Behavioral and hormonal pattern of repeat breeder cows around estrus

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Abstract

Repeat breeder (RB) cows were compared with normal (CTRL) ones with respect to behavioral estrus intensity, endocrine patterns and concentrations of plasma estradiol, progesterone and LH around estrus, and ovulation timing. A total of 27 and 31 cycles in 12 RB and 18 CTRL cows, respectively, were synchronized by means of the Ovsynch protocol followed by a single PG injection after 7 days. Behavioral estrus and ovulation were observed in 81.5 and 83.8% of the synchronized cycles in the RB and CTRL cows respectively. The RB and CTRL groups had similar estrus durations of 21.4 and 19.6 h respectively, but estrus was more intense in the RB, as indicated by numerically higher overall activity indexes and higher peak neck activity. The interval from PG injection to estrus onset (considered as proestrus) was 8.2 h shorter in RB than in CTRL cows, at 47.9 and 56.1 h respectively ($P < 0.007$), but the average preovulatory follicle size was similar. The estradiol concentration at peak was numerically higher (21%) and the AUC tended to be higher in the RB cows than in the CTRL cows. LH secretion during the period from 18 to 3 h before the LH peak was also lower in RB than in CTRL cows: 2.5 and 4.6 ng/ml respectively ($P < 0.01$). In conclusion, the behavioral estrus was more intense in the RB cows; nevertheless, short proestrus and subdued LH concentrations before the LH peak, which could impair oocyte competence and development, were first reported in RB cows.

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Introduction

Irrespective of the management system, repeat breeding in dairy cows remains a major cause of infertility; it leads to major economic losses because of reproductive wastage, culling, replacement costs, and loss of genetic gain (Bartlett *et al.* 1986). On an individual cow basis, an optimal strategy for treatment of repeat breeding often remains elusive because of its multifactorial etiology, which involves cows, semen quality, and inseminator or insemination technique (see review by Walsh *et al.* (2011)). In females, several interrelated factors such as estrous behavior and certain endocrine aspects have been investigated in modern high-yielding repeat breeder (RB) cows. Prolonged and/or silent estrus has been observed in up to 50% of RB cows (Perez-Marin & Espana 2007, Cummins *et al.* 2012). Several other extensive studies have indicated endocrine impairment in estrogen (E_2), progesterone (P_4), or luteinizing hormone (LH) as potential reasons for the repeat breeding phenomenon (Båge *et al.* 2002, Saumande & Humbolt 2005, Bloch *et al.* 2006) – prolonged estrus and extended estrus-to-ovulation interval were common to all these studies. Clinically, prolonged estrus can be

easily diagnosed and has been treated successfully (López-Gatius *et al.* 2001). However, RB cows with normal estrus duration and ovulation timings, which account for up to 37.8% of repeat breeding (Perez-Marin & Espana 2007), remain a serious challenge because of the elusive etiology. Thus, the objectives of this study were to investigate the quantitative and sequential differences in estrous activity, hormonal patterns around estrus, and ovulation time as reason(s) for infertility in RB cows.

Materials and methods

The procedures used in this study were approved by the Volcani Center Animal Care Committee. Israeli Holstein lactating cows (>60 days in milk) at the Volcani Center Experimental Farm (Bet Dagan, Israel) were used in this study, conducted during the winter (from mid-December 2013 to mid-March 2014), to avoid effects of heat stress. All the selected cows were normal cyclic, had normal estrus duration, and had no history of dystocia, retained placenta, or metritis, before the start of the study. The cows were grouped into two categories: control (CTRL) and RBs. The CTRL cows were >60 days in lactation, cycling, and not inseminated. A cow was considered as RB if it exhibited no clinically detected abnormality and did

not become pregnant after at least four successive inseminations during spontaneous estrus, with normal intervals between artificial inseminations (AIs). The average number of AIs in the RB cows was 7.0 ± 2.0 (range: 4–11). The reproductive health of the genital tracts of all the participating cows was reaffirmed by two successive examinations separated by a 10- to 11-day interval, by means of transrectal ultrasound examination with an Aquila 5-MHz linear array transducer (Pie Medical, Maastricht, The Netherlands).

