Association between ovarian follicle development and pregnancy rates in dairy cows undergoing spontaneous oestrous cycles

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Abstract

Ovarian follicle development continues in a wave-like manner during the bovine oestrous cycle giving rise to variation in the duration of ovulatory follicle development. The objectives of the present study were to determine whether a relationship exists between the duration of ovulatory follicle development and pregnancy rates following artificial insemination (AI) in dairy cows undergoing spontaneous oestrous cycles, and to identify factors influencing follicle turnover and pregnancy rate and the relationship between these two variables. Follicle development was monitored by daily transrectal ultrasonography from 10 days after oestrus until the subsequent oestrus in 158 lactating dairy cows. The cows were artificially inseminated following the second observed oestrus and pregnancy was diagnosed 35 days later. The predominant pattern of follicle development was two follicle waves (74.7%) with three follicle waves in 22.1% of oestrous cycles and four or more follicle waves in 3.2% of oestrous cycles. The interval from ovulatory follicle emergence to oestrus (EOI) was 3 days longer \( (P < 0.0001) \) in cows with two follicle waves than in those with three waves. Ovulatory follicles from two-wave oestrous cycles grew more slowly but were approximately 2 mm larger \( (P < 0.0001) \) on the day of oestrus.

Twin ovulations were observed in 14.2% of oestrous cycles and occurred more frequently \( (P < 0.001) \) in three-wave oestrous cycles; consequently EOI was shorter in cows with twin ovulations. Overall, 57.0% of the cows were diagnosed pregnant 35 days after AI. Linear logistic regression analysis revealed an inverse relationship between EOI and the proportion of cows diagnosed pregnant, among all cows \( (n = 158; P < 0.01) \) and amongst those with single ovulations \( (n = 145; P < 0.05) \). Mean EOI was approximately 1 day shorter \( (P < 0.01) \) in cows that became pregnant than in non-pregnant cows; however, pregnancy rates did not differ significantly among cows with different patterns of follicle development. These findings confirm and extend previous observations in pharmacologically manipulated cattle and show, for the first time, that in dairy cows undergoing spontaneous oestrous cycles, natural variation in the duration of post-emergence ovulatory follicle development has a significant effect on pregnancy rate, presumably reflecting variation in oocyte developmental competence.

Introduction

The growth and regression of ovarian follicles continues in a wave-like manner throughout the bovine oestrous cycle. During normal length oestrous cycles (18 to 24 days) two or three follicle waves generally develop (Ginther et al. 1989, Fortune 1993), although oestrous cycles with one (Savio et al. 1988) or four (Sirois & Fortune 1988, Rhodes et al. 1995) follicle waves have been reported. In oestrous cycles with two follicle waves, new waves typically emerge on the day of ovulation (day zero) and on day ten, while in three-wave oestrous cycles successive waves emerge on days 0, 9 and 16 (Ginther et al. 1989). Since it is the dominant follicle of the ultimate wave that ovulates, the interval from ovulatory follicle emergence to ovulation will be shorter in oestrous cycles with three follicle waves than in those with two waves (6 to 7 days compared with 10 to 11 days; Sirois & Fortune 1988, Ginther et al. 1989). The mechanisms that regulate the pattern of follicle development in spontaneous oestrous cycles remain to be elucidated although there is some evidence that the nutrition
of the animal may be important (Lucy et al. 1992, de la Sota et al. 1993, Diskin et al. 2003).

Progestagen treatments are routinely used to synchronise oestrus in cattle. However, when administered over long periods, or from close to the end of the oestrous cycle, pregnancy rates are reduced (Roche, 1974a,b; Beal et al. 1988, Brink & Kiracofe 1988). More recent studies suggest that this may be due to aberration of ovulatory follicle development. When cows are treated with progesterone in the absence of a functional corpus luteum (CL) the dominant follicle present at the start of the treatment period can persist and ovulate following progestagen withdrawal (Sirois & Fortune 1990, Adams et al. 1992, Savio et al. 1993b). However, conception rates are reduced by 20 to 50% and this decline in fertility has been attributed to the extended duration of dominance of the ovulatory follicle that may compromise oocyte quality (Sanchez et al. 1993, Savio et al. 1993b, Mihm et al. 1994a, Ahmad et al. 1995, 1996, Revah & Butler 1996, Austin et al. 1999).

