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Selection, dominance and atresia of follicles during the oestrous cycle of heifers

S. J. Sunderland[1], M. A. Crowe[1], M. P. Boland[2], J. F. Roche[1] and J. J. Ireland[3]

Faculties of [1]Veterinary Medicine and [2]Agriculture, University College Dublin, Ireland; and [3]Molecular Reproductive Endocrinology Laboratory, Department of Animal Science, Michigan State University, East Lansing, MI 48823, USA

This study examined the correlation between measurement of follicle growth by ultrasound, and measurement of intrafollicular ratios of oestradiol and progesterone concentrations and the serum concentrations of FSH during selection, dominance and atresia or ovulation of dominant follicles in heifers. Heifers were ovariectomized on days 0 (before LH surge), 1 (after LH surge, preovulation), 1 (postovulation), 3, 6 and 12 of the oestrous cycle. Blood samples were collected at 4–6 h intervals. After ovariectomy all follicles ≥ 5 mm were measured and follicular fluid was aspirated. Follicles were classified by size according to ultrasound (F1, largest; F2, second largest; F3, all remaining follicles ≥ 5 mm) and by the ratio of oestradiol:progesterone concentrations. During the follicular phase, a single dominant oestrogen-active follicle increased in diameter while serum concentrations of LH increased and FSH decreased ($P < 0.05$). On day 1 (after LH surge, preovulation), serum LH and FSH decreased to pre-surge concentrations ($P < 0.0001$), while follicle size and intrafollicular progesterone concentration increased and oestradiol concentration decreased ($P < 0.05$). A dominant nonovulatory follicle, classified as oestrogen-active on days 1, 3 and 6 and oestrogen-inactive on day 12, increased in size from day 1 to day 7 and lost dominance during days 10–12, coincident with the growth of multiple oestrogen-active follicles. The serum FSH concentration increased transiently ($P < 0.05$) before each new wave of dominant follicular growth. The overall correlation of ultrasound measurements of follicle diameter with measures of follicle size after ovariectomy was high. The ratio of oestradiol:progesterone concentrations, but not of size, reliably distinguished potential dominant from atretic follicles. The size of the follicle and the oestradiol concentration were not determinants of subsequent dominance during a selection phase. We conclude that: (1) ovarian follicles go through selection, dominance and atresia phases coincident with transient increases and decreases in FSH; and (2) ultrasound is an accurate measure of follicle growth, but that size alone is not a sufficient measure to ascribe dominance and both ultrasound and the intrafollicular ratio of oestradiol:progesterone concentrations are needed to monitor selection, dominance and atresia of follicles accurately.

Introduction

Antral follicles were originally considered to be in a continuous state of turnover without distinct patterns of growth and atresia during the oestrous cycle of heifers (Choudary *et al.*, 1968; Marion *et al.*, 1968; Dufour *et al.*, 1972). However, the classic histological study of Rajakoski (1960), coupled with the direct follicle-marking studies of Matton *et al.* (1981), indicated that at least two periods of turnover of antral follicles occur during the oestrous cycle of cattle. One follicle grows to ovulatory size (> 10 mm) and undergoes atresia during early dioestrus (days 6–12) and another follicle grows to ovulatory size from luteolysis (day 18) to oestrus (day 0) during the follicular phase and ovulates on day 1 of the cycle (Matton *et al.*, 1981). Ireland and Roche (1982, 1983a, b, 1987) demonstrated that the intrafollicular ratio of oestradiol:progesterone concentrations can be used to distinguish healthy growing from atretic bovine follicles (≥ 6 mm in diameter), and confirmed and extended the earlier studies on follicular turnover in heifers as follows: (i) a single large (> 10 mm) oestrogen-active follicle (ratio of oestradiol:progesterone concentrations > 1 in follicular fluid) is present during oestrus and early dioestrus of heifers; (ii) the number of LH receptors increases while the number of FSH receptors decreases during the growth of oestrogen-active follicles during oestrus and early dioestrus (Ireland and Roche, 1983a, b); (iii) the serum concentration of

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oestradiol increases in only one, rather than in both, utero–ovarian veins during oestrus, early dioestrus and mid-dioestrus (Ireland *et al.*, 1985); and (iv) oestrogen-active follicles should be classified as dominant because of their similarity to dominant follicles in primates (Goodman and Hodgen, 1983).

These results led to the hypothesis that heifers have three different periods of development of dominant follicles during an oestrous cycle (oestrus, early dioestrus and mid-dioestrus), and that each period of dominant follicle growth has three distinct phases: selection, dominance and atresia or ovulation (Ireland, 1987; Ireland and Roche, 1987). Selection is a hypothetical physiological process whereby 'excess' follicles are reduced to the ovulatory quota, whereas dominance is a process that enables the 'selected' follicle to suppress further growth of other follicles, escape initial atresia and continue to grow until ovulation or atresia (Goodman and Hodgen, 1983). In support of this model of a dominant follicle in heifers, workers in several laboratories have used ultrasound scanning to monitor daily individual follicle growth and confirm that heifers indeed have three (sometimes two and rarely one) different periods of turnover of dominant follicles (Savio *et al.*, 1988; Sirois and Fortune, 1988; Knopf *et al.*, 1989). Nevertheless, the precise correlation between ultrasound analysis of dominant follicle growth, which is now routinely used to monitor growth of antral follicles in both beef and dairy cows (Murphy *et al.*, 1990; Savio *et al.*, 1990; Crowe *et al.*, 1993), and changes in intrafollicular concentrations of oestradiol and progesterone, which is used to distinguish dominant from atretic follicles (Ireland and Roche, 1982, 1983a, b, 1987), has not been examined. The objectives of this experiment were therefore to: (i) examine the interrelationship of ultrasound measurements of follicle growth, intrafollicular ratios of oestradiol and progesterone concentrations and the serum concentrations of FSH during selection, dominance and atresia or ovulation of dominant follicles in heifers; (ii) evaluate the accuracy of ultrasound measurements of follicular size with postovariectomy measurements of follicle diameter and function; and (iii) use the results of this study to re-evaluate our original model for dominant follicle growth in heifers (Ireland, 1987; Ireland and Roche, 1987).

