Human Reproduction Vol.22, No.6 pp. 1687–1695, 2007 doi:10.1093/humrep/dem071
Advance Access publication on April 27, 2007

Follicle numbers are highly repeatable within individual animals but are inversely correlated with FSH concentrations and the proportion of good-quality embryos after ovarian stimulation in cattle

JJ.Ireland[1], F.Ward[2], F.Jimenez-Krassel[1], J.L.H.Ireland[1], G.W.Smith[3], P.Lonergan[2] and A.C.O.Evans[2,4]

[1]Molecular Reproductive Endocrinology Laboratory, Department of Animal Science, Michigan State University, East Lansing, MI 48824, USA; [2]School of Agriculture, Food Science and Veterinary Medicine, and Conway Institute of Biomedical and Biomolecular Research, College of Life Sciences, University College Dublin, Belfield, Dublin 4, Ireland; [3]Laboratory of Mammalian Reproductive Biology and Genomics, Department of Animal Science, Michigan State University, East Lansing, MI 48824, USA

[4]Correspondence address. ACO Evans, UCD Agriculture and Food Science Centre, Agriculture and Food Science Centre, University College Dublin, Belfield, Dublin 4, Ireland, Tel: +353 1 716 7731, Fax: +353 1 716 1103; E-mail: alex.evans@ucd.ie

BACKGROUND: The significance of the high variation in numbers of follicles produced during reproductive cycles in humans and cattle is unknown. METHODS: We selected beef heifers with high (≥25) or low (≤15) numbers of ovarian follicles and determined the association with alterations in FSH and estradiol concentrations, as well as responsiveness to superstimulation and embryo quality. The variation in follicle numbers was also compared with oocyte quality in natural cycles using IVF and abattoir sourced bovine ovaries. RESULTS: Results show that: (i) FSH was lower ($P < 0.03$) in animals with high compared with low follicle numbers per follicle wave; (ii) after superovulation, in the high versus low follicle number group, the number of oocytes/embryos recovered after insemination (10.6 ± 2.7 versus 4.7 ± 0.7) and the number of transferable embryos (5.4 ± 1.3 versus 3.8 ± 0.8) per animal were greater ($P < 0.05$), whereas the proportion of transferable embryos (50.7% versus 79.8%) was lower ($P < 0.05$); (iii) in unstimulated animals, the numbers of high-quality oocytes harvested and *in-vitro* fertilized oocytes developing into blastocysts were up to 4-fold greater ($P < 0.05$) for ovaries with high versus low numbers of follicles, but the proportions of oocytes developing into blastocysts were similar in the two groups. CONCLUSION: Phenotypic classification based on numbers of follicles may be useful to improve superovulation procedures. The lower proportion of transferable embryos following superovulation of ovaries with high numbers of follicles is probably not the result of differences in the quality of oocytes before superovulation.

Keywords: cattle; follicles; ovarian stimulation; oocyte; FSH

Introduction

The chronic rhythmic or wave-like growth and atresia of ovarian follicles is requisite for the continued development and ovulation of dominant follicles during reproductive cycles of single ovulating species like humans (Baerwald *et al.*, 2003a,b) and cattle (Ginther *et al.*, 1996; Ireland *et al.*, 2000). Nevertheless, despite the positive association of follicle numbers with a variety of measures of fertility in humans (Richardson *et al.*, 1987; Lass *et al.*, 1997; Tomas *et al.*, 1997; Chang *et al.*, 1998; Hsieh *et al.*, 2001; Huang *et al.*, 2001; Beckers *et al.*, 2002; Kupesic *et al.*, 2003; Scheffer *et al.*, 2003;) and cattle (Erickson, 1966a; Erickson *et al.*, 1976; Maurer and Echternkamp, 1985; Kawamata, 1994; Cushman *et al.*, 1999; Taneja *et al.*, 2000; Oliveira *et al.*, 2002; Singh *et al.*, 2004), the extent causes possible association with the ovarian reserve and fertility, and physiological significance of the variation, between animals, in the numbers of follicles growing during follicular waves has not been determined. Consequently, the well characterized bovine dominant follicle model (Sunderland *et al.*, 1994; Evans *et al.*, 1997; Ginther *et al.*, 1996; Ireland *et al.*, 2000) was recently used (Burns *et al.*, 2005) to establish the degree of variation both within and between animals in the number of antral follicles during the follicular waves of estrous cycles in dairy cattle. In this study, Burns *et al.* (2005) demonstrated that a 7-fold variation in number of follicles exists during follicular waves among

© *The Author 2007. Published by Oxford University Press on behalf of the European Society of Human Reproduction and Embryology. All rights reserved. For Permissions, please email: journals.permissions@oxfordjournals.org*

Downloaded from https://academic.oup.com/humrep/article/22/6/1687/610956 by guest on 20 August 2022

animals. Despite this variation and the marked differences in hormonal milieus associated with development of ovulatory and non-ovulatory follicles during the different follicular waves of an estrous cycle (Sunderland *et al.*, 1994), the number of follicles 3 mm in diameter or greater is very highly repeatable (0.95) within individuals (Burns *et al.*, 2005). In addition, the variation in follicle number during non-ovulatory waves is inversely associated with peripheral FSH concentrations (Burns *et al.*, 2005). Most importantly, these observations demonstrate that dairy cattle can be reliably phenotyped based on robust individual differences in numbers of follicles during follicular waves. For example, the peak number of follicles during different follicular waves of an estrous cycle may be consistently as low as eight in some cattle but as high as 56 in others (Burns *et al.*, 2005). Nevertheless, the high variation in follicle numbers among cattle, very high repeatability within individuals and inverse association of follicle numbers during waves with serum FSH concentrations observed in the previous study (Burns *et al.*, 2005) may be limited to dairy cattle, which have undergone long-term intensive genetic selection for milk production resulting in markedly different phenotypes and management protocols (DeJarnette *et al.*, 2004, Lucy, 2001) compared with beef breeds. In addition, the inverse association of FSH secretion with the variation in follicle numbers has only been examined during non-ovulatory waves of dairy cows (Burns *et al.*, 2005). Moreover, whether the variation in follicle numbers and FSH secretion patterns during follicular waves has a crucial role in regulation of oocyte quality, response to superovulation and subsequent embryo quality, and fertility is unknown. For example, it has not been determined whether the extensive variation in response to superovulation and resulting embryo quality (Lerner *et al.*, 1986; Looney, 1986; Mapletoft *et al.*, 2003) is associated with the very high variation in number of follicles during follicular waves in cattle (Burns *et al.*, 2005).

The present study, therefore, was designed to further validate the bovine dominant follicle model to study the physiological significance of the variation in numbers of follicles during follicular waves by using crossbred beef heifers to confirm previous findings on (i) the extent and repeatability of the variation in follicle numbers during follicular waves and (ii) the variation in follicle numbers during non-ovulatory and ovulatory waves being associated with alterations in secretion of FSH and estradiol. The present study had the novel aim of examining the relationship between the numbers of follicles and embryo quality both after superovulation and after *in vitro* fertilization of oocytes collected from unstimulated animals.