The primary objective of the present study was to test the hypothesis that there is an inverse relationship between the duration of ovulatory follicle development (from emergence to oestrus) and the likelihood of pregnancy being established following artificial insemination (AI) in lactating dairy cows undergoing spontaneous oestrous cycles. Additional aims of the present study were to identify factors (e.g. number of follicle waves, post partum interval to insemination, nutritional status, milk yield, parity) that influence follicle turnover and pregnancy rates among this group of cows and to determine the influence of these factors on the relationship between ‘emergence to oestrus interval’ and pregnancy rate. The incidence of twin ovulation within the cows studied has permitted a comparison of follicle turnover and pregnancy rate in twin and single ovulating cows and investigation of factors that may influence ovulation rate.

Materials and Methods

Animals

The present study was conducted during four successive breeding seasons at the Centre for Dairy Research (CEDAR), The University of Reading, Arborfield, Reading, Berks, UK. The lactating Holstein/Friesian cows (n = 189; one to eight parity) used in the study calved during August to January each season. The cows were between 30 and 125 days post partum at the time of selection for the study. Each cow was used only once during the 4 years of the study.

Oestrus detection and artificial insemination

Cows were observed for signs of oestrus at 0600 h, 1400 h and 2100 h each day from 3 weeks before the start of the service period and/or from 40 days post partum. Kamar oestrus detection aids (Kamar Inc., Steamboat Springs, CO, USA) were used to assist with oestrus detection. In addition, milk progesterone concentrations were determined in samples taken once weekly using a semi-quantitative ‘on-farm’ test (Ridgeway Science, Alvington, Glos, UK; as described by Sauer et al. 1986) to monitor ovarian cycles. Progesterone concentrations were defined as ‘low’ (<2 ng/ml) or ‘high’ (>2 ng/ml). Where behavioural signs of oestrus were not observed preceding ovulation (indicated by a subsequent increase in milk progesterone), the day of ‘low’ progesterone following two or three ‘high’ tests was taken as the day of oestrus for the purpose of determining when ultrasonographic scanning should commence. Cows enrolled in the study on the basis of milk progesterone concentrations were only included in the analyses if the development (regression, emergence, ovulation) of at least two follicle waves could be discerned. The cows were artificially inseminated (AI) using frozen–thawed semen by commercial AI technicians between 1000 h and 1200 h provided the cows had been detected in oestrus before 0600 h.

Ovarian and uterine observations

Ovarian follicular and luteal development were monitored daily (between approximately 0800 h and 1100 h) from approximately 10 days after oestrus (or 10 days after ‘low’ progesterone; oestrus 1) until the day of second oestrus (oestrus 2), on the day after AI (to confirm that ovulation had occurred) and 10 days after AI (for the presence of a CL in place of the ovulatory follicle) using the method of transrectal ultrasonography described by Pierson and Ginther (1988). Pregnancy was diagnosed ~35 and ~60 days after AI by uterine ultrasonography (Pierson & Ginther 1988). These two determinations of pregnancy rate were made to identify cases of embryo loss. Ultrasonography was carried out using a real-time B-mode ultrasound scanner fitted with a 7.5 MHz linear array probe (Concept 2000, Dynamic Imaging Ltd, Livingstone, Scotland). The ovarian images were recorded on videotape to facilitate subsequent sequential analysis of the growth and regression of follicles ≥5 mm diameter and corpora lutea (CL) ≥10 mm in diameter. Ovarian maps showing the relative positions and diameters of the follicles and CL on the ovaries were drawn to aid the identification of individual structures on successive days.

Milk production, diet and energy balance

The cows were milked twice per day and milk yield was recorded at each milking. The fat, protein and lactose content of milk were determined in samples taken at consecutive afternoon and morning milkings once a week. The cows were fed grass and maize based silage based diets supplemented with concentrates to support milk yields of 32 kg/day. The present study utilised 112 cows that were also being used in studies investigating the effects of diet.
on milk production. Daily dry matter intakes and weekly live weights of these cows were recorded from 4 weeks post partum facilitating the calculation of energy balance of these animals using the equations produced by AFRC (1993).

Statistical analysis

All statistical analyses were carried out using Genstat for Windows 5th edition (NAG Ltd, Oxford, UK; Lawes Agricultural Trust 2000). Characteristics of growth and regression of follicles and CL, daily milk yield during the week of AI (daily yield at AI), average daily milk yield from calving to AI (average yield to AI), 305-day lactation yield and mean calculated energy balance in (1) cows with two, three or \( \geq \) four follicle waves, (2) cows with single or twin ovulations and (3) pregnant or non-pregnant cows were compared by one-way analysis of variance (ANOVA). Fisher’s protected least significant difference test was used for post hoc comparisons. The relationships between (1) the number of follicle waves and (2) ovulation rate (single or twin ovulations) and the proportion of cows pregnant were examined using chi-squared analysis.