Materials and Methods

Animals, maintenance and synchronization of oestrus

Thirty-three cyclic Simmental crossbred, Hereford crossbred and Charolais crossbred beef heifers, 15–18 months of age and weighing 334–418 kg at the start of the experiment, were housed on slatted flooring, and had free access to grass silage and water and a daily supplement of 2 kg of a 16% crude protein concentrate. To synchronize oestrus, each heifer received a s.c. progestagen ear implant for 10 days (norgestomet: Crestar, Intervet Ireland Ltd, Finglas, Dublin). Two days before implant removal, a single injection (i.m.) of $PGF_{2\alpha}$ analogue ($PGF_{2\alpha}$: Prosolvin: Intervet Ireland Ltd) was administered to initiate luteolysis. After implant removal, heifers were observed for oestrous behaviour for 30 min at 06:00 h, 12:00 h, 16:00 h, 20:00 h and 00:00 h every day until oestrus was detected.

Treatments

Both ovaries were removed from six different groups of heifers (treatments I–VI) during the following phases or days of the oestrous cycle (day 0, the day of oestrus): (I) follicular phase ($n = 5$ heifers) 36 h after an injection of $PGF_{2\alpha}$, but before the preovulatory LH surge; (II) day 1 ($n = 6$ heifers) after the preovulatory LH surge and oestrus, but before ovulation ($-$ ov); (III) day 1 ($n = 5$ heifers) after ovulation ($+$ ov); (IV) day 3 ($n = 6$ heifers); (V) day 6 ($n = 5$ heifers) and (VI) day 12 ($n = 6$ heifers). The times for ovariectomy were chosen because they coincided with periods of the oestrous cycle when a dominant ovulatory follicle develops and ovulates (after $PGF_{2\alpha}$ injection until day 1) or when a dominant nonovulatory follicle develops, loses 'dominance' and begins to undergo atresia (days 1–12) (Ireland and Roche, 1982, 1987; Savio *et al.*, 1988). Loss of dominance was functionally defined as the day of the oestrous cycle when a new cohort of follicles ≥ 5 mm could first be identified by ultrasound scanning while the previous dominant follicle was present. In our study, loss of dominance for the early dioestrous dominant follicle occurred 11.8 ± 0.75 days (mean $\pm$ SEM, $n = 6$ heifers) after oestrus, referred to above as day 12 of the oestrous cycle (treatment VI).

Animals were not fasted before surgery and each heifer was prepared for surgery, anaesthetized and ovaries removed with an ecraseur, as described by Drost *et al.* (1992). All animal experimentation was performed in compliance with regulations set down by the BioMedical Centre, University College Dublin, and the Cruelty to Animals Act (Ireland), 1897. After ovariectomy, ovaries were placed in a 50 ml falcon tube containing ice-cold PBS, pH 7.4. Within 30 min of ovariectomy, all follicles ≥ 5 mm were counted and the diameter of each follicle was determined using a calliper. Follicular fluid was aspirated from each follicle, its volume recorded and the follicular fluid from each follicle stored separately and frozen at -20°C until hormone assays were performed. Concentrations of oestradiol and progesterone were determined for each follicular sample by radioimmunoassay and the ratio of oestradiol:progesterone in follicular fluid was used to classify follicles as oestrogen-active (oestradiol > progesterone) or oestrogen-inactive (oestradiol < progesterone).

Ultrasound scanning of follicles

From the time of prostaglandin injection until approximately 14 h before ovariectomy, the ovaries of each heifer were scanned daily with a transrectal 7.5 MHz linear transducer (Dynamic Imaging Ltd, Livingston), and the number, size and location of each follicle ≥ 5 mm was recorded daily, as described by Savio *et al.* (1988). Follicles were placed into three classes based on ultrasound analysis: F1, the largest or the dominant follicle (as defined by Savio *et al.*, 1988); F2, the second largest follicle; and F3, all other follicles ≥ 5 mm.

Collection of blood samples

The stage of the oestrous cycle was confirmed by collecting samples of blood (10 ml) daily to measure both oestradiol and

progesterone concentrations via jugular venepuncture from the time of $PGF_{2\alpha}$ injection (2 days before implant removal) until ovariectomy. In addition, samples were collected from each heifer every 4 or 6 h from 36 h after $PGF_{2\alpha}$ administration until the time of ovariectomy to establish when the preovulatory LH surge occurred and to determine changes in circulating concentrations of FSH. Each blood sample was maintained at room temperature for 60 min, at 4°C overnight, centrifuged at 700 *g* for 20 min and the serum was stored at −20°C until assays were performed.

