

## Follicular growth patterns in repeat breeder cows

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**ABSTRACT:** The aim of this study was to examine follicular development patterns in eighteen repeat breeder cows through natural oestrus cycles. Ovarian ultrasonographic examinations over 32 days after artificial insemination revealed that two follicular waves were the predominant patterns in animals with this syndrome (72.2%). Cycles with one or four waves rarely appeared. The ovulatory follicular diameter (day 0) was larger ( $P < 0.01$ ) in cycles with a small number of waves; no differences were detected between ovulatory and anovulatory dominant follicles. Progesterone plasmatic concentrations were determined by RIA and differences were not significant when cycles with two or three waves were compared. The number of follicular waves was higher (2 or 3 waves) with longer interovulatory intervals ( $22.3 \pm 1.89$  vs  $23.0 \pm 2.0$ ; n.s.) and older cows ( $7.0 \pm 2.64$  vs  $4.38 \pm 1.66$  years;  $P < 0.05$ ). Mean ovulatory follicular diameter was  $1.78 \pm 0.36$  cm. It can be concluded that cows with the RBC syndrome more frequently present two follicular waves, corresponding to longer cycles.

**Keywords:** repeat breeder; follicular waves; cattle

Folliculogenesis includes recurrent processes of follicular recruitment, selection, growth and maturation during the female's cycle, ending with ovulation. These processes are regulated by interactions between hormones, growth factors, cellular communication systems and genes (Roche and Boland, 1991).

Before real-time ultrasonography, knowledge of follicular development was based on the study of the cyclical patterns of gonadotropins and steroid hormones, and/or using laparoscopy for counting and measuring follicles after marking (Rajakoski, 1960; Dufour *et al.*, 1972; Matton *et al.*, 1981; Ireland and Roche, 1983; Fortune, 1993).

The ultrasound-scan has made it possible to establish follicular growth patterns in two-week-old pre-puberal cows (Evans *et al.*, 1994), in heifers (Savio *et al.*, 1988; Ginther *et al.*, 1989b; Sunderland *et al.*, 1994); in postpartum cows (Savio *et al.*, 1990); in pregnant cows (Pierson and Ginther, 1986; Ginther *et al.*, 1989c; Savio *et al.*, 1990; Thatcher *et al.*, 1991; Ginther *et al.*, 1996), in anovulatory cows (McDougall *et al.*, 1995); and after oestrus synchronisation treatments (Sirois and Fortune, 1990; Stock and Fortune, 1993).

Rajakoski (1960) described a model of “follicular waves” during the bovine oestrus cycle, reporting differences in the development of a follicular

population between one phase of growth, followed by a static phase, and subsequent follicular atresia. Other authors have argued that follicular growth is constant and independent of the cycle (Donaldson and Hansel, 1968; Dufour *et al.*, 1972; Spicer and Echtenkamp, 1986).

It is now well known that the most frequent patterns of follicular development in cows are the 2 follicular wave pattern (Rajakoski, 1960; Mariana and Nguyen, 1973; Pierson and Ginther, 1988; Ginther *et al.*, 1989b; Knopf *et al.*, 1989; Stock and Fortune, 1993; Carriere *et al.*, 1995) and the 3 follicular wave pattern (Ireland and Roche, 1983; Stock and Fortune, 1993; Carriere *et al.*, 1995; Taya *et al.*, 1996). Cycles with one or four follicular waves have been reported less frequently (Sirois and Fortune, 1988; Savio *et al.*, 1988; Carriere *et al.*, 1994).

Until now, no studies have focused on describing follicular dynamics in repeat breeding cows throughout their natural cycle (without hormonal treatment). Repeat breeder cows (RBC) show a repetition of cycles of normal length and gestation in them that has failed at least 3 times consecutively. The aetiology of this pathology is broad and is often described as multi-factor (Lafi and Kaneene, 1988; Bruyas *et al.*, 1993; Almeida, 1995; Pedroso and Roller, 1996). One possible reason for the lack of research in this field may be the difficulty in obtaining a group

of cows with these characteristics and without hormonally intervening in their sexual cycles.

