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Low numbers of ovarian follicles ≥3 mm in diameter are associated with low fertility in dairy cows

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ABSTRACT

The total number of ovarian follicles ≥3 mm in diameter (antral follicle count, AFC) during follicular waves varies among cattle of similar age, but AFC is highly repeatable within individuals. We hypothesized that lower AFC could be associated with reduced fertility in cattle. The AFC was assessed by ultrasonography for 2 d consecutively during the first wave of follicular growth of the estrous cycle, 4.6 ± 1.43 d (mean ± SD) after estrus, in 306 Holstein-Friesian dairy cows approximately 70 d postpartum. Cows were classified into 3 groups based on AFC: low (AFC ≤15), intermediate (AFC = 16 to 24), and high (AFC ≥25). During the cycle in which AFC was assessed and in subsequent cycles, cows were artificially inseminated (AI) following detection of estrus, and pregnancy status was assessed using ultrasonography. Cows with high AFC had 3.34 times greater odds of being pregnant at the end of the breeding season compared with cows with low AFC; the odds of a successful pregnancy at first service were 1.75 times greater in the intermediate compared with the low group. The predicted probability of a successful pregnancy by the end of the breeding period (length of breeding season was 86 ± 16.3 d) was 94, 88, and 84% for the high, intermediate, and low AFC groups, respectively. No difference was evident among groups in 21-d submission rate (proportion of all cows detected in estrus and submitted for AI in the first 21 d of the breeding season), but the interval from calving to conception was shorter in the high (109.5 ± 5.1 d) versus low (117.1 ± 4 d) group, and animals with intermediate AFC received fewer services during the breeding season (2.3 ± 0.1) compared with animals with low AFC (2.7 ± 0.1). Lactating cows with ≤15 ovarian follicles have lower reproductive performance compared with cows with higher numbers of follicles, but the existence of a positive association between high numbers of ovarian follicles and fertility is yet to be established.
Key words: follicle number, fertility, dairy cow

INTRODUCTION

In cattle, growth of ovarian follicles from approximately 0.3 to 3 to 5 mm in diameter takes more than 30 d, but subsequent development is rapid, with growth rates of up to 2 mm/d detected in larger antral follicles (Lussier et al., 1987). Antral follicles grow in a wave-like pattern (Sirois and Fortune, 1988; Ginther et al., 1989; Fortune et al., 1991) consisting of the synchronous development of a cohort of follicles preceded by a transient rise in serum FSH concentrations (Adams et al., 1992, 1994; Evans, 2003). The numbers of follicles ≥3 mm in diameter recruited in each wave have been counted (antral follicle count, **AFC**) on different days of the estrous cycle in both beef and dairy heifers and in postpartum dairy cows (Singh et al., 2004; Evans et al., 2011; Ireland et al., 2011). The AFC is highly variable among animals but very highly repeatable (0.85 to 0.95) within individuals (Jimenez-Krassel et al., 2009; Mossa et al., 2010a,b). Thus, cattle can be phenotyped reliably based on one AFC measure.

Although the proportion of healthy follicles (healthy divided by the total number of healthy and atretic follicles per animal) is similar, the total number of healthy and atretic follicles, number of healthy follicles, and number of healthy follicles per gram of ovary are lower in cattle with low compared with high AFC. Animals with a low AFC have smaller ovaries compared with age-matched cows with a higher AFC (Ireland et al., 2008). Moreover, cattle with a low AFC have a reduced response to superovulation (Singh et al., 2004; Ireland et al., 2007) and much lower circulating concentrations of progesterone and reduced endometrial thickness from d 0 to 6 of the estrous cycle (Jimenez-Krassel et al., 2009) compared with age-matched cattle with a higher

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AFC. Low circulating progesterone concentrations were associated with high rates of embryo mortality in cattle (Diskin and Morris, 2008). Consequently, cattle with a low AFC and correspondingly low circulating progesterone concentrations and diminished endometrial development may also have higher rates of embryo mortality.