The relationship between pregnancy rate (response variable; pregnant = 1, non-pregnant = 0) and the explanatory variables - (1) the number of days from ovulatory follicle emergence to oestrus 2 (EOI) and (2) the number of days from ovulatory follicle dominance to oestrus 2 (DOI) - were compared by linear logistic regression analysis (D Collett, personal communication; Collett 2003). Additionally, the influence of other explanatory variables, namely post partum interval to AI, ovulation rate, parity, daily yield at AI, average yield to AI, 305-day milk yield, mean daily, calculated energy balance and the number of follicle waves on this relationship was assessed using forward step-wise regression. This involved assessing the change in deviance on adding each of the selected variables in turn to a model including the constant term alone. The variables that were most associated with the response, i.e. the ones giving a significant change in deviance (chi-squared, 1 degree of freedom; \( P < 0.1 \)) were then added back into models that already included either the constant and EOI or the constant and DOI.

Results

Of 189 cows initially recruited to the study, 19 (10.1%) cows were excluded from the analyses because oestrus was not detected before ovulation at the end of the scanning period, so those cows were not inseminated. A further 12 (6.3%) cows were excluded as ovarian oestrous cycles were deemed abnormal. The abnormalities detected included the development of persistent anovulatory follicles (follicular cysts) following the onset of luteolysis (\( n = 3 \); 1.6%), an extended period (24 days) between the onset of luteolysis and oestrus during which time follicle turnover continued (\( n = 1 \); 0.5%), and ovulation of the dominant follicle (DF) from the first wave (DF1) despite the second wave dominant follicle (DF2) having been observed for 9 days before oestrus 2 (\( n = 1 \); 0.5%). Luteinised follicles were detected in two (1.1%) of the cows following oestrus 2. This condition was characterised by apparent ovulatory failure of the dominant follicle from the last wave (i.e. acute disappearance of the ‘ovulatory follicle’ was not observed following oestrus). This follicle continued growing with progressive thickening of its wall; the wall of the luteinised follicles developed the same echogenicity as that of a CL.

Prolonged oestrous cycles (\( \geq 30 \) days) were observed in five (2.6%) cows; these have been included in the analyses since follicle turnover appeared normal with regular periodic emergence and regression of follicle waves. The comparisons presented are for 158 cows (83.6% those enrolled) unless otherwise stated.

Follicle and luteal development

Of the 158 cows included in the analyses, 118 (74.7%) had two waves of follicle development and 35 (22.1%) had three waves. The remaining five cows (3.2%) with persistent CL had four (\( n = 3 \)) or six (\( n = 2 \)) follicular waves. The data from these five cows have been grouped together for all subsequent analyses (\( \geq 4 \) waves). The frequencies with which the different wave patterns were observed was similar in each of the years of the study and did not differ with parity. The post partum interval to oestrus 2 was similar among cows with different wave patterns (81.9 ± 1.4, 84.6 ± 2.8 and 92.4 ± 6.4 days

Table 1 Comparisons of the characteristics of ovulatory follicle development in 158 lactating dairy cows with two, three or \( \geq 4 \) waves of follicles during a spontaneous oestrous cycle. Values presented are means\( \pm \)S.E.M. unless otherwise stated.

<table>
<thead>
<tr>
<th></th>
<th>2 wave</th>
<th>3 wave</th>
<th>( \geq 4 ) wave</th>
<th>ANOVA P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cows</td>
<td>118</td>
<td>35</td>
<td>5</td>
<td>—</td>
</tr>
<tr>
<td>Percentage of total</td>
<td>74.7</td>
<td>22.1</td>
<td>3.2</td>
<td>—</td>
</tr>
<tr>
<td>Days from emergence to oestrus (EOI)</td>
<td>8.9 ± 0.1</td>
<td>5.9 ± 0.2</td>
<td>6.7 ± 0.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Days from dominance to oestrus (DOI)</td>
<td>6.2 ± 0.1</td>
<td>3.8 ± 0.2</td>
<td>4.0 ± 1.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Days from emergence to dominance</td>
<td>2.6 ± 0.1</td>
<td>2.2 ± 0.2</td>
<td>2.7 ± 0.4</td>
<td>0.06</td>
</tr>
<tr>
<td>Diameter on the day of emergence (mm)</td>
<td>6.0 ± 0.1</td>
<td>5.7 ± 0.1</td>
<td>5.2 ± 0.2</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Diameter on the day of oestrus 2 (mm)</td>
<td>16.0 ± 0.1</td>
<td>14.3 ± 0.3</td>
<td>13.8 ± 0.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Growth rate (mm/day)</td>
<td>1.3 ± 0.03</td>
<td>1.8 ± 0.05</td>
<td>1.8 ± 0.26</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Days from emergence to luteolysis</td>
<td>5.6 ± 0.2</td>
<td>2.2 ± 0.3</td>
<td>3.5 ± 0.9</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
respectively). Oestrous cycles with two follicle waves were shorter ($P < 0.01$) than those with three waves (21.9 ± 0.2 days vs 23.3 ± 0.5 days). Both two- and three-wave oestrous cycles were considerably shorter ($P < 0.0001$) than oestrous cycles with ≥ four waves (40.7 ± 3.3 days).