The aim of this study was to evaluate the pattern of follicular development in RBCs in their natural cycles by means of ovarian ultrasonography. Any study that helps to improve our knowledge of the follicular action in these animals is important since it will foster a better understanding of any changes that take place in their reproductive mechanisms, and will improve the yield of conceptive treatments.

## MATERIAL AND METHODS

Eighteen Holstein-Freisian RBCs between 3 and 10 years-old were studied. These animals had an average of 3.2 services/conception. They presented good corporal condition and had calved at least once. They were located in the south of Spain farm (38°02'N-4°10'W). Oestrus detection was performed by monitoring the daily oestrus behaviour of the animals, 3 times each day for 20 minutes.

From the day of insemination (day 0), manual and ultrasound exploration of the reproductive tract was performed in each cow on every fourth day; after 32 days of the study, a total of nine explorations had been carried out on each animal. An Aloka SSD-210 DX ultrasound scan with a 5 MHz, linear-array, rectal transducer was used, obtaining static images through a Sony UP-850 video-printer.

The ovulatory follicles were studied on day 0 – the first day of study ( $n = 18$ ) – and until those formed during the following cycle ( $n = 8$ ). Follicle size, location (RO/LO), and number of per ovary were studied in all of them; the individual follow-up of the follicles allowed follicular wave patterns to be established and the following parameters to be evaluated: the start day, day of maximum size, period of follicular growth, growth rate, duration of follicular atresia and atresia rate. The following formula was used to calculate the average diameter of follicles:  $\sqrt{\text{height} \times \text{width}}$ .

CL was monitored by ultrasonography and total luteal tissue was calculated ( $1/2 \text{ height} \times 1/2 \text{ width} \times \pi$ ; if the CL was cavitory, the area of this cavity was not taken into account in the measuring). Blood samples were taken in order to determine the level of plasmatic progesterone, as an indicator of CL functionality. The amount of released progesterone was analysed on the basis of the different days of study, and the area under the progesterone curve was determined using the trapezoidal

formula. Cows entering gestation were divided in two groups: *Rno* (repeating non-oestrus) when oestrus signs were not present, and *Ro* (repeating with oestrus) when this was detected within 19 and 28 days after AI.

The concentration of progesterone in plasma was measured using a RIA method in liquid phase with extraction. Ovine anti-progesterone serum was used, supplied by Dr. Butcher of West Virginia University. For extraction, 3 ml of hexane were mixture with 200 microliters of plasma. The progesterone dry residue obtained was mixture with buffer, anti-progesterone serum and  $^3\text{H}$ -progesterone, an incubated to 4°C for 24 hours. Separation of free from bound steroid was accomplished with dextran-coated charcoal. The sensitivity was 16 pg/ml, and the intra- and inter-assay variation coefficients were 10.4% ( $n = 8$ ) and 13.6% ( $n = 6$ ), respectively (López *et al.*, 1984).

Analyses of variance were performed in order to determine whether there were any differences between cycles with different follicular patterns in terms of: maximum follicular diameter, the day on which this size was reached, the duration of follicular growth, growth rate, follicular atresia, and atresia rate. Duncan's test was used when the *F* test was significant.

## RESULTS

The ovulatory follicle was ultrasonographically described as a rounded anecogenic structure, with very delimited edges, showing an external hypercogenic layer around the follicular antrum, which corresponds to the follicular wall. The mean diameter in the 26 ovulatory follicles studied was  $1.78 \pm 0.36$  cm, presenting wide variations between 1.2 and 2.5 cm.

According to the plasmatic progesterone profile, half of the RBCs once again entered gestation without displaying oestrus symptoms (*Rno* group), and the other half were confirmed as having entered oestrus within days 19 and 28 (average cycle length =  $23.3 \pm 2.7$  days). The size of the ovulatory follicle obtained in the *Rno* group was  $1.88 \pm 0.29$  cm ( $n = 12$ ); in the *Ro* group this was  $1.7 \pm 0.41$  cm ( $n = 14$ ) (non-significant differences were observed between *Ro* and *Rno*).

After ovulation, new dominant follicles began to grow; 2 follicular waves were detected in 72.2% of the total (13/18) and 3 follicular waves in 16.6%

(3/18). Patterns consisting in 1 and 4 follicular waves appeared in 5.6% and 5.6% of the cycles studied, respectively.