Taken together, these findings demonstrate that AFC is a reliable predictor of the size of the ovarian reserve (total number of healthy follicles and oocytes in ovaries) and is positively associated with ovarian size and ovarian function. It is unknown if cows with lower numbers of follicles have lower reproductive performance compared with those with a higher AFC. The hypothesis that AFC is positively associated with fertility in dairy cattle was tested. The objective was to determine if AFC and ovarian area were associated with several measures of reproductive efficiency in lactating Holstein-Friesian dairy cows.

MATERIALS AND METHODS

Animal experimentation was performed in compliance with protocols approved by the University College Dublin Animal Research Ethics Committee, the Cruelty to Animal Act (Ireland, 1876) and the European Union Directive 86/609/EC.

Animals and AFC

This study used a cohort of 306 Holstein-Friesian dairy cows during the spring (April to July 2008, n = 109; April to July 2009, n = 137) and autumn (November to February 2008, n = 60) breeding seasons in 2 farms at the Teagasc, Moorepark Animal and Grassland Research and Innovation Centre in Ireland (55°10′N, 8°16′W). At approximately 70 DIM, cows were observed for estrus using tail paint as a heat detection aid. During the first wave of follicular growth of the estrous cycle, 4.6 ± 1.43 d (mean ± SD) after estrus, transrectal ovarian ultrasonography (Aloka SSD-900 with a 7.5-MHz linear transducer; BCF Technology Ltd., Livingston, Scotland, UK) was performed by 1 of 2 operators on all cows for 2 d consecutively. All follicles ≥3 mm in both ovaries were counted on each day and a mean total number (for both ovaries) per animal was calculated. Because a positive association was reported between AFC and ovarian size (Ireland et al., 2008), the size of the ovary without the corpus luteum was measured in a subset of cows (n = 246), as described in previous studies (Ireland et al., 2008). Based on AFC, cows were classified into 3 groups: low (≤15 follicles), intermediate (16 to 24 follicles), and high (≥25 follicles). This classification was chosen because physiologically important phenotypic differences existed between young mature cattle with low versus high AFC, and many of these differences were reported for older, less-fertile cattle compared with their younger counterparts (Ireland et al., 2007, 2008; Mossa et al., 2010b).

Reproductive Management and Measures

Cows were inseminated either during the same estrous cycles in which AFC was assessed or in a subsequent cycle. For the entire breeding season, all cows were AI by the same technician following detection of estrus. Cows detected in estrus before morning milking (0630 h) were inseminated that morning, whereas cows detected later in the day were inseminated the following morning. Pregnancy status and overall pregnancy rates were assessed using ultrasonographic examinations of the uterus at approximately 30 to 36 and 60 to 66 d post-AI and at 150 d after the beginning of the breeding season (which started at approximately 70 DIM). Some cows were treated to synchronize their estrous cycles. Specifically, 7 cows (low, n = 3; intermediate, n = 3; high, n = 1) received a single injection of $PGF_{2\alpha}$ (500 μg of cloprostenol sodium; Estrumate, BP (Vet) Coopers, Berkhamsted, UK), whereas the following hormonal program was used on 9 cows (low, n = 4; intermediate, n = 1; high, n = 4): GnRH (10 μg of buserelin; Receptal, Intervet Ireland, Dublin) and controlled internal drug releasing-device (CIDR) insertion (Pfizer, Dublin, Ireland) on d 0, $PGF_{2\alpha}$ on d 7, and CIDR removal on d 8. The rest of the cows did not receive any estrous synchronization treatment. To study the association between AFC and fertility, the following reproductive measurements were calculated and analyzed: pregnancy rate at first service (detected by ultrasonography at 30 to 36 d post-AI), pregnancy rate at the end of the breeding season (86 ± 16.3 d; mean length of breeding season ± SD), calving to conception interval, 21-d submission rate (proportion of all cows detected in estrus and submitted for AI in the first 21 d of the breeding season), and the overall number of services per cow in all cows during the entire breeding season. The reproductive performance of only 1 breeding season was analyzed for each animal.

Repeatability of AFC

To test the repeatability of AFC across breeding seasons, a subset of cows (n = 44) was examined to assess AFC as described above in 2 consecutive breeding seasons (382 ± 15.1 d; mean interval between breeding seasons ± SD).

