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An Ultrasonographic Study of Ovarian Antral Follicular Dynamics in Prepubertal Gilts During the Expected Activation of the Hypothalamo-pituitary-ovarian Axis

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Abstract. Daily transrectal ultrasonography was carried out in eight 4–5-month-old Polish Large White × Polish Landrace gilts for 42 days to monitor the growth of individual ovarian antral follicles ≥2 mm in diameter. In total, 52.4 ± 16.2 and 123.0 ± 6.7 follicles per gilt (mean ± SD) that grew to ≥4 mm were identified during the first and second 21-day study periods, respectively (P<0.01). Four follicular waves (defined as the synchronous growth of a group of follicles from 2–3 mm to ≥4 mm) emerged during the first period, and five waves emerged during the second period. The maximum diameters attained by the largest follicles of waves were 5.7 ± 0.6 and 7.0 ± 0.5 mm (first and second periods, respectively; P<0.01). The present results provide direct evidence for the rhythmic, wave-like pattern of antral follicle recruitment in prepubertal gilts. The number of follicles and maximum diameter they attain increase significantly during the expected activation of the hypothalamo-pituitary-ovarian axis in prepubescent gilts.
Key words: Antral follicle, Follicular dynamics, Gilt, Pig, Prepubertal, Ultrasonography

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The ovaries of gilts become responsive to gonadotropins around day 60 after birth, during the period coinciding with the first appearance of follicle-stimulating hormone (FSH) receptors in ovarian follicular cells [1, 2]. However, the hypothalamo-pituitary-ovarian axis of gilts becomes fully operational only after ~100 days of age, as evidenced by the establishment of ovarian negative feedback on luteinizing hormone (LH) and FSH secretion [2]. Around this time, two morphological types of ovaries can be observed: the *grape type*, characterized by the presence of large antral follicles with a relatively low number of small follicles, and the *honeycomb type*, containing a large number of small follicles and no large antral follicles [3]. During the 20-day study in gilts, in which a diagnostic laparoscopy was performed every 5 days, at least one shift in ovarian morphological type was noted [4]; typically, the *honeycomb type* was observed for ~10 days and the *grape* type was observed for ~5 days. Because the changes in ovarian appearance and follicle numbers tended to occur synchronously, it was suggested that the growth of antral follicles in gilts exhibited a wave-like pattern [4]. In spite of the results of laparoscopic observations in gilts, the existence of an orderly pattern of antral follicle development in sows has generally been refuted [5]. It has been considered that the pig is one of the exceptions to the organized wave-like pattern of folliculogenesis and that antral follicular growth in this species is continuous [5].

Transrectal ovarian ultrasonography was adapted for use in pigs in the 1990s [6, 7]. In recent years, however, its application in research has become more widespread, which has created new possibilities for studying antral follicular dynamics [8]. The period encompassing the activation of the hypothalamo-pituitary-ovarian axis, after the animals have reached ~100 days of age, remains the least studied developmental phase in terms of antral follicular development in gilts. Thus, the purpose of the present study was to employ daily transrectal ultrasonography of ovaries to document the growth pattern of antral follicles in prepubertal gilts between the fourth and fifth month of life. Knowledge surrounding the patterns of ovarian activity in pre- and peripubertal gilts can help ameliorate breeding strategies in commercial operations (e.g., selection of animals with the early onset of puberty and high reproductive potential).

Although the gilts of the present study were age- and weight-matched, there were considerable variations in ovarian activity among individual animals. Over 180 growing antral follicles were identified during the 42-day observation period in 5 gilts, whereas in the 3 remaining gilts, the total number of the follicles was less than 160 (data not shown). The mean duration of the growing phase (3.2 ± 1.0 *vs.* 3.5 ± 1.0 days; first *vs.* second period, respectively; P<0.01) as well as the maximum diameter attained by the largest follicles of waves (5.7 ± 0.6 *vs.* 7.0 ± 0.5 mm; first *vs.* second period, respectively; P<0.05) were both greater in the second 21-day study period compared with the first 21-day study period (Table 1). The total numbers of growing antral follicles per gilt throughout the entire study period and per emerging follicular wave were significantly greater in the second study period compared with the first study period (Table 2).

