

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 Concentrations¹

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ABSTRACT

The extent, causes, and physiological significance of the variation in number of follicles growing during ovarian follicular waves in human beings and cattle are unknown. Therefore, the present study examined the variability and repeatability in numbers of follicles 3 mm or greater in diameter during the follicular waves in bovine estrous cycles, and we determined if the variation in number of follicles during waves was associated with alterations in secretion of FSH, estradiol, inhibin, and insulin-like growth factor I (IGF-I). Dairy cattle were subjected to twice-daily ultrasound analysis to count total number of antral follicles 3 mm or greater in diameter throughout 138 different follicular waves. In another study, blood samples were taken at frequent intervals from cows that consistently had low or very high numbers of follicles during waves and were subjected to immunoassays. Results indicate the following: First, despite an approximately sevenfold variation in number of follicles during waves among animals and marked differences in age, stage of lactation, and season of the year, a very highly repeatable (0.95) number of follicles 3 mm or greater in diameter is maintained during the ovulatory and nonovulatory follicular waves of individuals. Second, variation in number of follicles 3 mm or greater in diameter during waves and the inverse association of number of follicles during waves with FSH are not directly explained by alterations in the patterns of secretion of estradiol, inhibin, or IGF-I. Third, ovarian ultrasound analysis can be used reliably by investigators to identify cattle that consistently have low or high numbers of follicles during waves, thus providing a novel experimental model to determine the causes and physiological significance of the high variation in antral follicle number during follicular waves among single-ovulating species, such as cattle or humans.

estradiol, follicle, follicle-stimulating hormone, follicular development, inhibin

INTRODUCTION

Numbers of primordial follicles are highly variable at birth in cattle [1]. Because the ovarian reserve, which is comprised predominantly of primordial follicles, is depleted

during aging and never replenished [1], numbers of the different follicle types in the ovarian reserve vary greatly among individual adult cattle [1]. However, despite high variability and chronic age-related depletion of the ovarian reserve, cattle continue to ovulate one or, occasionally, two oocytes during each estrous cycle throughout most of their relatively long reproductive life spans. Nevertheless, it is unknown if the high variation in number of follicles in ovarian reserves among individuals causes a highly variable number of nonovulatory follicles to grow during estrous cycles. This question is especially important to resolve, because in cattle, relatively high numbers of antral follicles are positively associated with an increased responsiveness to gonadotropin treatments during superovulation [2–5], a larger number of oocytes recovered for in vitro fertilization [4], a higher percentage of normal oocytes [3, 4], a greater number of transferable embryos [4], higher pregnancy rates following in vitro fertilization [4], shorter calving intervals [6], and fecundity [1, 6, 7]. Taken together, these observations imply that variation in number of follicles that develop during estrous cycles may have an important, albeit poorly understood, role in regulation of fertility in single-ovulating species, such as cattle.

Numerous investigators have used ultrasonography to firmly establish that two to three FSH-induced “waves” of antral follicle growth occur at 7- to 10-day intervals during bovine estrous cycles [8–12]. During each wave, the increase in FSH stimulates growth of a cohort of 3- to 4-mm antral follicles, which is followed by development of a single dominant, ovulatory-sized (12–20 mm) follicle and atresia of the remaining follicles in the original cohort [11, 13]. The wave-like pattern of growth and atresia of antral follicles that culminates in development of dominant ovulatory follicles in cattle [12], coincident with a shrinking ovarian reserve during aging [1], represents an important, albeit poorly understood, biological rhythm that must be maintained for successful reproduction. Nevertheless, despite the aforementioned positive association between numbers of follicles and numerous measures of fertility, the degrees of variation both between and among cattle in the number of follicles that grow during follicular waves are unknown, as are the causes, possible association with the ovarian reserve, and physiological significance of such variation.

The well-characterized bovine dominant follicle model was used in the present study to examine variation in the number of follicles 3 mm or greater in diameter during follicular waves and to determine if variation in follicle number is associated with alterations in key hormones or growth factors known to regulate folliculogenesis. Specifi-

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cally, number of follicles in different follicular waves is hypothesized to be as variable within as it is among individual cattle because of the following: First, the size of the ovarian reserve, which may influence the number of follicles growing during waves, is highly variable among cattle and decreases as cattle age [1], and second, the different follicular waves during an estrous cycle occur in markedly different hormonal milieus [9]. To test this hypothesis, the first objective of the present study was to examine the variation in number of follicles during different nonovulatory and ovulatory follicular waves both among and within individual cattle of different ages and physiological stages of development. Furthermore, in cattle, FSH is the primary hormone that induces antral follicle growth during follicular waves [14], and antral follicles that grow during waves produce the chief FSH-negative feedback hormones, estradiol, and inhibin (INHA, or inhibin A, comprised of α and β A subunits) [15, 16]. Insulin-like growth factor I (IGF-I) also interacts with FSH to promote follicle growth in laboratory species [17, 18] and to mimic or enhance FSH action on bovine granulosa cells [19]. Thus, the second objective of the present study was to test the hypothesis that the variation in number of follicles during follicular waves is positively associated with secretion of FSH, estradiol, INHA, and IGF-I.

MATERIALS AND METHODS

Animals

Holstein heifers and lactating as well as nonlactating cows were obtained from the Michigan State University Dairy Cattle Teaching and Research Center. Heifers were housed in free stalls, whereas cows were housed in tie-stall barns. Animals were provided feed and water ad libitum. All rations met National Research Council requirements, and studies were conducted during the spring, summer, or fall of 2001 and 2002 and during the fall of 2003. The All University Committee on Animal Use and Care at Michigan State University sanctioned all procedures involving cattle.