Materials and Methods

Animals

Beef heifers (19.8 $\pm$ 0.7 months old, mean $\pm$ sem, ranging between 14 and 33 months of age and 350–450 kg, $n = 90$) were housed on slatted floors and provided feed and water ad libitum for the duration of the experiment (10 months). Animal experimentation was performed in compliance with protocols approved by the Animal Research Ethics Subcommittee, University College Dublin, the Cruelty to Animal Act (Ireland, 1876) and the European Union Directive 86/609/EC.

Ultrasound scanning procedure to count antral follicles ≥3 mm in diameter in ovaries

The ovaries of each heifer were monitored with an Aloka SSD-900 linear array *trans*-rectal probe (7.5-MHz transducer, BCF Ireland Ltd) and follicles were counted as previously described (Burns *et al.*, 2005). In brief, to standardize the counting of follicles, each ovary was scanned from end to end to identify the positions of the corpus luteum and antral follicles. Images for different ovarian sections were captured on a computer monitor, and the locations of the corpus luteum and each antral follicle $\geq$ 3 mm in diameter were drawn on an ovarian map. Two separate perpendicular measurements of diameter were averaged for each follicle, and the diameter and total number of follicles $\geq$ 3 mm in diameter per pair of ovaries for each animal was recorded.

Experiment 1: variability and repeatability of peak numbers of follicles ≥3 mm in diameter during follicular waves in beef heifers

Heifers ($n = 90$) were treated with two injections of 25 mg prostaglandin $F_{2\alpha}$ i.m. ($PGF_{2\alpha}$; Estrumate®, Loughrea, Co. Galway, Ireland) spaced 11 days apart to induce luteolysis and synchronize occurrence of estrus. Each animal was subjected to daily ultrasound analysis by the same operator beginning at the time of the second $PGF_{2\alpha}$ injection and continuing until 6 days after estrus. At each ultrasound session, the total number of antral follicles $\geq$3 mm in diameter in the ovaries of each animal was determined. Based on the data from at least two follicular waves, animals were segregated into three groups on the basis of the average peak number of follicles during a wave: low ($\leq$15 follicles, $n = 14$ animals), intermediate ($>$15 and $<$25 follicles, $n = 65$ animals) and high ($\geq$25 follicles, $n = 11$). The categories were chosen based on previous work in dairy cattle (Burns *et al.*, 2005). Animals in the intermediate group were not studied further. To examine the repeatability of peak number of follicles, animals classified as low or high were scanned daily during at least three different follicular waves in the same or consecutive estrous cycles.

Experiment 2: association of patterns of secretion of FSH and estradiol with number of follicles ≥3 mm in diameter during follicular waves

To determine the association of follicle numbers with serum hormone concentrations during follicular waves, the estrous cycles of animals in the low ($n = 11$) and high ($n = 8$) groups (from Experiment 1) were synchronized with $PGF_{2\alpha}$ as explained above. Each animal was subjected to daily ultrasound analysis beginning the day after the second $PGF_{2\alpha}$ injection and continuing until 1 day after ovulation. Number of follicles during the first or ovulatory wave was determined during each scan as previously described.

Blood samples (10 ml) were taken by jugular venipuncture beginning 48 h after the second $PGF_{2\alpha}$ injection and continuing every 8 h until Day 7 of the cycle. Based on previous results (Sunderland *et al.*, 1994), this blood-sampling regimen should span the days of the cycle that coincide with the latest stages of development of a dominant ovulatory follicle and the initial stages of development of the first-wave dominant non-ovulatory follicle. In a subset of animals in the low ($n = 5$) and high ($n = 5$) groups, jugular blood samples (20 ml) were taken at 8-h intervals from Day 6 to 16 of the estrous cycle then at 4-h intervals until 1 day post-ovulation. Based on previous results (Sunderland *et al.*, 1994), this blood-sampling regimen should span the days of the cycle that coincide with emergence of the ovulatory wave and development and ovulation of the dominant ovulatory follicle.

Downloaded from https://academic.oup.com/humrep/article/22/6/1687/610956 by guest on 20 August 2022

Immunoassays

Concentrations of FSH in duplicate 100-μl serum samples for each animal were determined using a previously validated heterologous radioimmunoarray (Bame *et al.*, 1999). Results of the FSH radioimmunoarray correlate with those of an *in vitro* bioassay of FSH activity (Crowe *et al.*, 1997). All samples were analysed in a single FSH assay. The intra-assay coefficient of variation (CV) was 10.5%, and sensitivity of the assay was 0.03 ng/ml. Concentrations of LH in duplicate 300-μl serum samples obtained during the ovulatory wave for each animal were determined using a previously validated homologous radioimmunoarray (Matteri *et al.*, 1987). Intra- and inter-assay CVs ($n = 6$ assays) were <6%, and assay sensitivity was 0.02 ng/ml. Estradiol concentrations were determined in duplicate 500-μl serum samples previously extracted with ether, using a modified version of the commercial MAIA Kit (Biodata, S.P.A. Rome, Italy) (Prendiville *et al.*, 1995). Sensitivity of the assay was 0.04 ± 0.01 pg/ml. Inter- and intra-assay ($n = 8$ assays) CVs ranged between 10% and 22% for reference samples that averaged 5.1 and 0.88 pg/ml.

Experiment 3: relationship between peak number of antral follicles during follicular waves, superovulatory response and embryo quality

The estrous cycles of animals in the high ($n = 11$) and low ($n = 14$) follicle number groups (from Experiments 1 and 2 above) were synchronized by administration of $PGF_{2\alpha}$ as explained above. Detection of estrus was initiated 12 h after the second $PGF_{2\alpha}$, and the day estrus was detected was defined as Day 0. Animals not responding to the second $PGF_{2\alpha}$ were not superovulated. Beginning on Day 10, animals were superovulated with a total of 12 ml (420 i.u) of FSH (Folltropin®, Bioniche, Inverin, Co. Galway, Ireland) given as twice daily injections over 4 days on a decreasing dose schedule. An i.m. injection of $PGF_{2\alpha}$ was administered at the time of the fifth and sixth FSH injection to induce luteolysis. Heifers were inseminated with frozen-thawed semen 12 and 24 h after the onset of estrus. On Day 7 post-insemination, the number of ovulations was recorded by estimating the number of corpora lutea (CL) by palpation and ultrasound. Embryos were recovered by nonsurgical transcervical flushing with 500 ml phosphate-buffered saline (PBS) supplemented with 10% fetal calf serum (FCS) by an experienced commercial operator (Bovi Genetics, Straffen, Co. Kildare, Ireland). Recovered fluid was filtered through an Emcon® filter (Veterinary Concepts, Spring Valley, WI, USA) and searched under a stereomicroscope. The recovered oocytes/embryos were assessed for developmental stage based on their morphology using the guidelines of the International Embryo Transfer Society (Anonymous, 1998). This procedure was repeated once at an interval of 6 weeks and the combined results were analysed statistically (some animals did not respond to estrus synchronization and were not superovulated a second time).