Statistical analyses revealed significant differences between the peaks and nadirs in the mean daily numbers of identified growing follicles (Fig. 1A–D); during the 42-day study period, there were eight, nine and four peaks in daily follicle numbers for follicles

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Table 1. Characteristics (mean ± SD) of antral follicular development in eight prepubertal gilts that underwent daily transrectal ultrasonography of ovaries during two consecutive 21-day periods between the fourth and fifth month of age

Variable	Period 1	Period 2	Periods 1 and 2
Growth rate (mm/day)	0.95 ± 0.24	0.95 ± 0.22	0.95 ± 0.22
Regression rate (mm/day)	0.91 ± 0.22	0.90 ± 0.22	0.91 ± 0.20
Duration of growing phase (days)	3.2 ± 1.0^{a}	3.5 ± 1.0^{b}	3.4 ± 1.0
Duration of regressing phase (days)	3.4 ± 0.6	3.6 ± 0.9	3.6 ± 0.8
Maximum diameter of the largest follicles of waves (mm)	5.7 ± 0.6^{A}	7.0 ± 0.5^{B}	6.4 ± 0.9

Values with different superscripts differ significantly between periods 1 and 2. ^{ab} P<0.05; ^{AB} P<0.01.

Table 2. Characteristics (mean ± SD) of growing ovarian antral follicles in three different categories and detected in eight prepubertal gilts that underwent daily transrectal ultrasonography of ovaries during two consecutive 21-day periods between 4 and 5 months of age

Variable	Period 1	Period 2	Periods 1 and 2
Follicles growing to ≥4 mm and <5 mm	40.4 ± 11.0^{a}	62.4 ± 14.0^{b}	102.8 ± 14.9
Follicles growing to ≥5 mm and <6 mm	10.8 ± 8.0^{A}	45.2 ± 12.8^{B}	56.0 ± 9.1
Follicles growing to ≥6 mm	1.2 ± 1.8^{A}	15.4 ± 5.3^{B}	16.6 ± 5.4
Total number of growing follicles	52.4 ± 16.2^{A}	123.0 ± 6.7^{B}	175.4 ± 15.2
Follicles growing to ≥4 mm and <5 mm/wave	9.1 ± 3.9^{A}	13.2 ± 6.1^{B}	11.4 ± 5.6
Follicles growing to ≥5 mm and <6 mm/wave	2.5 ± 2.4^{A}	9.2 ± 4.0^{B}	6.2 ± 4.7
Follicles growing to ≥6 mm/wave	0.3 ± 0.8^{A}	3.1 ± 2.5^{B}	1.8 ± 2.4
Total number of growing follicles/wave	11.9 ± 5.3^{A}	25.5 ± 6.0^{B}	19.5 ± 8.8

Values with different superscripts differ significantly between periods 1 and 2. ^{ab} P<0.05; ^{AB} P<0.01.