Ultrasound Scanning Procedure to Count Antral Follicles 3 mm or Greater in Diameter in Ovaries

Ovaries in each cow were scanned with an Aloka SSD-900 linear array *trans*-rectal probe (7.5-MHz transducer; Aloka Ultrasound, Wallingford, CT). To standardize counting of follicles, each ovary was scanned from end to end to identify the positions of the corpus luteum and antral follicles. Video images for different ovarian sections were captured on a computer monitor, and the locations of the corpus luteum and each antral follicle 3 mm or greater in diameter in each section were drawn on an ovarian map. Two separate measurements of diameter were averaged for each follicle and recorded next to the appropriate follicle on each ovarian map. The scanning procedure was repeated to confirm the locations of follicles on each ovarian map. Total number of antral follicles 3 mm or greater in diameter per pair of ovaries for each animal was determined by counting the number of follicles 3 mm or greater in diameter on each map for each animal. It should be noted that total number of follicles counted during follicular waves reflects not only follicles growing in response to the transient increase in FSH that precedes each follicular wave [12] but also may include growing, nongrowing, and/or atretic follicles that may or may not be part of the FSH-induced follicular wave.

Validation of Ultrasound Scanning to Count Total Number of Antral Follicles 3 mm or Greater in Diameter in Ovaries

To examine the variability of counting follicles, repeatability of a single-ultrasound-operator counting of the total number of antral follicles 3 mm or greater in diameter in ovaries for five nonlactating, 3- to 5-yr-old cows on three separate occasions (10 min apart) twice daily (0800 and 1800 h) for 11 consecutive days beginning at random stages of the estrous cycle was calculated. In this study, number of follicles (range, 4–22 follicles/cow) counted by a single ultrasound operator at each different ultra-

sound session in individual cattle was highly repeatable (0.97, $n = 330$ counts). In addition, the correlation of the number of follicles counted in ovaries between two different operators during ultrasound analysis on each of 11 randomly chosen days during estrous cycles of five nonlactating cows was determined. The daily ultrasound session for each cow by each operator was approximately 1 h apart, and the results of counting follicles by each operator were not disclosed until the study ended. In this study, the daily follicle counts (range, 6–25 follicles/cow) between the two ultrasound operators were highly correlated (correlation, 0.92; $P < 0.01$; $n = 55$ counts/operator).

Study 1: Variability and Repeatability of Maximal Numbers of Follicles 3 mm or Greater in Diameter During Follicular Waves

Part A. To examine the repeatability of number of follicles during different follicular waves in the same or consecutive estrous cycles, fourteen 10- to 12-mo-old heifers and sixteen 4- to 7-yr-old cows (nine cows at late stages of lactation, seven cows not lactating) were subjected to ultrasound analysis. All animals were treated with two i.m. injections of 25 mg of prostaglandin $F_{2\alpha}$ (PGF_{2 α} ; Lutalyse; Pharmacia-Upjohn Co., Kalamazoo, MI) spaced 11 days apart to induce luteolysis and synchronize occurrence of estrus. Each animal was subjected to twice-daily (0800 and 1800 h) ultrasound analysis by the same ultrasound operator (D.S.B.) for an entire estrous cycle beginning at the time of the second PGF_{2 α} injection and continuing until 2 days after spontaneous ovulation during the subsequent estrous cycle. At each ultrasound session, total number of antral follicles 3 mm or greater in diameter in the ovaries of each animal was determined.

Part B. This study determined the repeatability of number of follicles during follicular waves of individual cattle using a different ultrasound operator (F.J.-K.). In this study, three heifers (age, 13 mo) and two lactating Holstein cows (age, 3–5 yr) were treated with PGF_{2 α} and subjected to twice-daily ultrasound analysis to count the number of follicles as explained in part A.

Part C. In the first experiment, repeatability of the number of follicles during follicular waves in estrous cycles spaced 3 mo apart for the same four nonlactating cows (age, 4–7 yr) was examined. Estrous cycles were either spontaneous or synchronized with two or three injections of PGF_{2 α} spaced 11 days apart. Numbers of follicles were counted twice daily either throughout all the different follicular waves during each estrous cycle for two cows or only during the first wave of three consecutive estrous cycles for the remaining two cows.

In the second experiment, repeatability of follicle number during follicular waves in estrous cycles spaced approximately 15 mo apart for the same individuals was examined. To complete this study, seven of the original 10- to 12-mo-old heifers from part A were treated at 27 to 28 mo of age with two injections of PGF_{2 α} spaced 11 days apart. Each cow was subjected to twice-daily ultrasound analysis to determine repeatability of the maximal number of follicles during each of three to five waves in consecutive estrous cycles as explained above. At the beginning of the treatment period, the cows were approximately 2 mo postcalving and near peak lactation. The results from the ultrasound analysis of these cows were then compared with the previous results generated from ultrasound analysis of three to four waves for the same individuals as heifers.

In addition to repeatability, differences in the overall average for the maximal numbers of follicles during waves in estrous cycles compared with estrous cycles for the same animals 3 or 15 mo later were determined.