Statistical Analysis

The correlation between AFC on the first and second consecutive days of scanning was r = 0.88; therefore, the mean of both measures was used in the subsequent analyses. Factors associated with both AFC and ovarian area were determined using a fixed-effects linear model; AFC was $\log_{10}$ transformed before analysis, but back transformed values are reported. Fixed effects tested for an association with AFC and ovarian area were parity (1, 2, 3, 4, and ≥5), days from calving to scanning (continuous variable), year of scanning, and week of the year at scanning. Nonlinear associations with days since calving were tested. When the dependent variable was AFC, ovarian area was tested for an association with AFC, after adjusting for other fixed effects in the model.

The association between both ovarian AFC and ovarian area and calving to first service interval were quantified using a fixed effects linear model, where calving to first service interval was the dependent variable and parity, day of the year at calving, and AFC group were included as fixed effects in the model. The association between ovarian AFC and ovarian area and the remaining reproductive performance were determined using nonlinear models. Logistic regression, utilizing a logit link function, was used to quantify the association between AFC group and ovarian area (predictor variables) and both pregnancy rate to first service and pregnancy rate by the end of the breeding season (outcome variables); the odds of a successful pregnancy was modeled. The odds ratio (**OR**) is an estimation of the relative odds of an event (e.g., likelihood of a pregnancy) occurring in the exposed group relative to a reference group. The low group was used as the reference group; therefore, an OR of 1 represents an equal likelihood of pregnancy in a particular group compared with a contemporary in the low group, whereas an OR >1 indicates a greater likelihood of that particular event (pregnancy ensuing) occurring relative to the low group. The associations between ovarian AFC and pregnancy rate to first service, pregnancy rate by the end of the breeding season, 21-d submission rate, and overall number of services per cow during the entire breeding season were quantified. For all models, fixed effects adjusted for in the model were, where significant ($P < 0.05$), month of calving, year of calving, and parity of the cow; AFC and ovarian area were separately forced into each model. Calving to first service interval was included as a continuous fixed effect when the dependent variable was pregnant to first service.

Survival analysis was used to determine the association between both group and ovarian area and calving to conception interval. Animals that did not conceive were assumed not pregnant (censored) at the last day of the breeding season. Survival was expressed as the relative hazard of a cow conceiving at day t, given that it had not conceived at day t – 1, in the exposed group relative to the reference group. The low AFC group was used as the reference group. Therefore, a hazard ratio (**HR**) of 1 represents an equal likelihood of a cow conceiving at time t compared with a contemporary in the reference group, whereas a lower HR indicates a decreased likelihood of an animal conceiving at time t compared with the low AFC group. Ordinal regression, using a cumulative logit link function, was used to determine the association between AFC group and ovarian area and number of services per cow.

RESULTS

Follicle Numbers

The mean AFC (±SD) in all cows was 18.5 ± 9.0, ranging from 4 to 61 follicles per cow; the frequency distribution of number of antral follicles is in Figure 1. Based on the AFC counted on 2 d consecutively, 126 (41%) cows were assigned to the low group (≤15 follicles), 122 (40%) to the intermediate group (16 to 24 follicles), and 58 (19%) to the high (≥25 follicles) group (Table 1).

Lactation number (Table 1) was associated ($P < 0.01$) with AFC. The mean AFC (±SEM) increased from 16.5 ± 0.7 in parity 1 animals to 18.2 ± 1.2 in parity 2 animals and was 22.7 ± 1.4 in parity 3 animals, after which it stayed constant (Table 2). Week of the year at scanning was associated ($P < 0.01$) with AFC, but we failed to observe a consistent association between AFC and week of year at scanning. Cows in

