for 20 min at 4°C and stored at −20°C until assayed for hormones (E2, P4, FSH and LH). For determining LH surge, blood samples were collected every 15 to 30 min from onset to end of oestrus in heparinized 5 ml vacutainer tubes, plasma was extracted and stored at −20°C until assayed.

The commercially available ELISA kits of estradiol-17β (E2) (Cat# 501890, Cayman chemical; analytical sensitivity: 20 pg/ml; intra- and inter-assay coefficients of variation: 9.12% and 13.45%, respectively), progesterone (P4) (Cat# 582601, Cayman chemical; analytical sensitivity: 10 pg/ml; intra- and inter-assay coefficients of variation: 5.46% and 9.37%, respectively), bovine follicle stimulating hormone (FSH) (Cat# MBS7606382, MyBioSource; analytical sensitivity: 0.938 mIU/ml; intra- and inter-assay coefficients of variation: 7.05% and 12.52%, respectively) and bovine luteinizing hormone (LH) (Cat# MBS7606383, MyBioSource; analytical sensitivity: 0.938 mIU/ml; intra- and inter-assay coefficients of variation: 6.25% and 12.07%, respectively) were used for hormone estimation in 96-well microplate using Multiskan™ FC microplate photometer (ThermoFisher Scientific).

2.5 | Ovulation time

Ovaries were examined every 3 h from mid-oestrus (determined by arborization pattern) until ovulation as previously described (Berg et al., 2020). The time of ovulation after the end of oestrus was considered to be the midpoint between the detected disappearance of the DF and previously detected intact follicle, that is ultrasonographic determination of ovulation time minus 1.5 h.

2.6 | Data handling and statistical analysis

The follicular data were aligned to the hormone data as previously described (Evans et al., 1994). Briefly, the follicular data were initially normalized by aligning the growth profiles of the largest follicles to the mean day of wave emergence for respective follicular waves. The concentrations of hormones were normalized to the mean FSH surges that preceded follicular waves. The hormonal data were aligned to the follicular data, such that they matched the normalized follicle profiles.

Statistical analysis was carried out using SPSS version 20, (IBM Corporation). The data of oestrous cycles were grouped according to the number of follicular waves and tested for normality by Shapiro–Wilk statistics and the outliers were removed. Descriptive statistics were calculated for oestrous duration, inter-ovulatory interval (IOI), ovulation time after the end of oestrus, and characteristics of the dominant and largest subordinate follicle and corpus luteum. Differences among means of characteristics of follicular waves, DF and SF between 2 W and 3 W, differences between means of the diameter of DF and SF within the same waves in 2 W and 3 W and hormone profiles between 2 W and 3 W were compared using Student's *t* test. The results are presented as mean ± SEM and considered statistically significant when $p < .05$.

3 | RESULTS

3.1 | Characteristics of follicular waves and dominant follicles (DFs)

The follicular dynamics evaluated in Mithun cows were characterized by two, three and four follicular wave cycles. Out of eighteen oestrous cycles analysed, the three wave (3 W) follicular cycles were maximum (n = 12: 66.66%) followed by two (2 W) (n = 4: 22.22%) and four (4 W) (n = 2: 11.11%). Two animals exhibited repetitive 3 W follicular cycles in three consecutive oestrous cycles. Mean ± SEM (range) duration of oestrus (h) and IOI (days) for two, three and four waves were 54 ± 6 (48–72), 50.16 ± 2.64 (36–72) and 66 ± 18 (48–84) and, 23.5 ± 0.64 (22–25), 23.5 ± 0.72 (20–27) and 30 ± 2 (28–32), respectively. The IOI was greater in the 4 W cycles and no differences were observed between 2 W and 3 W cycles. The characteristics of follicular waves and dominant follicles (DFs) for different follicular wave cycles are presented in Table 1. Days of wave emergence did not differ between 2 W and 3 W cycles ($p > .05$). The duration of ovulatory waves was shorter ($p < .05$) than anovulatory waves. The maximum diameter of ovulatory DF was greater than the anovulatory DF in the 2 W cycles and the diameter of mid-wave DF was lower ($p < .05$) than the first anovulatory and ovulatory DF in 3 W cycles. Neither the maximum diameter of ovulatory DFs nor the growth rates of ovulatory and anovulatory DFs differed in the observed cycles ($p > .05$).

3.2 | Characteristics of largest subordinate follicles (SFs)

The characteristics of the largest subordinate follicles (SFs) for different follicular wave cycles are presented in Table 2. Statistical comparison between 2 W and 3 W cycles revealed no significant ($p > .05$) differences in the diameter of the emergence of DFs and SFs; however, SFs were detected 24 h later after the identification of DFs. The diameter of DFs was greater ($p < .05$) than SFs in ovulatory and anovulatory waves in 2 W, 3 W (Figure 2), and greater in 4 W cycles. The SFs could not be identified in the second and third waves in two oestrous cycles. The representative daily ultrasound images of both ovaries indicating DFs and SFs during the three follicular wave cycles are presented in Figure 3.