Hormone assays

Previously validated radioimmunoassays were used to quantify oestradiol (Moran *et al.*, 1991), progesterone (Ronayne and Hynes, 1990) and LH (Niswender *et al.*, 1969) concentrations. The sensitivities of the progesterone, oestradiol and LH assays were 0.2 ng ml^{-1}, 1.5 pg ml^{-1} and 0.2 ng ml^{-1}, respectively. Serum FSH concentrations were quantified using a heterologous assay as described by Glencross *et al.* (1992) using the NIDDK-anti-oFSH antibody and bovine FSH standard preparation (NIH B1 bFSH). The sensitivity of the assay was 1.6 ng FSH ml^{-1}. Interassay coefficients of variation (CV) for the oestradiol assays averaged 11.3 and 15.4% for serum samples containing 0.5 and 20.2 pg oestradiol ml^{-1}, respectively. Intra-assay CV for the same serum pools were 10.9 and 8.8%, respectively. Interassay CV for the progesterone assays averaged 9.3 and 6.5% for serum samples containing 0.9 and 3.0 ng progesterone ml^{-1}, respectively. Intra-assay CV for the same serum pools were 6.5 and 5.7%, respectively. Interassay CV for the LH assays averaged 15.7 and 12.6% for serum samples containing 3.9 and 26 ng LH ml^{-1}, respectively. Intra-assay CV for the same serum pools were 12.9 and 10.8%, respectively. Interassay CV for the FSH assays for three serum pools containing 13.9, 27 and 84 ng FSH ml^{-1} averaged 9.9, 11.9 and 11.2%, respectively. Intra-assay CV for the same serum pools were 4.7, 6.9 and 9.5%, respectively.

Statistical analyses

Follicular development during the oestrous cycle was evaluated using several different statistical analyses with the computer programs of SYSTAT (1990) and the general linear model of SAS (1986).

A split-plot repeat measure analysis was used to examine whether diameter of follicles determined by the last ultrasound measurement before ovariectomy and measurement of diameter by calliper, volume of follicular fluid, intrafollicular concentrations of oestradiol and progesterone, and the ratio of oestradiol:progesterone concentrations in follicular fluid of the same follicles established after ovariectomy differed ($P < 0.05$) among the six groups (I–VI) of heifers and the three follicle classes. If a significant ($P < 0.1$) statistical interaction was observed, the Bonferroni *t* test was used to test whether means for the F1 follicle class differed ($P < 0.05$) from the F2 and F3 follicle classes for each group of heifers. There were usually 1–3 follicles ≥ 5 mm per pair of ovaries the growth of which was accurately monitored by ultrasound. Because the SEM values increased with means, all data were log-transformed (base 10) before statistical analysis; arithmetic means are reported in the text.

Regression analysis was used to determine the correlation of the last ultrasound measurement of follicle size (taken approximately 14 h before ovariectomy) with measurements of diameter, volume of follicular fluid, intrafollicular concentrations of oestradiol and progesterone, and the ratio of oestradiol: progesterone concentrations in follicular fluid from the same follicles established after ovariectomy from each of the six groups of heifers, for each follicle class (F1, F2, F3) and overall (F1 + F2 + F3).

To determine whether follicle size on days 1–6 could be used to predict whether a follicle would become dominant was investigated using regression analysis. This evaluated whether the proportion of the largest follicles on days 1–6 that were classified as dominant by ultrasound on days 6 and 12 varied during days 1–6.

McNemar's test (Gill, 1978) was used to determine whether there was a difference between the proportion of the largest follicles determined by ultrasound before ovariectomy and the proportion of largest follicles measured after ovariectomy that had the greatest concentration of oestradiol, progesterone, oestradiol:progesterone or progesterone:oestradiol ratios in follicular fluid ($P < 0.05$).

Changes in hormone concentrations throughout the study were evaluated by comparing mean hormone concentrations at different time points using paired *t* test analysis.