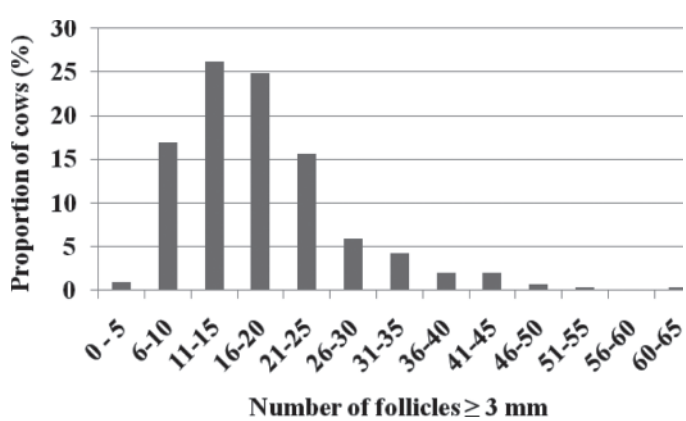

Figure 1. Frequency distribution of the total number of ovarian follicles ≥3 mm in diameter detected using ovarian ultrasonography on 2 d consecutively during the first wave of follicular growth of the estrous cycle in 306 lactating dairy cows.

Table 1. Characteristics at ultrasound scanning and reproductive performance in dairy cows with high (≥25), intermediate (16 to 24), and low (≤15) numbers of ovarian follicles ≥3 mm in diameter (antral follicle count)

	Antral follicle count				
Variable	Low	Intermediate	High	SEM[1]	*P*-value[2]
Characteristic at scanning					
Number	126	122	58		
Antral follicle count	11.4[a]	19.6[b]	33.2[c]	0.45	<0.0001
Lactation number	1.9[a]	2.1[a]	2.6[b]	0.13	0.004
DIM	71.5	71.1	69.5	1.80	NS
Reproductive trait					
Pregnancy rate at first service (%)	32[a]	45[b]	38[ab]		0.12
Pregnancy rate overall (%)	84[a]	88[ab]	94[b]		0.06
21-d submission rate (%)	71	81	83		0.09
Median calving to conception interval (d)	114[a]	100[b]	100[b]		0.09
Number of services overall	2.7[a]	2.3[b]	2.4[ab]		0.09

[a–c]Values within a row with no common superscript differ ($P < 0.05$).

[1]SEM = pooled SEM.

[2]Level of significance (*P*-value) is based on fixed effects linear model for calving to first service interval, survival analysis for calving to conception interval, ordinal regression for number of services and logistic regression for the remaining traits. Rate variable calculations are based on the odds ratio output, whereas least squares means are provided for the remaining variables.

the 3 AFC groups were at similar DIM when AFC was assessed (Table 1).

Ovarian Dimensions

The mean (±SD) area of the cross-section of the ovary was 3.09 ± 1.60 cm^2, and the correlation between ovarian area and AFC was r = 0.37 ($P < 0.001$). Similar to AFC, ovarian area was associated with lactation number ($P < 0.001$); it increased with parity until parity 3, after which it did not change (Table 2). Ovarian area was associated with AFC ($P < 0.001$) and increased from (mean ± SEM) 2.8 ± 0.09, 3.1 ± 0.10, and 3.7 ± 0.14 cm^2 in the low, intermediate, and high groups, respectively. Ovarian area was not associated with any of the fertility measures analyzed (Table 3).

Association Between AFC and Fertility

The association between AFC and fertility is summarized in Tables 1 and 4. The OR of a successful pregnancy at first service were lower ($P < 0.05$) in animals with low versus intermediate AFC, whereas no difference existed between animals in the high versus the low group (Table 4). The predicted probability of a successful pregnancy at first service for animals in the low, intermediate, and high groups was 32, 45, and 38%, respectively (Table 1), and the actual pregnancy rates at first service were 35.2% in the low, 47.1% in the intermediate, and 34.5% in the high group.

Animals with a high AFC had a 3.34 times greater OR of being pregnant at the end of the breeding season compared with animals with low AFC ($P < 0.05$), whereas no difference was detected between the intermediate and high or between the intermediate and low groups (Table 4). The predicted probability of a successful pregnancy by the end of the breeding period tended to be different ($P = 0.06$) between AFC groups and was 84, 88, and 94% in the low, intermediate, and high groups, respectively (Table 1). The actual pregnancy rates overall were 83.2, 86.7, and 91.2% in the low, intermediate, and high groups, respectively.