Experiment 4: in vitro development of oocytes recovered from ovaries of beef heifers with low versus high numbers of follicles

Pairs of ovaries were collected at a commercial abattoir from non-pregnant animals under 30 months of age and at unknown stages of the estrous cycle. Numbers of antral follicles ≥ 3 mm were counted per pair of ovaries, and those with low (≤15 follicles, $n = 68$ pairs of ovaries) and high (≥25 follicles, $n = 37$) numbers of follicles were retained. Oocytes were recovered by manual aspiration from all visible 3 to 10 mm follicles (Rizos *et al.*, 2002). Cumulus–oocyte complexes (COCs) were classified into four morphological categories: (i) compact multilayered cumulus investment, (ii) compact with two to three layers of cumulus investment, (iii) denuded and (iv) expanded cumulus investment (de Loos *et al.*, 1989). All category 1 and 2 COCs obtained each day were pooled. COCs were matured and fertilized with spermatozoa from a single bull in groups of ~50 in 500 μl maturation medium and subsequently cultured for 7 days in groups of 25 in 25 μl droplets of medium (containing serum) under oil as previously described (Rizos *et al.*, 2002). Five replicates (1 replicate = 1 day of collection of COCs) were conducted per treatment. The proportion of zygotes cleaved by 48 h post-insemination and the proportion developing to the blastocyst stage on days 6–8 (Day 0 = day of IVF) were recorded.

Statistical analysis

Repeatability (range = 0–1, 1 = perfect) is defined as the proportion of the total variance that could be attributed to animal variance, which is calculated as follows:

ϕ^2 animal/(ϕ^2 animal + ϕ^2 error) (Boni *et al.*, 1997). Variance components were estimated using the PROC MIXED model approach of SAS (SAS, 1995). A mixed model repeated measures approach was used to determine if serum FSH and estradiol concentrations were different ($P < 0.05$) for heifers with low versus high numbers of follicles during waves (SAS, 1995). Main effects included: groups of heifers with low or high follicle numbers during waves, time and an interaction of the groups with time. Serum hormone concentrations and numbers of follicles were considered dependent variables. Time of blood sampling was treated as a repeated measure across individual heifers. Differences in serum LH concentrations between the two groups of animals were not examined because of the episodic nature of LH secretion and infrequent blood sampling regimen in the present study. However, the LH values were used to align data relative to peak of the pre-ovulatory LH surge for the two groups of animals for statistical analysis. Data were log transformed before statistical analysis. The Bonferroni *t*- test was used to determine whether statistical differences existed among individual means (SAS, 1995). Proportions of oocytes recovered in different morphological categories, and developmental parameters for the *in vitro* study were analysed using the χ^2-test (SAS, 1995).

Results

Although the peak number of follicles per wave ranged from 9 to 45 among animals, the repeatability of peak (0.84) and average (0.89) number of follicles per wave within individual heifers during different follicular waves in different estrous cycles was very high. Numbers of follicles during the first non-ovulatory and ovulatory wave were ~2-fold higher ($P < 0.01$), whereas sizes of the dominant and largest subordinate follicle were similar ($P > 0.10$), in the high compared with the low follicle number group of heifers (Fig. 1 and 2).

Serum FSH concentrations were higher ($P < 0.05$) −1, −0.3 and 0 days before the post-ovulatory peak in FSH concentrations, and basal FSH concentrations tended ($P < 0.10$) to be higher from 3 days after the peak in FSH concentrations, in the low compared with the high follicle number group of animals (Fig. 1). In contrast, there was no difference in serum estradiol concentrations between the two groups (Fig. 1).

During the ovulatory wave, heifers were categorized as having had either two ($n = 5$) or three ($n = 5$) follicle waves during the cycle based in retrospective daily ultrasonographic observations of the ovaries. In heifers with two follicle waves, serum FSH and estradiol concentrations were not different ($P > 0.05$) between heifers with low ($n = 2$) or high ($n = 3$) numbers of follicles. However, in heifers with three follicle

Downloaded from https://academic.oup.com/humrep/article/22/6/1687/610956 by guest on 20 August 2022

waves serum FSH concentrations were higher ($P < 0.05$) at numerous time points before and after emergence and tended ($P = 0.13$) to be higher before and after the pre-ovulatory LH surge in the low ($n = 3$) compared the high ($n = 2$) follicle number group of cattle. In contrast, serum estradiol was not different between groups, although a statistical interaction ($P < 0.05$) was detected. Specifically, estradiol concentrations were higher initially (-2.67 and -1.67 days) before the pre-ovulatory LH surge in the low group, but lower thereafter, in the low compared with the high follicle number group (Fig. 2).

After ovarian stimulation, the number of CL, the number of unfertilized oocytes or early embryos recovered on Day 7, and the number of transferable embryos per animal and proportion of fertilized non-transferable embryos (arrested in early stages of cleavage) were significantly higher ($P < 0.05$) for animals in the high versus the low follicle number group, but recovery rate of embryos (number of oocytes/embryos recovered per corpus luteum) was similar for the two groups (55.9% versus 60.0%, Table 1). However, the proportion of transferable embryos (79.8% versus 50.7%) was greater in the animals in the low versus the high follicle number group ($P < 0.05$, Table 1).

In the *in vitro* study of unstimulated cycles, the number of oocytes recovered in each category, total number of oocytes recovered or subjected to IVM, cleavage rates, number of embryos at blastocyst stage on Days 6–8 and number of hatched blastocysts per animal were 3- to 4-fold greater ($P < 0.05$) for animals with high compared with low numbers of follicles (Table 2). However, the proportions of