Study 2: Relationship of Variation in Maximal Number of Follicles 3 mm or Greater in Diameter During Follicular Waves with Development Dynamics of Dominant and Subordinate Follicles

The animals used in study 1 (parts A and B) were arbitrarily segregated into the following four groups based on the overall average for the maximal number of follicles during waves: low (≤ 15 follicles during waves; $n = 30$ waves, 9 animals), intermediate (16–20 follicles; $n = 29$ waves, 10 animals), high (21–25 follicles; $n = 25$ waves, 8 animals), and very high (> 25 follicles; $n = 30$ waves, 8 animals). The number of follicles (average \pm SEM) during waves for cattle segregated into the low, intermediate, high, or very high groups was 12.8 ± 1.5 , 16.8 ± 1.7 , 22.1 ± 1.9 , and 33.8 ± 1.5 , respectively. To determine if numbers of follicles during waves were associated with alterations in the growth dynamics of dominant and subordinate follicles, differences in day of emergence for each follicular wave, day of deviation, length of dominance, interval be-

tween ovulations, and maximal diameter of the dominant or subordinate follicle at deviation or during dominance for cattle with low, intermediate, high, and very high numbers of follicles during waves were examined. Definitions used to characterize follicular waves and dominant follicle growth are as follows: *day of emergence*, determined retrospectively (after growth of the dominant follicle) as the first day of the new wave that a follicle 4 mm or greater in diameter is detected by ultrasound minus 1 day; *day of deviation*, first day of a new wave that the largest or dominant follicle is consistently 1 mm or greater in diameter than the next largest or subordinate follicle throughout the wave minus 1 day; and *length of dominance*, interval from first day of deviation in a new wave until emergence of the next wave.

Study 3: Association of Patterns of FSH, Estradiol, INHA, and IGF-I Secretion with Number of Follicles 3 mm or Greater in Diameter During Follicular Waves

Animal selection. A group of 3- to 5-yr-old cows ($n = 25$) at similar stages of late lactation were subjected to ultrasound analysis for two to three consecutive days, chosen at random during an estrous cycle, to identify cows with a low (≤ 15) or a very high (> 25) number of follicles. Three animals in the low group and four in the very high group were identified for blood sampling. To determine number of follicles during waves, estrous cycles for these animals were synchronized with three consecutive injections of PGF_{2 α} spaced 11 days apart. Each animal was subjected to daily (0800 h) ultrasound analysis after each of the first two PGF_{2 α} injections and twice-daily (0800 and 1800 h) ultrasound analysis after the third PGF_{2 α} injection. Ultrasound analysis began at the time of each injection of PGF_{2 α} and ended 4 days after ovulation. Number of follicles during the first follicular wave after each PGF_{2 α} injection was determined as explained above. The day of ovulation after each PGF_{2 α} injection was defined as the first day that the ultrasound operator was unable to detect the dominant ovulatory follicle during scanning.

Blood collection. Blood samples (5 ml) were removed from each animal's tail vein beginning 48 h after the third PGF_{2 α} injection and continuing every 4 h until 24 h after ovulation. Thereafter, blood was collected every 8 h until 96 h after ovulation. Based on previous results [9], this blood-sampling regimen should span the days of an estrous 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 nonovulatory follicle.

Immunoassays

Concentrations of FSH, estradiol, and INHA were determined for all serum samples, whereas IGF-I concentrations in serum were determined at 8- to 24-h intervals.

Concentrations of FSH in duplicate 100- μ l serum samples for each cow were determined using a previously validated, heterologous RIA [20]. Results of the FSH RIA correlate with those of *in vitro* bioassay of FSH bioactivity [21]. Ovine FSH (U.S. Department of Agriculture [USDA] oFSH-19-SIAFP-I-2) was used as radio-iodinated tracer, bovine FSH (USDA bFSH-I-2) as standard, and National Institute of Diabetes and Digestive and Kidney Diseases anti-oFSH-1 (AFP-C5288113; Rockville, MD) as antiserum. All samples were analyzed in a single FSH assay. The intra-assay coefficient of variation (CV) was 10.5%, and the sensitivity of the assay was 0.03 ng/ml.

Estradiol concentrations were determined in duplicate 500- μ l serum samples previously extracted with ether using a modified version [22] of the commercial MAIA Kit (Polymedco, Inc., Courtlandt Manor, NY). Standard curves ranged from 0.20 to 50 pg/ml (0.04–10 pg/tube), and the median effective dose (mean \pm SEM) for standard curves ($n = 4$) averaged 4.8 ± 0.8 pg/ml. Sensitivity of the assay was 0.04 ± 0.01 pg/ml. Inter- and intra-assay ($n = 4$ assays) CVs were 17.5% and 9.6%, respectively, for serum samples that averaged 5.1 pg/ml and 16.5% and 24.4%, respectively, for samples ($n = 4$) that averaged 0.88 pg/ml.

Concentration of IGF-I in serum samples was determined with use of a commercial two-site immunoradiometric assay (Diagnostic Systems Laboratories, Inc., Webster, TX) previously validated for bovine serum [23]. Serum samples (50 μ l) were acid-ethanol extracted per the manufacturer's instructions and run in duplicate in a single assay. Assay of different volumes of cow serum (10–50 μ l) was parallel with the standard curve (data not shown). The intra-assay CV for controls containing low (45.7 ± 1.8 ng/ml) or high (161.4 ± 13.6 ng/ml) concentrations of IGF-I was 7.8% and 16.8%, respectively, and the sensitivity of the assay was 5 ng/ml.