Table 2. Effect of parity on the least squares means for antral follicle count, ovarian area, calving to conception interval, and overall number of services and on the predicted probability of pregnancy rate at first service, overall pregnancy rate, and 21-d submission rate

Variable	Parity 1	Parity 2	Parity 3	Parity 4	Parity 5	*P*-value	SEM[1]
Antral follicle count	16.5	18.2	22.7	20.9	21.0	0.0003	1.06
Ovarian area (cm^2)	2.77	3.01	3.75	3.45	3.45	<0.001	0.13
Calving to conception interval (d)	96.8	109.3	100.3	111.1	118	0.023	5.14
Number of services overall	2.04	2.47	2.18	2.90	2.72	NS	0.19
Pregnancy rate at first service (%)	64.5[a]	46.7[b]	61.2[ab]	41.6[b]	36.8[b]	0.022	
Pregnancy rate overall (%)	90.7[a]	82.5[ab]	72.5[b]	63.0[b]	62.0[b]	0.002	
21-d submission rate (%)	46.9	35.4	37.3	34.7	41.7	NS	

[a,b]Values within a row with no common superscript differ ($P < 0.05$).

[1]SEM = pooled SEM.

Table 3. Association between total ovarian area and odds ratios (pregnancy rate overall and at first service, 21-d submission rate, number of services) and hazard ratio (calving to conception interval) for fertility performance parameters in dairy cows (95% CI in parentheses)

	Total ovarian area (cm^2)			
Variable	≤2	2.1 to 3.9	≥4	*P*-value
Pregnancy rate to first service	1	0.56	0.66	0.16
	0	(0.30 to 1.03)	(0.35 to 1.20)	
Overall pregnancy rate	1	0.50	0.77	0.33
	0	(0.19 to 1.29)	(0.30 to 1.94)	
Calving to conception interval	1	0.76	0.80	0.20
	0	(0.55 to 1.04)	(0.58 to 1.09)	
21-d submission rate	1	0.95	1.41	0.43
	0	(0.48 to 1.86)	(0.91 to 2.64)	
Overall number of services	1	1.59	1.55	0.16
	0	(0.93 to 2.71)	(0.72 to 2.74)	

No difference in 21-d submission rate was evident among groups (Tables 1 and 4). The HR of a cow conceiving at time *t*, given she had not conceived by time $t - 1$, was 1.43 times greater ($P < 0.05$) in the high compared with the low group (i.e., reference class), although no difference was evident between the intermediate group and either of the other 2 groups (Table 4). The median calving to conception interval was 114 d in the low group and 100 d in both the intermediate and high groups (Table 1).

Animals with a low AFC received more services during the overall breeding season compared with animals with an intermediate AFC (2.7 vs. 2.3; $P < 0.05$), whereas no difference was detected in the number of inseminations received by cows in the high versus low group (Tables 1 and 4).

Repeatability of AFC

A correlation of r = 0.65 ($P < 0.0001$) existed between AFC recorded in 2 different seasons in the same cows (n = 44). The mean (±SD) change within cow in AFC was −1.65 ± 7.5, and no linear correlation was detected between seasons.

DISCUSSION

Previous studies show that cattle with a relatively low AFC have numerous phenotypic characteristics usually associated with reduced fertility (Ireland et al., 2011). Young adult cattle with compared with a high AFC have relatively low concentrations of circulating progesterone (Jimenez-Krassel et al., 2009), which are associated with high rates of embryo mortality in cattle (Stronge et al., 2005; McNeill et al., 2006; Diskin and Morris, 2008). These findings provide indirect evidence for an association between low fertility and low AFC. The association between low AFC, enhanced FSH secretion, and decreased progesterone production (Evans et al., 2010; Ireland et al., 2011) may result in increased rates of embryo mortality. This could explain the lower fertility in cattle with a low AFC in our study.