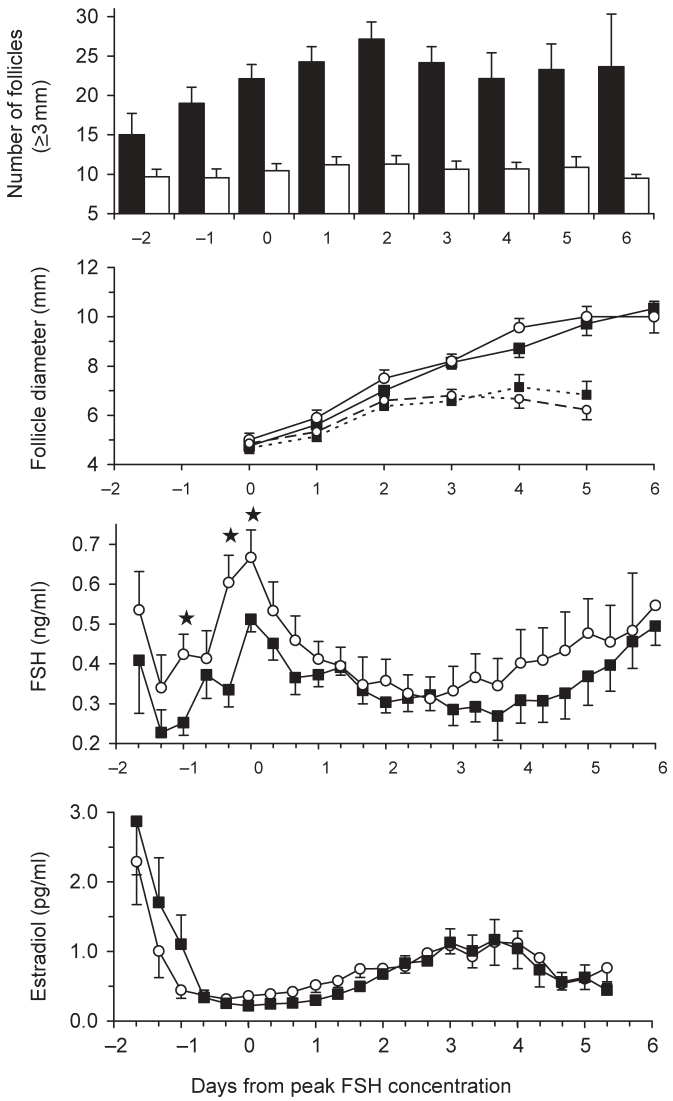

Figure 1: Peak number of follicles, diameter of dominant (solid line) and largest subordinate (dotted line) follicle, and serum concentrations of FSH and estradiol for beef heifers that had low (≤15 follicles, $n = 11$ animals) versus high peak numbers of follicles (≥25 follicles, $n = 8$) ≥ 3 mm in diameter during the first non-ovulatory follicular wave-Symbols represent the average ± SEM for heifers with low (open symbols) or high (solid symbols) numbers of follicles during waves. Data were aligned relative to the peak of the first post-ovulatory increase in serum FSH concentrations

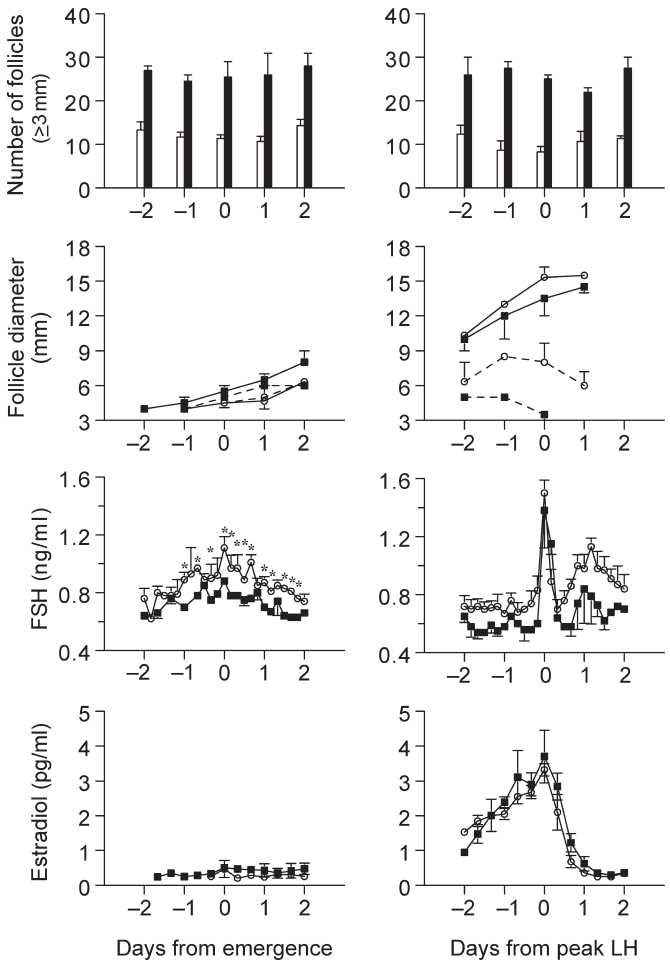

Figure 2: Peak number of follicles, diameter of dominant (solid line) and largest subordinate (dotted line) follicle, and serum concentrations of FSH and estradiol for beef heifers with three follicle waves per cycleAnimals with low (≤15 follicles, $n = 3$ animals) versus high peak numbers of follicles (≥25 follicles, $n = 2$) ≥ 3 mm in diameter during ovulatory follicular waves were compared. Symbols represent the average ± SEM for heifers with low (open symbols) or high (solid symbols) number of follicles during waves. For some data points, the SEM is smaller than the symbol thus not visible. Data were aligned relative to day of emergence (first day of ultrasound scanning when a follicle ≥4 mm in diameter was detected in the ovulatory wave) or peak of the pre-ovulatory LH surge

Downloaded from https://academic.oup.com/humrep/article/22/6/1687/610956 by guest on 20 August 2022

oocytes classified into each quality grade and the cleavage rate, blastocyst yield and numbers developing into hatched blastocysts were not different when expressed as a percentage of the oocytes cultured.

Discussion

The most significant findings of the present study indicate that: (i) ultrasound analysis can be used to reliably identify beef cattle with relatively high or low number of follicles during follicular waves (ii) serum FSH but not estradiol concentrations are inversely associated with the variation in follicle numbers during non-ovulatory and ovulatory follicular waves in beef heifers, (iii) part of the variability of responsiveness of cattle to superovulation is attributable to the variation in numbers of follicles during waves, (iv) cattle with relatively high numbers of follicles during follicular waves respond best to superovulation and (v) the lower proportion of transferable embryos following superovulation of cattle with high numbers of follicles during waves is probably not the result of differences in quality of oocytes before superovulation.

The number of primordial follicles, which comprise >98% of the ovarian reserve (total numbers of follicles per pair of ovaries), is highly variable at birth ranging from ~350 000 to 1 100 000 in human beings (Block, 1953; Forabosco *et al.*, 1991; Gougeon *et al.*, 1994) and from ~16 000 to 240 000 in cattle (Erickson, 1966a,b). During aging, the ovarian reserve is depleted and never replenished, thus numbers of primordial, growing preantral and antral follicles contained in the ovarian reserve vary enormously throughout reproductive cycles of individual adult humans (Block, 1951, 1952) and cattle (Erickson, 1966a). Whether this inherently large variation in size of the ovarian reserve contributes to the decline in oocyte quality and fertility in women (Block, 1951, 1952, 1953b; Te Velde *et al.*, 1998; Faddy, 2000; Te Velde and Pearson, 2002; Ottolenghi *et al.*, 2004;) or cattle (Erickson, 1966a,b; Erickson *et al.*, 1976; Royal *et al.*, 2000; Lucy, 2001; Sartori *et al.*, 2002a,b; Washburn *et al.*, 2002) is unknown. The results clearly demonstrated that peak number of follicles (≥3 mm in diameter) during follicular waves varied 5-fold among animals, but was very highly repeatable (0.84) within individuals. Others report moderate to high repeatability or correlations in number of antral follicles at emergence of two consecutive follicular waves (Singh *et al.*, 2004) or following ultrasound-guided needle aspiration of antral follicles at various intervals in individual cattle (Boni *et al.*, 1997), and from one menstrual cycle to the next in human beings (Scheffer *et al.*, 1999). In addition, repeatability of follicle numbers during waves remains very high in individual cattle for at least 1 year (Burns *et al.*, 2005), and others report numbers of growing preantral and antral follicles remain relatively constant until cattle are 7–9 years old (Erickson, 1966a; Katska and Smorag, 1984) and human beings are 35–40 years old (Gougeon *et al.*, 1994; Scheffer *et al.*, 1999; Tufan *et al.*, 2004). Taken together, these observations imply that numbers of antral follicles during waves may remain relatively constant during extended periods of ovarian aging in individuals, despite significant loss of primordial follicles in the ovarian reserve. The chronic maintenance of high repeatability of antral follicle growth during waves may possibly be explained by the increased rate of initiation of growth of primordial follicles [recruitment, (Hodgen, 1982)] as the ovarian reserve is depleted, as demonstrated in classical histological studies in several species (Krarup *et al.*, 1969; Peters *et al.*, 1978; Driancourt, 1987; Hirshfield, 1994; Shirota *et al.*, 2003). One of the major findings of the present study, coupled with our previous results (Burns *et al.*, 2005), is that beef and dairy cattle can be used as a novel experimental model to determine the causes and physiological significance not only of the high variation in follicle numbers during follicular waves, but also the compensatory mechanism that maintains the chronic high repeatability of antral follicle growth during follicular waves in single-ovulating species like cattle or human beings.