In total, 18 CTRL and 12 RB cows were used in the study, and the experiment was designed in three clusters, each comprising both CTRL and RB cows. A few cows were included in more than one cluster. The cows were fed according to NRC (2001) recommendations and were housed in the same covered loose pen with an adjacent outside yard. The animals were milked three times a day, and milk yields and body weights were automatically recorded daily with the AfiFarm System (SAE, Afikim, Israel). At the initiation of the synchronization protocol, body condition score (BCS) was determined in all animals by the same technician, according to Edmondson *et al.* (1989). One-week averages of body weight, milk, and fat-corrected milk (FCM 4%) before the investigated estrus were considered as baseline data.

Estrus synchronization, detection of ovulation, and bleeding schedule

A schedule for synchronization and blood collection is presented schematically in Fig. 1. Following selection, the cows were synchronized using the Ovsynch protocol, followed by PG treatment 7 days later (Fig. 1). The GNRH1 and GNRH2 injected on days 0 and 9, respectively, comprised 200 µg of gonadorelin acetate (Gonabreed; Parnell Living Science, Alexandria, NSW, Australia). The PG injected on day 7 (PG1) and day 16 (PG2) comprised 500 µg of cloprostenol sodium (Estroplan; Parnell Living Science). After the PG2 injection, the cows were under intensive observations for the following 6 days; two persons visually monitored the cows round the clock for mounting activity or other behavioral signs typical of estrus (van Eerdenburg 2008). Starting at 15 h from estrus onset, the animals were monitored for ovulation time by repeated 6 h-interval examinations by transrectal ultrasonography, and ovulation was considered to occur 3 h before the last examination, when the preovulatory follicle disappeared from the ovary. The diameter of the preovulatory follicle was also recorded at the time of the first ultrasound screening for ovulation, i.e., 15 h after onset of estrus.

Figure 1 Schematic representation of estrus synchronization, estrus detection, and bleeding schedule.

From the GNRH2 injection on day 9 until the PG2 injection on day 16, one blood sample was collected every 48 h, and from PG2 injection onward every 8 h till estrus onset. From estrus onset, blood samples were collected every 3 h for 24 h, i.e., a total of nine samples. The blood samples were collected from the jugular vein into vacuum tubes containing lithium heparin (Becton Dickinson System, Cowley, UK), and plasma was immediately separated by centrifugation for 15 min at 1000 g and was stored at -32°C pending analysis.

Quantification of estrous activity

Estrous activity was quantified according to neck movements and pedometer readings. However, because neck movements were recorded every 2 h and pedometer readings approximately every 8 h, the former were used for detailed analysis. The cows were fitted with collar-mounted tags (H-tag; SCR Engineers, Hadarim, Netanya, Israel) equipped with a three-dimensional accelerometer. Neck movements were analyzed and filtered by means of complex algorithms in an on-board central processing unit (CPU). The result was a dimensionless 'activity index' that was stored in 12 2-h memory cells inside the H-tag and was recorded three times daily at the milking parlor. Moreover, for estrus quantification by locomotion assessment, the cows were fitted with a pedometer system (AfiFarm System; SAE). Pedometer readings were taken thrice daily at 8-h intervals at the milking parlor, and analyzed automatically by the herd management computer program.

The neck activity index, recorded at 2-h intervals, was considered in terms of actual values, and also by calculating percentage deviations of the actual values from the average of the base values that were recorded on the same clock timings during the 7 days preceding estrus. Estrus onset and duration, as detected visually, coincided with the increase in percentage deviation; therefore, the first increase in activity by $\geq 50\%$ and the last observation indicating decreased activity to $\leq 50\%$ of the base values were considered as the beginning and end of estrus respectively. The percentage deviation data were used for calculating area under the curve (AUC) for plots vs time of mean estrous activity for all 2-h intervals during estrus, which signified estrus intensity, peak activity, time from estrus onset to peak activity, and estrus duration. The pedometer activity index was calculated in a way similar to neck activity, except that a 75% difference from base activities was taken to indicate estrus.