Comparisons of the characteristics of follicle and luteal development in cows with different wave patterns are presented in Table 1. The mean EOI and DOI were shorter ($P < 0.0001$) in cows with three waves than in those with two waves. Among cows with ≥ four follicle waves, both EOI and DOI were approximately 2 days shorter ($P < 0.005$) than in cows with two waves but were similar to those in cows with three waves. Ovulatory follicles grew more slowly in cows with two waves than in those with either three or ≥ four waves but were approximately 2 mm larger on the day of oestrus (two vs three waves, $P < 0.0001$; two vs ≥ four waves, $P < 0.005$). The interval from emergence of the ovulatory follicle to the onset of luteolysis was 3.4 days longer in cows with two waves of follicle development compared with those with three waves ($P < 0.0001$), and 1.3 days longer in cows with ≥ four follicle waves compared with those with three waves ($P < 0.05$). The interval from luteolysis to oestrus 2 did not differ significantly with follicle wave pattern (3.3 ± 0.1 days, 3.7 ± 0.2 days, 3.2 ± 0.2 days in two-, three- or ≥ four-wave oestrous cycles respectively). Mean CL and luteal tissue areas were similar during the 10 days before oestrus 2 among cows with the different patterns of follicle development.

**Comparison of follicle and luteal development in cows with single or twin ovulations**

Of the 189 cows initially included in the study, 27 (14.3%) cows had one or two incidences of twin ovulations i.e. twin ovulations following oestrus 1 (indicated by the presence of two CL during the period of ultrasonography) or oestrus 2 (indicated by the development of two ovulatory follicles during the final wave and the presence of two CL 10 days after oestrus) or following both. Twin ovulations were observed in 5 of 37 (13.5%) first parity, 5 of 38 (13.2%) second parity, 7 of 43 (16.3%) third parity and 10 of 40 (25.0%) fourth to eighth parity cows. Among the cows with twin ovulations following oestrus 2, two were excluded from the analyses as either the follicular phase was prolonged or oestrus 2 was not observed before ovulation. Twin ovulatory follicles were both derived from the final wave of the oestrous cycle in all but one of the cows studied. In this cow, despite the emergence of a third wave and apparent dominance of DF3, this follicle ovulated together with DF2. Her data have been excluded as the conflicting origin of follicles confounds analyses.

Twin ovulations were observed more frequently ($P < 0.001$) in cows with three (10 of 35) or ≥ four follicle waves per oestrous cycle (two of five) than in cows with two waves per oestrous cycle (2 of 118). Comparisons of the characteristics of ovulatory follicle development in cows with twin or single ovulations derived from the same wave are presented in Table 2. EOI was approximately 3 days shorter in cows that ovulated twin follicles than in those with single ovulations. Thus, despite being of similar diameter on the day of emergence and having slower rates of growth, the diameter of the ovulatory follicle on the day of oestrus was approximately 3 mm greater in single than in twin ovulating cows. While the interval from emergence of the ovulatory follicle to the onset of luteolysis was 3.1 days shorter in twin ovulating cows, there was no difference in the interval from the onset of luteolysis to oestrus.