It is well established that FSH is required for folliculogenesis, especially growth of antral follicles (Richards and Midgley, 1976; Kumar *et al.*, 1999) during follicular waves in cattle (Turzillo and Fortune, 1990). The well accepted positive role of FSH on folliculogenesis implies that the variation in number of growing follicles during follicular waves would be expected to be positively associated, at least during a portion of a wave, with serum FSH concentrations. One study has reported that cows with more dominant follicles per follicle wave had higher FSH concentrations than cows with fewer dominant follicles (Lopez *et al.*, 2005), although the total

Downloaded from https://academic.oup.com/humrep/article/22/6/1687/610956 by guest on 20 August 2022

Table 1: Recovery rate and morphological classification of embryos recovered after after ovarian stimulation of heifers with consistently low (≤15) or high (≥25) peak numbers of follicles ≥3 mm in diameter during follicular waves

	Low Follicle Numbers		High Follicle Numbers	
	Total	Per animal	Total	Per animal
Number of animals	12		11	
Number of flushes	21		19	
Estimated number of CL	177	8.5 ± 1.1[a]	335	17.6 ± 3.6[b]
Embryos/oocytes recovered	99	4.7 ± 0.7[a]	201	10.6 ± 2.7[b]
Transferable embryos	79	3.8 ± 0.8[a]	102	5.4 ± 1.3[b]
Proportion transferable (%)	79/99(79.8)[a]		102/201(50.7)[b]	
Fertilized non-transferable (%)	9/99(9.1)[a]	0.4 ± 0.2	54/201(26.9)[b]	2.8 ± 1.4
Non-fertilized (%)	11/99(11.1)	0.5 ± 0.2	35/201(17.4)	1.8 ± 0.8

[a,b]Values in the same row with different superscripts differ significantly ($P < 0.05$). Transferable embryos are defined as those graded as Class 1 or 2 as explained in Methods. Two heifers in the low group were not superovulated because they did not respond to the second $PGF_{2\alpha}$ injection. The vast majority of non-transferable embryos were arrested at early cleavage stages.

Table 2: Classification of oocytes isolated from ovaries with low (≤15 follicles per pair of ovaries per animal, $n = 68$ pairs of ovaries) or high (≥25 follicles per pair of ovaries per animal, $n = 37$ pairs of ovaries) follicle numbers collected at an abattoir, and subsequent embryo development following IVF

Oocyte isolation and IVF results	Low Follicle Numbers		High Follicle Numbers	
	Total	Per animal	Total	Per animal
Total oocytes recovered	511	7.5[a]	1093	29.5[b]
Category 1	190 (37.2)	2.8[a]	409 (37.4)	11.1[b]
Category 2	135 (26.4)	2.0[a]	219 (20.1)	5.9[b]
Category 3	85 (16.6)	1.3[a]	139 (12.7)	3.8[b]
Category 4	101 (19.8)	1.5[a]	326 (29.8)	8.8[b]
Oocytes subjected to IVM	308 (60.3)	4.5[a]	586 (53.6)	15.8[b]
Oocytes cleaved after IVF	228 (74.0)	3.4[a]	438 (74.7)	11.8[b]
Blastocyst development				
Day 6	48 (15.6)	0.7[a]	77 (13.1)	2.1[b]
Day 7	91 (29.6)	1.3[a]	181 (30.9)	4.9[b]
Day 8	100 (32.5)	1.5[a]	196 (33.5)	5.3[b]
Hatched blastocysts	43 (14.0)	0.6[a]	87 (14.8)	2.4[b]

[a,b]Values in the same row with different superscripts differ significantly ($P < 0.05$) and numbers in parenthesis represent percentages. The percentages for cleavage rate, blastocyst development and hatched blastocysts are calculated based on total oocytes cultured. Only Category 1 and 2, oocytes were subjected to oocyte culture and IVF. Some oocytes are lost and degenerate oocytes are discarded when they are denuded after IVF, whereas the remaining oocytes are transferred to the *in vitro* culture medium.

numbers of follicles were not recorded. However, the second major finding of the present study was that serum FSH concentrations are inversely associated with number of follicles during ovulatory and non-ovulatory follicular waves in 18-month-old crossbred beef heifers. This observation supports our recent results in 2–5-year old late lactation dairy cows (Burns *et al.*, 2005). An inverse relationship between follicle numbers and FSH concentrations for 18–36-month-old dairy heifers (Haughian *et al.*, 2004) and lactating 2–8-year-old beef cows (Singh *et al.*, 2004) has been reported previously, but animals were at unknown stages of follicular waves. These findings could lead to the suggestion that rather than FSH concentrations dictating numbers of antral follicles in the ovaries, the negative feedback of hormones from the ovaries regulates FSH concentrations. Unfortunately, the present estradiol data and our previous estradiol and inhibin-A data (Burns *et al.*, 2005) do not support this notion. However, we can not rule out the possibility that pituitary sensitivity to negative feedback is more sensitive than our ability to detect differences in hormone secretion. Nonetheless, we can conclude that the inverse relationship between FSH concentrations and follicle numbers is not dependent on breed, age, management or physiological stage of development of cattle.