Schedule, methods, and components of endocrine events in blood plasma

Estradiol 17B (E_2) was determined in the first three samples collected every 8 h preceding estrus, and in the first five samples after estrus onset. The E_2 concentration was determined with RIA (DSL4800; Beckman Coulter, Inc., Brea, CA, USA) after extraction of 1 ml of plasma by elution with methanol (Shore *et al.* 1998), on C-18 solid-phase columns (Bond Elut-C18, 500 mg 3 ml; Varian, Lake Forest, CA, USA). The antibody for E_2 bonded 100% to E_2 and 50% to estrone. Sensitivity of the E_2 assay was 2 pg/ml. Progesterone was determined in the nine samples collected from the time of PG1

until 48 h after PG2, and in three samples taken at estrus onset (0 h), and 12 and 24 h after estrus onset. The P₄ concentration was determined by RIA (Diagnostic Products, Los Angeles, CA, USA), with a P₄ detection threshold of 0.2 pg/ml. The LH was determined in all nine samples collected after estrus onset; LH concentrations were measured using the LH EIA kit (INRA, Nouzilly, France), with a detection threshold of 0.6 ng/ml. The inter- and intra-assay coefficient of variation values, respectively, were 4.1 and 3.6% for E₂, and 9.2 and 8.5% for P₄.

The E₂ and LH were analyzed for peak concentrations and also after normalizing the data to the respective peaks, and for the time of peak in relation to PG2 injection and to estrus onset. Values of E₂ and LH plasma concentrations were also transformed to AUC against time (hours) by the trapezoid method (Altman 1991).

Statistical analysis

The E₂ and LH concentrations and AUC in plasma, estrous activity, and timing were analyzed as repeated measurements by the MIXED procedure, version 9.2 (SAS 2002). The effects of treatment, cow, and cluster were included in the model. The autoregressive order 1 (AR 1) was used as a covariance structure in the model.

Distributions of cows with differing intervals from PG administration to E₂ and LH estrus peaks were analyzed by the χ^2 procedure of SAS (2002).

Least-squares means and adjusted s.e.m.s are presented in tables; the significance level was $P < 0.05$ unless otherwise stated.

Results

The general information on the cows included in the study was as follows. The average values (mean $\pm$ s.d.) in the CTRL and the RB group, respectively, were: milk, 44.3 ± 9.8 and 34.6 ± 6.5 kg/day; FCM (4%), 41.3 ± 7.5 and 33.4 ± 6.0 kg/day; parity, 2.4 ± 1.6 and 2.1 ± 1.3 ; body weight, 637.2 ± 80.1 and 682.4 ± 66.1 kg; day in milk (DIM), 107.5 ± 33.6 and 349.7 ± 59.7 days; and BCS, 2.8 ± 0.2 and 2.9 ± 0.1 . Twenty-six out of 31 (83.8%) CTRL cows and 22 out of 27 (81.5%) RB cows responded to the estrus synchronization, exhibited behavioral estrus, and ovulated.

The average activity index, evaluated as actual points of neck activity during estrus, did not differ between the two groups in any of the 2-h intervals. However, when considered in terms of percentage deviation from the base values, the average neck activity was higher in the RB cows in most of the 2-h intervals (Table 1 and Fig. 2A). Consequently, the AUC for the percentage increases and the average deviation for all the 2-h-interval records tended to be higher in the RB cows than in the CTRL cows ($P < 0.1$; Table 1). The peak activity was also 30% higher in the RB cows than in the CTRL cows ($P < 0.04$). However, the interval from estrus onset to peak activity was 1.6 h shorter in the CTRL cows than in the RB cows ($P < 0.1$). Estrus duration did not differ

Table 1 Comparison between average (mean $\pm$ s.e.m.) estrous activity parameters of control (CTRL) and repeat breeder (RB) cows.