Serum INHA concentration was determined using a two-site ELISA, which was developed specifically for bovine/ovine samples [24]. The sen-

sitivity of the assay was improved by using a different biotinylated detection antibody (PPG 14/6) as recently described [25]. This antibody was raised against a synthetic peptide corresponding to amino acid sequence 1–32 of the bovine α C subunit. The immobilized "capture" antibody (E4; raised against synthetic peptides corresponding to the amino acid sequence 82–114 of the human and ovine β A subunit) was the same as that employed in the original assay. Sensitivity of the assay was 0.04 pg/tube, and within- and between-plate CVs were both less than 12%. The antibody used in the INHA assay does not cross-react with the bovine inhibin α subunit pro α C, recombinant human (rh) INHB (inhibin B, comprised of α and β B subunits), rh-INHBA (activin A, comprised of two β A subunits), rh-INHBB (activin B, comprised of two β B subunits), or rh-FST (follicle-stimulating hormone) [25].

Statistical Analysis

Repeatability (range, 0–1, where 1 = perfect) is defined as the proportion of the total variance that could be attributed to animal variance, which is calculated as σ^2 animal/ σ^2 animal + σ^2 error [26]. Variance components were estimated using the PROC MIXED model approach of SAS [27].

In study 1, repeatability of maximal numbers of antral follicles 3 mm or greater in diameter during different follicular waves in the same or consecutive estrous cycles or in estrous cycles spaced 3 or 12 mo apart for individuals was determined as explained above. Also, the paired *t*-test [27] was used to determine if the overall average maximal number of follicles during waves in estrous cycles was significantly ($P \leq 0.05$) different compared with that during estrous cycles of the same individuals 3 or 15 mo later.

In study 2, a mixed-model, repeated-measures approach was used to determine whether growth of dominant and subordinate follicles during follicular waves was similar for cattle that consistently had low, intermediate, high, or very high numbers of follicles during waves [27]. The cattle used in parts A and B of study 1 were used for this analysis. Main effects included technician; groups of cattle with low, intermediate, high, or very high maximal number of follicles during waves; lactation status; age of animals; number of follicular waves per estrous cycle; and ovulatory and nonovulatory waves. Dependent variables included maximal follicle number during waves, length of dominance, interval between ovulations, and diameter of the dominant or subordinate follicles at deviation or during dominance. Number of waves subjected to ultrasound analysis per animal was treated as a repeated measure across individual cows for analyses. Least-squares means were calculated for all main effects in the model.

In study 3, repeatability of maximal numbers of follicles during waves in the three synchronization periods was determined as explained above. A mixed-model, repeated-measures approach was used to determine if serum FSH, estradiol, IGF-I, and INHA concentrations and number of antral follicles were different for cows with low versus cows with very high numbers of follicles during waves [27]. Main effects included groups of cows with low or very high numbers of follicles during waves, time, and an interaction of the follicle groups with time. Serum hormone concentrations and number of follicles were considered to be dependent variables. Time of blood sampling and number of follicles during waves per animal were treated as a repeated measure across individual cows. Data were log₁₀-transformed before statistical analysis. Least-squares means and SEMs of nontransformed data were calculated for all main effects in the model and used to plot the data in Figure 2.

When main effects were significant ($P < 0.05$) in study 2 or 3, a Bonferroni *t*-test was used to determine whether statistical differences existed among individual means [27].

RESULTS

Study 1: Variability and Repeatability of Number of Follicles 3 mm or Greater in Diameter During Follicular Waves among and Within Individual Cattle

In part A, numbers of follicles during waves for four representative cows that consistently had low, intermediate, high, or very high numbers of follicles during the different follicular waves in an estrous cycle are depicted in Figure 1. These results typified the remarkable similarity in the maximal number of follicles during different follicular waves of individual animals and the great variation among animals. Despite the high variability in maximal number of

follicles during waves among cattle (range, 11–54 follicles), repeatability of the maximal number of follicles during different follicular waves in the same or consecutive estrus cycles within each individual was very high overall (0.95; $n = 98$ follicular waves, 30 animals).

In part B, a separate study by another ultrasound operator (F.J.-K.), the maximal number of follicles during waves for five animals ranged from 9 to 39 ($n = 18$ waves). Repeatability of numbers of follicles during waves for individuals was 0.86.

In part C, for estrous cycles 3 mo apart, the maximal number of follicles during waves ranged from 9 to 33 ($n = 21$ waves) among animals. Repeatability of the maximal number of follicles during waves was 0.92 in individuals, and the overall means \pm SEMs for the maximal number of follicles during waves were similar (20.4 ± 1.9 vs. 21.2 ± 2.3 , $P > 0.20$). For estrous cycles 15 mo apart, the maximal number of follicles during waves ranged from 9 to 36 ($n = 53$ waves) among animals, and repeatability of the maximal number of follicles during waves was 0.85. However, in contrast to estrous cycles spaced 3 mo apart, the overall mean for the maximal number of follicles during waves was 22% lower ($P < 0.01$) during estrous cycles of cows compared with the same individuals as heifers (16.4 ± 1 vs. 21.1 ± 1). The decrease in maximal numbers of follicles during waves for each of the seven cows compared with the same individuals as heifers was significant ($P < 0.05$ – 0.01) for five animals (15%, 20%, 24%, 40%, and 46%) but not for the others (0% and 12%).

Regarding overall follicular dynamics and variability and repeatability of maximal numbers of follicles 3 mm or greater in diameter during follicular waves, when the 35 animals in parts A and B were combined, seven (four cows, three heifers) had three follicular waves, whereas 28 (14 cows, 14 heifers) had two follicular waves during an estrous cycle. Others [28–30] also report that most Holsteins have two rather than three waves during estrous cycles. The maximal number of follicles during follicular waves averaged 21.5 ± 0.8 (range, 8–54 follicles) and was similar ($P > 0.20$) for heifers (20 ± 1.1 ; range, 8–42 follicles; $n = 17$ animals) and cows (22.8 ± 1.3 ; range, = 11–54; $n = 18$). Nevertheless, as previously reported [31] (also see the summary of 15 studies by Sartori et al. [30]), cows had longer ($P < 0.05$) intervals between ovulations (23.7 ± 0.6 vs. 21.3 ± 0.8 days), larger ($P < 0.05$) dominant follicles (15.8 ± 0.3 vs. 14.7 ± 0.5 mm) and longer ($P < 0.05$) periods of dominance (7.6 ± 0.3 vs. 6 ± 0.6 days) compared with heifers.