3.3 | Growth characteristics of corpus luteum (CL)

The corpus hemorrhagicum was identified immediately after ovulation in only two 3 W cycles. Both compact and cavitary CL was observed during oestrous cycles irrespective of waves. Typical growing, static and regression phases of CL were observed. Table 3 represents the characteristics of the growth of the corpus luteum, and no differences ($p > .05$) were observed between 2 W and 3 W cycles.

3.4 | Concentrations of hormones (E2, P4, FSH and LH) during the oestrous cycles

The concentrations of E2, P4, FSH and LH hormones for 2 W and 3 W cycles are presented in Figure 4. The peak concentrations and amplitudes of E2 in presence of DF were non-significantly ($p > .05$) greater in the ovulatory waves than in anovulatory waves in 2 W and 3 W (Figure 4) and greater in 4 W (Table S1) cycles. The E2 concentrations increased gradually as the DF increased in size and observed maximum on day of maximum diameter of DF in all waves. The P4 concentrations decreased to ≤0.5 ng/ml at the onset of oestrus, during the oestrous phase and before ovulation irrespective of wave cycles and increased sharply to the maximum levels (≥3.8 ng/ml) on the days when CL attained maximum diameter in static phase (Figure 4; Table S1). The FSH (mIU/ml) peaks appeared 1 or 2 days before the emergence of the wave and the peak concentrations and amplitudes of FSH were non-significantly ($p > .05$) greater in the ovulatory waves than in anovulatory waves in 2 W and 3 W cycles (Figure 4) and decreased at the emergence of third wave in 4 W cycles (Table S1). The LH (mIU/ml) hormone increased and attained a surge on the day before ovulation in all different wave cycles (Figure 4) and peaks of 2 W and 3 W cycles did not differ ($p > .05$) (Table S1). The concentration of LH hormone reached a nadir on the day of ovulation in all cycles.

3.5 | Ovulation time

The ovulation time (mean ± SEM) irrespective of follicular waves was 10.5 ± 0.64 h with a range of 7.5–16.5 h after the end of oestrus. Figure 5 represents the mean ± SEM of ovulation time (h) for 2 W, 3 W and 4 W cycles. The percentage of right-sided ovulations was greater than left-sided ovulation (55% vs 44%).

TABLE 1 Follicular wave and dominant follicle characteristics (mean ± SEM) throughout oestrous cycle in two, three and four follicular wave cycles in Mithun cows. Day 0 was the day of ovulation

Parameter	Two wave	Three wave	Four wave
Oestrous cycles (%)	22.22 (*n* = 4)	66.66 (*n* = 12)	11.11 (*n* = 2)
Follicular wave characteristics			
Emergence (day)			
First wave (day)	0.25 ± 0.25	0.16 ± 0.11	0
Second wave (day)	9.5 ± 0.64^{a}	7.83 ± 0.27^{a}	7.5 ± 1.5
Third wave (day)	–	14.58 ± 0.41^{b}	15 ± 2
Fourth wave (day)	–	–	21 ± 3
Wave length (days)			
First wave (day)	15.25 ± 0.47^{aA}	11.8 ± 0.33^{bA}	11.5 ± 1.5
Second wave (day)	8.25 ± 1.25^{aB}	7.23 ± 0.48^{aB}	7.2 ± 0.5
Third wave (day)	–	5.92 ± 0.65^{bC}	6.65 ± 1
Fourth wave (day)	–	–	5.1 ± 1
Number of recruited follicles (NRF) (≥3 mm)			
NRF at emergence of first wave	9.75 ± 1.03	11.16 ± 0.77^{A}	10 ± 1
NRF at emergence of second wave	7.75 ± 0.47	8.75 ± 0.62^{B}	7.5 ± 0.5
NRF at emergence of third wave	–	10.66 ± 0.64^{AB}	7.5 ± 1.5
NRF at emergence of fourth wave	–	–	9
Dominant follicle (DF) characteristics (growing phase)			
Diameter at emergence (mm)			
1st DF	5.7 ± 0.33	5.51 ± 0.26	5.91 ± 0.59
2nd DF	5.64 ± 0.25	5.44 ± 0.14	5.72 ± 0.71
3rd DF	–	5.76 ± 0.17	6.05 ± 0.24
4th DF	–	–	6.37 ± 0.12
Linear growth rate (mm/day)			
1st DF	0.97 ± 0.08	0.98 ± 0.05^{AB}	1.08 ± 0.18
2nd DF	0.87 ± 0.01	0.78 ± 0.05^{A}	0.75 ± 0.14
3rd DF	–	1.094 ± 0.08^{B}	0.84 ± 0.05
4th DF	–	–	0.84 ± 0.19
Day at maximum diameter			
1st DF	9.5 ± 1.19^{a}	6.83 ± 0.27^{b}	4.5 ± 0.5
2nd DF	22.25 ± 0.62^{a}	13.66 ± 0.44^{b}	14 ± 3
3rd DF	–	22.33 ± 0.69^{a}	19.5 ± 3.5
4th DF	–	–	28 ± 2
Maximum diameter (mm)			
1st DF	13.87 ± 0.80	11.80 ± 0.41^{A}	11.1 ± 0.7
2nd DF	14.27 ± 0.31^{a}	9.36 ± 0.29^{bB}	8.93 ± 0.48
3rd DF	–	12.91 ± 0.40^{aA}	8.45 ± 0.35
4th DF	–	–	12.05 ± 0.25
Lifespan/persistence (days)			
1st DF	19.5 ± 1.19^{A}	14.66 ± 0.73^{A}	12 ± 2
2nd DF	14 ± 1^{aB}	11.1 ± 0.50^{aB}	11 ± 1
3rd DF	–	8.83 ± 0.54^{bC}	9.5 ± 0.5
4th DF	–	–	8.5 ± 0.5
Dominant follicle characteristics (regressing phase)			
Onset of regression (day)			