Parameter	Group		S.E.M.	P <
	CTRL	RB		
Neck activity ^a				
Intensity ^b	159.5	186.8	9.9	0.11
AUC	1520.8	1914.1	117.0	0.10
Peak activity	331.3	428.1	29.3	0.04
Estrus onset to peak (h)	11.3	12.9	0.73	0.13
Estrus duration (h)	19.6	21.4	1.05	0.25
Pedometer ^c				
Intensity ^d	283.3	319.8	19.2	0.24

^aPertains to percentage of increase from the base values at 2-h intervals.

^bCombined activity for all 2-h intervals. ^cPertains to percentage of increase from the base values at 8-h intervals. ^dCombined activity for all 8-h intervals.

between the two groups. Better estrus expression in the RB cows was also confirmed by numerically higher pedometer activity during estrus (Table 1).

The average plasma E₂ concentrations for most of the surge timings were numerically higher in RB cows than in CTRL cows (Fig. 2B), which led to a tendency for a higher AUC of E₂ in the RB cows than in the CTRL cows (Table 2; 105.2 vs 85.6 pg/ml $\times$ h respectively; $P < 0.13$). The average peak plasma LH concentration did not differ between the groups. However, before the ascending limb of the LH peak (-18 to -3 h), the pooled LH concentrations were lower ($P < 0.01$) in RB cows than in CTRL cows, at 2.5 ± 0.1 and 4.6 ± 0.6 ng/ml respectively (Fig. 2C). The intervals from E₂ peak to LH peak did not differ between the two groups of cows (Table 2).

Average plasma P₄ concentrations at various points during the synchronization procedure and estrus are presented in Fig. 3. Except for the time of PG1, which was sampled blindly, the plasma P₄ concentrations were similar between the two groups during the synchronization protocol and estrus.

The intervals from PG2 injection to ovulation, endocrine-related events, and estrus onset are given in Table 2. Unlike the RB cows, all of which were in estrus within 60 h after PG2 injection, $\sim 31\%$ of the CTRL cows started behavioral estrus after more than 60 h ($P < 0.004$; Fig. 4A). Furthermore, almost all the RB cows exhibited E₂ (Fig. 4B) and LH (Fig. 4C) peaks within 70 h after PG2 injection ($P < 0.009$ and $P < 0.02$, for E₂ and LH respectively), whereas approximately one-third of the CTRL cows took more than 70 h to reach the respective endocrine peaks (Fig. 4B and C respectively). The onset of estrus relative to the PG2 injection was on average 8.2 h earlier in the RB cows than in the CTRL cows ($P < 0.007$; Table 2 and Fig. 5A). Furthermore, times from PG2 administration to E₂ ($P < 0.08$; Fig. 5B) and LH ($P < 0.009$; Fig. 5C) peaks were ~ 8 h earlier in the RB cows.

The time from estrus onset to ovulation did not differ between the two groups. The average pre-ovulatory follicular diameter, recorded 15 h after estrus onset, was

Figure 2 Average (mean $\pm$ S.E.M.) percentage increase from base values of neck activity (A) at 2-h intervals during estrus in CTRL (filled square) and RB (filled circle) cows; average E₂ concentration in blood relative to E₂ peak (B) in CTRL (filled square) and RB (filled circle) cows; average LH concentration in blood relative to LH peak (C) in CTRL (filled square) and RB (filled circle) cows.

also similar between the CTRL (17.7 ± 0.5 mm) and the RB (17.0 ± 0.6 mm) cows.

Discussion

A prerequisite for optimal timing of AI and oocyte fertilization is the cow's ability to exhibit behavioral estrus and manifest a successful ovulation. In this study,

we investigated the quantitative and sequential events with respect to behavioral estrus intensity and endocrine patterns around estrus of RB cows compared with normal cows. The behavioral estrus was more intense in the RB cows and ovulation timing was within the normal range in both groups. However, short proestrus and subdued LH secretory pattern before the LH peak, which could impair oocyte competence and development, could be the potential reasons for reproductive failure in RB cows.