### Milk production and calculated energy balance

Average milk production was 7063 ± 97 kg in 305 days with peak daily yields of 32.4 ± 0.5 kg among the 154 cows included in the study that completed their lactations (four cows were culled before completing a 305-day lactation). Average yield to AI, daily yield at AI and 305-day lactation yields were greater ($P < 0.05$) in cows with two follicle waves than in those with three or ≥ four waves (29.7 ± 0.6, 27.4 ± 0.9, 25.7 ± 2.0 kg per day; 29.8 ± 0.5, 27.3 ± 1.0, 24.9 ± 1.4 kg per day, 7209 ± 109 vs 6694 ± 218 and 6117 ± 247 kg in 305 days respectively). Calculated energy balance tended ($P = 0.06$) to vary with follicle wave pattern. Mean calculated energy balances from 5 weeks post partum to the day of oestrus 2 were 26.4 ± 3 MJ/day, 28.4 ± 6.5 MJ/day and 58.3 ± 12.3 MJ/day in cows with two, three or ≥ four waves respectively. There were weak positive linear relationships between both EOI and 305-day milk yield ($r = 0.20; P < 0.05$) and DOI and 305-day milk yield ($r = 0.17; P < 0.05$). Daily yield at AI (29.1 ± 0.5 kg vs 28.8 ± 1.5 kg respectively), average yield to AI (28.9 ± 0.5 kg vs 29.9 ± 1.2 kg respectively) 305-day yield (7083 ± 101 kg

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Table 2. Comparison of the characteristics of ovulatory follicle development in spontaneous oestrous cycles of 158 dairy cows with twin or single ovulations. Values presented are means ± S.E.M. unless otherwise stated.

<table>
<thead>
<tr>
<th></th>
<th>Twin ovulations</th>
<th>Single ovulation</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cows</td>
<td>13</td>
<td>145</td>
<td>—</td>
</tr>
<tr>
<td>Percentage of total</td>
<td>8.2</td>
<td>91.8</td>
<td>—</td>
</tr>
<tr>
<td>Days from emergence to oestrus (EOI)</td>
<td>5.5 ± 0.4</td>
<td>8.4 ± 0.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diameter on the day of emergence (mm)</td>
<td>5.5 ± 0.2</td>
<td>5.9 ± 0.1</td>
<td>NS</td>
</tr>
<tr>
<td>Diameter on the day of oestrus (mm)</td>
<td>12.7 ± 0.5</td>
<td>15.8 ± 0.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Growth rate (mm/day)</td>
<td>1.69 ± 0.10</td>
<td>1.41 ± 0.03</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Days from luteolysis to oestrus</td>
<td>3.5 ± 0.4</td>
<td>3.3 ± 0.1</td>
<td>NS</td>
</tr>
<tr>
<td>Days from emergence to luteolysis</td>
<td>1.9 ± 0.5</td>
<td>5.0 ± 0.2</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

NS, not significant.

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vs 6826 ± 345 kg respectively) and mean energy balance (15.3 ± 11.1 MJ/day vs 28.7 ± 2.8 MJ/day respectively) were not significantly different among single and twin ovulating cows.

**Pregnancy rates**

There was no significant difference in pregnancy rates during the 4 years of study. Overall, 90 of 158 cows (57.0%) were diagnosed pregnant 35 days after AI. Following two cases of embryo loss between 35 and 60 days after AI - one in a cow with two follicular waves, the other in a cow with three follicular waves - pregnancy rates at day 60 were 55.7%. The results presented relate to pregnancy status 35 days after AI.

The post partum interval to oestrus 2 was longer ($P < 0.01$) in cows that were pregnant 35 days after AI (85.6 ± 1.6 days) compared with cows diagnosed as non-pregnant (79.1 ± 1.8 days). Pregnancy rates were not influenced by parity of the cows. Of the cows with twin ovulations, 76.9% (10 of 13) were diagnosed pregnant 35 days after AI compared with 55.2% (80 of 145) of those with single ovulations ($P = 0.13$).

Both EOI (7.8 ± 0.2 days vs 8.6 ± 0.2 days; $P < 0.01$) and DOI (5.4 ± 0.2 days vs 6.0 ± 0.2 days; $P < 0.05$) were significantly shorter in cows that became pregnant than in those that did not. However, pregnancy rate was not significantly influenced by follicle wave pattern. Of 158 cows studied, 55.1% (65 of 118) of those with two follicular waves, 62.9% (22 of 35) with three waves and 60% (3 of 5) with ≥ four waves became pregnant ($P = 0.7$). Other parameters of follicle and luteal development compared did not differ significantly with subsequent pregnancy status. The relationship between the duration of the later stages of ovulatory follicle development and pregnancy rates was confirmed by logistic regression analysis (Table 3). There were negative linear relationships between both EOI ($P < 0.01$) and DOI ($P < 0.05$) and the proportion of cows diagnosed pregnant 35 days after AI (Fig. 1).