(Continues)

TABLE 1 (Continued)

Parameter	Two wave	Three wave	Four wave
1st DF	11 ± 1.22^{a}	8.08 ± 0.28^{b}	6.5 ± 0.5
2nd DF	–	14.83 ± 0.45^{a}	15.5 ± 3.5
3rd DF	–	–	20.5 ± 3.5
4th DF	–		–
Linear regression rate (mm/day)			
1st DF	0.81 ± 0.07	0.83 ± 0.04	0.74 ± 0.04
2nd DF	–	0.90 ± 0.07	0.52 ± 0.01
3rd DF	–	–	0.51 ± 0.04
4th DF	–	–	–

Note: Rows with different small caps letters (a, b, c) and columns with large caps letters (A, B, C) differ significantly ($p < .05$).

4 | DISCUSSION

The duration of oestrus was longer in Mithun cows with no distinct visible signs of oestrus (silent oestrus) and agrees with an earlier study in Mithun cows with a mean duration of oestrus of 67.2 h (Dhali et al., 2006a). In the present study, 2 W, 3 W and 4 W cycles were identified with a predominance of 3 W cycles; however, the study included only eighteen oestrous cycles and a lower number of 2 W and 4 W cycles were identified. Mithun cows exhibited a greater number of 3 W cycles similar to *B. indicus* (Mollo et al., 2007; Sartorelli et al., 2005; Viana et al., 2000); yet, the occurrence of 2 W or 3 W has been reported with the predominance of 2 W in *B. taurus* and Nellore cows (Sartori et al., 2004; Townson et al., 2002). The 4 W cycles are reported in Brahman heifers (8/117, 6.8%), Brahman cows (3/32, 9.3%) and Gir cows (4/15, 26.7%) (Rhodes et al., 1995; Viana et al., 2000; Zeitoun et al., 1996). The longer mean IOI in the 4 W cycle is attributed to the longer luteal phase with delayed onset of regression of CL (Sirois & Fortune, 1988) and it is a consequence of smaller size and the short period of dominance of DF of the first wave. Furthermore, elevated Insulin-like growth factor-1 (IGF-1) but lower FSH may result in less persistent dominant follicles; therefore, more follicular waves develop during an oestrous cycle (Alvarez et al., 2000). No difference was observed in the mean IOI interval between the 2 W and 3 W as they had a similar luteal phase. In contrast, the studies have recorded a longer luteal phase with longer IOI in 3 W than 2 W cycles (Chasombat et al., 2014; Jaiswal et al., 2009). Ginther et al. also observed 2 days longer luteal phase in 2 W than in 3 W IOIs (Ginther et al., 2014). In the present study, similar mean IOI between 2 W and 3 W cycles is probably due to the lower sample size in the 2 W cycle; however, the above four oestrous cycles had extended lifespan for the first dominant follicle (Jaiswal et al., 2009; Savio et al., 1988) which delayed peak concentrations of circulating FSH (Adams et al., 1992). In addition, the early onset of regression of CL in presence of oestrogen producing dominant follicle has a negative effect on the lifespan of the CL; therefore, the viable dominant follicle present at the time of luteolysis becomes the ovulatory follicle (Figueiredo et al., 1997; Rhodes et al., 1995).

