

# Causes and consequences of the variation in the number of ovarian follicles in cattle

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## Summary

In cattle we have noted that the antral follicle count (AFC, follicles  $\geq 3$  mm in diameter) varies greatly among animals (from 5 to 50), is repeatable within animals, and is highly correlated with the total number of healthy follicles in ovaries. Also, animals with low AFC have higher serum concentrations of FSH and LH, but lower concentrations of Anti-Mullerian Hormone, progesterone and androgens than animals with high AFC. We have investigated the effect of maternal environment during gestation on their offspring AFC by restricting maternal nutrition to 60% of maintenance requirements (compared with 100% in controls) during the first third of gestation. Calves born to nutritionally restricted mothers had 60% lower AFC compared with calves born to mothers fed control diets. In other studies we have evidence to indicate that fertility may be compromised in animals with low AFC due to effects on oocytes, progesterone and the endometrium compared with animals with high AFC. To examine this directly we assessed AFC in post-partum dairy cows and found that cows with a high AFC had higher pregnancy rates, shorter calving to conception intervals and received fewer services during the breeding season compared with cows with a low AFC. In addition, the high variation in follicle numbers in adults may not only be reflective of reproductive disorders and suboptimal fertility, but there is evidence to indicate that it may be associated with alterations in the function of other non-reproductive systems (e.g. cardiovascular) that may have profound effects on the animal's health and welfare.

## Introduction

In cattle, growth of ovarian antral follicles from about 300  $\mu\text{m}$  to 5 mm in diameter takes more than 30 days (Lussier *et al.* 1987). Subsequent follicle growth to 15 to 20 mm in diameter is rapid and occurs in a wave-like pattern over the next 4 to 6 days with two or three waves occurring during the normal course of oestrous cycles in cattle (Evans 2003). The wave-like pattern of follicle growth was first proposed by Rajakoski in 1960 after a large study examining the number of follicles  $> 1$  mm in diameter in the ovaries of slaughtered cattle on different days of the cycle (Ireland *et al.* 2000). Rajakoski's observation, that the numbers of follicles increased and decreased in a wave-like pattern, gave rise to the two follicle wave hypothesis (Rajakoski 1960). Ireland and Roche used intrafollicular concentrations of oestradiol to progesterone to classify antral follicles  $\geq 6$  mm in diameter and proposed the three-wave hypothesis for dominant follicle development in cattle (Ireland & Roche 1982, Ireland & Roche 1983b, Ireland & Roche 1983a, Ireland & Roche 1987). The development of ultrasonography as a tool to noninvasively monitor the growth and regression of individual ovarian follicles repeatedly in the same animal (Pierson & Ginther 1984) firmly established that antral follicles grow in cohorts, in two or three wave-like patterns during oestrous cycles in cattle (Pierson & Ginther 1988, Savio *et al.* 1988, Sirois & Fortune 1988). This finding was later extended to other reproductive periods in cattle and to other species (Ireland *et al.* 2000, Evans 2003). Numerous subsequent studies examined the associations among reproductive hormones and follicle growth (Mihm *et al.* 2002). However, the observations that the number of primordial follicles is highly variable in cattle at birth (Erickson 1966b), that the number of different follicle types vary greatly among adult cattle, and that they reliably ovulate one, or occasionally two, follicles during each oestrous cycle prompted further investigations into the variation in ovarian follicle numbers in cattle.

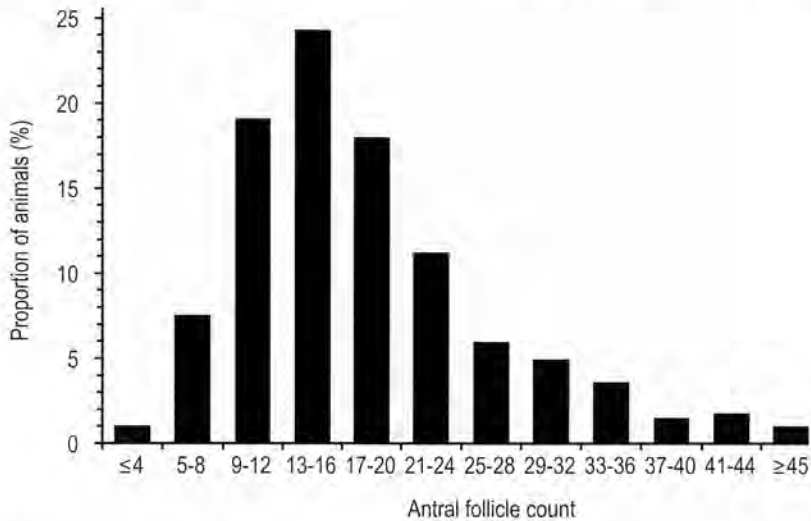
### Variation in the numbers of ovarian follicles and associated reproductive hormones