When animals in parts A and B of study 1 and of study 3 (described below) were combined ($n = 44$ animals, 138 waves), maximal number of follicles during waves ranged from 8 to 54 among animals, whereas repeatability of maximal number of follicles during waves for individuals was 0.95 (Table 1). In addition, repeatability of maximal number of follicles during waves in individuals was very high (0.86–0.96) regardless of season of year, number of follicular waves per estrous cycle, stage of lactation, technician who conducted the ultrasound examination, or age of cattle (Table 1). Repeatability of basal or the minimal numbers of antral follicles 3 mm or greater in diameter during waves in individuals (range, 2–31) also was high overall (0.85; $n = 68$ waves, 30 animals).

Although follicle diameters ranged from 3 to 20 mm during follicular waves, the vast majority of the total number of follicles counted on most days during waves for each animal had diameters between 3 and 3.9 mm (data not

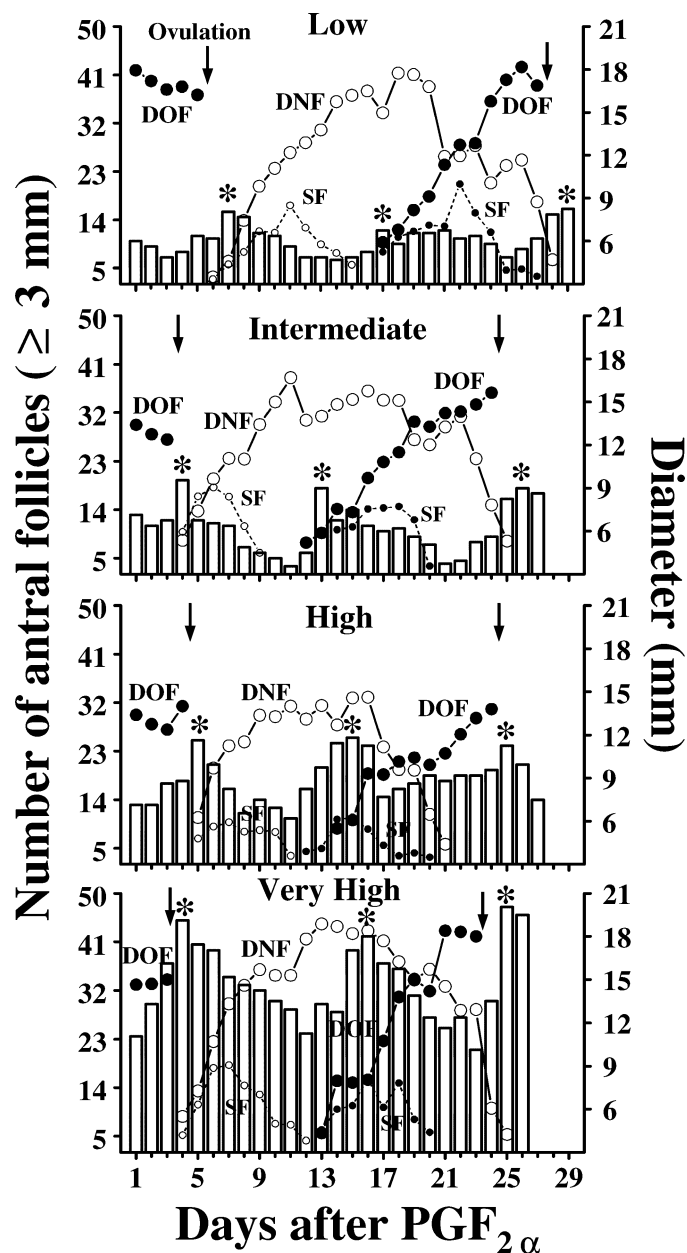


FIG. 1. Differences in total number of antral follicles (diameter, ≥ 3 mm) during the first and ovulatory follicular waves of an estrous cycle and during the early stages of the first follicular wave of the next estrous cycle of Holstein cows with low ($n \leq 15$), intermediate ($n = 16$ – 20), high ($n = 21$ – 25), or very high ($n > 25$) numbers of follicles during follicular waves. Each bar represents the average for the total number of follicles in both ovaries determined at each of two daily ultrasound sessions (0800 and 1800 h). An asterisk above a bar indicates maximal number of follicles during each wave. Arrows indicate ovulation. Lines represent changes in diameter of the dominant and the largest subordinate follicle for the first and ovulatory wave. DNF, Dominant nonovulatory follicle (large open circles); DOF, dominant ovulatory follicle (large solid circles); SF, largest subordinate follicle (small open or solid circles).

shown). For example, the peak number of follicles in the 3- to 3.9-mm category during waves was at least three- to fivefold greater than the peak number of follicles in any other size categories. Thus, differences in numbers of follicles during waves among cattle (Fig. 1 and Table 1) were primarily the result of differences in numbers of 3-mm follicles.