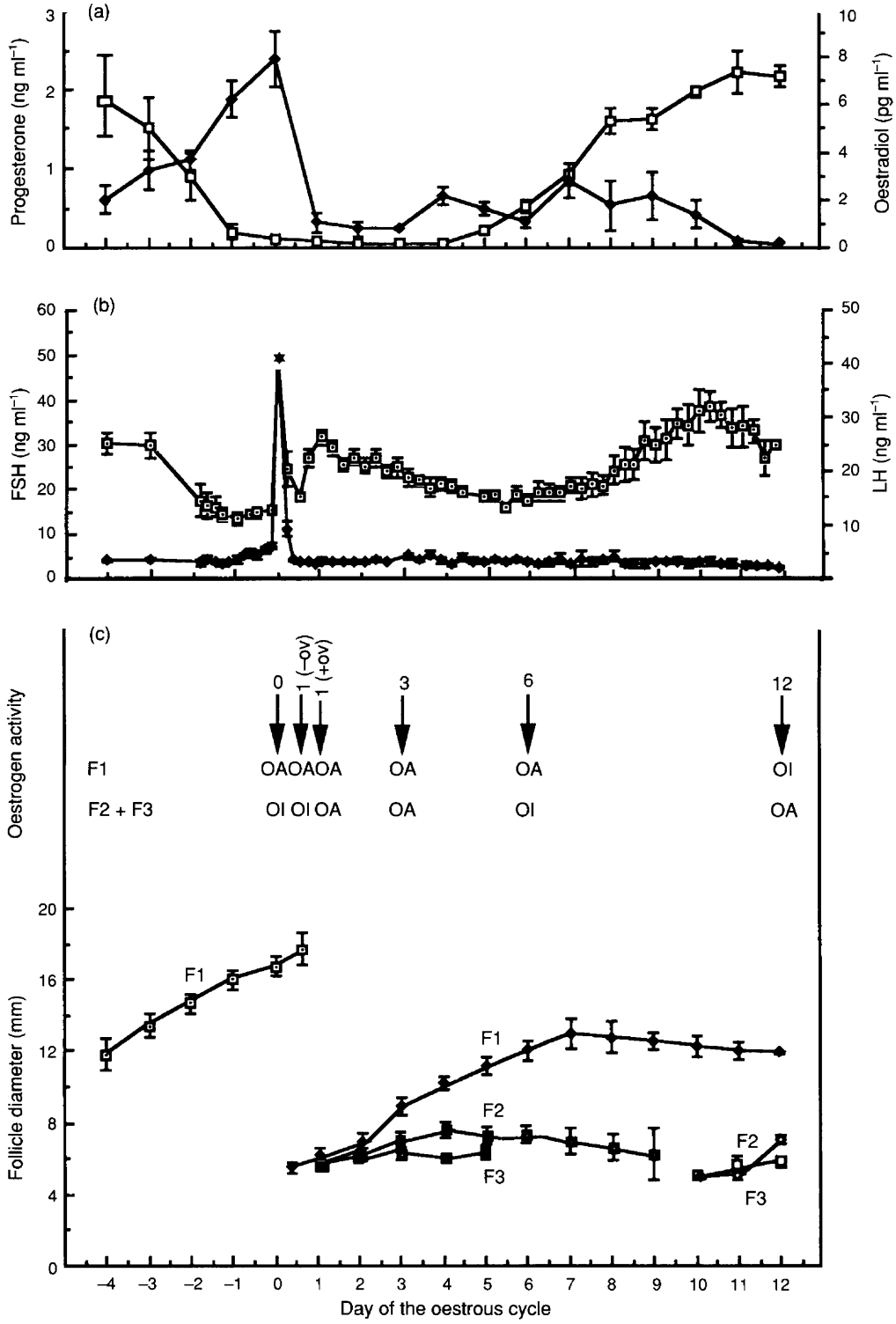
Results

The follicular phase of the oestrous cycle of heifers began coincident with luteolysis on day −4 (4 days before oestrus), when the mean serum concentration of progesterone was < 2.0 ng ml^{-1}, and ended on day 1 (−ov), concomitant with ovulation (Fig. 1a). Day 0 was defined as the time of oestrus and the preovulatory gonadotrophin surges. From day −4 to day −1 each heifer had a single dominant ovulatory follicle (F1) that increased ($P < 0.05$) from 11.6 ± 0.9 mm to 16 ± 0.6 mm in diameter coincident with a sustained basal increase ($P < 0.05$) in the serum concentration of oestradiol, a decrease ($P < 0.05$) in the serum concentration of FSH and progesterone and with no change in LH concentration. After the occurrence of the preovulatory LH and FSH surges on day 0, the serum concentration of LH, FSH and oestradiol decreased ($P < 0.0001$) to presurge values within 12 h, and the diameter of the dominant ovulatory follicle increased to its maximum size of 18 ± 0.8 mm. As the F2 and F3 follicles were < 5 mm in diameter between days −4 and 0, their growth patterns were not measured.

On days 0 and 1 (−ov), dominant ovulatory follicles (F1) were classified as oestrogen-active, whereas F2 and F3 follicles were oestrogen-inactive (Figs 1c, 2). Diameter, oestradiol concentrations, and the ratio of oestradiol:progesterone concentration in follicular fluid were greater ($P < 0.01$) for dominant ovulatory follicles compared with F2 follicles (Figs 1, 2). In contrast, the concentration of progesterone was higher ($P < 0.01$) in F2 than in dominant ovulatory follicles (Fig. 2). Concentrations of oestradiol and the ratio of oestradiol: progesterone concentrations in F1 follicles were two and five

times lower ($P < 0.02$), respectively, whereas the progesterone concentration was higher ($P < 0.01$) in dominant ovulatory follicles on day 1 (− ov) after the preovulatory gonadotrophin surge compared with values on day 0 (Fig. 2). Concentrations of oestradiol, progesterone and the ratio of oestradiol: progesterone concentrations in follicular fluid were highly correlated ($r > 0.75$; $P < 0.05$) with changes in size of dominant ovulatory follicles on day 0, but not on day 1 (− ov) (Table 1).

The luteal phase of the oestrous cycle of heifers began on day 1 (+ ov) after ovulation, when serum concentrations of progesterone were less than 0.5 ng ml^{-1}, and ended on day 12. Each heifer had a single dominant nonovulatory (F1) follicle that increased ($P < 0.05$) in diameter from 6.2 ± 0.3 mm on day



1 (+ ov) to 13 ± 0.8 mm by day 7. Thereafter, the size of this F1 follicle did not change ($P > 0.05$) up to the time of ovariectomy on day 12 (Fig. 1c). Coincident with development of the dominant nonovulatory follicle were two transient increases ($P < 0.02$) in the serum concentration of FSH between days 0.5 and 1.5, and days 8 and 10.5 of the oestrous cycle. During the early luteal phase, the serum LH concentration remained unchanged ($P < 0.05$), whereas transient increases ($P < 0.05$) in serum concentration of oestradiol occurred during days 3–10 when FSH concentrations were low. In contrast to the time from day −4 to day −1 of the follicular phase, F2 and F3 follicles were both ≥5 mm during the early dioestrous phase. In addition, the diameter of F2 and F3 follicles increased ($P < 0.05$) during days 1–4 and then decreased to < 5 mm in diameter during days 5–10. A new group of F2 and F3 follicles emerged between days 10 and 12 (Fig. 1c).

Similar to the dominant ovulatory follicle, the dominant nonovulatory follicle on days 1, 3 and 6 was classified as oestrogen-active (Figs 1c, 2). It should be noted that the F1, or the largest follicle was not always the dominant nonovulatory follicle until after day 4 (Fig. 3). Unlike F2 and F3 follicles on day 0 and day 1 (− ov), F2 and F3 follicles on days 1 (+ ov) and 3 of the early luteal phase were oestrogen-active (Figs 1c, 2). The emerging medium follicles of the second wave were also all oestrogen-active on day 12.