#### Follicle numbers

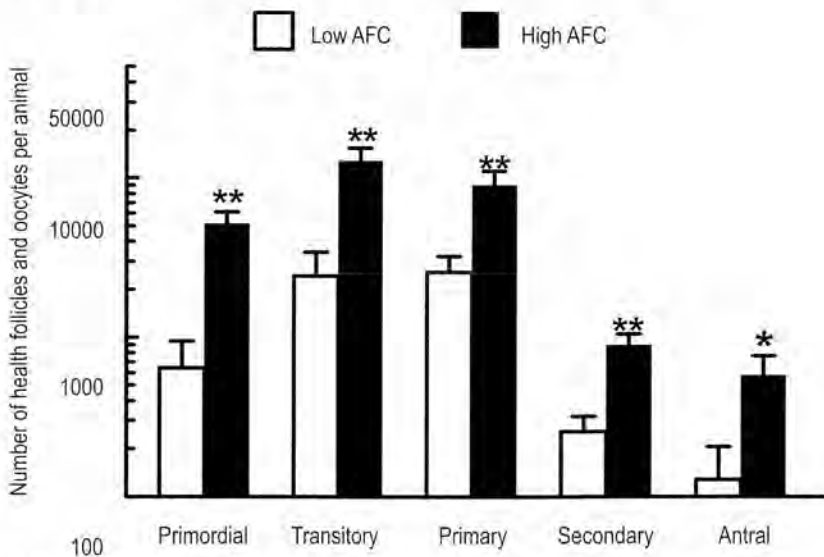
Our research groups have now conducted a number of studies that have systematically counted and catalogued the numbers of antral follicles on different days of the oestrous cycle in both beef and dairy heifers and in post-partum dairy cows. We have established that the numbers of follicles in ovarian follicular waves of the oestrous cycle are highly variable among animals but very highly repeatable within individuals (Burns *et al.* 2005, Ireland *et al.* 2007, Ireland *et al.* 2008, Jimenez-Krassel *et al.* 2009) (Mossa *et al.* 2010b). This observation holds true when considering the peak or nadir numbers associated with waves or the mean numbers across all days of the cycle. However, the count must include all the follicles  $\geq 3$  mm in diameter in both ovaries (the antral follicle count or AFC) and explains why many studies in the last 20 years that have focused on follicles  $\geq 5$  or 6 mm in diameter have not noted the high variability of follicle numbers growing during follicular waves among animals nor the remarkably high repeatability of follicle numbers during waves in individuals. For example, we have noted that the AFC in both ovaries during different follicular waves of an oestrous cycle may be consistently lower than 5 during follicular waves in some animals and greater than 50 in others (Burns *et al.* 2005, Ireland *et al.* 2007, Mossa *et al.* 2010b) (Figure 1). Moreover, this high repeatability of follicle numbers during waves persists for at least one year (Burns *et al.* 2005).

The observation of the variation in AFC raises questions about the possibility for variation in the total numbers of follicles in the ovaries. To address this, age matched heifers (adult cycling animal 12 to 18 months of age) that had high ( $\geq 25$ ) or low ( $\leq 15$ ) AFC were identified, and the numbers of all follicles in the ovaries were counted in histological sections (Ireland *et al.* 2008).

This study showed that the AFC reliably predicted the numbers of morphologically healthy follicles in all classes of the stages of folliculogenesis (Ireland *et al.* 2008) (Figure 2). For example, cattle with a low AFC also had a very low total number of healthy primordial, preantral and antral follicles in ovaries compared with cattle with a high AFC (Ireland *et al.* 2008).