The emergence of waves in all different wave cycles occurred on the day of ovulation or the day after ovulation because of preceding FSH peaks 1 or 2 days before wave emergence. In some oestrous cycles, the recruitment of follicles started before ovulation that appeared as a small cohort of follicles of ≤2 mm. In the 2 W cycle, (M. F. Martínez, G. A. Bo, M. Caccia, R. J. Mapletoft, unpublised data) have reported day 0 as the emergence of the first wave and early emergence of the second wave in *B. indicus* (day 8.0 ± 0.3) and *B. taurus* (day 8.4 ± 0.3) compared with Mithun cows. In contrast, there is a delay in the emergence of both first (1.53 ± 0.17) and second waves (11.02 ± 0.2) in Thai native heifers (Chasombat et al., 2014). In the 3 W cycle, all three waves emerged earlier in Mithun cows compared with the Nellore cattle (Figueiredo et al., 1997) and Holstein heifers (Sirois & Fortune, 1988). The emergence of waves corresponds to the early FSH peaks in Mithun cows. In the 4 W cycle, the emergence of all waves was late in Mithun cows compared with Gir cattle (Viana et al., 2000) due to the longer oestrous cycle length for the four-wave cycle in Mithun cows. In the present study, in all different wave cycles, the wave length of ovulatory waves was shorter compared with anovulatory waves as the existing dominant follicle ovulates when CL undergoes luteolysis. The shorter ovulatory waves are reported in all three different wave cycles in cattle (Chasombat et al., 2014; Figueiredo et al., 1997; Viana et al., 2000). In the 2 W cycle, the wavelengths (days) of the first and second waves in Mithun cows are similar to those reported in Nellore cattle (Figueiredo et al., 1997). In the 3 W cycle, wavelengths (days) of three waves are similar to those reported in Friesian x Hereford heifers (Savio et al., 1988). For 4 W cycles, the wavelengths were shorter in Mithun cows as compared to Gir cattle (Viana et al., 2000). The differences in the wave lengths are due to the difference in the lengths of IOIs in different species and breeds of cattle.

The number of recruited follicles (NRF) ≥3 mm in each wave for different follicular waves was lower in Mithun cows as compared to the *B. taurus* (Mossa et al., 2012) and *B. indicus* (Castilho et al., 2000). The low NRF in Mithun cows is possibly due to the low number of primordial follicles or species differences (Lucci

TABLE 2 Largest subordinate follicle characteristics (mean ± SEM) throughout oestrous cycle in two, three and four follicular wave cycles in Mithun cows. Day 0 was the day of ovulation

Parameter	Two wave	Three wave	Four wave
Oestrous cycles (%)	22.22 (*n* = 4)	66.66 (*n* = 12)	11.11 (*n* = 2)
Subordinate follicle (SF) characteristics (growing phase)			
Detection day			
1st SF	1.25 ± 0.47	1.33 ± 0.22	1 ± 1
2nd SF	12.5 ± 0.86	9.63 ± 0.47	9 ± 2
3rd SF	–	15.81 ± 0.64	16.5 ± 2.5
4th SF	–	–	22 ± 3
Diameter at emergence (mm)			
1st SF	5.72 ± 0.29	5.14 ± 0.27	6.13 ± 0.86
2nd SF	5.33 ± 0.30	5.36 ± 0.22	5.49 ± 0.29
3rd SF	–	5.41 ± 0.23	4.8 ± 0.19
4th SF	–	–	5.76 ± 0.16
Linear growth rate (mm/day)			
1st SF	0.80 ± 0.10	0.76 ± 0.08	1.23 ± 0.24
2nd SF	0.86 ± 0.16	0.78 ± 0.06	0.86 ± 0.24
3rd SF	-	0.80 ± 0.04	0.86 ± 0.08
4th SF	–	–	0.71 ± 0.1
Day at maximum diameter			
1st SF	4.5 ± 0.28	4.33 ± 0.35	2.5 ± 0.5
2nd SF	15.75 ± 1.49	10.81 ± 1.15	12 ± 1
3rd SF	–	18.81 ± 0.77	18 ± 3
4th SF	–		25 ± 2
Maximum diameter (mm)			
1st SF	7.88 ± 0.26	7.31 ± 0.33	7.52 ± 0.12
2nd SF	7.91 ± 0.23	7.08 ± 0.20	7.88 ± 0.38
3rd SF	–	7.77 ± 0.25	6.68 ± 0.62
4th SF		–	8.14 ± 0.14
Lifespan/persistence (days)			
1st SF	7.25 ± 0.47	6.83 ± 0.42	5.5 ± 0.5
2nd SF	7.5 ± 0.5	5.45 ± 0.45	5.5 ± 0.5
3rd SF	–	6.33 ± 0.43	3.5 ± 0.5
4th SF	–	–	7.5 ± 0.5
Subordinate follicle characteristics (regressing phase)			
Onset of regression (day)			
1st SF	5.75 ± 0.25	5.63 ± 0.38	4
2nd SF	16.75 ± 1.49	12.45 ± 0.82	13 ± 1
3rd SF	–	19.90 ± 0.80	19 ± 3
4th SF	–	–	26 ± 2
Linear regression rate (mm/day)			
1st SF	0.93 ± 0.15	0.82 ± 0.08^{AB}	0.81 ± 0.08
2nd SF	0.67 ± 0.06	0.74 ± 0.03^{A}	0.83 ± 0.08
3rd SF	–	0.98 ± 0.07^{B}	0.84 ± 0.08
4th SF	–	–	1.07 ± 0.06