Although concentrations of progesterone in follicular fluid were greater ($P < 0.05$) in the F2 and F3 follicles than in the F1 follicle on day 1 (+ ov), size, oestradiol concentration, and the ratio of oestradiol:progesterone concentrations were similar for all follicle classes (Figs 1c and 2), and oestradiol, progesterone and the ratio of oestradiol:progesterone concentrations were not correlated with the size of follicles (Table 1). On day 3, only diameter (by ultrasound) and the volume of follicular fluid were greater for F1 compared with F2 and F3 follicles, and the size of follicles was correlated ($r = 0.59$; $P < 0.03$) with oestradiol concentrations in follicular fluid. On day 6, all indices of follicle size, oestradiol concentration in follicular fluid and the ratio of oestradiol:progesterone concentrations in follicular fluid were greater ($P < 0.05$) for dominant nonovulatory follicles than for F2 follicles, and size of follicles was highly correlated ($r > 0.70$; $P < 0.02$) with the ratio of oestradiol: progesterone concentrations in follicular fluid (Table 1). Although all indices for size were greater ($P < 0.05$) for dominant nonovulatory follicles compared with F2 or F3 follicles on day 12, the concentration of oestradiol and the ratio of oestradiol:progesterone concentrations in follicular fluid were greater ($P < 0.05$) in F2 and F3 follicles than in the dominant nonovulatory follicle (F1; Figs 1c, 2). In contrast, the concentration of progesterone was greater in dominant nonovulatory follicles (F1) than in F2 follicles on day 12 (Fig. 2). The size of follicles on day 12 was correlated ($r = 0.56$; $P < 0.02$) only with progesterone in follicular fluid (Table 1).

The overall correlation between ultrasound and calliper measurements of diameter ($r = 0.92$; $P < 0.0001$) and volume ($r = 0.88$; $P < 0.0001$) of F1, F2 and F3 follicles after ovariectomy was very high (Table 1). However, ultrasound measurements of diameter were not correlated ($P > 0.05$) with diameter and volume of follicles after ovariectomy on day 1 (+ ov) or day 3, or for F3 follicles.

The proportion of the largest follicles with the greatest concentrations of oestradiol, progesterone, oestradiol:progesterone or progesterone:oestradiol ratios was similar regardless of whether follicle size was measured by ultrasound or with callipers after ovariectomy (Table 2). The predictability of the proportion of the largest follicles that became dominant increased ($P < 0.002$) from 0% on day 1 to 100% by day 5 of the oestrous cycle (Fig. 3).

Discussion

This is the first report to evaluate the relationship between ultrasound monitoring of follicular growth and measures of follicular growth and function after ovariectomy. Our results indicate that ultrasound and postovariectomy measurements of follicle size are highly correlated, implying that ultrasound is an accurate method for monitoring follicular growth. However, the greatest correlation of ultrasound and postovariectomy measures of follicular growth occurred when a single dominant follicle was present during the follicular and luteal phases of the oestrous cycle. In contrast, when a clear hierarchy of follicles was not established, as on days 1 and 3 after ovulation, ultrasound measurements were not significantly correlated with postovariectomy measures of follicle size. While the reason for this finding is unclear, results of correlation analysis have indicated that ultrasound and postovariectomy measurements of follicular growth are least correlated when follicles are classified as F3 (usually 5–6 mm in diameter). Thus, the accuracy of measuring these follicles, regardless of the method used to estimate size, may be diminished compared with measurements of larger antral follicles.

In support of the accuracy of ultrasound measurements for large follicles, both ultrasound and two different

Fig. 1. Mean (± SEM) changes in (a) serum concentrations of progesterone (□; ng ml^{-1}) and oestradiol (◆; pg ml^{-1}), (b) serum concentrations of FSH (⊡) and LH (✱) (ng ml^{-1}), and (c) size of antral follicles during oestrus and early dioestrus of the oestrous cycle of heifers. Daily ultrasound measurements were made on each heifer beginning 4 days before oestrus until ovariectomy. Each point either represents (c) the daily mean (± SE) for diameter of F1, F2 and F3 follicles, or (b) means (± SE) every 6 h for serum concentrations of LH and FSH or (a) daily mean (± SE) serum concentrations for progesterone and oestradiol for 5–33 heifers. Arrows in (c) indicate that groups of heifers ($n = 5$–6 per treatment) were ovariectomized on day 0, day 1 after the preovulatory LH surge, but before ovulation [1 (− ov)], day 1 after ovulation [1 (+ ov)], day 3, day 6 and day 12 of the oestrous cycle. Follicles were separated into three classes based on ultrasound measurement of diameter (F1, largest follicle; F2, second largest; F3, all the remaining follicles ≥5 mm), and oestrogen activity [oestradiol:progesterone ratio > 1; oestrogen-active (OA) or oestradiol:progesterone ratio < 1; oestrogen inactive (OI)]. The oestrogen activity for the F1, F2 and F3 follicles on the six times of ovariectomy is indicated below the arrows for the respective days. On days 0 and 1 (− ov), F1 is the dominant ovulatory follicle. On days 1 (+ ov) to day 3, ultrasound measurements of follicle size cannot be used accurately to predict which follicle is dominant. However, on days 4–12, F1 is the dominant nonovulatory follicle. Note that all 33 heifers are included between 4 days before oestrus and ovariectomy (day 0); thereafter, numbers of heifers were reduced by 5–6 after each ovariectomy (indicated by arrows).