TABLE 1. Effect of various parameters on repeatability of maximal numbers of antral follicles (≥ 3 mm in diameter) during follicular waves.^a

	No. of animals	No. of waves	Range of numbers of antral follicles per follicular wave	Repeatability
Season				
Spring	5	18	8–39	0.86
Summer	30	96	11–54	0.95
Fall	9	24	12–38	0.94
No. of follicular waves per estrous cycle				
Two	28	86	8–54	0.93
Three	7	28	11–44	0.95
Lactational status				
Lactating	20	58	12–44	0.94
Non-lactating	24	80	8–54	0.93
Technician				
DB	39	120	11–54	0.95
FJ	5	18	8–39	0.86
Age ^b				
Heifers	17	56	8–42	0.92
Cows	27	82	11–54	0.96
Overall	44	138	8–54	0.95

^a Cattle from study 1 (parts A and B) and/or study 3 were combined and used to generate the data depicted in Table 1.

^b Heifers were 10 to 13 mo old, whereas cows were 3 to 7 yr old.

Study 2: Relationship of Number of Follicles 3 mm or Greater in Diameter During Waves and Development Dynamics of Dominant and Subordinate Follicles

The numbers of heifers or cows with two or three follicular waves per estrous cycle were equally distributed ($P > 0.10$) in the low, intermediate, high, or very high classifications for number of antral follicles during waves (data not shown).

Age of cattle, stage of lactation, length of dominance, interval between ovulations, day of emergence, day of deviation, largest diameter of the dominant and subordinate follicles during dominance, and diameter of the dominant and subordinate follicles at deviation were similar ($P > 0.10$) for cattle with low, intermediate, high, or very high numbers of follicles during waves (data not shown).

Study 3: Association of Patterns of FSH, Estradiol, INHA, and IGF-I Secretion with Number of Follicles 3 mm or Greater in Diameter During Follicular Waves

The three cows in the low group averaged 15.1 ± 0.9 follicles during waves ($n = 9$ follicular waves; range, 11–18; age, 3 yr), whereas the four cows in the high group averaged 34.9 ± 1.1 follicles during waves ($n = 10$ follicular waves; range, 29–44; ages, 3–4 yr). Repeatability of maximal number of follicles during waves in three consecutive estrous cycles was very high for individuals (0.90; $n = 19$ waves, 7 cows) and similar to the results of study 1.

For statistical analysis, all values for numbers of follicles and hormone or growth factor concentrations were aligned for each cow relative to the peak FSH value during the first postovulatory FSH surge. Hereafter, this peak FSH value ($t = 0$ on the x -axis in Fig. 2) is referred to as the peak of the postovulatory FSH surge. Because of limited numbers of observations ($n = 0$ –2) per time point, values for follicle numbers or each hormone from 20 to 40 h (pool 1), 44 to 64 h (pool 2), and 68 to 88 h (pool 3) after the peak of the postovulatory FSH surge were pooled for each animal. The midway point for each of the aforementioned blood-sam-

pling periods for pool 1, 2, or 3 is depicted along the x -axis in Figure 2 at 30, 54, and 78 h, respectively.

Although serum LH concentrations were not determined in the present study, timing of the preovulatory LH surge was assumed to be coincident with the peak FSH value occurring 20 ± 0 h before the peak of the postovulatory FSH surge (Fig. 2B), which is similar to results of others [25]. Time of ovulation (estimated to be midway between the time of the ultrasound analysis at which the dominant ovulatory follicle was last observed until it was no longer observed) and emergence of the first nonovulatory follicular wave occurred 8.6 ± 2 and 2.3 ± 3 h, respectively, before the peak of the postovulatory FSH surge, whereas deviation and development of the first-wave dominant nonovulatory follicle occurred 41 ± 5.6 h after the peak of the postovulatory FSH surge (Fig. 2A, arrows). No differences were observed in the aforementioned values when cows with low versus cows with very high numbers of follicles during waves were compared. Numbers of follicles during waves were greater ($P < 0.001$) (Fig. 2A), whereas FSH was lower ($P < 0.02$) (Fig. 2B) throughout the sampling period for cows with very high versus cows with low numbers of follicles during waves. The INHA level tended to be higher ($P < 0.07$) (Fig. 2C) primarily before ovulation for cows with very high versus cows with low numbers of follicles during waves. Cows with low versus cows with very high numbers of follicles during waves had similar ($P > 0.50$) serum concentrations of estradiol (Fig. 2D) and IGF-I (data not shown). During the sampling period, the number of follicles ($P < 0.01$) increased from 48 to 0 h before the peak of the first postovulatory FSH surge and then declined thereafter (Fig. 2A). In contrast, serum concentrations of FSH ($P < 0.001$) (Fig. 2B), INHA ($P < 0.02$) (Fig. 2C), and estradiol ($P < 0.001$) (Fig. 2D) decreased from 48 h to approximately 24 h before the peak of the postovulatory FSH surge and then increased thereafter. Serum IGF-I concentrations remained unchanged ($P > 0.70$, data not shown) during the sampling period.