Significant differences ( $P < 0.01$ ) were observed between the mean size of the preovulatory follicle (day 0) and the number of follicular waves developing during the sexual cycle. Therefore, when the preovulatory follicle was larger, a smaller number of follicular waves developed during the cycle: in 2-wave cycles, the average diameter of the preovulatory follicle was  $1.88 \pm 0.2$  cm; in 3 wave cycles, this was smaller ( $1.45 \pm 0.2$  cm); and significant differences were only observed in the two cycles with just one (2.5 cm) and four follicular waves (1.3 cm).

Ovulatory follicles were slightly larger (n.s.) when any follicle larger than 15 mm was present in any ovary ( $1.9179 \pm 0.09$  cm vs  $1.759 \pm 0.06$  cm). The location of any large companion follicles had no effect on the final diameter of the ovulatory follicle (companion ipsilateral follicles:  $2.0 \pm 0.09$  cm; companion contralateral follicles:  $1.856 \pm 0.14$  cm).

The characteristics observed in the different follicular growth patterns are shown in the Tables 1, 2, 3 and 4.

Depending on the number of waves (cycles with one, two, three or four waves), significant differences were observed between the day of maximum size in the first and second dominant follicles ( $P < 0.01$ ). Significant differences were also observed in the rate of atresia ( $P < 0.03$ ) for the second dominant follicles. Significant differences were also recorded in terms of the lengths of periods in which each of the follicular waves achieved the maximum diameter ( $P < 0.001$ ). In cycles with three follicular waves, the first dominant follicle (anovulatory) achieved a larger size than the following follicle, whereas in 2-wave cycles the dominant follicle was smaller than the next follicle, which was ovulatory. When

Table 1. Follicular characteristics in RBC ( $n = 1$ ) with one-wave interovulatory interval

	1st wave
Day of first detection	
Maximun diameter (cm)	1.6
Day of maximun diameter	19
Duration of growth follicular period (days)	11
Growth rate (cm/day)	0.145
Duration of atresia follicular period (days)	–
Atresia rate (cm/day)	–

comparing cycles with 2 or 3 follicular waves, the first DF achieved a similar diameter in both cases ( $1.69 \pm 0.35$  vs  $1.7 \pm 0.13$  cm), but appeared earlier in cows with a larger number of waves (8.0 vs 11.0 days). The ovulatory follicle forming at the end of the oestral cycle emerged later in cows with 3-wave cycles (day 15 vs day 11.62) and the ovulatory diameter was smaller when compared with 2-wave cycles ( $1.25 \pm 0.48$  vs  $1.71 \pm 0.19$  cm).

No differences were observed in terms of plasmatic progesterone levels and total luteal tissue between cows with 2 and 3 follicular waves patterns (Figures 1 and 2); however, significant differences ( $P < 0.05$ ) were observed with respect to the area under the progesterone curve, starting at day 8 and indicative of the amount of hormone released since the first day of the study (Figure 3).

No significant differences were observed in terms of the age of cows and oestrus cycle length. However, cows with 2 follicular waves were younger ( $4.38 \pm 1.66$  years) than those with 3 follicular waves ( $7.0 \pm 2.64$  years) ( $P < 0.05$ ).

Table 2. Follicular characteristics in RBC ( $n = 13$ ) with two-wave interovulatory intervals

	1st wave	2nd wave
Day of first detection		11.62
Maximun diameter (cm)	$1.69 \pm 0.35$	$1.71 \pm 0.19$
Day of maximun diameter	$11 \pm 2.83$	$22.08 \pm 2.47$
Duration of growth follicular period (days)	$9.31 \pm 3.47$	$10.46 \pm 5.14$
Growth rate (cm/day)	$0.205 \pm 0.08$	$0.207 \pm 0.09$
Duration of atresia follicular period (days)	$11.62 \pm 6.4$	–
Atresia rate (cm/day)	$0.196 \pm 0.12$	–

Table 3. Follicular characteristics in RBC ( $n = 3$ ) with three-wave interovulatory intervals