Estrus intensity and ovulation time

In general, the average duration and range of the estrous period recorded in the RB and CTRL cows in this study were within the normal range and similar to the 20.3-h duration recorded previously (Lyimo *et al.* 2000). Reductions in both intensity and duration of estrus form a major contributing factor to the decline in reproduction efficiency in modern dairy cows (Law *et al.* 2009). Stevenson *et al.* (1983) found a positive correlation between estrus intensity and fertility in cattle, and Cummins *et al.* (2012) found that, in Holstein cows grouped according to the high and low fertility history, the peak estrous activity was 41% higher in those with a high fertility history. Previous investigations of estrus expression and ovarian function in RB cows or those with low fertility history revealed that $\sim 22\%$ of them exhibited silent heat (Perez-Marin & Espana 2007, Cummins *et al.* 2012). However, these findings contradict those of this study, in which more intense estrus and higher peak activity were observed in RB cows than in CTRL cows. The discrepancy between these findings could be explained as follows: within a certain group of cows, differences in estrus intensity could be related to fertility performance; however, in this study, by design, we compared two distinct groups that comprised RB and normal cows respectively. No other studies

Table 2 Comparison between control (CTRL) and repeat breeder (RB) cows with regard to average (mean $\pm$ S.E.M.) estrus- and endocrine-related characteristics, as related to PG and estrus onset.

Parameter	Group			
	CTRL	RB	S.E.M.	P<
PG to estrus onset (h)	56.1	47.9	1.95	0.007
PG to E ₂ peak (h)	58.0	50.2	3.04	0.08
PG to LH peak (h)	65.2	57.5	1.91	0.009
PG to ovulation (h)	84.1	79.6	2.4	0.20
Estrus onset to E ₂ peak (h)	2.1	2.5	1.93	0.97
Estrus onset to LH peak (h)	9.3	9.5	1.3	0.90
E ₂ peak to LH peak (h)	7.2	7.2	1.96	0.98
LH peak to ovulation (h)	20.2	21.7	1.5	0.48
Estrus onset to ovulation (h)	28.9	31.5	1.58	0.25
E ₂ concentration at peak (pg/ml)	18.8	22.7	2.13	0.21
AUC – E ₂ (pg/ml $\times$ h)	85.6	105.2	7.48	0.13
LH concentration at peak (ng/ml)	10.5	10.1	0.58	0.63
AUC – LH (ng/ml $\times$ h)	34.2	32.4	1.76	0.48

Figure 3 Average (mean $\pm$ S.E.M.) plasma progesterone concentration at various stages of synchronization and estrus in CTRL (filled square) and RB (filled circle) cows (// separates pre-estrous from estrous periods). E (1), E (5), and E (9) are the first, fifth, and ninth samples collected at estrus onset, and 12 and 24 h after estrus onset respectively.

have quantified estrus intensity in RB cows with normal estrus duration.

Several other studies attributed the failure of fertility in RB cows or those with low fertility history to delayed ovulation (Båge *et al.* 2002, Saumande & Humbolt 2005, Bloch *et al.* 2006) or ovulation failure (López-Gatius *et al.* 2005a, Demetrio *et al.* 2007, Cummins *et al.* 2012). However, in this study, in spite of slightly longer estrus-to-ovulation interval in the RB cows than in the CTRL cows (31.5 vs 28.9 h; NS), all the CTRL and RB respondent cows ovulated within the normal range. Thus, in this study, estrus intensity and ovulation perturbations were, at least, not the underlying causative factors of infertility in the RB cows.

Hormones and behavioral estrus intensity

In several studies, the intensity and duration of estrus were positively related to circulating E_2 concentration (Katz *et al.* 1980, Britt *et al.* 1986, Lyimo *et al.* 2000, Lopez *et al.* 2004). However, examination of ovariectomized progesterone-primed cows revealed that those cows maintained on higher circulating E_2 (12 vs 6 pg/ml) exhibited increased estrus duration (17.1 vs 8.8 h), but similar estrus intensities (Reames *et al.* 2011). In contrast, in this study, the RB cows that had higher circulating E_2 exhibited higher estrus intensity, but similar estrus duration to the CTRL cows. Some other reports, however, suggested a threshold level rather than circulating concentrations of E_2 to trigger 'complete' estrus, and that the increasing E_2 concentration increased neither the duration nor the intensity of estrus (Glencross *et al.* 1981, Cook *et al.* 1986, Coe & Allrich 1989, Allrich 1994). Furthermore, variation in estrus characteristics at an individual or herd level cannot be precluded (Bertilsson *et al.* 1998).