Step-wise logistic regression analysis indicated that pregnancy outcome to AI was also positively associated with post partum interval to AI ($P < 0.01$) and negatively associated with both 305-day milk yield ($P < 0.05$) and daily yield at AI ($P < 0.01$). When these variables were added to the model that already included the constant with either EOI or DOI, both 305-day milk yield and daily yield at AI (but not post partum interval to AI) significantly reduced the deviance further.

The regression equations for these models were:

$$\text{logit}(p) = 3.59 - 0.187(\text{EOI}) - 0.0003 \times (305 - \text{day yield}); \ P = 0.01;$$

$$\text{logit}(p) = 3.16 - 0.160(\text{DOI}) - 0.0003 \times (305 - \text{day yield}); \ P = 0.02;$$

$$\text{logit}(p) = 3.16 - 0.21(\text{EOI}) - 0.04 \times (\text{yield at AI}); \ P = 0.01;$$

$$\text{logit}(p) = 2.56 - 0.18(\text{DOI}) - 0.04 \times (\text{yield at AI}); \ P = 0.03.$$

Although both daily yield at AI (28.4 ± 0.6 kg and 29.9 ± 0.6 kg respectively) and average yield to AI (28.5 ± 0.7 kg and 29.7 ± 0.6 kg respectively) were similar among cows subsequently diagnosed pregnant and those that were not, 305-day lactation yield was

<table>
<thead>
<tr>
<th>Variable</th>
<th>Regression coefficient</th>
<th>s.e.</th>
<th>Constant</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EOI</td>
<td>−0.22</td>
<td>0.09</td>
<td>2.10</td>
<td>0.01</td>
</tr>
<tr>
<td>DOI</td>
<td>−0.19</td>
<td>0.01</td>
<td>1.36</td>
<td>0.03</td>
</tr>
<tr>
<td>Waves</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0.20</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>0.32</td>
<td>0.40</td>
<td>0.20</td>
<td>0.42</td>
</tr>
<tr>
<td>4+</td>
<td>0.20</td>
<td>0.93</td>
<td>0.20</td>
<td>0.83</td>
</tr>
<tr>
<td>Post partum interval to oestrus</td>
<td>0.03</td>
<td>0.01</td>
<td>−2.12</td>
<td>0.01</td>
</tr>
<tr>
<td>Parity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0.73</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>−0.31</td>
<td>0.48</td>
<td>0.73</td>
<td>0.53</td>
</tr>
<tr>
<td>3</td>
<td>−0.69</td>
<td>0.46</td>
<td>0.73</td>
<td>0.14</td>
</tr>
<tr>
<td>4+</td>
<td>−0.73</td>
<td>0.47</td>
<td>0.73</td>
<td>0.12</td>
</tr>
<tr>
<td>Mean energy balance ($n = 112$)</td>
<td>−0.009</td>
<td>0.007</td>
<td>0.65</td>
<td>0.19</td>
</tr>
<tr>
<td>Daily yield at AI</td>
<td>−0.05</td>
<td>0.03</td>
<td>1.69</td>
<td>0.10</td>
</tr>
<tr>
<td>Average yield to AI</td>
<td>−0.03</td>
<td>0.03</td>
<td>1.28</td>
<td>0.20</td>
</tr>
<tr>
<td>305-day milk yield ($n = 154$)</td>
<td>−0.0003</td>
<td>0.0001</td>
<td>2.42</td>
<td>0.03</td>
</tr>
<tr>
<td>Ovulation rate (0 = single; 1 = twin)</td>
<td>1.00</td>
<td>0.68</td>
<td>0.21</td>
<td>0.12</td>
</tr>
<tr>
<td>EOI (single ovulating cows; $n = 145$)</td>
<td>−0.195</td>
<td>0.10</td>
<td>1.85</td>
<td>0.04</td>
</tr>
</tbody>
</table>

EOI = interval (days) from ovulatory follicle emergence to oestrus; DOI = interval (days) from ovulatory follicle dominance to oestrus.

$P$ value indicates significant change in deviance.
of the CEDAR herd during the study period. The improvement in reproductive performance is likely to be a result of the increased observation and handling of the cows involved in the present study and perhaps the more accurate timing of insemination in cows in which ovarian function was being monitored daily during the period leading up to oestrus 2.