Note: Rows with different small caps letters (a, b, c) and columns with large caps letters (A, B, C) differ significantly ($p < .05$).

et al., 2002). Buffalo has low primordial follicles (VanTy et al., 1989) and subsequent low antral follicles (Baldrighi et al., 2014; Baruselli et al., 1996) compared to cattle. In cattle, NRF numbers in each wave are highly repeatable within individual females irrespective of age, breed, location, season and stage of lactation (Mossa et al., 2012). In Mithun cow, NRF in the ovulatory wave was lower

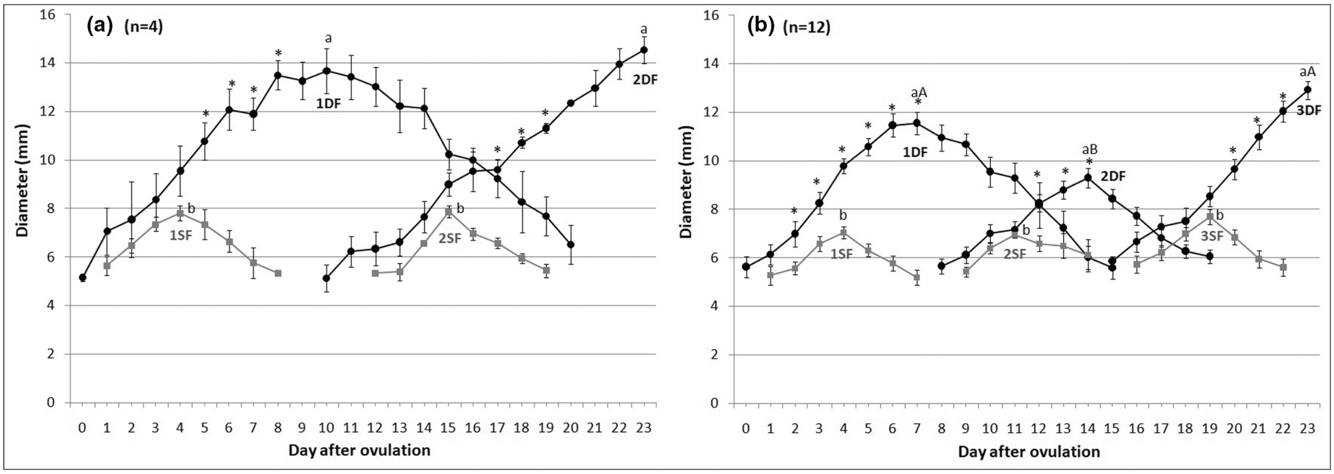

FIGURE 2 Mean (±SEM) diameter (mm) of dominant follicles (DFs) and subordinate follicles (SFs) during the oestrous cycle with two (a) ($n = 4$) and three (b) ($n = 12$) follicular waves. Values with asterisks (*) indicate significant ($p < .05$) differences between DF and SF within the same waves. Values with (a,b) and (A,B) indicate significant ($p < .05$) differences between the maximum diameter (mm) of DF and SF in the same waves and between DF of different waves, respectively. The follicular data were normalized by aligning the growth profiles of the largest follicles to the mean day of wave emergence. Day 0 was the day of ovulation