A negative correlation between estrus intensity and P_4 concentration around ovulation might be another factor that influences estrus intensity (Waldmann *et al.* 2001). Davidge *et al.* (1987) found that heat signs were depressed in a dose-dependent manner after P_4

supplementation, and suggested that P_4 blocked the estrus-inducing action of E_2 . However, no differences in P_4 concentrations between groups were observed in this study (Fig. 3); therefore, we hypothesize that higher estrus intensity in the RB cows was linked directly to higher E_2 concentrations. Moreover, a lack of P_4 differences between the groups during the estrus in this study also rules out the suprabasal P_4 -related behavioral, ovarian, and endocrine disturbances such as prolonged estrus, weak estrus expression, a prolonged interval between estrus onset and LH peak, or delayed ovulation, that had been blamed for repeat breeding in cattle (Duchens *et al.* 1994, Båge 2003, Singh *et al.* 2005).

Figure 4 Distribution (%) of cows among time intervals from PG administration to estrus onset (A), estradiol peak (B), and LH peak (C) after PG administration in CTRL (filled square) and RB (filled circle) cows.

Figure 5 Average percentage increase from base values of neck activity (i.e., estrus onset (A)), E_2 (B), and LH (C) blood concentrations relative to the time of PG2 injection in CTRL (filled square) and RB (filled circle) cows.

Timing of estrus onset, E_2 , and LH peaks

In this study, the interval between PG2 injection and estrus onset, E_2 , and LH peaks was ~ 8 h shorter in the RB cows than in the CTRL cows (Fig. 5A, B and C respectively) and, furthermore, all the RB cows entered estrus within 60 h after PG2 administration compared with only 69.3% of the CTRL cows (Fig. 4A). A variation

in the interval from PG administration to the onset of estrus has previously been analyzed from three different viewpoints: i) the stage of estrous cycle at the time of PG injection; ii) variations in P_4 concentration; and iii) differences in the size and maturity of the dominant follicle. In the earlier studies, the stage of estrous cycle at the time of PG injection was considered in itself as the reason for variation. When PG was given between days 5 and 8 of the estrous cycle, the mean time to estrus ranged from 48 to 72 h (Tanabe & Hann 1984, Watts & Fuquay 1985); this was, however, extended to 70 h when PG was injected between days 8 and 11 of the cycle (King *et al.* 1982, Stevenson *et al.* 1984). If the day of GNRH2 injection was considered as day 0 in this study and PG2 administered 7 days later, the time taken for estrus onset was similar to that found in the studies cited earlier but, characteristically, all the RB cows responded within the lower levels of the timing range. Variations of P_4 concentration could be another factor regulating estrus onset (Larson & Ball 1992). However, in this study, similar P_4 concentrations at the time of PG2 administration were found in both groups (Fig. 3), which excluded P_4 variation as a reason for early estrus onset in the RB group. The third reason could be differences in the size and maturity of the most recently emerged dominant follicle at the time of PG administration (Kastelic & Ginther 1991, Twagiramungu *et al.* 1992, 1995, Ferguson & Galligan 1993). The GNRH2 in the Ovsynch protocol ovulates 86–100% of the pre-ovulatory follicles generated from GNRH1 in cyclic cattle (Wiltbank 1998). In such a case, it can be assumed that most, if not all, of the cows in the present experiment were in a similar follicular status following GNRH2, and this assumption is also supported by a similarity in P_4 concentrations and the diameters of pre-ovulatory follicles recorded 15 h after estrus onset. The similar diameters of preovulatory follicles also indicate that the rate of follicular development could have been faster in the RB cows; the average sizes mentioned earlier in this study were reached within 62.9 and 71.1 h after PG2 administration in RB and CTRL cows respectively. Thus, the only plausible explanation for earlier estrus onset in the RB cows when compared with the CTRL cows must be the higher E_2 concentration at the time of estrus onset.