	1st wave	2nd wave	3rd wave
Day of first detection		3	15
Maximun diameter (cm)	$1.7 \pm 0.13$	$1.43 \pm 0.4$	$1.25 \pm 0.48$
Day of maximun diameter	$8 \pm 0$	$14.67 \pm 4.62$	$23.67 \pm 1.15$
Duration of growth follicular period (days)	$9 \pm 0$	$11.67 \pm 2.31$	$8.67 \pm 4.04$
Growth rate (cm/day)	$0.189 \pm 0.015$	$0.132 \pm 0.06$	$0.185 \pm 0.15$
Duration of atresia follicular period (days)	$7.33 \pm 2.89$	$12 \pm 2.65$	–
Atresia rate (cm/day)	$0.274 \pm 0.16$	$0.119 \pm 0.01$	–

Table 4. Follicular characteristics in RBC ( $n = 1$ ) with four-wave interovulatory intervals

	1st wave	2nd wave	3rd wave	4th wave
Day of first detection		11	15	22
Maximun diameter (cm)	1	1.9	1.9	1.3
Day of maximun diameter	4	16	20	27
Duration of growth follicular period (days)	5	5	5	5
Growth rate (cm/day)	0.2	0.38	0.38	0.26
Duration of atresia follicular period (days)	3	9	13	–
Atresia rate (cm/day)	0.333	0.211	0.146	–

The sexual cycle was shorter in cows with one follicular wave (19 days) than in cows with 4 waves (27 days), although these differences were not statistically significant (probably due to the small number of data). No significant differences were recorded with respect to the duration of sexual cycles for cows with 2 or 3 follicular waves, although this was longer in the latter ( $23.0 \pm 2.0$  days vs.  $22.3 \pm 1.89$  days).

## DISCUSSION

The average ovulatory diameter observed in this study for RBCs was  $1.78 \pm 0.36$  cm. In RBCs, Dovensky *et al.* (2000) and Taponen *et al.* (1999) recorded slightly smaller follicular diameters than those achieved in this study (1.69 and 1.58–1.67 cm, respectively). According to the data obtained here,

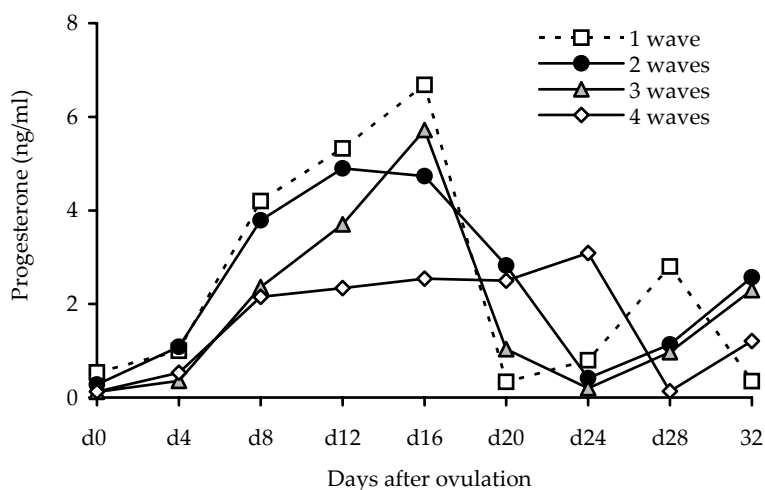


Figure 1. Mean daily plasma concentration of progesterone in cows with one ( $n = 1$ ; ---□---), two ( $n = 16$ ; —●—), three ( $n = 3$ ; —▲—) and four ( $n = 1$ ; —◇—) waves of follicular development