Numerous studies report that increased serum FSH concentrations are associated with depletion of the ovarian reserve, diminished responsiveness to gonadotropin stimulation, reduced success of IVF or decreased fertility during aging of women (Block, 1953; Richardson *et al.*, 1987; Forabosco *et al.*, 1991; Gougeon *et al.*, 1994; Lass *et al.*, 1997; Tomas *et al.*, 1997; Chang *et al.*, 1998; Prior, 1998; Hsieh *et al.*, 2001; Huang *et al.*, 2001; Beckers *et al.*, 2002; Kupesic *et al.*, 2003; Scheffer *et al.*, 2003) and cattle (Erickson, 1966a; Maurer and Echternkamp, 1985; Bryner *et al.*, 1990; Kawamata, 1994; Cushman *et al.*, 1999; Taneja *et al.*, 2000; Singh *et al.*, 2004; Wolfenson *et al.*, 2004; Malhi *et al.*, 2005). Moreover, relatively high FSH concentrations in the presence of insulin decrease capacity of oocytes to develop into blastocysts following IVF in rodents (Eppig *et al.*, 1998). However, it is unknown whether the relatively high serum FSH concentrations in young sexually mature cattle with low numbers of follicles during follicular waves are detrimental to oocyte quality and reflective of a diminished ovarian reserve, reduced responsiveness to superovulation or low fertility, as reported for older compared with younger cattle (Erickson, 1966a; Maurer and Echternkamp, 1985; Bryner *et al.*, 1990; Kawamata, 1994; Cushman *et al.*, 1999; Taneja *et al.*, 2000; Singh *et al.*, 2004; Wolfenson *et al.*, 2004; Malhi *et al.*, 2005) or women (Block, 1953; Richardson *et al.*, 1987; Forabosco *et al.*, 1991; Gougeon *et al.*, 1994; Lass *et al.*, 1997; Tomas *et al.*, 1997; Chang *et al.*, 1998; Prior, 1998; Hsieh *et al.*, 2001; Huang *et al.*, 2001; Beckers *et al.*, 2002; Kupesic *et al.*, 2003; Scheffer *et al.*, 2003) and, although controversial (Abdalla & Thum, 2004), for younger women with relatively high serum FSH concentrations (Chuang *et al.*, 2003, El-Toukhy *et al.*, 2002).

Extreme variation in superovulatory response remains one of the biggest problems in the embryo transfer industry in cattle. In general, a small proportion (30%) of cows yield most (70%) embryos and one-quarter of treated cows produce no embryos (Lerner *et al.*, 1986; Looney, 1986). The results of the present study demonstrate convincingly that cattle with consistently high follicle numbers during waves respond best to superovulation and produce more high-quality embryos for transfer into recipients, and that ovaries obtained from cattle with relatively high number of antral follicles produce many more high-quality oocytes capable of developing into blastocysts compared with animals with relatively low follicle numbers. These findings not only extend previous reports that high numbers of antral follicles at unknown stages of follicular waves are positively associated with an increased responsiveness to gonadotropin treatments during superovulation (Cushman *et al.*, 1999, Kawamata, 1994, Singh *et al.*, 2004), but also support the conclusion of Singh *et al.* (2004) that a simple ultrasound scan to count number of follicles ≥2 mm

Downloaded from https://academic.oup.com/humrep/article/22/6/1687/610956 by guest on 20 August 2022

at wave emergence can be used to predict the superovulatory response in cattle. Given the high repeatability of follicle numbers during follicular waves observed in individual cattle, our results suggest that use of ultrasound to phenotype cattle based on follicle numbers during waves may be a useful predictor of the likelihood of whether or not an animal will be a productive embryo donor.

The total number of high-quality embryos in the present study was much greater following superovulation of cattle with relatively high follicle numbers during waves, but the proportion of high-quality embryos relative to the total number of fertilized oocytes and embryos recovered was greater in animals with relatively low follicle numbers. This observation supports previous reports that the proportion of high-quality embryos is inversely associated with responsiveness of cattle to superovulation (Shaw *et al.*, 1995), and the percentage of embryos transferred declines as number of oocytes harvested increases following IVF and embryo transfer in women (Meniru and Craft, 1997). Since the recovery of embryos and oocytes after artificial insemination was similar for animals with low or high follicle numbers during waves in the present study, the inverse association between response to superovulation and proportion of high-quality embryos could imply that the proportion of follicles with high-quality oocytes during waves may be greater for cattle with low follicle numbers and relatively high circulating FSH concentrations during waves. Nevertheless, the *in vitro* component of the present study (using ovaries obtained at an abattoir) demonstrated that recovery rates and proportion of high-quality oocytes or fertilized oocytes developing into blastocysts were similar for animals with high or low number of antral follicles on the ovarian surface. This finding implies that proportion of follicles with high-quality oocytes is probably similar before superovulation of cattle, regardless of the variation in follicle numbers or serum FSH concentrations during follicular waves. If oocyte quality is indeed similar between animals with different follicle numbers during waves, as implied by *in vitro* results of the present study, then the higher proportions of transferable embryos observed in cattle with low follicle numbers during waves could have resulted from positive or negative effects of superovulation on oocyte quality in cattle with low or high follicle numbers during waves, respectively. Because superovulation diminishes developmental competence of bovine oocytes (Lonergan *et al.*, 1994; Blondin *et al.*, 1996), the results of the present study indicate that the high gonadotropin dose regimen used for superovulation may be more detrimental to oocyte quality in cattle with high versus low follicle numbers during waves. Nevertheless, the reason superovulation treatments have potentially greater negative effects on oocyte quality in cattle with relatively high follicle numbers and low serum FSH concentrations during follicular waves is unknown. From a practical viewpoint, the use of ultrasound to identify and group cattle based on follicle numbers during follicular waves followed by optimization of responsiveness of each group to gonadotropin treatments should improve yield of transferable embryos following superovulation.

Based on the results of the present study, we concluded that: (i) ultrasound analysis can be used to identify reliably (i.e. phenotype) cattle with consistently high follicle numbers and low circulating FSH concentration or low follicle numbers and high circulating FSH concentrations during the follicular waves of estrous cycles, (ii) phenotypic classification of cattle based on the variation in follicle numbers during waves may be useful for improving superovulation procedures and (iii) the lower proportion of transferable embryos following superovulation of cattle with high numbers of follicles during waves is probably not the result of differences in quality of oocytes before superovulation. We suggest that these conclusions may also have significant implications for assisted human reproduction.

Acknowledgements

The authors thank Andrew Grant, Managing Director, Bioniche Animal Health Europe for the generous gift of Folltropin. This work was supported by grants from the Higher Education Authority (HEA-PRTLI Cycle 5) to FW; National Research Initiative Competitive Grants 2000-02391, 2001-002255 and 2004-35203-14781 from the USDA Cooperative State Research, Education and Extension Service and by Funds from the Agriculture Experiment Station to J.J.I. and Science Foundation Ireland under Grant 02/IN1/B78 to A.C.O.E. and P.L. (The opinions, findings and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the Science Foundation Ireland.)

References

Abdalla H, Thum MY. An elevated basal FSH reflects a quantitative rather than qualitative decline of the ovarian reserve. *Hum Reprod* 2004;**19**:893–898.

Anonymous; In: Stringfellow DA, Seidel SM eds *Manual of the International Embryo Transfer Society*. International Embryo Transfer Society, 1998.

Baerwald AR, Adams GP, Pierson RA. Characterization of ovarian follicular waves in women. *Biol Reprod* 2003a;**69**:1023–1031.

Baerwald AR, Adams GP, Pierson RA. A new model for ovarian follicular development during the human menstrual cycle. *Fertil Steril* 2003b;**80**:116–122.

Bame J, Dalton JC, Degelos SD *et al.* Effect of long-term immunization against inhibin on sperm output in bulls. *Biol Reprod* 1999;**60**:1360–1366.