**Fig. 1.** Frequency distribution of the number of follicles  $\geq 3$  mm in diameter (Antral Follicle Count) in post-partum Holstein-Friesian dairy cows ( $n = 383$ ) measured using ultrasonography (Mossa *et al.* unpublished)



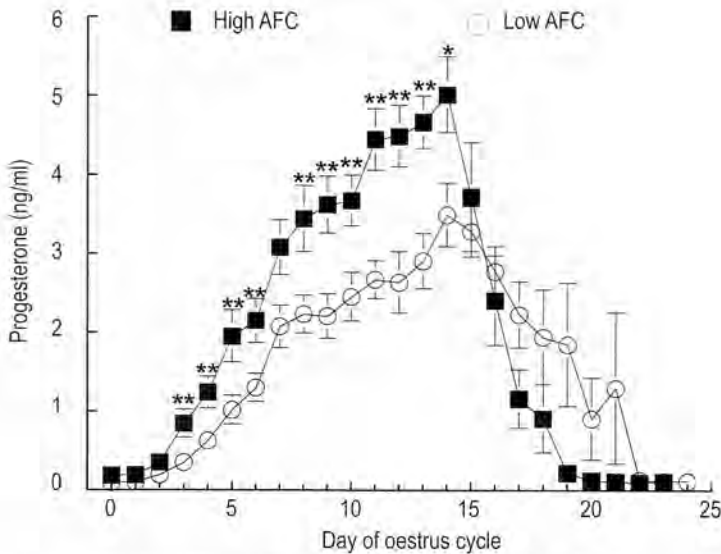
**Fig. 2.** Number of morphologically healthy primordial, transitory, primary, secondary, and antral follicles in ovaries of cattle with a low ( $\leq 15$  follicles,  $n = 5$ ) versus a high ( $\geq 25$  follicles,  $n = 5$ ) numbers of follicles  $\geq 3$  mm in diameter (Antral Follicle Count, AFC) during ovarian follicular waves. The ovary contralateral to the recent ovulation was removed surgically 1–2 days after ovulation and histologically examined. Asterisks ( $*P < 0.05$ ,  $**P < 0.01$ ) indicate difference between means for the low versus high group. From (Ireland *et al.* 2008).

### Hormones

Transient rises in FSH concentrations precede and stimulate the emergence of follicle waves (Adams *et al.* 1992). A number of studies have now shown that FSH concentrations are lower in animals with high AFC compared with animals with low AFC (Burns *et al.* 2005, Ireland *et al.* 2007, Mossa *et al.* 2010b). In addition, circulating LH concentrations during the estrous cycle are chronically higher in young adult cattle with low versus high AFC (Jimenez-Krassel *et al.* 2009). This observation leads to the question as to whether pituitary function differs between the two groups of animals. We tested this notion by examining the response to ovariectomy of cows with high or low AFC and then challenging them with GnRH and follicular fluid (rich source of inhibin) on separate occasions 30 days after ovariectomy (Mossa *et al.* 2010b). The study showed that there was no difference between the two groups in basal FSH or LH secretion or response to the treatments leading to the conclusion that differences in gonadotrophin secretion between cows with high versus low AFC is due to differences in negative feedback hormones and not differences in pituitary function (Mossa *et al.* 2010b).

Evidence that the total number of healthy follicles in ovaries contributes to the endocrine environment is provided by examining Anti-Müllerian Hormone (AMH) concentrations. AMH is produced primarily by granulosa cells of healthy growing follicles (La Marca & Volpe 2006) and is positively associated with follicle numbers in mice (Kevenaar *et al.* 2006) and women (Jayaprakasan *et al.* 2009, van Disseldorp *et al.* 2009). It has recently been shown that AMH concentrations can reliably predict the AFC and the number of healthy follicles and oocytes in age matched young adult cattle (Ireland *et al.* 2008). Furthermore, AMH serum concentrations before superovulation are highly positively correlated with the numbers of ovulations after treatment (Rico *et al.* 2009) and are repeatable within animals (Ireland *et al.* 2008, Rico *et al.* 2009). In comparison to AMH, serum concentrations of both oestradiol and inhibin-A, the main feedback regulators of FSH secretion, are not different between groups of animals with high and low AFC (Burns *et al.* 2005, Ireland *et al.* 2007). This may be explained by the fact that individual follicles from cattle with low AFC have higher follicular fluid concentrations and produce more oestradiol than follicles from animals with high AFC (Ireland *et al.* 2009) and we have speculated that this difference may be a consequence of higher circulating FSH concentrations in the low versus the high AFC groups (Ireland *et al.* 2009). Nonetheless it appears that the total oestradiol negative feedback is similar between the high and low groups as fewer follicles produce more oestradiol in the low AFC animals compared with more follicles producing lower oestradiol in the high AFC group (at least within the limits of sensitivity of our oestradiol assay). Despite not being able to measure a difference in circulating oestradiol concentrations between high and low AFC groups, we have recently shown that circulating androgen concentrations are indeed higher in cattle with high compared with low AFC (Jimenez-Krassel *et al.* 2009). In the absence of an alternative explanation, we suggest that the difference in circulating gonadotrophin concentrations associated with variable AFC is due to differences in ovarian feedback hormones produced by the follicles.

