

# Endocrinology of increased ovarian folliculogenesis in cattle selected for twin births<sup>1,2</sup>

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## Abstract

Long-term genetic selection in cattle, using an index of ovulation and twinning rate (Twinner), has increased the frequency of twin births to > 50% as a result of enhanced ovarian follicular development. Ovaries of Twinner females had a twofold greater density of secondary preantral follicles, 50% more small ( $\leq 5$  mm) and medium (6 to 12 mm) surface antral follicles, and a > 70% frequency of twin or multiple ovulatory follicles than did unselected (Control) females. Density of primordial and of primary follicles did not differ between genetic populations. Blood FSH concentrations at proestrus and estrus, after GnRH (25 or 150  $\mu$ g) treatment, or after follicle ablation did not differ between females in the Control and Twinner populations. Blood LH concentrations did not differ between populations at proestrus and estrus or after follicle ablation, but LH release after both doses of GnRH was increased in Twinners. Follicular fluid concentrations of estradiol and progesterone did not differ between dominant follicles from cattle selected and unselected for twinning, whereas blood concentrations of estradiol and progesterone were related positively to the number of functional dominant follicles and corpora lutea, respectively, on the ovaries. The IGF-I concentrations were consistently greater in blood and follicular fluid of Twinner cows. Aspiration of all follicles  $\geq 5$  mm in Twinner and Control cyclic cows initiated a transient increase in FSH, but not LH, followed by increased numbers of growing follicles and selection of twin or triplet dominant follicles in Twinners; however, the FSH response did not differ between Twinners and Controls. Circulating concentrations of GH and insulin did not differ between the two cattle populations. However, blood cholesterol was greater in Controls. Increased follicular recruitment and selection of twin dominant or ovulatory follicles in cattle selected for dizygotic twinning are likely associated with increased intraovarian and(or) systemic IGF-I production. Conversely, the increased ovarian folliculogenesis in Twinner females is not accompanied by changes in gonadotropin secretion.

*Key Words: Cattle, Twinning, Ovulation Rate, Gonadotropins, Insulin-like Growth Factor*

## Introduction

Cattle are primarily monovulatory, and the frequency of dizygotic (fraternal) twin births ranges from < 1% for British breeds to 2 to 4% for Continental breeds and > 4% for dairy breeds (Rutledge, 1975). About 10% of the twin births are monozygotic. A research project was initiated 20 yr ago at the U. S. Meat Animal Research Center (MARC) to evaluate the feasibility of increasing the incidence of twin births in cattle through genetic selection (Gregory et al., 1990). The foundation herd was composed of 307 cows (96 from private herds and 211 from research herds at MARC) with a history of twin births.

Because twin ovulations are the first prerequisite for dizygotic twins, the selection criterion was expanded in 1984 to include measurement of ovulation rate for 8 to 10 estrous cycles in all female progeny starting at 12 mo of age (Echternkamp et al., 1990a). Use of ovulation rate as a predictor of twinning rate also reduced the generation interval and the necessity to retain all females for several parturitions to evaluate twinning rate. Potential herd sires were progeny-tested by obtaining ovulation information from 10 to 12 daughters/sire. A multiple-trait repeated records model was implemented in 1990 to predict breeding value for twinning rate for each individual animal in the herd; the procedure combines information for the individual and all relatives for ovulation and twinning rate (Gregory et al., 1997). Twinning rate increased linearly from 4% in 1984 to 35% in 1996. In Proceedings of the American Society of Animal Science, 1999

1997, herd size was reduced from 750 to 250 calving cows annually. Culling cows with the lowest estimated breeding value increased annual twinning rate from 35 to > 50%. The purpose of this paper is to describe changes in ovarian follicular development and in circulating hormone concentrations associated with the selection of cattle for twin ovulations and dizygotic twin births.