Examination of the relationship between follicle turnover and pregnancy rate by linear logistic regression analysis revealed a significant inverse relationship between EOI (or DOI) and the proportion of cows diagnosed pregnant following AI. That is, as the interval between ovulatory follicle emergence (or dominance) and the subsequent oestrus increases so pregnancy rates decline. As far as we are aware this is the first study to demonstrate a relationship between the duration of ovulatory follicle development and fertility in cattle undergoing spontaneous oestrous cycles. Previous studies involving progestagen-treated cattle have likewise shown that an extended duration of dominance of a persistent ovulatory follicle has a detrimental effect on pregnancy rate (Sanchez et al. 1993, Savio et al. 1993b, Mihm et al. 1994a, Ahmad et al. 1996, Austin et al. 1999). Given the above, it is perhaps surprising that pregnancy rates were not significantly influenced by the observed pattern of follicle development during the oestrous cycle before AI, a finding in agreement with Ahmad et al. (1997) but contradicted by the more recent report of Townson et al. (2002). However, this may be explained by the considerable variation in EOI among cows with different wave patterns. In the present study, cows with twin ovulations contribute in the most part to the population of cows with a shorter EOI (those cows with higher pregnancy rates). This may be due to the advantage of releasing two oocytes at ovulation or to the shorter EOI. However, a significant inverse relationship between EOI and pregnancy rates was also present among single ovulating cows (Fig. 2). There was a tendency for pregnancy rates to decline particularly steeply where EOI exceeds 9 days although it was not possible to test this formally using the linear logistic regression method used to analyse these data.

Studies in which persistent follicles were induced during progestagen treatment suggest that reduced fertility is associated with premature maturation of the oocyte (Mihm et al. 1994b, Revah & Butler 1996). Oocytes from persistent follicles had expanded cumulus oocyte complexes, condensed chromatin and some evidence of germinal vesicle breakdown due to the increased luteinising hormone (LH) pulse frequency during the period of progestagen treatment, sufficient to advance oocyte maturation but not to induce ovulation. This may then influence embryo survival since Ahmad et al. (1995) found that despite high fertilisation rates following artificial insemination, embryo recovery was reduced. It remains to be determined whether oocyte viability is similarly affected in follicles with longer EOI developing.

**Figure 1** Relationship between the interval (days) from ovulatory follicle emergence to oestrus (EOI) and pregnancy rate in 158 cows undergoing spontaneous oestrous cycles. Each data point represents the proportion of cows pregnant for a given EOI. The shading of each data point indicates whether contributing cows had two (○), three or more (●) or two or three or more (⊗) waves of follicle development during the oestrous cycle before AI. The numbers in parentheses indicate the number of cows contributing to each data point.

Discussion

Of 189 cows recruited into the study, only 158 (83.6%) were included in the final analyses. The principal reason for excluding cows was failure to detect oestrus 2 before ovulation (10% of cows). This was despite close monitoring of the cows involved in the study and the advantage of daily ovarian ultrasonography to assist in predicting when oestrus was likely to occur. However, these findings are supported by the results of a recent study at CEDAR that suggested oestrus expression may be particularly poor in 20 to 25% of the dairy herd (H L Pearse and E C L Bleach, unpublished data). The 90% oestrus detection rate was significantly better than current estimates for commercial UK dairy herds (52%; Esslemont & Kossaibati 2002). The incidence of abnormal ovarian function among cows in the present study (approximately 6%) was similar to that reported by Royal et al. (2000) (approximately 10%) for cows with extended ‘inter luteal phase’ intervals having previously recommenced oestrous cycles. This grouping would have included the cows with follicular cysts and prolonged follicular phase in the present study.

Overall pregnancy rates among the cows used in the present study were substantially higher (57.0%) than the current average for UK dairy herds (37 to 47%; Royal et al. 2000, Esslemont & Kossaibati 2002) and in the remainder approximately 400 kg higher in non-pregnant than in pregnant cows (7302 ± 136 kg vs 6889 ± 134 kg respectively; P < 0.05).
Ovarian follicle development and pregnancy rates

627

Figure 2 Relationship between the interval (days) from ovulatory follicle emergence to oestrus (EOI) and pregnancy rate in 145 single ovulating cows undergoing spontaneous oestrous cycles. Each data point represents the proportion of cows pregnant for a given EOI. The shading of each data point indicates whether contributing cows had two (O), three or more (●) or two or three or more (○) waves of follicle development during the oestrous cycle before AI. The numbers in parentheses indicate the number of cows contributing to each data point.

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during spontaneous oestrous cycles although we would predict that this is the case.