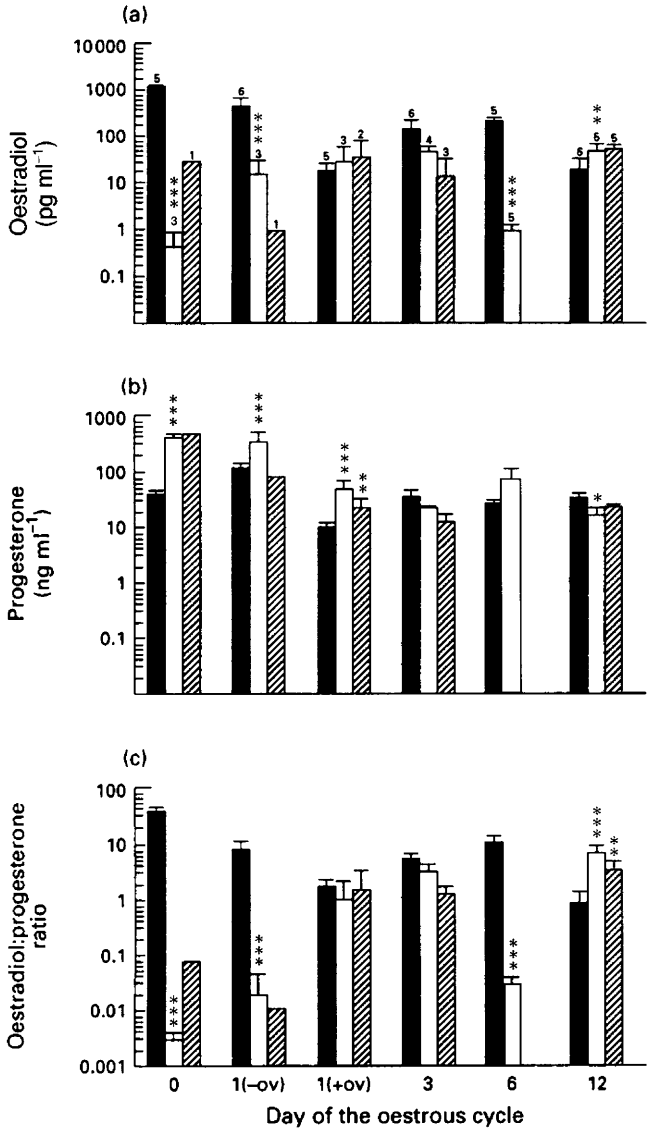


Fig. 2. Changes in the concentrations of (a) oestradiol, (b) progesterone and (c) ratio of oestradiol:progesterone concentrations in follicular fluid for follicles ≥ 5 mm in diameter during oestrus and early dioestrus of the oestrous cycle of heifers. Ultrasound was used to monitor growth and classify follicles as F1 (largest; ■), F2 (second largest; □) or F3 (remaining follicles ≥ 5 mm; ▨) in six different groups of heifers ($n = 5$–6 heifers per group). Bars represent means (± SE) and asterisks above bars indicate whether means for F1 follicles differed statistically (*$P < 0.10$, **$P < 0.05$, ***$P < 0.01$) from F2 or F3 follicles within a certain day. The number of follicles measured is shown in the top panel. − ov: preovulation; + ov: postovulation.

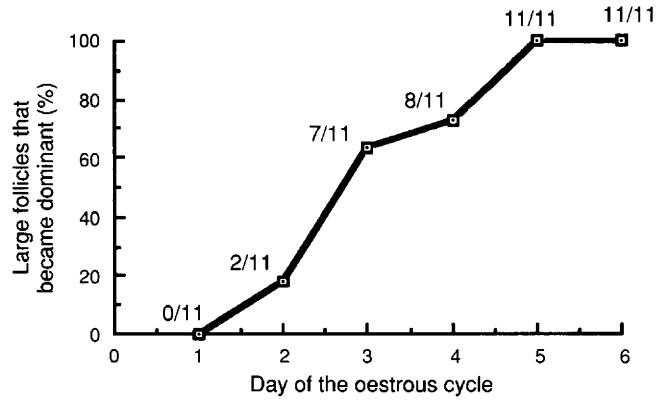


Fig. 3. Proportion of dominant follicles on days 6 and 12 of the oestrous cycle of heifers identified by ultrasound that were also the largest follicles on days 1–6. Follicle growth was monitored daily by ultrasound for 11 heifers from 4 days before oestrus until ovariectomy on days 6 and 12. Data for heifers ovariectomized on days 1 and 3 were excluded because ultrasound measurements could not be accurately used on these days to establish which follicle would become dominant. Numbers represent the number of dominant follicles on days 6 and 12 identified as the largest follicles on days 1–6, divided by the total number of dominant follicles.

postovariectomy measurements of follicle size, diameter and volume were used to determine whether the proportion of the largest follicles (excluding the largest follicle on day 12) with the highest concentration of oestradiol, progesterone, oestradiol:progesterone or progesterone:oestradiol ratios differed. Regardless of which method was used to measure follicle size, the results were similar. Finally, the pattern of turnover of follicles of heifers during days 0–12 is similar to previous reports (Savio *et al.*, 1988; Sirois and Fortune, 1988; Knopf *et al.*, 1989; Adams *et al.*, 1992; Badinga *et al.*, 1992). On the basis of results of this study and those of others (Sirois and Fortune, 1988; Knopf *et al.*, 1989; Adams *et al.*, 1992; Badinga *et al.*, 1992), ultrasound analysis is an accurate and consistent procedure for monitoring the size and turnover of follicles ≥ 5 mm during the oestrous cycle of heifers.