## Ovarian Follicular Development in Twin-Producing Cattle

**Distribution of Twin Ovulations.** The first prerequisite for dizygotic twins is the occurrence of twin or multiple ovulations. Table 1 reports the distribution of ovulations (i.e., corpora lutea [CL]) between the right and left ovary in yearling Twinner heifers evaluated from 1994 to 1999. Just as the right ovary had a higher ( $P < 0.01$ ) frequency of ovulations than the left ovary in estrous cycles with a single ovulation (Table 1), the right ovary also had a higher frequency of twin ovulations than the left ovary. Thus, the distribution of twin ovulations between the two ovaries deviated ( $P < .01$ ) from the theoretical 1:1:2 ratio (Table 1). Because migration of bovine embryos between uterine horns is rare, distribution of twin embryos between the left and right uterine horns is similar to the distribution of CL between the left and right ovaries (Echternkamp et al., 1990a).

Table 2 reports the change in twinning rate from 1984 to 1996 as a result of using a selection criterion that included  
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both ovulation and twinning rate. The linear increase in twinning rate was 2.7 percentage points per year. The increase in annual twinning rate from 35 to > 50% resulted from a reduction in herd size in 1997 from 750 to 250 calving cows per year, retaining cows with the highest estimated breeding value for twinning.

**Ovarian Follicular Waves.** The existence of waves of ovarian follicular development during the bovine estrous cycle was first proposed by Rajakoski (1960). Since the confirmation of ovarian follicular waves by ultrasonography (Sirois and Fortune, 1988; Ginther et al., 1989), the characteristics and regulation of these follicular waves have been investigated extensively. Such follicular waves are characterized by recruitment of a cohort of growing follicles from which one follicle is selected to become the dominant or ovulatory follicle, and the other follicles in the cohort become atretic and regress. Daily ultrasonographic monitoring of follicular development during the estrous cycle revealed that Twinner cows can exhibit either two- or three-wave estrous cycles. In addition, comparisons of follicular patterns between Twinners and Controls suggest that follicular waves of Twinner cows recruit more follicles into the cohort of growing medium-sized follicles and select two or multiple dominant (ovulatory) follicles. Patterns of follicular development for a two-wave estrous cycle in a Control cow and a Twinner cow are contrasted in Figure 1A and 1B, respectively. Growth and regression of individual follicles are represented by a single line. Ultrasound monitoring began on d 3 or 4 of the estrous cycle (estrus = d 0) and continued through the subsequent estrus. The pattern in the Control cow (Panel A) was characterized by formation of a dominant and a subordinate follicle on the right ovary (right panel) in both waves and a single ovulation at the end of the second wave. In the Twinner cow (Panel B), two or three dominant follicles formed on the left ovary (left panel) in the first wave and two ovulatory follicles formed on the right ovary in the second wave to yield twin ovulations. Figure 1C illustrates a three-wave estrous cycle with twin ovulations. Multiple large follicles formed on both ovaries in the first wave; two large follicles on the right ovary in the second wave; two ovulatory follicles (twin ovulations) on the right ovary in the third wave; and then a dominant follicle formed on each ovary in the first wave of the new estrous cycle.

Few Twinner cows express exclusively twin ovulations. In 1998, means for ovulation rate in mature cows in the spring- and fall-breeding Twinner herds were 1.75 and 1.78, respectively. Figure 1D illustrates a two-wave cycle in a Twinner cow with the formation of multiple large follicles in the first wave but, in the second wave, one of the two large follicles became the ovulatory follicle and the other regressed to become the subordinate follicle. Two dominant follicles and one subordinate follicle formed in the first wave of the subsequent cycle.

Table 3 provides comparisons of follicular wave length and of diameter of the two largest follicles between Twinner cows with twin vs single ovulations and two- vs three-wave cycles. For cows with twin ovulations in a two-wave cycle, the interval from estrus to maximal diameter of the dominant

follicle was 8.5 d for the first wave and 20.0 d for the second wave, intervals similar to those for a single ovulation. The three-wave estrous cycle had a longer first follicular wave, which increased the overall cycle length. Diameter of the largest and second-largest follicle (Table 3) did not differ in cows with twin ovulations, although the second ovulatory follicle is sometimes smaller. Variation in size of the ovulatory follicles may also account for observed variation in CL size (mass) between twin CL. Because the single ovulators were also from the twinner population, the two largest follicles of the first wave did not differ in diameter, but the subordinate follicle in the second wave was smaller ( $P \leq .01$ ) than the ovulatory follicle.