DISCUSSION

The remarkably high repeatability of the numbers of antral follicles 3 mm or greater in diameter during follicular waves in individual dairy cattle observed in the present study is indirectly supported by the results of other investigators. For example, numbers of 2- to 6-mm follicles at emergence for two successive follicular waves in individual lactating beef cows are highly positively correlated ($r = 0.77$) [5]. Numbers of growing antral follicles following serial ultrasound-guided needle aspiration of antral follicles every 7–10 days are repeatable (0.58) in individual cattle [26], and numbers of antral follicles are moderately reproducible in individual human beings from one menstrual cycle to the next [32]. The high repeatability of antral follicle growth probably explains why the overall average for maximal number of antral follicles during waves was similar for young compared to older animals in the present study, despite the permanent and steep loss of follicles as cattle age [1]. Indeed, previous reports indicate that the number of antral follicles in ovaries remains relatively constant until cattle are 8 to 10 yr of age [1, 33] and until humans are 35 to 40 yr of age [32]. Thereafter, coincident with the near exhaustion of primordial follicles, the number of antral follicles decrease in cattle [1] and humans [32]. Taken together, these observations indicate that a chronic compensatory mechanism must exist to maintain the high repeatability of numbers of follicles 3 mm or greater in diameter during follicular waves as cattle age. This compensatory mechanism could explain why classical histological studies in several species [34–38] show that the rate of initiation of growth of primordial follicles is inversely associated with the number of growing follicles. For example, the rate of initiation of growth of primordial follicles (recruitment) may have to increase as the ovarian reserve dwindles during aging to maintain a constant number of antral follicles during follicular waves. It is unknown, however, whether this putative chronic compensatory mechanism is similar to the acute compensatory mechanism that maintains the species-specific ovulation rate after unilateral ovariectomy of multiple-ovulating species [39, 40] or has a role in fertility or reproductive longevity.

The maximal number of follicles 3 mm or greater in diameter during waves was slightly, but significantly, lower in the nearly 2-yr-old cows at their peak stages of lactation compared with the same individuals as 10- to 12-mo-old heifers in the present study. We interpret these seemingly contradictory results to indicate that although follicle numbers during waves are highly repeatable in individuals, different physiological states, such as lactation [41], heat stress [42], pregnancy [43], and/or inadequate nutrition [41], may transiently suppress numbers of follicles during waves. The physiological significance and causes of temporary declines in number of follicles during waves, however, are unknown.

Our findings imply that the marked variation among cattle, primarily in number of 3-mm follicles during follicular waves, does not affect development or function of dominant follicles. However, dominant follicle development and se-

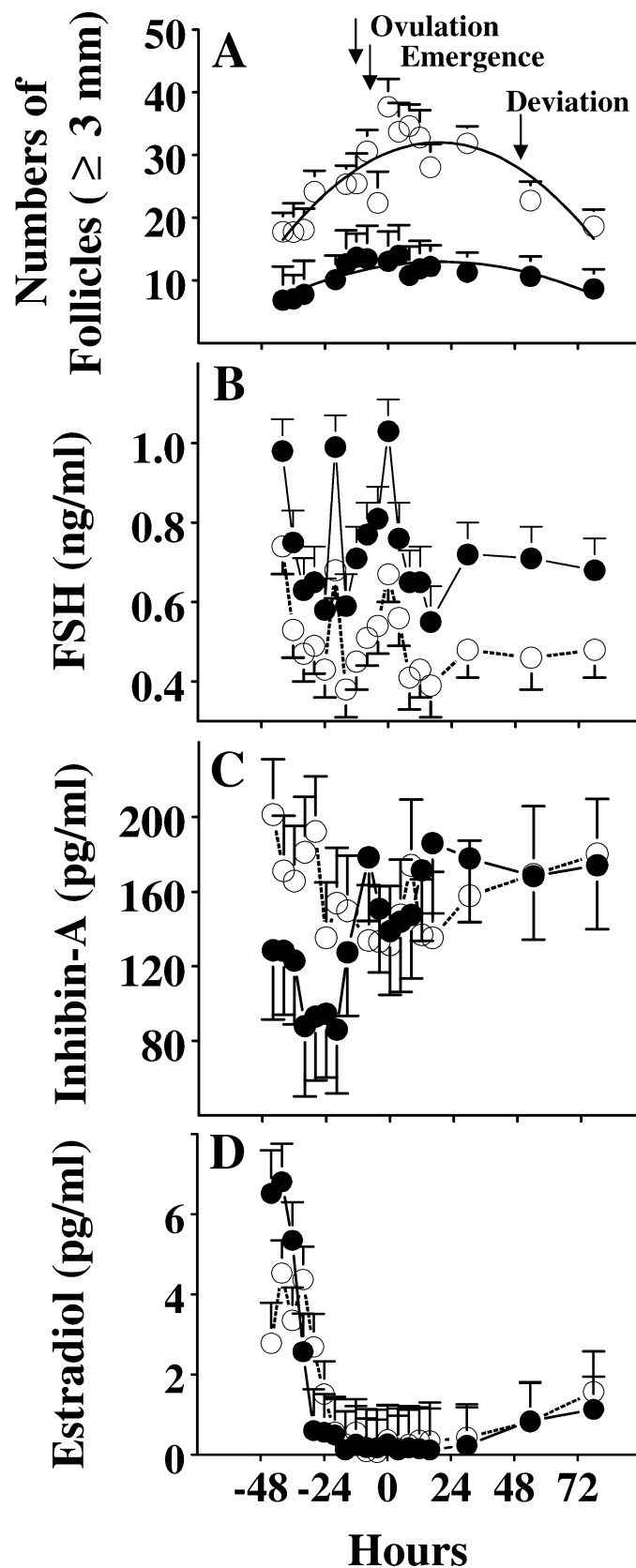


FIG. 2. Number of follicles and serum concentrations of FSH, INHA, and estradiol for Holstein cows that consistently had low ($n \leq 15$ follicles, $n = 3$ cows) versus cows with very high ($n > 25$ follicles, $n = 4$ cows) peak numbers of follicles 3 mm or greater in diameter during follicular waves. Symbols represent the average \pm SEM for the total number of antral follicles 3 mm or greater in diameter in both ovaries (A) or serum FSH (B), inhibin A (C), or estradiol concentrations (D), respectively, for

cows with low (solid circles) versus cows with very high (open circles) numbers of follicles during waves. Data were aligned for statistical analysis relative to the peak of the first postovulatory FSH surge. The arrows in A indicate average times of ovulation, emergence of the first nonovulatory follicular wave, and deviation in growth of dominant versus the largest subordinate follicles (not shown). Follicle number and hormone values at 30, 54 and 78 h after the peak of the postovulatory FSH surge represent pooled values as explained in Results.