Due to the considerable interest in the contribution of early/mid luteal phase progesterone concentrations for the successful establishment of pregnancy, we compared progesterone concentrations between groups of animals and found that animals with low AFC have much lower progesterone concentrations during oestrous cycles than animals with high AFC (Jimenez-Krassel *et al.* 2009) (Figure 3). Also the ability of both granulosa and luteal cells from animals with low AFC to produce progesterone *in vitro* was diminished compared with animals with high AFC (Jimenez-Krassel *et al.* 2009). The possible consequences of this difference in progesterone concentrations are discussed below.



**Fig. 3.** Alterations in serum concentrations of progesterone during the bovine oestrous cycle (Day 0 = oestrus). Each symbol represents the daily mean ( $\pm$ SEM) progesterone value for animals with a consistently low ( $\leq 15$  follicles per wave,  $n = 32$  oestrous cycles for 25 animals) versus high ( $\geq 25$  follicles per wave,  $n = 30$  cycles for 22 animals) AFC during follicular waves. Asterisks indicate significant differences (\*\*  $P < 0.01$ ) between groups. ANOVA indicated an overall significant effect of group (Low versus High,  $P < 0.02$ ), day of cycle ( $P < 0.001$ ) and group  $\times$  day interaction ( $P < 0.001$ ). From (Jimenez-Krassel *et al.* 2009).

### Causes of the variation in the numbers of ovarian follicles

The cause of the inherently high variation in number of follicles is unknown but a number of explanations are plausible. Firstly, proliferation of oogonia during early embryonic development (that give rise to a fixed pool of non proliferating oocytes within primordial follicles for the lifetime of the animal) may be highly variable resulting in a highly variable pool of follicles. Secondly, proliferation of oogonia may be relatively constant (similar among females) but depletion of this pool may proceed at different rates among individuals giving rise to females with differing AFC at the same age. Thirdly, the variation in follicle numbers among age matched females occurs as a result of a combination of the first two possibilities.

David Barker (the Barker Hypothesis) suggests that environmental influences early in human foetal life are reflected in impaired growth, development and metabolism leading to increased risk for diseases in adulthood (Barker 1992). The hypothesis proposes that some diseases originate through foetal adaptations to malnutrition that permanently alter body function. This hypothesis is supported by animal models (McMillen & Robinson 2005). Most studies have examined the link between malnutrition with cardiovascular disease, obesity and diabetes, but few studies have examined potential links with reproduction. However, poor foetal growth due to the manipulation of maternal diet alters gonadotrophin gene expression in the pituitary and number of follicles in the ovaries of late-gestation lambs (Da Silva *et al.* 2002). In addition, maternal under-nutrition during the first trimester retards foetal ovarian development in sheep (Borwick *et al.* 1997). Hence, it is reasonable to speculate that maternal nutrition during gestation, at the time of ovarian development in their foetuses, may impact oogonia proliferation and thus follicle numbers postnatally. We have recently started to test this hypothesis by restricting nutrition of beef heifers to 0.6 of their maintenance energy requirements, from shortly before

conception to the end of the first trimester of pregnancy (period encompassing the peak in oocyte numbers in foetuses (Erickson 1966a)). Results show that calves born to nutritionally restricted mothers have a 60% lower peak, minimum and mean AFC during follicular waves compared with calves born to mothers fed control diets (Mossa *et al.* 2009) (Table 1). In addition, calves born to dams nutritionally restricted also show higher resting arterial blood pressure compared with those born to control mothers (Mossa *et al.* 2009). In addition, similar studies in *Bos indicus* cross heifers support the notion that prenatal maternal nutrition affects ovarian measures in their offspring (Sullivan *et al.* 2009).

**Table 1.** Mean ( $\pm$  SEM) number of follicles  $\geq 3$  mm in diameter (Antral Follicle Count) during follicle waves in heifer calves born to mothers fed a control diet (Control) or who were nutritionally restricted (Restricted, 60% energy requirement) for the first 110 days of gestation. From (Mossa *et al.* 2009).