**Ovarian Follicle Numbers.** Comparisons of number of surface antral follicles present on ovaries collected during proestrus further confirmed enhanced follicular development in cattle selected for twinning (Echternkamp et al., 1990a; Echternkamp and Gregory, 1998). In Figure 2, ovaries were collected from Twinner and contemporary Control cows injected with PGF<sub>2 $\alpha$</sub>  (Lutalyse, Pharmacia & Upjohn, Kalamazoo, MI) on d 17 of the estrous cycle and slaughtered at 0, 24, 48, or 72 h after PGF<sub>2 $\alpha$</sub> . Visible antral follicles were grouped into three size categories: small,  $\leq 5$  mm; medium, 6 to 12 mm; and large,  $> 12$  mm. Ovaries of the Twinner cows ( $n = 24$  cows) had more antral follicles (Figure 2) than ovaries from Control cows ( $n = 24$  cows) in all three size categories: small ( $P \leq .01$ ), medium ( $P \leq .05$ ), and large ( $P \leq .01$ ). The distribution of follicles was not affected ( $P \geq .1$ ) by time after PGF<sub>2 $\alpha$</sub> . Paired ovarian weight without CL (22.7 vs 16.1 g; SEM = 1.2) and ovulation rate (1.5 vs 1.1 CL; SEM = .1) were also greater ( $P \leq .01$ ) for the Twinner than for the Control group (Echternkamp and Gregory, 1998).

A recent histological evaluation of microscopic follicular development in ovaries from Twinner and Control cows (Cushman et al., 2000) suggested that the greater number of antral follicles in Twinner cows resulted from maintenance of a larger ( $P \leq .05$ ) number of growing follicles at the secondary stage of preantral development (Table 4). Reported means are the total number of follicles found in 200 fields ( $2 \times 2$  mm) from one ovary of each cow. Densities of primordial and of primary follicles were not different ( $P > 0.1$ ) between the two genetic populations, whereas the number of tertiary follicles within the 200 fields was too small to assess population differences. Classification criteria for the microscopic follicles were as follows: primordial (oocyte surrounded by a single layer of pregranulosa cells), primary (oocyte surrounded by a single layer of granulosa cells), secondary (oocyte surrounded by two or more layers of granulosa cells), or tertiary follicles (oocyte surrounded by multiple layers of granulosa cells with initiation of antrum formation to  $\leq 1$  mm in diameter). Means for the number of antral follicles present on the surface of both ovaries of the same cows are reported in Table 5. Ovaries of the Twinner cows also tended ( $P \leq .1$ ) to have more small antral follicles and a higher incidence of twin ovulations (i.e., CL). In this study, the ovaries were collected on d 6 or 7 of the estrous cycle, rather than at proestrus as in the previous studies (Figure 2), which would

account for the smaller number of medium and large follicles in the latter study (Table 5).