cretion of FSH, estradiol, INHA, and IGF-I were not evaluated throughout the ovulatory wave or during the portion of the first nonovulatory follicular wave when estradiol secretion is maximal [9]. Nevertheless, INHA concentrations tended to be higher in cows with very high versus cows with low numbers of follicles during waves on the last day of development of ovulatory follicles, but not during the first nonovulatory follicular wave. This observation supports the possibility that the number of follicles during waves may influence, or be associated with, alterations in the secretion of growth factors by dominant follicles. It also suggests that small antral follicles make a significant contribution to circulating INHA levels. Further studies will be necessary to determine if the variation in number of follicles during follicular waves is associated with alterations in dominant follicle function.

The general patterns of secretion of FSH, estradiol, and INHA during the later stages of development of the dominant ovulatory follicle and during the early stages of development of the first nonovulatory follicular wave are similar to previous reports for cattle [9, 25, 44, 45]. However, concentrations of FSH were inversely, rather than positively, associated with the number of follicles 3 mm or greater in diameter during follicular waves in the present study, as recently reported for dairy heifers [46] and lactating beef cows [5]. Although estradiol and INHA would be expected to be produced primarily by the largest antral follicles during waves [47], it was somewhat surprising that serum concentrations of these negative-feedback hormones were similar for cows with low versus cows with very high numbers of follicles during waves. This observation supports the speculation that factors other than FSH and its chief negative-feedback hormones, estradiol and INHA, have a role in regulating the number of follicles growing during waves. For example, despite treatment of cattle with a GnRH1 agonist to significantly reduce secretion of FSH and LH [48], antral follicles grow to at least 4 mm in diameter. Growth hormone [49] and IGF-I [50] promote growth of antral follicles. Thus, alterations in secretion of either of these hormones could explain the high variability, primarily in number of 3-mm follicles, during waves among the cattle in the present study. Nevertheless, serum IGF-I concentrations were similar for cows with very high versus cows with low numbers of follicles during waves in the present study, implying that other mechanisms, such as size of the ovarian reserve, may regulate the variation in numbers of follicles during follicular waves.

Indirect evidence indicates that the number of follicles during follicular waves probably reflects the size of the ovarian reserve in humans [32, 51] and cattle [1, 3, 52]. Thus, the cattle with high serum FSH concentrations and relatively low numbers of follicles during waves in the present study also may have had relatively smaller ovarian reserves. In support of this possibility, serum FSH is higher in older women [53] and cows [54] compared to their younger counterparts. Factors that regulate variation in antral follicle numbers during waves may include not only size of the ovarian reserve, differential responsiveness of antral follicles to FSH [55], differential secretion of hormones and growth factors that alter FSH responsiveness (e.g., LH [56]), and genetic mechanisms [52, 55] but also growth factors that regulate recruitment and growth of preantral follicles, such as antimüllerian hormone [57–59], KIT ligand [60, 61], INHBA [62], and growth differentiation factor 9 [60, 63, 64]. Moreover, our findings imply that estradiol and INHA may not be the only feedback regulators of

FSH secretion during follicular waves. Rather, control of FSH secretion during follicular waves may involve other follicular (or intrapituitary) factors, such as INHBA, which stimulate FSH secretion; FST, which inhibits INHBA action; or inhibin B, which inhibits FSH secretion [15]. In addition, differential secretion of GnRH1, differential sensitivity to GnRH1 or to the negative feedback actions of INHA, and/or estradiol could explain our results. Most importantly, the high repeatability of the number of follicles 3 mm or greater in diameter during waves of individuals, coupled with the high variation in follicular number among cattle, enables use of ultrasound to identify reliably groups of cattle that consistently have low, intermediate, high, or very high numbers of follicles during waves. Consequently, classification of cattle based on the number of antral follicles 3 mm or greater in diameter during waves should provide a novel model not only to elucidate the potentially complex role of the aforementioned factors in regulation of FSH secretion and the dynamics of follicular growth but also to examine the relationship of the variation in numbers of follicles during waves among cattle to size of the ovarian reserve, reproductive life span, superovulation, and fertility.

We conclude the following: First, despite an approximately sevenfold variation in maximal number of follicles 3 mm or greater in diameter during waves among cattle, development of two or three follicular waves in different hormonal milieus during estrous cycles, and a decline in the number of antral follicles during waves in association with aging and lactation, a compensatory mechanism exists to maintain a very highly repeatable (0.95) number of follicles during waves in estrous cycles of individuals. Second, variation in the numbers of follicles 3 mm or greater in diameter during waves and the inverse association of the number of follicles during waves with FSH are not directly explained by alterations in the secretion patterns of estradiol, INHA, or IGF-I. Third, use of ultrasound to identify cattle with consistently low or high numbers of follicles 3 mm or greater in diameter during waves provides a novel experimental model to determine the causes and physiological significance of the high variation in follicle number during follicular waves among single-ovulating species, such as cattle and humans.

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