The predominant pattern of follicle turnover within the group of cows used in the present study was two waves (74.7%). This is in common with other studies of follicle turnover in Holstein heifers (Ginther et al. 1989, Knopf et al. 1989) and dairy cows (Taylor & Rajamahendran 1991) where 80 to 90% of cows had two-wave oestrous cycles, but differs from the observations of Savio et al. (1988) and Sirois and Fortune (1988) who found that 70 to 80% of heifers had oestrous cycles with three waves of follicles. As indicated in the present study, the incidence of two or three waves of follicle development does not appear to be influenced by parity or post partum interval. The reason why a given population of cattle has a predominant pattern of follicle turnover remains obscure.

The characteristics of follicle and luteal development observed in the present study are comparable with those reported by others in normal, spontaneous oestrous cycles (Savio et al. 1988, Sirois & Fortune 1988, Ginther et al. 1989, Ahmad et al. 1997). The ovulatory follicle emerged 3 to 4 days later in oestrous cycles with three follicle waves than in those with two waves. The incidence of twin ovulations was 8.2%, similar to the incidence reported by Al-Dahash and David (1977). This is about twice the rate of twin births observed in UK dairy herds (4%; Esslemont & Kossaibati 2002) and at CEDAR during the 5 years of the study (3–6%).

Interestingly, twin ovulations were detected with greater frequency in cows with three waves of follicle development than in those with two or ≥ four waves. Twin ovulatory follicles were both derived from the final wave in all but one cow in which DF2 and DF3 ovulated. The latter phenomenon appears to be common in sheep with multiple ovulations (Bartlewski et al. 1998) although it has also been seen in cattle (Savio et al. 1993a). This finding suggests that the process of atresia of the DF is gradual and that despite the apparent loss of functional dominance (indicated by new wave emergence) some previously dominant follicles may retain ovulatory capacity following luteolysis.

Where twin ovulatory follicles were derived from the same wave, the EOI was ~ 3 days shorter than in single ovulating cows. In these cows the interval from emergence to luteolysis was approximately 2 days compared with 5 days in single ovulating cows. Since dominance is not attained until at least 2 days after emergence in the present study, it may be of significance that in twin ovulating cows the selection of the dominant follicle would be coincident with the onset of luteolysis. The association between twin ovulations and the emergence of the follicle wave close to luteolysis would appear to be supported by the observations of others. Twin ovulations have also been reported where the ovulatory wave emerged following induced luteolysis (Kastelic et al. 1990), withdrawal of progesterone treatment (Savio et al. 1993a), and in cows spontaneously recovering from follicular cysts (Savio et al. 1990). In each of these examples peripheral progesterone concentrations would have been low (and LH pulse frequency increasing) at the time of emergence of the ovulatory wave.

Another interesting finding from the present study was that cows with two follicle waves during the oestrous cycle before insemination produced more milk than those with three waves. Each of the measures of milk production considered were 8 to 9% higher in cows with two waves. There was also a positive linear relationship between EOI and 305-day milk yield. The latter observations may be explained by delayed conception in cows with an extended period of ovulatory follicle growth. However, there may also be underlying mechanisms that associate the level of milk production with follicle turnover pattern that remain to be elucidated. In the present study the level of energy balance between weeks 5 and 17 post partum was similar among cows with two or three waves of follicles. In this regard, it may have been pertinent to commence assessment of energy balances from parturition. Other studies suggest that negative energy balance is at its nadir during the first 3 weeks of lactation (Butler et al. 1981, Staples et al. 1990, Gardner et al. 2001). It is estimated that a preovulatory follicle takes in excess of 40 days to complete its growth from the early antral stage (Lussier et al. 1987). Since a follicle ovulating ~ 80 days post partum will have begun its growth during the early post partum period, nutritional status during these early

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stages of follicle development may be important in influencing (via altered circulating metabolic hormone and metabolite levels; Nebel & McGilliard 1993, Webb et al. 1999) follicle turnover during the final stages of development observed by ultrasonography.

In conclusion, these findings support the hypothesis that increased duration of ovulatory follicle development from the time of emergence (or dominance) to oestrus is associated with reduced pregnancy rates following AI in dairy cows undergoing spontaneous oestrous cycles. In addition, the occurrence of longer EOI and of two-wave oestrous cycles are positively associated with higher milk yields, which may go some way to explaining why increased milk production is associated with decreased pregnancy rates. Further work is needed to identify the mechanisms by which increased milk yield may influence follicle dynamics and also to develop practicable methods for favouring ovulation of those follicles with the shortest EOI without pharmacological manipulation.

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