The natural progression of ovarian follicular development in cattle begins with the recruitment of resting primordial follicles, with their dormant oocyte, into a pool of actively growing follicles. The single layer of flattened, squamous-shaped granulosa cells surrounding the oocyte in the primordial follicle is transformed into a single layer of cuboidal granulosa cells in the primary follicle. Proliferation of these cuboidal granulosa cells yields the secondary follicle with its oocyte surrounded by multiple layers of granulosa cells. The mechanism(s) by which a pool of primordial follicles is triggered to initiate transformation into primary follicles and subsequently into secondary follicles is unknown. Also, it is unknown whether activation occurs by stimulation of the primordial follicles to initiate growth, or by the release of primordial follicles from inhibitory stimuli that maintain them in a quiescent state. Early folliculogenesis is likely initiated by the oocyte and maintained by a combination of intra- and extraovarian factors. For example, activation of the primordial follicle in immature mice is reportedly initiated by an activin-mediated, follicle-organizing factor produced by the oocyte (Li and Mather, 1997), whereas activin is reported to inhibit *in vitro* development of preantral follicles from mature mice (Mizunuma et al., 1999). Inhibition of primordial follicle development is also the speculated role for the presence of anti-mullerian hormone (AMH), another member of the transforming growth factor- $\beta$  (TGF- $\beta$ )-activin family, in ovarian granulosa cells of adult mammalian females. For example, ovaries of AMH null mice show a relatively early depletion of their stock of primordial follicles compared to wild-type female mice (Durlinger et al., 1999). Another member of the TGF- $\beta$  superfamily of growth and differentiating factors that has been associated with early preantral follicular development is growth differentiating factor-9 (GDF-9). Transcription of GDF-9 mRNA has been found in both bovine and ovine oocytes at the primordial stage (Bodensteiner et al., 1999), whereas transcription of GDF-9 mRNA does not appear in human and murine ovarian follicles until the primary stage (Fitzpatrick et al., 1998). Likewise, the GDF-9 protein is present in the growing oocyte of primary and secondary, but not primordial, preantral follicles (Hayashi et al., 1999). Apparently GDF-9 is required for early ovarian folliculogenesis in mice; disruption of the GDF-9 gene (i.e., deletion of exon 2) resulted in arrest of ovarian follicles at the primary stage, abnormal organization of the single layer of granulosa cells, no theca cell components, and impaired oocyte development (Dong et al., 1996). Treatment of cultured early preantral follicles from ovaries of immature rats with recombinant GDF-9 enhanced growth and differentiation of the follicles (Hayashi et al., 1999).

Androgens and IGF-I have also been implicated in the regulation of the early stages of follicular growth and development. Treatment of female rhesus monkeys with testosterone or dihydrotestosterone for 3 to 10 d enhanced the transition of primordial follicles to primary follicles, which was accompanied by an increase in IGF-I and IGF-I receptor mRNA, suggesting that androgens promote initiation of pri-

mordial follicle growth in the primate ovary and implicating a role for oocyte-derived IGF-I in this activation process (Vendola et al., 1999). In contrast, Wandji et al. (1992) reported low IGF-I binding in the granulosa and theca cells of preantral bovine follicles but a significant increase in granulosa IGF-I binding with antral formation. Treatment of farm animals with GH increases systemic IGF-I concentrations, and the chronic treatment of swine (Spicer et al., 1992; Echternkamp et al., 1994), cattle (Gong et al., 1993), or sheep (Gong et al., 1996) with pST or bST increases the number of small and/or medium antral follicles. Because GH receptor mRNA has been detected in membrana granulosa and oocytes of both preantral and small antral ovine ovarian follicles (Eckery et al., 1997), some GH effects on ovarian follicular development may be direct rather than mediated through increased IGF-I secretion. The role of IGF-I, GDF-9, activin, AMH, and/or androgens in the initiation of ovarian folliculogenesis and their contribution to increased numbers of secondary preantral follicles and of antral follicles in Twinner cows is being further evaluated. Current observations do indicate elevated IGF-I, but not GH, concentrations in the blood and ovarian follicular fluid of Twinner females.

Although bovine (Wandji et al., 1992) and ovine (Eckery et al., 1997) preantral follicles contain gonadotropin receptors, the role of gonadotropins (i.e., FSH and LH) in early ovarian follicular development seems to be permissive rather than regulatory; for example, ovarian follicles undergo early stages of development in mice (Kumar et al., 1998) and humans (Aittomaki et al., 1995) with genetic deficiencies in FSH or the FSH receptor. Similarly, bovine primordial follicles initiate and maintain growth in culture in a serum-free medium without gonadotropins (Braw-Tal and Yossefi, 1997). However, with the initiation of antrum formation, growth and maturation of ovarian follicles do become gonadotropin-dependent, with the primary effect being prevention of apoptosis in small and medium-size antral follicles by FSH. Differences in FSH and LH secretion have not been detected between bovine females selected and unselected for twin ovulations and dizygotic twins (Figure 3). Previous studies have shown that both *in vivo* and *in vitro* treatment of follicular cells with IGF-I increases granulosa and theca cell proliferation, increases FSH and LH receptor expression, and enhances steroidogenesis by granulosa and theca cells (see review by Spicer and Echternkamp, 1995). Thus, the increased intraovarian IGF-I concentrations in Twinner females may enhance gonadotropic stimulation of folliculogenesis.