attaining ≥4 and <5 mm, ≥5 mm and <6 mm and ≥6 mm in diameter, respectively (Fig. 1A–C). In the first study period, daily numbers of follicles attaining a maximum diameter of ≥6 mm were very low; the follicles began to reach this size range on day 13, and their daily numbers increased to levels that allowed for detection of statistically significant differences after day 26 (Fig. 1C). The existence of significant differences in daily numbers of recruited antral follicles (Fig. 1) led to detection of the nine consecutive waves of follicle growth (WI-IX) defined as the groups of follicles growing synchronously to ≥4 mm in diameter before regression (Fig. 1D) (i.e., total number of growing antral follicles). In the first period, there were 4 waves, and during the second period, the emergence of 5 waves was seen. The mean number of follicles in a wave was greater (P<0.01) in the second period compared with the first period (Table 2). The number of follicles that reached ≥4 and <5 mm in size as well as the total number of growing antral follicles increased significantly at the outset of the second 21-day period (Wave V) and then did not vary significantly (P>0.05) until the end of the study (Fig. 2). The number of follicles growing to ≥5 mm and <6 mm in diameter decreased (P<0.05) transiently between Wave I and Wave II, increased (P<0.05) between Wave II and III and again between Wave III and V, and then remained relatively stable (P>0.05) until the end of the study (Fig. 2). Follicles attaining a maximum diameter of ≥6 mm were first detected in Wave III, and their number rose significantly by Wave VII (Fig. 2). Overall, the greatest total number of growing antral follicles was recorded in Wave V (first wave of the second 21-day observation period) and the last two waves of the study period (Waves VIII and IX; Fig. 2).

The tempo of changes in antral follicular development is so rapid that its monitoring requires daily or more frequent, noninvasive observations. Currently, the best available technique to achieve this goal is ultrasonography [6–8]. The present study was conducted during the period encapsulating the physiological “turning point” associated with the activation of the hypothalamo-pituitary-ovarian axis in gilts [2]. Ovarian activity during the first 21-day study period (~4 months of age) was moderate; the number of growing antral follicles was rather small, there were no follicles attaining ≥6 mm in size until approximately 2 weeks into the first observation period, and the maximum follicular diameter rarely exceeded 5 mm. This was most likely due to diminished ovarian responsiveness to gonadotropic stimuli (i.e., low level of FSH receptors in ovarian follicular cells [2]). Alternatively, a shift in the biological activity of FSH, as observed during the prepubertal period in small ruminants [9], may have initiated processes leading to the recruitment of a larger number of antral follicles in peripubertal gilts aged ~5 months.

Fluctuations in daily numbers of growing antral follicles in the gilts of the present study may be interpreted to suggest that cohorts of small antral follicles in prepubertal gilts begin to grow in a synchronized manner, producing recurrent follicular waves. Notably, these shifts in daily follicle numbers were detected in data aligned to the chronological age of animals, suggesting that the emergence of antral follicles in prepubertal gilts is very tightly regulated and age dependent. Interestingly, the main characteristics of antral follicular waves in ultrasonographically monitored gilts were similar to those previously described in sheep and goats; emerging follicles had similar growth and regression rates (0.9–1 mm/day), they grew and