The peak E_2 and LH concentrations in this study were within the range of those reported previously (Ribadu & Nakao 1999, Saumande & Humbolt 2005). The sequence and time relationships among the various endocrine/ovulation events in this study were also consistent with most of the related data published thus far (Schams *et al.* 1977, Dieleman *et al.* 1986, Larsson 1987, Rajamahendran *et al.* 1989, Stevenson *et al.* 1998, Saumande & Humbolt 2005), but there were some exceptions. In seven CTRL and five RB cows, the E_2 peak preceded estrus onset and, in four CTRL and four RB cows, the LH peak preceded the E_2 peak. In the latter

group, both peaks occurred after estrus onset; occurrence of the LH peak before estrus reduces fertility (Saumande & Humbolt 2005, Bloch *et al.* 2006). The exceptions described earlier in this study are not uncommon and were recorded in previous studies (Cook *et al.* 1986, Coe & Allrich 1989, Reames *et al.* 2011).

Proestrous period

Early estrus onset in the RB cows could have two implications. First, the short fertile half-life of gametes restricts the period of breeding and fertilization (Nebel *et al.* 2000); therefore, adopting a fixed time for AI could not yield similar conception rates in the two groups of cows, as arranged in this study. Hence, estrus detection should be included in the AI protocol of programed breeding, or the timing of blind AI should be adjusted in light of the new findings. The second implication is the duration of proestrous period, which begins with the luteolytic pulses of PG and CL lysis. Early return to estrus, as demonstrated in the RB cows in this study, can be considered as a short proestrous period. There are several compelling findings that associate lower conception rates in cows with reduced proestrous duration: Dadarwal *et al.* (2013) found that cows with proestrus of 12 and 36 h had conception rates of 11 and 46% respectively. Ribeiro *et al.* (2012) found a similar trend of lower conceptions rates in cows with shorter proestrous period. Although not consistent among various studies, smaller follicles and low E_2 contents in cows with short proestrus have, in themselves, been the probable reasons for reproduction failure. Mussard *et al.* (2003a) reported that a proestrus of 52.8 ± 2.4 h and the corresponding follicular diameter of 13.6 ± 0.2 mm resulted in a conception rate of 57%; and Mussard *et al.* (2003b) found that a proestrus of 24.0 ± 2.4 h with the corresponding follicular diameter of 11.1 ± 0.2 mm resulted in a conception rate of 8%. The same group reaffirmed the importance of proestrus length for conception rates, irrespective of follicular diameter (Mussard *et al.* 2007), which was also found by Bridges *et al.* (2010).

It is known that priming of the reproductive tract with ovarian steroids for an optimal period benefits reproduction, for example, after synchronization with a double PG system, injected 13 days apart, fertility increased by $\sim 10\%$ in the cows supplemented with P_4 for 5 days before the second PG administration (Xu *et al.* 1996). Accordingly, we hypothesize that short proestrus-linked E_2 exposure of the reproductive tract limits its direct roles in production of the oviductal secretory glycoproteins (Buhi 2002) and in uterine receptivity (Ozturk & Demir 2010) required to increase the success of fertilization and improve embryo quality and viability (Atkins *et al.* 2013, Jinks *et al.* 2013). Furthermore, the frequency of abnormal embryos was enhanced in the oviducts of RB cows (Linares *et al.* 1980). Whether the

latter observation is linked to altered E_2 exposure-mediated production of oviductal secretory glycoproteins needs further investigation; evaluation of oocyte competence can provide some clues regarding the etiology of repeat breeding.