During the oestrous cycle, follicles are recruited into a growing pool and the number selected to continue growing to become dominant follicles is equivalent to the species-specific ovulatory quota (Goodman and Hodgen, 1983). However, the factors involved in selection of dominant follicles are unknown. The completion of a selection phase is defined in cattle by both ultrasound measurements (Savio *et al.*, 1988; Sirois and Fortune, 1988) and the intrafollicular ratio of oestradiol:progesterone concentrations (Ireland and Roche, 1982, 1983a, b) as the time when an oestrogen-active follicle promotes its own growth and inhibits the growth of other follicles. In our study, we examined whether the size of a follicle influenced whether it would become dominant and whether the largest follicles had the highest concentration of oestradiol. The results indicated that the early dioestrus dominant nonovulatory follicle was usually not the largest follicle (2 of 22 measurements) on days 1 and 2 of the oestrous cycle, but was usually the largest after day 2 (37 of 44). Although the concentration of oestradiol and the ratio of oestradiol:progesterone concentrations were not different for each follicle ≥ 5 mm on day 1 after ovulation, concentrations of progesterone were greater in the F2 and F3 than in the F1 follicles. These results indicate that factors other than size and oestradiol concentrations, such as progesterone, may be important for establishing which follicle becomes dominant during a selection phase.

Although the largest follicle with the greatest concentration of oestradiol during a selection phase does not always become dominant, functioning dominant follicles during the follicular phase and on day 6 of the oestrous cycle were always the

Table 1. Correlation of ultrasound measurements of follicle diameter with calliper measurements of diameter, volume of follicular fluid and concentrations of oestradiol, progesterone and the ratio of oestradiol:progesterone concentrations in follicular fluid during the oestrous cycle of heifers[a]

Day of cycle	*n*	Diameter	Volume	Oestradiol	Progesterone	Oestradiol:progesterone
0	9	0.94[b] (0.0001)[c]	0.93 (0.0001)	0.90 (0.001)	0.83 (0.006)	0.76 (0.02)
1 (− ov)	10	0.96 (0.001)	0.88 (0.001)	0.40 ns	0.37 ns	0.38 ns
1 (+ ov)	10	0.51 ns	0.14 ns	0.17 ns	0.05 ns	0.27 ns
3	13	0.43 ns	0.45 ns	0.59 (0.03)	0.00 ns	0.49 ns
6	10	0.88 (0.001)	0.88 (0.001)	0.74 (0.01)	0.11 ns	0.70 (0.02)
12	16	0.92 (0.0001)	0.91 (0.0001)	0.25 ns	0.56 (0.02)	0.29 ns
Overall[d]						
F1	33	0.93 (0.0001)	0.89 (0.0001)	0.47 (0.005)	0.57 (0.001)	0.32 ns
F2	23	0.46 (0.03)	0.68 (0.0001)	0.03 ns	0.40 ns	0.07 ns
F3	12	0.13 ns	0.09 ns	0.28 ns	0.68 (0.01)	0.68 ns
F1 + F2 + F3	68	0.92 (0.0001)	0.88 (0.0001)	0.55 (0.0001)	0.09 ns	0.43 (0.0001)

[a]Groups of five or six heifers were ovariectomized on six different days of the oestrous cycle and follicles were classified as follows: F1, largest follicle; F2, second largest; F3, all remaining follicles ⩾5 mm. The last ultrasound measurement of diameter of follicles ⩾5 mm was made approximately 14 h before ovariectomy and correlated with measures of diameter, volume of follicular fluid and concentrations of oestradiol, progesterone and the ratio of oestradiol:progesterone concentrations in follicular fluid determined from the same follicles after ovariectomy. Each heifer had 1–3 follicles ⩾5 mm.
[b]Regression coefficient.
[c]*P* value.
[d]Data for all days of the oestrous cycle were combined and correlations within each follicle class examined: F1, largest follicle; F2, second largest follicle; and F3, all other follicles ⩾5 mm.
n: number of follicles; ns: not significant ($P > 0.05$); − ov: preovulation; + ov: postovulation.

Table 2. Proportion of largest follicles of heifers determined by ultrasound or at ovariectomy that had the highest concentrations of oestradiol, progesterone and ratio of oestradiol:progesterone or progesterone: oestradiol concentrations in follicular fluid[a]

Parameter	Ultrasound diameter[b]	At ovariectomy Diameter	Volume
Oestradiol	22/27 (81%)[c]	25/27 (93%)[c]	25/27 (93%)[c]
Progesterone	12/27 (44%)[d]	14/27 (52%)[d]	12/27 (44%)[d]
Oestradiol:progesterone	25/27 (93%)[c]	25/27 (93%)[c]	25/27 (93%)[c]
Progesterone:oestradiol	11/27 (41%)[d]	11/27 (41%)[d]	11/27 (41%)[d]

[a]All heifers were included in this analysis except those ovariectomized on day 12 of the oestrous cycle. Heifers on day 12 were excluded because all the largest follicles were atretic.
[b]The last ultrasound measurement was taken approximately 14 h before ovariectomy, and was used to determine the largest follicle.
[c, d]Values with different superscripts are significantly different ($P < 0.05$) between proportions in columns.

largest follicles with the highest concentration of oestradiol (Ireland and Roche, 1983a; Martin *et al.*, 1991). However, the F1 follicle on day 12, despite being the largest follicle, had lost its 'functional dominance' as early as day 10, based on the emergence of a new wave of follicular development. The dominant follicle on day 12 was hormonally classified as

oestrogen-inactive and therefore atretic. Atresia of the dominant nonovulatory follicle is characterized by a significant decrease in the number of granulosa cells, a decrease in both LH and FSH receptors (Ireland and Roche, 1983b) and a diminished capacity to produce oestradiol between days 7 and 13 of the oestrous cycle in heifers (Badinga *et al.*, 1992). The factors involved in the regulation of atresia of follicles are not clear, but it has been demonstrated that decreasing the LH pulse frequency to luteal concentrations results in the faster atresia of the dominant follicle (Sirois and Fortune, 1990), suggesting that the dominant ovulatory follicle fails to undergo atresia because it is subjected to a higher LH pulse frequency in the follicular phase. These results, coupled with those of earlier studies showing that the dominant follicle was not always the largest during a selection phase, clearly indicate that only functioning dominant follicles that are present during finite periods of an oestrous cycle are the largest follicles with the greatest concentration of oestradiol. Although measuring the size or the oestradiol concentration alone is not indicative of whether a follicle will become dominant or whether it is functionally dominant (e.g. preventing growth of other follicles), the ratio of oestradiol:progesterone concentrations can be reliably used to distinguish growing from atretic follicles ≥ 5 mm in diameter in heifers.