Heifer age (weeks)	Antral Follicle Count		P <
	Control (n = 13)	Restricted (n = 10)	
7	23.9 $\pm$ 2.1	14.1 $\pm$ 0.9	0.01
18	26.1 $\pm$ 2.9	16.2 $\pm$ 1.1	0.01
35	23.9 $\pm$ 2.2	16.6 $\pm$ 1.2	0.01

The Barker Hypothesis, and supporting data in a variety of animal models, indicates that a negative uterine environment can influence the development of different organs and systems in the foetus including the reproductive system. This observation may have relevance for the dairy industry, since it is well established that selecting dairy cows for increased milk production potential has been associated with a concomitant decrease in fertility and an increase in the susceptibility to some diseases (Beckers *et al.* 2002). In order to have a 365-day calving interval, cows must conceive during the period of their peak lactation. This period of peak metabolic stress is coincident with follicle growth, ovulation, fertilization, early embryonic development and early foetal development. According to the Barker Hypothesis, animals conceived and developing in this nutritionally stressed maternal environment may have compromised development that could affect them for the rest of their lives. We have investigated the relationship between maternal milk production (a proxy for maternal stress) on performance indicators in the first, second, and third lactations of their female offspring using a large Irish database and results have shown that greater milk yield preconception and during gestation is associated with reduced survival and milk yield and greater somatic cell count in their progeny (Berry *et al.* 2008). This shows that maternal environment during gestation in dairy cows does have long-term effects on their female offspring but we have not yet investigated the effects of AFC.

### Consequences of the variation in the numbers of ovarian follicles

In women undergoing IVF treatment, the number of antral follicles present before ovarian stimulation is considered a predictor of the ovarian responsiveness to gonadotrophin stimulation, since low numbers of follicles are associated with lower numbers of oocytes recovered and pregnancy rates (Tomas *et al.* 1997, Chang *et al.* 1998, Hsieh *et al.* 2001, Huang *et al.* 2001, Beckers *et al.* 2002). Similarly in cattle the number of follicles prior to superovulatory treatment is correlated with the numbers of follicles and corpora lutea after superovulation, and total ova and transferable embryos recovered (Kawamata 1994, Cushman *et al.* 1999, Taneja *et al.* 2000, Singh *et al.* 2004). Moreover, intrafollicular oestradiol concentrations are about 2-fold higher in animals with a low versus a high AFC (Ireland *et al.* 2009) and this may have detrimental effects on oocyte maturation and developmental competence in cattle with low follicle

numbers because high physiological concentrations of oestradiol block maturation of bovine oocytes *in vitro* (Beker-van Woudenberg *et al.* 2004). In addition, lower circulating concentrations of progesterone in cattle with low AFC are associated with a much lower endometrial thickness from Day 0 to 6 of the oestrous cycle (Jimenez-Krassel *et al.* 2009) and endometrial thickness is positively associated with implantation and pregnancy rates in humans subjected to *in vitro* fertilization programs (Raga *et al.* 1999). Taken together these findings indicate that AFC is positively associated with fertility in cattle. To test this hypothesis we performed ovarian ultrasonography on 306 dairy cows (aged  $3.48 \pm 1.66$  years) during the first wave of follicular development 1 to 4 months post-partum and recorded their reproductive performances during the breeding season. Cows with a high AFC had higher pregnancy rates, shorter calving to conception intervals and received fewer services during the breeding season compared with cows with a low AFC (Mossa *et al.* 2010a). This is supported by a study in beef heifers showing higher pregnancy rates in heifers with high AFC versus low AFC (Cushman *et al.* 2009).

### Conclusion

Improved understanding of the pattern of follicle development in the last 20 years has led to the development of more effective treatments to synchronize (and induce) oestrus behaviour in cattle. The recent studies reviewed here indicate that the variation in number of follicles growing during follicle waves, and *in toto* in ovaries, may be an important consideration when new methods are developed to manipulate and improve superovulation and fertility in cattle. Moreover, the variation in number of oocytes and follicles in offspring may be determined by the maternal environment during foetal development coincident with development of all the organ systems. Thus, the high variation in follicle numbers in adults may not only be reflective of reproductive disorders and suboptimal fertility, but also alterations in other non-reproductive systems that may have profound effects on the animal's health and welfare.

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