LH curve

Higher E_2 stimulated higher LH (Short *et al.* 1979). However, in the RB cows, in this study, in spite of increased E_2 we recorded low LH before its peak (Fig. 2C). This again could be a consequence of a short proestrus. A short E_2 exposure influenced neither the timing of the LH surge onset nor the amplitude of the GnRH surge, but less LH was released, which suggests a requirement for longer E_2 priming at the pituitary gland, to enable the full amplitude of LH secretion (Evans *et al.* 1997). The LH surge, which lasts for 8–10 h in cows (Chenault *et al.* 1975), triggers oocyte maturation and, accordingly, the LH surge has been the focus of nearly all investigations. However, the changes within the oocyte begin much earlier, and low pre-surge LH concentrations may prevent the development of a normal oocyte. For instance, at 2 h pre-LH surge, the gene expression of the cumulus cells is set to receive the last major induction of final oocyte maturation. In cows, under normal LH secretory mechanisms, mainly two genes – tribbles homolog 2 (*TRIB2*) and ERBB receptor feedback inhibitor (*ERRFI1*) – had significantly higher expression in the cumulus cells around oocytes at 2 h before the LH surge than those evaluated 6 h after the LH surge. In cattle, a stronger expression of these genes along with a few others terminated the progression/proliferation of cumulus cells, so that they could undergo rapid extracellular matrix expansion after the LH surge (Assidi *et al.* 2010). Thus, a lower LH concentration before the LH peak may prevent normal changes in the cumulus cells in the RB cows. Furthermore, a low pre-surge LH concentration is also a reason for a slightly longer estrus-to-ovulation interval in the RB cows than in the CTRL ones (31.5 vs 28.9 h), as observed previously (Bloch *et al.* 2006). It is worth mentioning that in all previous studies that related length of proestrus to fertility, the duration of proestrus was experimentally manipulated, and it was truncated by generating an LH surge using exogenous GnRH, in contrast to the spontaneous LH surge in this study. Moreover, the LH concentrations before the induced surge were beyond the scope of previous studies; therefore, the present findings are unique.

As the RB cows had been inseminated up to 11 times and had a much longer *post partum* period, there were conspicuous, but inevitable differences between the CTRL and the RB cows in DIM, BCS, and milk yields. These differences need to be discussed in light of the present findings. It has been observed that, after the first silent estrus *post partum*, the DIM and the number of previous estruses did not affect the estrus intensity

(Yániz *et al.* 2006). Similarly, variation in BCS does not affect the duration of estrus in beef cows (Castro *et al.* 2013). In this study, the average milk yield in the CTRL cows was significantly higher than that in the RB cows: 44.3 and 34.6 kg/day respectively. The E₂ concentration was also higher in the RB animals, whereas the P₄ concentrations in the two groups remained similar. There are conflicting findings on the effect of milk yield on estrus expression in cows. van Eerdenburg (2008) suggested no correlation between milk yield and estrus expression, while several other studies considered that increased metabolic clearance of ovarian steroids in high yielders reduced the behavioral manifestations of estrus (Lopez *et al.* 2004, López-Gatius *et al.* 2005b). Lopez *et al.* (2004) reported that cows with higher milk yield had a lower serum E₂ concentration on the day of estrus and greatly reduced estrus duration compared with those with lower yields. Similarly, in another study on estrus events, each 1-kg increase in milk yield was associated with a 1.6% decrease in walking activity (López-Gatius *et al.* 2005b). These results fit with the findings of this study, in which the CTRL had higher milk yields, and lower E₂ concentration and estrus intensity than the RB cows. Furthermore, increased E₂ in the RB cows might be due to more steroidogenically active follicle, as, in another experiment, we have found higher E₂ concentrations in the follicular fluid from E₂-active follicles in a group of RB cows than in normal cows (P Sood, unpublished data).

A limitation to our study is the possibility of CTRL cows to fall into the RB category. However, as stated earlier in this study, we have taken a few means to exclude cows with any history (as dystocia, retained placenta, etc.) and monitored ovaries and uterus to minimize the likelihood of having potential RB in the CTRL group. Similar to that, we have defined the RB as cows that did not conceive after at least four AIs, although there was a chance of these cows to conceive in the following AI. We were well aware of these limitations when planning the study, and also believe that this is inevitable in such a study.

In conclusion, the occurrence of better estrus expression, similar follicular diameters, and higher E₂ in RB cows when compared with CTRL cows indicates that the etiology of repeat breeding lies beyond these parameters. However, a short proestrus and subdued LH secretory pattern, especially before the LH peak, could be the potential reasons for reproductive failure in RBs. Subsequent validation of the curative measures *per se* would be of immense importance.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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