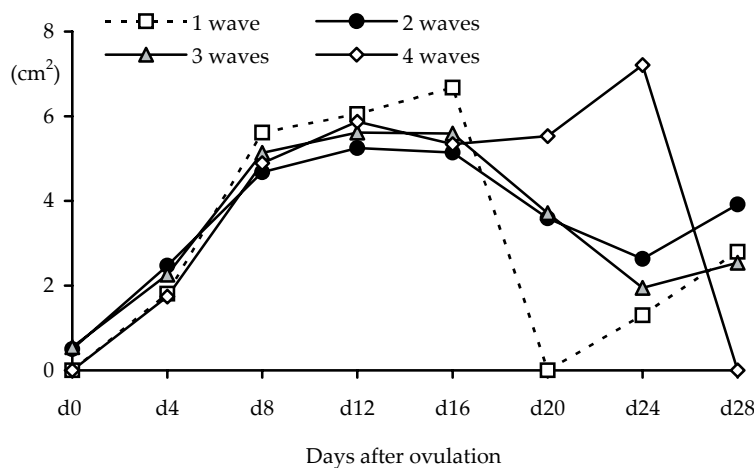


Figure 2. Area of luteal tissue in cows with one ( $n = 1$ ;  $-\square-$ ), two ( $n = 16$ ;  $-\bullet-$ ), three ( $n = 3$ ;  $-\nabla-$ ) and four ( $n = 1$ ;  $-\diamond-$ ) waves of follicular development

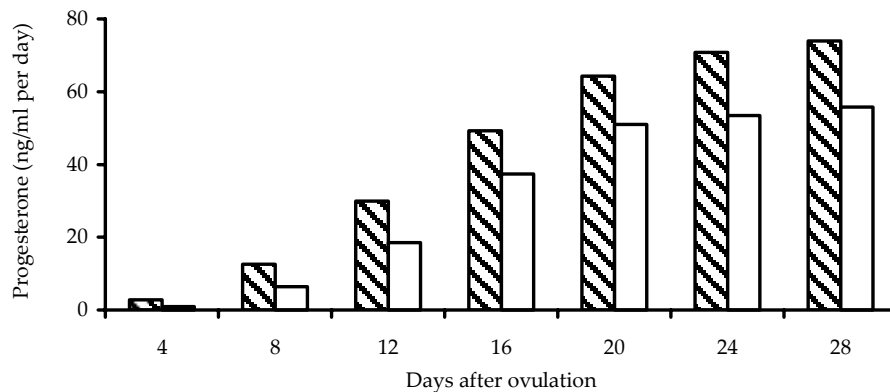


Figure 3. Area under the curve of progesterone in cows with 2 (contour bar) and 3 (white bar) follicular waves

it may be concluded that the ovulatory diameter observed in RBCs is highly variable. Therefore, while some cows present very large follicular diameters (2.5 cm), relatively small values (1.2 cm) were recorded in others, possibly suggesting that this feature is not related to the appearance of this syndrome.

The methodology followed in this study seemed to be suitable for studying follicular waves in RBCs with natural cycles; a predominance of cycles with 2 follicular waves (72.2%) was observed with respect to those consisting in 3 waves (16.6%).

It has been shown that an increase in plasmatic FSH precedes each follicular growth wave in cattle, indicating the start of this process (Adams, 1999), while a decrease in FSH coincides with an increase in DF inhibin and estradiol secretion, associated with the regression of non-dominant follicles. FSH plays a critical role in DF development, and it is possible that inhibin and other follicular liquid factors act directly on it (Law *et al.*, 1992); i.e. an increase in IGF bioactivity may be involved in DF selection (Mihm *et al.*, 1996; Rhodes *et al.*, 1997). Ovulatory follicular diameter (day 0) varied, probably due

to the number of follicular waves post ovulation. Consequently, the more waves in the cycle, the smaller the follicular diameter.

Statistical analyses revealed differences in terms of the day on which maximum diameter was reached within each wave. The cow with a single follicle during the oestrus cycle, dominant and ovulatory at the same time, achieved a maximum diameter on day 19; this coincides with the findings of Savio *et al.* (1988). In cows with 2 follicular waves, maximum follicular diameter was achieved on days 11 and 22, respectively, as reported by Ginther *et al.* (1989a). In animals with 3 waves, maximum diameter was achieved days 8, 14 and 23, respectively. Lastly, in the cow that presented 4 follicular waves, maximum diameter was recorded on days 4, 16, 20 and 27.