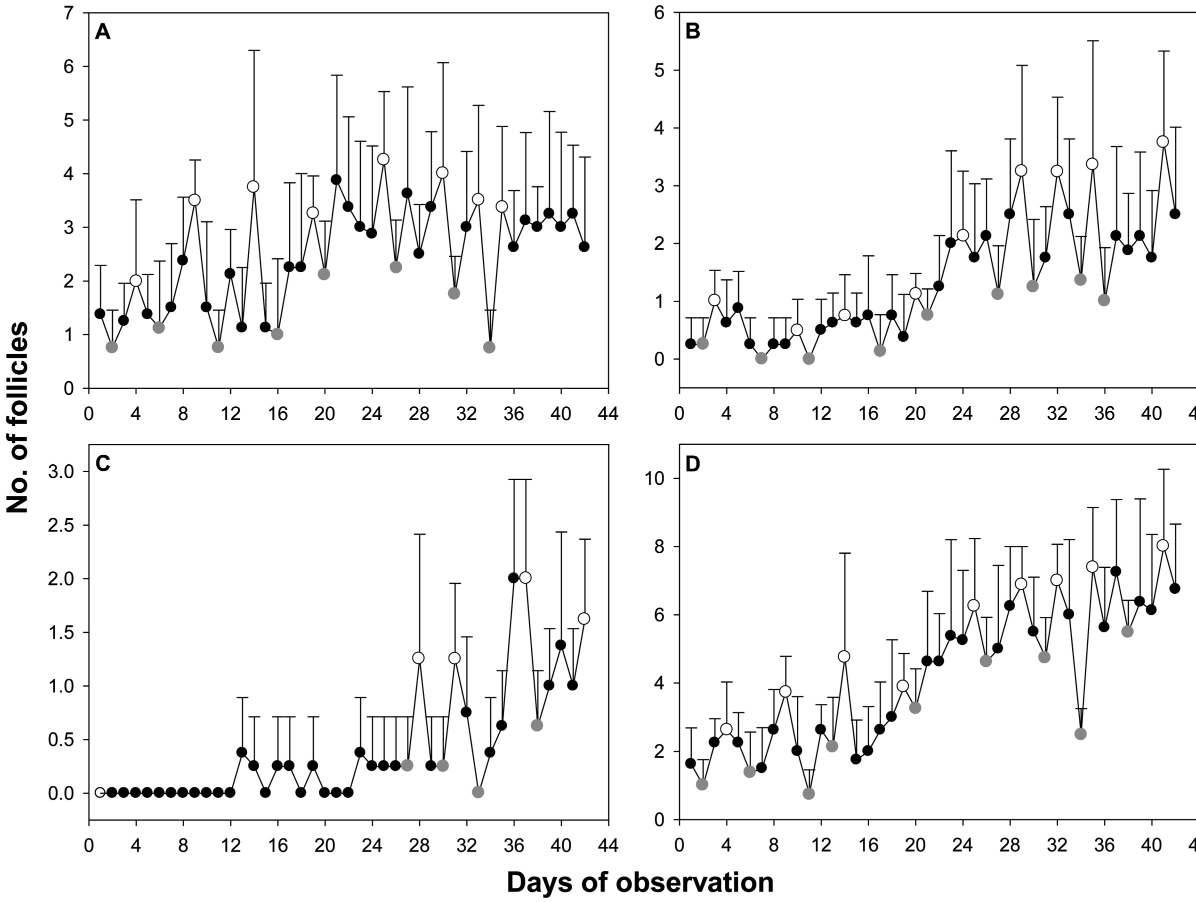

Fig. 1. Mean (± SD) numbers of follicles that started to grow on each day during the 42-day study period in eight gilts examined between the fourth and fifth month of age. Separate panels depict the numbers of follicles growing to (size ranges): (A) ≥4 mm and <5 mm, (B) ≥5 mm and <6 mm, (C) ≥6 mm in diameter before regression and (D) the total number of growing antral follicles. Successive statistically different (P<0.05) nadirs and peaks in mean daily numbers of follicles are denoted by light grey and white circles, respectively.

regressed over the same period of time, and the largest follicles of waves attained similar maximum diameters to those seen in small ruminants [10–16]. However, a major difference in comparison with other species is the total number of growing follicles in gilt ovaries.

The second period of the present study was associated with more than a doubling of the numbers of growing follicles that consistently attained >6 mm in diameter. Numerous studies demonstrated that injections of exogenous gonadotropins stimulated the growth of ovarian antral follicles to ovulatory sizes in prepubertal gilts [17–22]. This level of endogenous stimulation is typically observed just prior to attainment of sexual maturity, before the first pubertal ovulation [17]. However, previous longitudinal studies in prepubescent gilts revealed that circulating concentrations of estradiol higher than the levels observed during the luteal phase of the estrous cycle can be detected very early in life [2, 23]. This would suggest that endogenous stimulation of antral follicular growth and steroidogenesis in gilts can occur long before the attainment of puberty. None of the gilts reached puberty until the end of the present study (5 months of age), but in the second period, follicles with diameters >6 mm were detected in all animals. The appearance of follicles with larger diameters did not disrupt the wave-like nature of antral follicle recruitment, suggesting that follicular dominance is absent in prepubertal gilts [23–25]. In the second period, approximately three follicles per wave reached a diameter of >6 mm, and the number of these follicles increased with the age of gilts; in the last two waves, approximately five follicles ≥6 mm in diameter per wave were detected.