Beckers NGM, Macklon NS, Eijkemans MJC *et al.* Women with regular menstrual cycles and a poor response to ovarian hyperstimulaitn for in vitro fertilzation exhibit follicular phase characteristics suggestive of ovarian aging. *Fertil Steril* 2002;**78**:291–297.

Block E. Quantitative morphological investigations of the follicular system in women. *Acta Endocrinol* 1951;**8**:33–54.

Block E. Quantitative morphological investigation of the follicular systen in women. *Acta Anat* 1952;**14**:108–123.

Block E. A quantitative morphological investigation of the follicular system in newborn female infants. *Acta Anat* 1953;**17**:201–206.

Blondin P, Coenen K, Guilbault LA *et al.* Superovulation can reduce the developmental competence of bovine embryos. *Theriogenology* 1996;**46**:1191–1203.

Boni R, Reoelofsen MWM, Pieterse MC *et al.* Follicular dynamics, repeatability and predictability of follicular recruitment in cows undergoing repeated follicular puncture. *Theriogenology* 1997;**48**:277–289.

Bryner RW, Garcia-Winder M, Lewis PE *et al.* Changes in hormonal profiles during the oestrous cycle in old lactating beef cows. *Dom Anim Endocrinol* 1990;**7**:181–190.

Burns DS, Jimenez-Krassel FJ, Ireland JLH *et al.* Numbers of antral follicles during follicular waves in cattle: evidence for high variation among animals, very high repeatability in individuals, and an inverse association with serum follicle-stimulating hormone concentrations. *Biol Reprod* 2005;**73**:54–62.

Chang MY, Chiang CH, Hsieh TT *et al.* Use of the antral follicle count to predict the outcome of assisted reproductive technologies. *Fertil Steril* 1998;**69**:505–510.

Downloaded from https://academic.oup.com/humrep/article/22/6/1687/610956 by guest on 20 August 2022

Chuang C-C, Chen C-D, Chao K-H *et al.* Age is a better predictor of pregnancy potential than basal follicle-stimulating hormone levels in women undergoing in vitro fertilization. *Fertil Steril* 2003;**79**:63–68.

Crowe MA, Padmanabhan V, Hynes N *et al.* Validation of a sensitive radioimmunoassay to measure serum follicle-stimulating hormone in cattle: correlation with biological activity. *Anim Reprod Sci* 1997;**48**: 123–136.

Cushman RA, DeSouza JC, Hedgpeth VS *et al.* Superovulatory response of one ovary is related to the micro- and macroscopic population of follicles in the contralateral ovary of the cow. *Biol Reprod* 1999;**60**:349–354.

de Loos FVC, van Maurik P, Kruip TA. Morphology of immature bovine oocytes. *Gamete Res* 1989;**24**:197–204.

DeJarnette JM, Marshall CE, Lenz RW *et al.* Sustaining the fertility of artificially inseminated dairy cattle: the role of the artificial insemination industry. *J Dairy Sci* 2004;**87**:E93–E104

Driancourt MA. Ovarian features contributing to the variability of PMSG-induced ovulation rate in sheep. *J Reprod Fert* 1984;**80**:207–212.

El-Toukhy T, Khalaf Y, Hart R *et al.* Young age does not protect against the adverse effects of reduced ovarian reserve–an eight year study. *Hum Reprod* 2002;**17**:1519–1524.

Eppig JJ, O'Brien MJ, Pendola FL *et al.* Factors affecting the developmental competence of mouse oocytes grown In vitro: follicle-stimulating hormone and insulin. *Biol Reprod* 1998;**59**:1445–1453.

Erickson B. Development and senescence of the postnatal bovine ovary. *J Anim Sci* 1966a;**25**:800–805.

Erickson BH. Development and radio-response of the prenatal bovine ovary. *J Reprod Fert* 1966b;**10**:97–105.

Erickson BH, Reynolds RA, Murphree RL. Ovarian characteristics and reproductive performance of the aged cows. *Biol Reprod* 1976;**15**: 555–560.

Evans ACO, Komar CM, Wandji S-A *et al.* Changes in androgen secretion and luteinizing hormone pulse amplitude are associated with the recruitment and growth of ovarian follicles during the luteal phase of the bovine oestrous cycle. *Biol Reprod* 1997;**57**:394–401.

Faddy MJ. Follicle dynamics during ovarian ageing. *Mol Cell Endocrinol* 2000;**163**:43–48.

Forabosco A, Sforza C, De Pol A *et al.* Morphometric study of the human neonatal ovary. *Anat Rec* 1991;**231**:201–208.

Ginther OJ, Wiltbank MC, Fricke PM *et al.* Selection of the dominant follicle in cattle. *Biol Reprod* 1996;**55**:1187–1194.

Gougeon A, Ecochard R, Thalabard JC. Age-related changes of the population of human ovarian follicles: increase in the disappearence rate of non-growing and early-growing follicles in aging women. *Biol Reprod* 1994;**50**:653–663

Haughian JM, Ginther OJ, Kot K *et al.* Relationship between FSH patterns and follicular dynamics and the temporal associations among hormones in natural and GnRH-induced gonadotropin surges in heifers. *Reproduction* 2004;**127**:23–33.

Hirshfield AN. Relationship between supply of primordial follicles and the onset of follicular growth in rats. *Biol Reprod* 1994;**50**:421–428.

Hodgen GD. The dominant ovarian follicle. *Fertil Steril* 1982;**38**:281–300.

Hsieh Y-Y, Chang C-C, Tsai H-D. Antral follicle counting in predicting the retrieved oocyte number after ovarian hyperstimulation. *J Assist Reprod Genet* 2001;**18**:320–324.

Huang F-J, Chang S-Y, Tsai M-Y *et al.* Determination of the efficiency of controlled ovarian hyperstimulation in the gonadotropin-releasing hormone agonist-suppression cycle using the initial follicle count during gonadotropin stimulation. *J Assist Reprod Genet* 2001;**18**:91–96.

Ireland JJ, Mihm M, Austin E *et al.* Historical perspective of turnover of dominant follicles during the bovine oestrous cycle: key concepts, studies, advancements, and terms. *J Dairy Sci* 2000;**83**:1648–1658.

Katska L, Smorag Z. Number and quality of oocytes in relation to age in cattle. *Anim Reprod Sci* 1984;**7**:451–460.

Kawamata M. Relationship between the number of small follicles prior to superovulatory treatment and superovulatory response in Holstein cows. *J Vet Med Sci* 1994;**56**:965–970.

Krarup T, Pederson T, Faber M. Regulation of oocyte growth in the mouse ovary. *Nature* 1969;**224**:187–188.

Kumar TR, Palapattu G, Wang P *et al.* Transgenic models to study gonadotropin function: the role of follicle-stimulating hormone in gonadal growth and tumorigenesis. *Mol Endocrinol* 1999;**13**:851–865.

Kupesic S, Kurjak A, Bjelos D *et al.* Three-dimensional ultrasonographic ovarian measurements and in vitro fertilization outcome are related to age. *Fertil Steril* 2003;**79**:190–197.

Lass A, Silye R, Abrams D-C *et al.* Follicular density in ovarian biopsy of infertile women: a novel method to assess ovarian reserve. *Hum Reprod* 1997; **12**:1028–1031.