Table 4. Association between number of ovarian follicles ≥3 mm in diameter (antral follicle count, AFC) in dairy cows with high (≥25), intermediate (16 to 24), and low (≤15) AFC and the odds ratios (pregnancy rate overall and at first service, 21-d submission rate, number of services) and hazard ratio (calving to conception interval) for fertility performance parameters (95% CI in parentheses)

	AFC			
Fertility measure	Low	Intermediate	High	*P*-value
Animals (no.)	126	122	58	
Pregnancy rate at first service	1	1.75*	1.29	0.12
		(1.01 to 3.01)	(0.63 to 2.59)	
Overall pregnancy rate	1	1.49	3.34*	0.06
		(0.70 to 3.26)	(1.12 to 9.90)	
21-d submission rate	1	1.73	2.00	0.10
		(0.94 to 3.17)	(0.91 to 4.33)	
Calving to conception interval	1	1.25	1.43*	0.10
		(0.94 to 1.65)	(1.01 to 2.01)	
Overall number of services	1	0.60*	0.88	0.09
		(0.37 to 0.95)	(0.49 to 1.55)	

*Significant difference ($P < 0.05$) compared with the low group.

For the first time we provide evidence that a phenotypic ovarian measurement may be predictive of reproductive performance in dairy cattle. Conception rate at first service was not associated with the number of follicles ≥4 mm in diameter growing during follicular waves in beef cattle (Starbuck-Clemmer et al., 2007). This latter study reported atypically high conception rates, and the authors indicated this as a possible factor precluding detection of differences. Two-dimensional real-time ultrasonography is currently performed in cattle, primarily for early pregnancy diagnosis and to assess ovarian and uterine pathologies, and this technique has been feasible in a farm environment (Fricke, 2002). Based on our results, such a tool could be used to provide relevant information on potential fertility in cattle. We have established that the number of follicles recruited in each wave is highly repeatable within individuals, regardless of age, breed, country of origin, season or stage of lactation, span of time between AFC measurements in the same individuals (up to 15 mo), or the ultrasound technician conducting the ovarian ultrasonography (Burns et al., 2005; Ireland et al., 2007).

The observation in the current study that cows with a low AFC have smaller ovaries than those with a high AFC supports previous publications (Burns et al., 2005; Ireland et al., 2008; Mossa et al., 2010b) and is an important finding of practical significance, as less time and skill are required for an ultrasound technician to determine ovarian size than AFC. This measurement is a less accurate proxy for AFC than direct follicle counting, which may explain why, under the conditions of the current study, we found no evidence to support the hypothesis that ovarian size is a predictor of reproductive performance.

Finally, we observed an increase in AFC from parity 1 to 3; this finding was unexpected because the ovarian reserve diminishes with age in cattle (Erickson, 1966). It could be interpreted in support of the notion that cows with a high AFC are more fertile than cows with a lower AFC. For example, the less-fertile animals with a low AFC may not survive in the herd because they are culled earlier than cows with a higher AFC. Therefore, animals with a relatively high AFC may become more prevalent in the herd when animals are stratified by age. On the other hand, this may not be the case, as pregnancy rates at first service and overall declined and the length of calving to conception interval increased with parity in this study. Decreased fertility with aging has been documented in cattle (Malhi et al., 2005), and one study reported that 55% of the herd was infertile by 13 yr of age (Erickson et al., 1976). Here, we examined cows up to fifth parity and the number of subjects may have been too limited to elucidate the effects of lactation number on fertility.

This study examined for the first time the possible association between AFC and fertility in vivo in dairy cows without the use of superovulatory treatments. Evidence for a positive association between AFC and reproductive performance is provided by lower pregnancy rates at first service and at the end of the breeding season, longer interval from calving to conception, and higher number of services received during the breeding season in cows with low compared with higher AFC. Other findings are inconsistent with this hypothesis, such as the highest pregnancy rates at first service and the lowest numbers of services during the entire breeding season recorded in cows with an intermediate AFC. Collectively, the results presented indicate that dairy cows with a low number of ovarian follicles (≤15) have lower reproductive performance compared with cows with higher numbers of follicles growing during follicular waves, but the existence of a positive association between a high numbers of ovarian follicles and fertility is yet to be established.

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