The rate of antral follicular growth and regression did not differ throughout the study. Regardless of the degree of maturity of the reproductive organs and varying levels of gonadotropic stimulation in prepubescent gilts, the rate of antral follicle turnover appears to remain constant. Similar observations were made in different physiological states in small ruminants; the rate of follicular growth and atresia was consistent throughout the breeding season [10–12, 16], seasonal anestrus [26, 27] and early pregnancy [28, 29].

To recapitulate, the growth of ovarian antral follicles reaching a diameter of ≥4 mm in gilts aged 4–5 months exhibits a distinct wave-like pattern. A rise in the number and maximum diameter of emerging antral follicles suggests the occurrence of a gradual increase

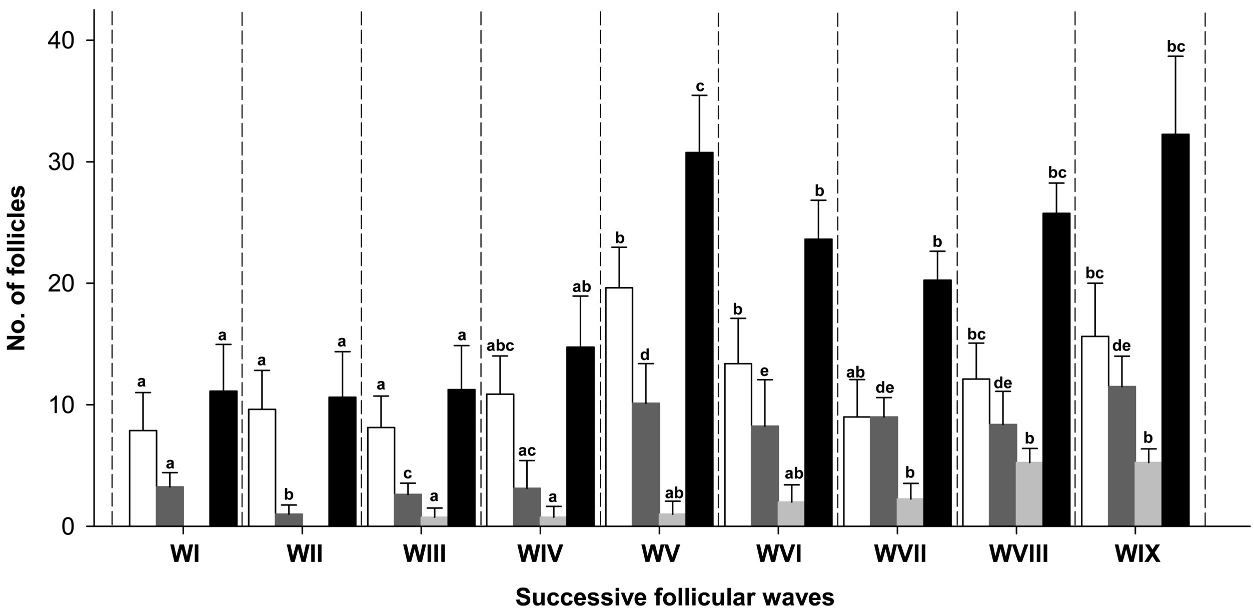

Fig. 2. Mean (± SD) numbers of identified growing follicles in consecutive waves (WI-IX) detected during the entire study period. Within each follicle size category, values denoted by different letters vary significantly ([a-e] P<0.05). Follicles growing to ≥4 mm and <5 mm (white), ≥5 mm and <6 mm (grey) and ≥6 mm (light grey) before regression, and the total number of growing antral follicles (black).