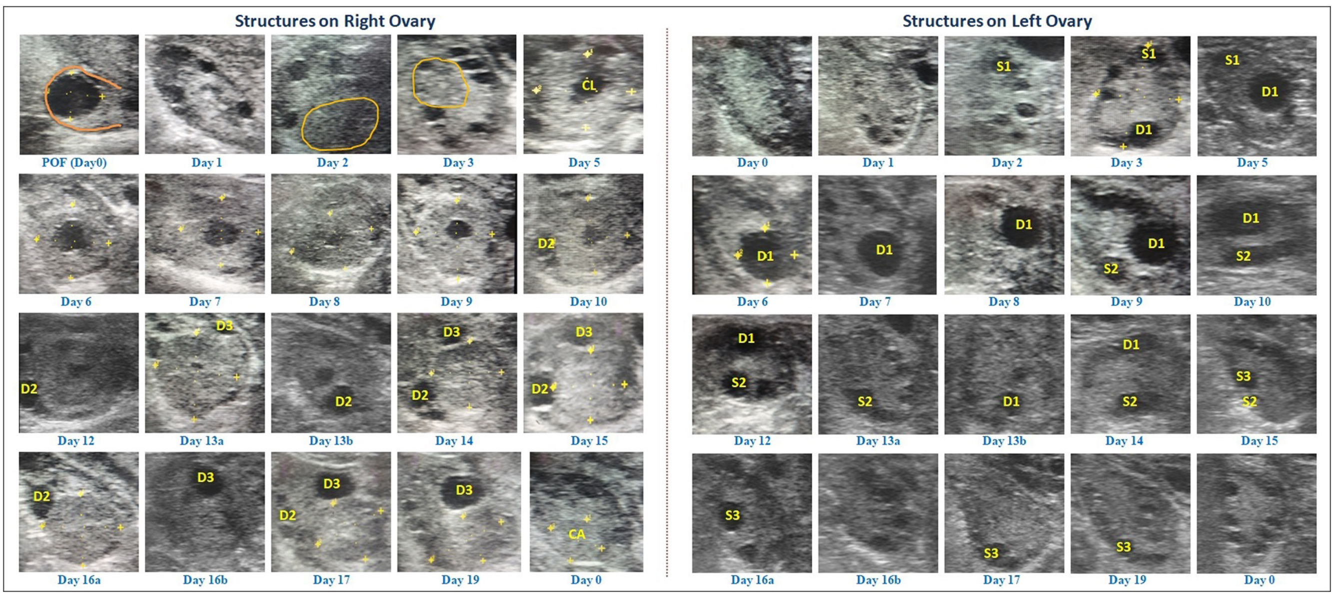

FIGURE 3 Representative daily ultrasound images of Mithun cows ovarian structures during the estrous cycle (three-wave cycle, preovulatory follicle; D1, dominant follicle of first wave; D2, dominant follicle of second wave; D3, dominant follicle of third wave; S1, subordinate follicle of first wave; S2, subordinate follicle of second wave; S3, subordinate follicle of third wave; CL, corpus luteum and CA, corpus albicans. Day 0 was the day of ovulation

compared to the first wave in all different wave cycles. This is in agreement with the findings of a previous study (Chasombat et al., 2014). In addition, low NRF in anovulatory waves is associated with a temporal relationship between the surge in circulating FSH concentrations and the growth of small follicles (Jaiswal et al., 2004). Likewise, the low surge in FSH concentrations during the onset of these waves is a factor for the low number of NRF in anovulatory waves. The factors other than FSH, negative-feedback regulation from estradiol and inhibin-A have a role in the recruitment of a variable number of follicles during each wave (Burns et al., 2005).

In Mithun cows, the maximum diameter of the ovulatory DF is comparable with *B. indicus* cattle (10–12 mm) (Bó et al., 2003; Figueiredo et al., 1997; Rhodes et al., 1995); however, it was smaller than that of *B. taurus* cattle (15–20 mm) (Sartori et al., 2004; Savio et al., 1988). The size of DF and CL was smaller in Mithun cows; yet, the pattern of growth and deviation of DF were similar to cattle. These differences are important for practical applications such as palpation of CL and differentiating the normal ovulatory follicles from cystic follicles. Under the P4 in the dioestrous phase (period from complete development of CL to beginning of the regression of CL), the anovulatory DF in

TABLE 3 Growth characteristics of corpus luteum (CL) (mean ± SEM) throughout oestrous cycle in two, three and four follicular wave cycles in Mithun cows. Day 0 was the day of ovulation

Parameter	Two wave	Three wave	Four wave
Oestrous cycles (%)	22.22 (*n* = 4)	66.66 (*n* = 12)	11.11 (*n* = 2)
Corpus hemorrhagicum diameter (mm)	–	12.5 ± 1.5 (*n* = 2)	–
Compact CL (%)	50	25	50
Cavitary CL (%)	50	75	50
Growing phase (days)	10.5 ± 0.95	11.83 ± 0.42	13.5 ± 0.5
Growth rate (mm/day)	1.15 ± 0.03	0.90 ± 0.05	0.74 ± 0.08
CL maximum diameter (mm)	20.2 ± 0.5	20.47 ± 0.35	20.05 ± 1.55
Day at CL maximum diameter	9.75 ± 0.75	11 ± 0.44	13
Onset of regression (day)	11.75 ± 0.75	12.66 ± 0.46	15.5 ± 0.5
Regression phase (days)	11.75 ± 0.62	11 ± 0.77	15.5 ± 0.5
Regression rate (mm/day)	0.66 ± 0.06	0.84 ± 0.08	0.89 ± 0.14
CL lifespan/persistence (days)	22 ± 0.70	22.25 ± 0.67	28.5 ± 2.5