In cycles with 3 follicular waves, the first DF was larger than the following dominant follicles, probably due to the FSH wave post ovulation (Ginther *et al.*, 1989b; Adams *et al.*, 1992; Sunderland *et al.*, 1994; Mihm *et al.*, 1996). However, this was not the case in cows with 2 waves, perhaps due to the ovulatory status in the second DF, which provided the stimulus for stronger growth. The first DF in the

cows with 3 waves had an average diameter similar to that recorded in cows with 2 waves, thus coinciding with the observations reported by Ginther *et al.* (1989b), but different to those described by Fortune (1993); however, this size was achieved earlier, suggesting the action of an additional follicular growth stimulus (Ahmad *et al.*, 1997), probably due to the above-mentioned increase in FSH or estradiol, or to a decrease in inhibin concentration. The physiological role of the anovulatory DF during oestrus cycles is unknown, and it is claimed to bear some influence on luteal regression (Fricke, 1999).

It has been shown that these follicles have an ovulatory capacity when stimulated with hCG (Fricke *et al.*, 1993). The absence of dominant follicles during the mid or late luteal phase delays luteal regression, since estradiol released from these follicles stimulates luteal regression. The CL exerts a negative effect on ovulation through its progesterone production. This steroid prevents the frequent release of LH surge, required to increase follicular estradiol and achieve a LH peak, a stimulus that is essential for ovulation. The regular development of follicular waves continues until the CL returns, and it is more likely to have 3-wave cycles when the CL lifetime is prolonged.

No significant differences were observed in terms of plasma concentrations of progesterone between cows with two and three follicular waves (Figure 1); this coincides with the results obtained by Custer *et al.* (1994) and Ahmad *et al.* (1997). However, Adams *et al.* (1992) reported that cows with higher progesterone concentrations during the early and mid-luteal phase had a tendency to develop 3 follicular waves. The results of this study suggest an opposite situation, since cows with 3 follicular waves presented lower progesterone levels, although the area under the curve was significantly different from day 8 onwards in cows with 3 waves (Figure 3). In cows with 2 follicular waves, progesterone concentrations, the preovulatory growth period and ovulatory size were also higher, as reported by Ginther *et al.* (1989a,b) and Ahmad *et al.* (1997), and may be involved in compromising the viability of the oocyte, as in cases of persistent follicles. However, no significant differences were observed between the luteal tissues forming in cycles with 2 or 3 waves (Figure 2).

The length of oestral cycles was shorter when the number of follicular waves was smaller, but the differences were not significant, as reported by Fortune *et al.* (1988), Savio *et al.* (1988), and Ginther *et al.*

(1989b). The longer duration in the three follicular waves cycles might be due to the delay in the second DF, which only grows when the uterus is sensitive to estradiol, and, on the other hand, the concentration and the release time of estradiol in the second DF may be not enough to trigger the luteolitic cascade (Salfen *et al.*, 1999). Fortune (1993) states that both the cycle and the luteal phase length are important factors that affect the number of follicular waves per cycle.

We observed that cows with two follicular waves were younger than cows with three waves, although there are no reports confirming the age influence (Fortune, 1993). A possible explanation might be that age has a negative effect on the functionality of the hypothalamic-hypophyseal-gonadal axis and, consequently, on the synthesis and release of different hormones and ovarian factors. Furthermore, older cows present metabolic defects more often; this could prevent the final follicular growth by hypothalamic negative feedback of estradiol, reducing GnRH pulses, and prompting a decrease in LH pulses that prevents the follicles from achieving the ovulatory size; appropriate estradiol quantities are therefore released to develop a LH peak and ovulation (Wiltbank, 1999).

Studies on ovarian follicular dynamics will enable the development of new methods to improve fertility, achieve more accurate synchronisation of oestrus and improve superovulatory results (Roche *et al.*, 1999). In order to develop new oestrus synchronisation methodologies, better knowledge of cow follicular dynamics is needed, focused mainly on controlling the number of follicular waves per cycle. In fact, in GnRH treatments after the implementation of PGF $2\alpha$ , the best results are achieved when treatments are applied in females with 2 follicular waves as compared with cows with 3 waves; this is so due to the fact that GnRH does not produce any effect when applied prior to DF selection (Roche *et al.*, 2000). The present study shows that the most common follicular growth pattern in RBCs is the 2-wave pattern; good therapeutic results may therefore be expected using a combination of PGF $2\alpha$  and GnRH to treat this syndrome.

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