in the activity of the hypothalamo-pituitary-ovarian axis during that period. The rate of growth and regression of antral follicles ≥4 mm in diameter seems to be independent of the maximum diameter achieved by these follicles or the age of gilts. Approximately five antral follicles with a diameter of ≥6 mm growing in a single wave are insufficient to induce estrus and preovulatory LH discharge in gilts. The present observations warrant further studies of the pattern and endocrine control of follicular wave emergence in pre- and peripubertal gilts and sexually mature pigs. The use of novel approaches to ovarian imaging and data analyses may help procure evidence showing that the pig is indeed a species exhibiting the wave-like pattern of ovarian follicular dynamics.

Methods

Experimental animals

All experimental procedures used were in compliance with the EC guidelines for animal experimentation and had been approved by the local animal care committee. The present study utilized 8 prepubertal cross-bred gilts of the F1 generation (Polish Large White × Polish Landrace) housed at the Experimental Station of the Department of Swine and Small Ruminant Breeding, University of Agriculture, Cracow, Poland (longitude 19°57'E, latitude 50°04'N). On the first day of the 42-day study period, the average age of the gilts was 109.0 ± 4.1 days, and the average weight was 55.8 ± 8.1 kg (mean ± SD). On the last day the study, the average weight of the gilts was 78.8 ± 6.4 kg. The animals were kept under natural conditions of light and ambient temperature, in a pen with shallow bedding and unlimited access to an extensive outdoor range. Twice a day, gilts were fed a complete ration adjusted to their age and supplied by Provimi Polska (Provimi Polska, Warsaw, Poland).

Ultrasonographic imaging

Daily transrectal ultrasonography of ovaries was performed with the Aloka ProSound 2 scanner (Hitachi Aloka Medical, Tokyo, Japan) connected to a stiffened, 7.5-MHz linear array transducer that had been adapted and validated for rectal examinations in gilts [6–8]. Before the beginning of ultrasonographic examinations, the gilts were habituated to the procedure for 20 days, during which time they were subjected to short transrectal examinations without recording the results. Daily ovarian ultrasonography was performed for a total of 42 days in June and July; the duration of the present study was chosen to monitor the ovarian activity in prepubertal gilts over the time corresponding to approximately two consecutive estrous cycles of sexually mature pigs. During scanning, gilts were restrained in a specially constructed pen, which permitted immobilization of animals with minimal stress.

Sequential still images of the ovary (Fig. 3) were captured after having scanned the entire organ. The scanning loop of the Aloka PS2 spans approximately 20 sec, and 450 sequential frames are saved during that time. About 20 frames per ovary, from different regions of the gonad, were collected for analyses; an attempt was made to maintain equal spatiotemporal intervals between consecutive frames. A typical ovary of the prepubertal gilt is about 2.5–3 cm (length) × 1.5–2 cm (width). Therefore, the two consecutive frames captured ovarian cross sections separated by ~1.5 mm, which permitted the retrospective detection and enumeration of follicles ≥2 mm in diameter. The diameters of all visible follicles were measured using built-in electronic calipers to the nearest 0.1 mm. The positions of all identified follicles were determined using points of reference, which