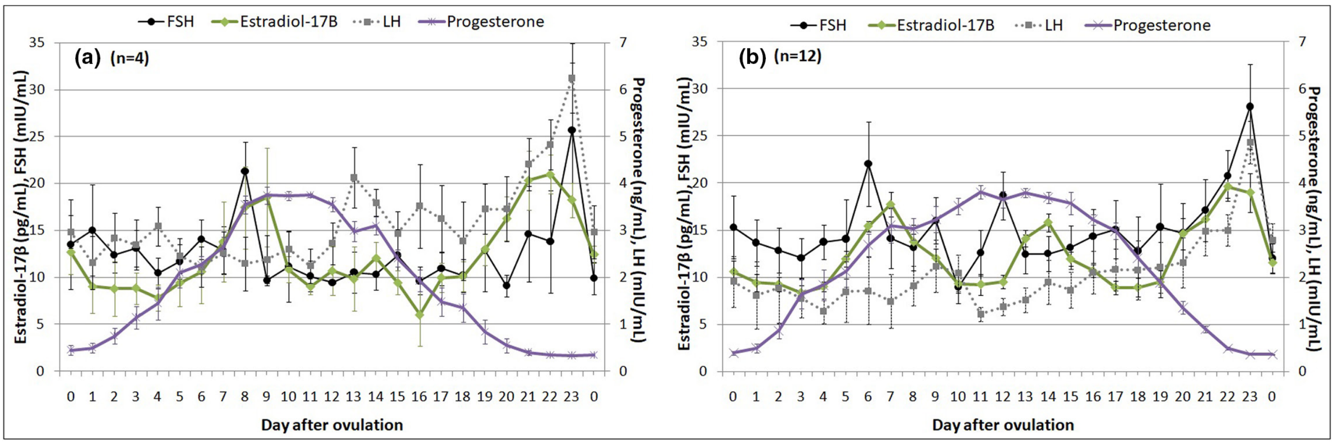

FIGURE 4 Mean (±SEM) concentrations of estradiol-17β (E2), progesterone (P4), follicle stimulating hormone (FSH) and luteinizing hormone (LH) in plasma during the oestrous cycle in two (*n* = 4) and three (*n* = 12) follicular waves. The concentrations of hormones were normalized to the mean FSH surges that preceded follicular waves. Day 0 was the day of ovulation

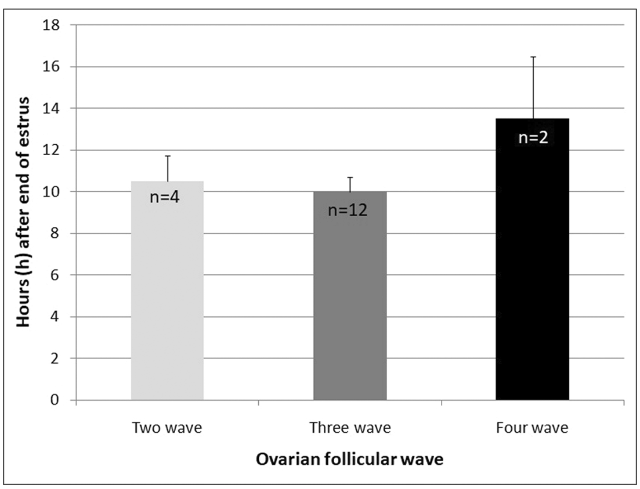

FIGURE 5 Mean (±SEM) ovulation time (h) for two wave, three wave and four wave cycles in Mithun cows

Mithun cows reaches a maximum of 8–10 mm for a shorter period before atresia. The linear growth rates (mm/day) of ovulatory and anovulatory DF (0.8–1 mm) in Mithun cows were similar to the earlier findings in *B. indicus* (0.92 mm) but slower than that of *B. taurus* cattle (1.4–2.00 mm; 1.8–2.2 mm and 1.2–1.8 mm) (Ginther et al., 1989; Knopf et al., 1989; Murphy et al., 1990). A relatively, smaller maximum diameter of ovulatory and anovulatory DF in Mithun cows, explains the slower growth rates. The largest subordinate follicle (SF) increased in diameter for a few days after detection and underwent atresia. There was a clear deviation of DF from SF in each wave; however, SF could not be tracked in a few oestrous cycles. In Mithun cows, the maximum diameter of SF is similar to diameters recorded in Holstein heifers (7.4 and 8–9 mm) (Adams et al., 1993; Fortune, 1994) and Nellore cows (6–7 mm) (Figueiredo et al., 1997). In Mithun cows, linear growth rates of SF were lower compared to DF as they achieved a lower maximum diameter than DF.