Lerner SP, Thayne WV, Baker RD *et al.* Age, dose of FSH and other factors affecting superovulation in Holstein cows. *J Anim Sci* 1986;**63**:176–183.

Lopez H, Sartori R, Wiltbank MC. Reproductive hormones and follicular growth during development of one or multiple dominant follicles in cattle. *Biol Reprod* 2005;**72**:788–795.

Lonergan P, Monaghan P, Rizos D *et al.* Effect of follicle size on bovine oocyte quality and developmental competence following maturation, fertilization and culture in vitro. *Mol Reprod Dev* 1994;**37**:48–53.

Looney CR. Superovulation in beef heifers. Proc 5th Am Conv. AETA, . 16–29.

Lucy MC. Reproductive loss in high-producing dairy cattle: where will it end? *J Dairy Sci* 2001;**84**:1277–1293.

Malhi PS, Adams GP, Singh J. Bovine model for the study of reproductive aging in women: follicular, luteal, and endocrine characteristics. *Biol Reprod* 2005;**73**:45–53.

Mapletoft RJ, Steward KB, Adams GP. Recent advances in the superovulation in cattle. *Reprod Nutr Dev* 2003;**42**:601–611.

Matteri RL, Roser JF, Baldwin DM *et al.* Characterization of a monoclonal antibody which detects luteinizing hormone from diverse mammalian species. *Domest Anim Endocrinol* 1987;**4**:157–165.

Maurer RR, Echternkamp SE. Repeat-breeder females in beef cattle: influences and causes. *J Anim Sci* 1985;**61**:624–636.

Meniru GI, Craft IL. Utilization of retrieved oocytes as an index of the efficiency of superovulation strategies for in-vitro fertilization treatment. *Hum Reprod* 1997;**12**:2129–2132.

Oliveira JF, Neves JP, Moraes JC *et al.* Follicular development and steroid concentrations in cows with different levels of fertility raised under nutritional stress. *Anim Reprod Sci* 2002;**73**:1–10.

Ottolenghi C, Uda M, Hamatani T *et al.* Aging of oocyte, ovary, and human reproduction. *Ann NY Acad Sci* 2004;**1034**:117–131.

Peters H, Byskov AG, Grinsted J. Follicular growth in fetal and prepubertal ovaries of humans and other primates. *Clin Endocrinol Metab* 1978;**7**:469–485.

Prendiville DJ, Enright WJ, Crowe MA *et al.* Immunization of heifers against gonadotropin-releasing hormone: Antibody titers, ovarian function, body growth and carcass characteristics. *J Anim Sci* 1995;**73**:2382–2389.

Prior JC. Perimenopause: the complex endocrinology of the menopausal transition. *Endocrinol Rev* 1998;**19**:397–428.

Richards JS, Midgley ARJ. Protein hormone action: a key to understanding ovarian follicular and luteal cell development. *Biol Reprod* 1976; **14**:82–94.

Richardson SJ, Senikas V, Nelson JF. Follicular depletion during the menopausal transition: evidence for accelerated loss and ultimate exhaustion. *J Clin Endo Metab* 1987;**65**:1231–1237.

Rizos D, Ward F, Duffy P *et al.* Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. *Mol Reprod Dev* 2002;**61**:234–248.

Royal M, Mann GE, Flint APF. Strategies for reversing the trend towards subfertility in dairy cattle. *Vet J* 2000;**100**:53–60.

Sartori R, Rosa GJM, Wiltbank MC. Ovarian structures and circulating steroids in heifers and lactating cows in summer and lactating and dry cows in winter. *J Dairy Sci* 2002a;**85**:2813–2822.

Sartori R, Sartor-Bergfelt R, Mertens SA *et al.* Fertilization and early embryonic development in heifers and lactating cows in summer and lactating and dry cows in winter. *J Dairy Sci* 2002b;**85**:2803–2812.

SAS. SAS Institute Inc. In Cary NC (ed). *SAS User's Guide: Statistics*. SAS Institute Inc., 1995.

Scheffer GJ, Broekmans FJ, Dorland M *et al.* Antral follicle counts by transvaginal ultrasonography are related to age in women with proven natural fertility. *Fertil Steril* 1999;**72**:845–851.

Scheffer GJ, Broekmans FJM, Looman CWN *et al.* The number of antral follicles in normal women with proven fertility is the best reflection of reproductive age. *Hum Reprod* 2003;**18**:700–706.

Shaw DW, Farin PW, Washburn SP *et al.* Effect of retinol palmitate on ovulation rate and embryo quality in superovulated cattle. *Theriogenology* 1995;**44**:51–58.

Shirota M, Soda S, Katoh C *et al.* Effects of reduction of the number of primordial follicles on follicular development to achieve puberty in female rats. *Reproduction* 2003;**125**:85–94.

Singh J, Dominguez M, Jaiswal R *et al.* A simple ultrasound test to predict the superstimulatory response in cattle. *Theriogenology* 2004;**62**:227–243.

Downloaded from https://academic.oup.com/humrep/article/22/6/1687/610956 by guest on 20 August 2022

Sunderland SJ, Crowe MA, Boland MP *et al.* Selection, dominance and atresia of follicles during the estrous cycle of heifers. *J Reprod Fertil* 1994; **101**:547–555.

Taneja M, Bols PE, Van de Velde A *et al.* Developmental competence of juvenile calf oocytes in vitro and in vivo: influence of donor animal variation and repeated gonadotropin stimulation. *Biol Reprod* 2000;**62**:206–213.

te Velde ER, Pearson PL. The variability of female reproductive ageing. *Hum Reprod Update* 2002;**8**:141–154.

Te Velde ER, Scheffer GJ, Dorland M *et al.* Development and endocrine aspects of normal ovarian aging. *Mol Cell Endocrinol* 1998;**145**: 67–73.

Tomas C, Nuojua-Huttunen S, Martikainen H. Pretreatment transvaginal ultrasound examination predicts ovarian responsiveness to gonadotropins in in-vitro fertilization. *Hum Reprod* 1997;**12**:220–223.

Tufan E, Elter K, Durmusoglu F. Assessment of reproductive ageing patterns by hormonal and ultrasonographic ovarian reserve tests. *Hum Reprod* 2004;**19**:2484–2489.

Turzillo AM, Fortune JE. Suppression of the secondary FSH surge with bovine follicular fluid is associated with delayed ovarian follicular development in heifers. *J Reprod Fertil* 1990;**89**:643–653.

Washburn SP, Silvia WJ, Brown CH *et al.* Trends in reproductive performance in southeastern Holstein and Jersey DHI herds. *J Dairy Sci* 2002;**85**:244–251.

Wolfenson D, Inbar G, Roth Z *et al.* Follicular dynamics and concentrations of steroids and gonadotropins in lactating cows and nulliparous heifers. *Theriogenology* 2004;**62**:1042–1055.

Submitted on October 2, 2006; resubmitted on February 15, 2007; accepted on February 21, 2007

Downloaded from https://academic.oup.com/humrep/article/22/6/1687/610956 by guest on 20 August 2022