Both ultrasound analysis and the ratio of oestradiol:progesterone concentrations indicate that there are two different periods of growth of multiple follicles ≥ 5 mm between days 1 and 3 and days 10 and 12. The results reported here indicate that days 1–3 are the selection phase for development of the early dioestrus dominant nonovulatory follicle, whereas days 10–12 are not only the selection phase for development of the next dominant follicle, but are also the period when the first dominant follicle ceases to function (loses dominance), becomes oestrogen-inactive and begins to undergo atresia. Functional dominance of a follicle begins when a selection phase ends (Goodman and Hodgen, 1983) and is defined as those periods of the oestrous cycle when only a single antral follicle > 5 mm grows to ovulatory size. In our study, use of ultrasound indicated that there were periods of functional dominance during the follicular phase (4 days before oestrus up to the day of ovulation) and from day 4 to day 9 of the oestrous cycle, a finding consistent with previous studies examining follicular dynamics in heifers (Savio *et al.*, 1988; Sirois and Fortune, 1988; Knopf *et al.*, 1989). These periods of functional dominance identified by ultrasound corresponded with the presence of a single large (≥ 10 mm) oestrogen-active follicle that had greater concentrations of oestradiol and ratio of oestradiol: progesterone concentrations than those for other coexisting follicles (F2, F3). Although our results do not indicate precisely when the selection phase begins or ends and when dominance begins, an easily distinguishable hierarchy of follicles was clear by day 4 of the oestrous cycle. In contrast, despite the presence of multiple potential dominant follicles on days 1 (after ovulation), 3 and 12 (based on the ratio of oestradiol:progesterone concentrations), a functional dominant follicle was not present on these days of the oestrous cycle. This finding is supported by both ultrasound and hormonal analysis indicating that multiple, rather than a single, oestrogen-active follicles were growing during these times and that the first dominant follicle was oestrogen-inactive and undergoing atresia by day 12. Ultrasound analysis also indicates that the F2 and F3 follicles on days 4–10, which were present when the functional dominant ovulatory follicle was present on the ovary, and the dominant (F1) nonovulatory follicle on day 12 were either not growing or had lost functional dominance and were also identified as oestrogen-inactive.

This is the first study to characterize the relationship between changing FSH concentrations and intrafollicular changes in the ratio of oestradiol:progesterone concentrations as follicles develop and regress. Both selection periods of follicular growth, days 1–3 and days 10–12, are preceded by a rise in serum concentrations of FSH. Our results, using samples taken every 6 h and both ultrasound and the ratio of oestradiol: progesterone concentrations as methods of monitoring follicular growth, confirm the findings of Adams *et al.* (1992), who used daily blood samples and ultrasound to demonstrate that an increase in FSH occurred 2–4 days before a new wave of follicle development. FSH has a deterministic role in all stages of follicular development (Richards, 1980); thus, it is hypothesized that increases in circulating concentrations of FSH initiate the emergence phase for dominant follicle growth.

This hypothesis is supported by recent data which demonstrate that delaying the first rise in FSH after ovulation between days 0 and 3 by administering bovine follicular fluid delays the emergence of a new dominant follicle (Adams *et al.*, 1992). Removal of the inhibitory influences of the dominant follicle by ovariectomy on days 3, 5 and 8 of the oestrous cycle increases both the FSH concentration and the number of small follicles, and advances the emergence of a new dominant follicle (Adams *et al.*, 1992). Thus, our data provide compelling evidence for a cyclic pattern of FSH secretion during the oestrous cycle of cattle, and this FSH secretion is responsible for the emergence and selection of follicle growth. Once selection is complete as indicated by the presence of one oestrogen-active follicle, the circulating concentrations of FSH have decreased, suggesting that the dominant follicle secretes some inhibitory substance(s) to decrease FSH. It is now well established that both oestradiol (Price and Webb, 1988) and inhibin (Beard *et al.*, 1990; Rivier and Vale, 1991; Robertson *et al.*, 1991), produced by the follicle, control the release of FSH in a negative fashion. The relative importance of both hormones in the regulation of FSH concentration is not clear, but the endocrine data generated in this experiment (Fig. 1) indicate that there is not a clear negative association between serum concentrations of FSH and oestradiol at all stages of the oestrous cycle. These findings suggest that inhibin is important for the regulation of FSH, but owing to the lack of a specific inhibin assay to measure biologically active inhibins (Ireland *et al.*, 1994), it is not yet possible to demonstrate such an effect. Despite this decrease in the circulating FSH concentration after selection and the atresia of all but one follicle, the dominant follicle continues growing, demonstrating that large amounts of FSH are not necessary to sustain follicular dominance.

We conclude that (1) ovarian follicles go through distinct phases of selection, growth, dominance and atresia resulting in the cyclic development of dominant ovulatory and dominant nonovulatory follicles throughout the bovine oestrous cycle, and FSH is probably the physiological 'trigger' for this cyclic follicle growth pattern; (2) the methods of ultrasound and the intrafollicular ratio of oestradiol:progesterone concentrations

must be combined to monitor dominant follicle growth, function and atresia accurately; and (3) the original model for dominant follicle growth (Ireland and Roche, 1987) was a valid physiological representation of dominant follicle turnover during the oestrous cycle of heifers.

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