Similar to other bovine species, the E2 concentration in Mithun cows started to increase during the follicular deviation of each wave and continued further till the formation of the DF and decreased when the DF has undergone atresia (Evans et al., 1997). In Mithun cows, greater E2 concentrations were observed during oestrus followed by a decline to 5–10 pg/ml 3 days later. The ovulatory wave had greater E2 concentrations in all wave cycles corresponding to the largest dominant follicle. A similar increase in the E2 concentrations corresponded to the emergence of each wave in Mithun cows (Mondal et al., 2006a) with maximum levels observed on the day of oestrus (25–30 pg/ml). The lower E2 concentrations are associated with the smaller diameter of ovulatory DF and have been related to weaker oestrous expression in Mithun cows (Lyimo et al., 2000; Mondal et al., 2006a). The earlier studies of endocrine profile in Mithun cows reported the appearance of only two peaks of E2 corresponding to two-wave cycles (Dhali et al., 2005; Mondal et al., 2006a). However, these studies did not track follicular growth by real-time ultrasonography.

The plasma P4 concentration was low during oestrus (<0.5 ng/ml) and corroborated well with earlier studies in Mithun cows (Dhali et al., 2005; Mondal et al., 2006a) and cattle (Suthar et al., 2011). The plasma P4 concentration reached its peak on the day corresponding to the maximum diameter of CL and remained static for a few days until the onset of luteolysis. The concentration of P4 was lower during ovulation and mid-cycle in Mithun cows (<0.5 and <4 ng/ml) than *B. indicus* (0.83 and 6.4 ng/ml) (Hassan et al., 2017), Holstein cows (0.5 and 5.7 ng/ml) and Brahman cows (0.5 and 9.3 ng/ml) (Díaz et al., 1986). The lower concentration of P4 may be due to the lesser functionality of CL. The CL size of *B. indicus* and Mithun cows is similar (17–21 mm); yet, the reported greater P4 concentration in *B. indicus* than in Mithun cows may be due to species differences. It seems that the decrease in circulating concentration of P4 starting from days 12, 14 and 16 in two, three and four waves, respectively, may have stimulated LH surge before ovulation in Mithun cows.

In the current study, the biphasic pattern of FSH in Mithun cows confirmed the pattern of FSH reported previously (Dhali et al., 2005) with the exception that LH did not follow a pattern of FSH. Similar to other bovine species, the plasma LH concentrations remained low for most of the part of the oestrous cycle and LH surge was observed on the day before ovulation in Mithun cows (Peters & Ball, 1995). The FSH concentrations showed peaks in between the oestrous cycle depending on the number of waves per cycle. The concentrations remained low in between the oestrous cycles and increased at the onset of oestrus. The FSH concentrations were basal during and 24–96 h after deviation similar to *B. taurus* cattle where the FSH remained basal during and after deviation (Ginther et al., 2003; Kulick et al., 2001). In Mithun cows and cattle, FSH concentrations attain peak levels at the onset of oestrus (Adams et al., 1992; Dhali et al., 2005). The maximum concentrations of FSH at the onset of oestrus are responsible for greater NRF immediately after ovulation in Mithun cows. LH pulse frequencies in the follicular phase will promote the final maturation of the ovulatory follicle and makes the environment for functional CL development following ovulation (Aerts & Bols, 2010).

5 | CONCLUSION

The follicular growth occurs in a wave-like fashion during the oestrous cycle in Mithun cows and is comprised of two, three and four follicular waves. The three follicular waves were predominant with little difference between the two- and three- follicular waves. The pattern of growth and deviation of DF from SF were comparable with cattle. The profiles of E2 and FSH hormones were closely related to the number of waves per cycle in Mithun cows. Ovulation occurred 10.5 h after the end of oestrus in Mithun cows.

AUTHOR CONTRIBUTION

The experimental protocol was designed by VR and MHK. The experimets were performed by VR, LSD and MM. The experimental animals were managed by KK. The manuscript was written by VR and revised by VJ and MHK.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICAL COMPLIANCE

The experiment was approved by the Institutional Animal Ethics Committee (IAEC) of ICAR-National Research Centre on Mithun, Nagaland, India (NRCM/IAEC/2020[01]/07, Sl. No. 07).

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