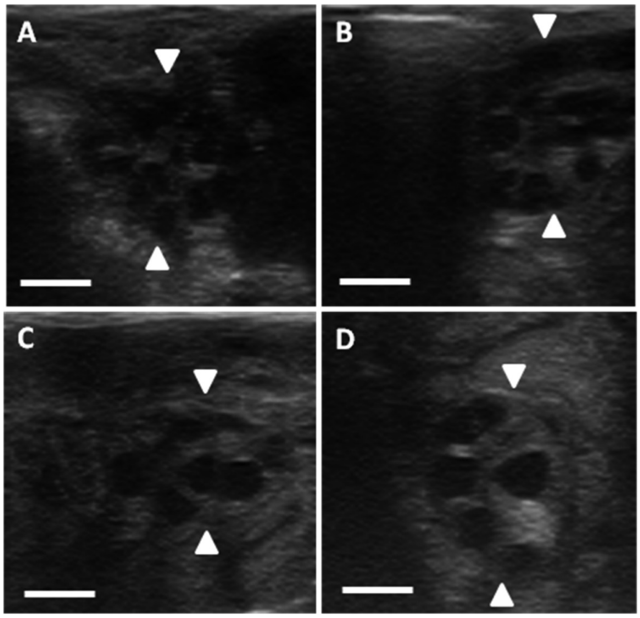

Fig. 3. Photographic reproductions of the ultrasonograms depicting porcine ovaries and obtained with a stiffened 7.5-MHz linear-array transducer connected to a B-mode scanner. (A) A group of small (2–3 mm) antral follicles, (B) an ovary containing follicles that attained 4 mm in diameter, (C) an ovary with the largest follicles that grew to 5 mm in diameter and (D) ovarian follicles of 6 mm in size. White arrowheads delineate the boundaries of the ovary. Scale bars in the bottom left corner represent 10 mm.

were, in the absence of corpora lutea, follicles ≥4 mm in diameter (first 21-day period) or ≥5 mm in diameter (second period).

Data analyses

The growth curves of individual follicles that reached a diameter of ≥4 mm before regression were determined using the methods described for sheep and goats by Ginther *et al.* [10] and Ginther and Kot [11], respectively, and by Jaiswal *et al.* [30] for bovine ovaries. The day of follicular recruitment (or emergence) was defined as the day on which a follicle that attained ≥4 mm in diameter could be retrospectively identified in ovarian images and sketches at its smallest diameter (2–3 mm). The duration of the growth phase was defined as the period taken by such follicles to grow to their maximum size, and the duration of the regressing phase was regarded as the period during which the identified follicles regressed to their smallest identifiable size. The rate of growth/regression was defined as the mean daily increase/decline in antrum diameter.

Ovarian data were analyzed on a per animal basis, with the observations for both ovaries combined. The average growth and regression rates as well as mean numbers of growing antral follicles in three size categories (attaining ≥4 mm and <5mm, ≥5 mm and <6 mm, and ≥6 mm in diameter before regression) that began to grow on each day of the study period were calculated for each gilt. On the basis of significant differences in the daily numbers of identified growing follicles, the distinct periods of intensified antral follicular growth (i.e., follicular waves) were detected in the gilts of the present study. The groups of follicles that grew from 2–3 mm to ≥4 mm in size were regarded as waves of ovarian follicular growth; ovarian antral follicles that emerged during a maximum period of 3 days were included in a wave. For all follicular waves, the mean number of growing follicles in the three size categories was calculated.

Statistical analyses utilized the following tests: 1) General Linear Model (GLM) procedures in repeated-measures (RM) analysis of variance (ANOVA) for analysis of daily numbers of identified growing follicles, 2) one-way ANOVA for analysis of the numbers of follicles in different size categories in each wave (the Duncan test was used as a post-ANOVA test for comparisons of individual mean values), 3) the Student's *t*-test for independent groups to compare the rate and duration of follicular growth and regression phases as well as maximum diameters of the largest follicles of waves between the two study periods and 4) the paired Student's *t*-test to compare the total and daily numbers of recruited follicles between the two study periods.

During the 42 days of observation, there were seven days on which one of the ovaries could not be detected with ultrasonography. In such instances, the diameters of growing follicles were determined using the measurements obtained at 2-day intervals. Images of 22 ovaries were "blurred" (i.e., the boundaries of antral follicles could not be easily delineated), which made the measurement and identification of small antral follicles difficult. If on the next day, one or more new follicles ≥4 mm in size were identified in those ovaries, the approximate diameter of such follicles on the previous day was calculated using the mean growth rate of follicles. Follicles ≥5 mm in size could be identified even in the blurred images.

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