### Evaluation of Endocrine Profiles Between Cattle Selected and Unselected for Twin Births

The induction of ovarian follicular development and of twin or multiple births in cattle with exogenous gonadotropins (e.g., FSH, PMSG, human menopausal gonadotropin, and LH) suggested the possibility that cattle producing dizygotic twins may have greater gonadotropin production. Because of the low twinning frequency, the relationship be-

tween gonadotropin secretion and ovulation rate has not been evaluated in cattle but has been studied extensively in comparisons between high- and low-prolificacy genetic lines of sheep. Unfortunately, many of the results from the ovine comparisons are either contradictory or inconclusive.

**Preovulatory FSH and LH Release.** As illustrated in Figure 3, differences in blood FSH concentrations (Panel A) were not detected ( $P > .1$ ) between cows with (Twinner) and without (Control) a history of twinning for either the preovulatory FSH release or the postovulatory FSH release. Similarly, magnitude of the preovulatory LH release (Figure 3, Panel B) did not differ ( $P > .1$ ) between cows in the two populations. Blood samples were collected at 4-h intervals starting on d 18 of the estrous cycle and continuing until d 3 after onset of estrus. Data were standardized among cows to the peak LH concentration. Similarly, comparisons of the preovulatory LH surge among high- and low-prolificacy Finnsheep and Galway ewes (Adams et al., 1988) or between Booroola and noncarrier Merino ewes (Bindon et al., 1984) revealed no differences in the preovulatory LH release among the genetic lines, except possibly a longer interval from onset of estrus to LH peak in prolific ewes (Bindon et al., 1979). In contrast, magnitudes of the preovulatory and secondary FSH surges between low- and high-prolificacy populations of sheep range from significantly smaller preovulatory and secondary FSH surges in prolific Finnsheep ewes (Adams et al., 1988), to increased (McNatty et al., 1987) or unaffected (Bindon and Piper, 1986) FSH secretion in Booroola vs noncarrier Merino ewes, to a greater secondary FSH surge in prolific Romanov ewes (Cahill et al., 1981), to greater preovulatory and secondary FSH surges in high-prolificacy D'Man (Lahlou-Kassi et al., 1984) and in low-prolificacy Ile-de-France ewes (Bindon et al., 1979). Pituitary content of FSH is greater in Booroola than in noncarrier Merino ewes (Robertson et al., 1984; McNatty et al., 1987).

**FSH and LH Response to GnRH.** The FSH and LH responses to either a low (.033  $\mu\text{g GnRH/kg BW}$ ) or high (.22  $\mu\text{g GnRH/kg BW}$ ) dosage of GnRH were compared between Twinner and Control cows ( $n = 20$  cows / population) in a switch-back experimental design (Figure 4). The single i.m. injection of GnRH was administered 36 h after an injection of PGF<sub>2 $\alpha$</sub>  on d 12 to 16 of the estrous cycle. Blood samples were collected at 15-min intervals for 4 h before and after the injection of GnRH. Magnitude of both the FSH (Figure 4, Panel A) and LH (Figure 4, Panel B) release was greater ( $P < .01$ ) with the larger GnRH dosage as measured by either peak amplitude or total area under the response curve. However, the FSH release did not differ ( $P > .1$ ) between the two cattle populations. Conversely, Twinner cows released more ( $P < .05$ ) LH to both dosages of GnRH. The increased LH response in Twinner cows may have been influenced by pretreatment estrogen priming, because the Twinners tended to have higher circulating estradiol concentrations (Table 6). Pretreatment FSH, LH, and progesterone concentrations did not differ ( $P > .1$ ) between the two populations. Similarly, FSH release to GnRH did not differ between Booroola and noncarrier Merino ewes, whereas the LH response was lower in the prolific Booroola ewes (McNatty et al., 1987). In sup-

port of a positive LH effect on ovulation rate, the increased ovulation rate in sheep actively immunized against androstenedione coincides with an increase in blood LH and progesterone concentrations; blood FSH concentrations are unaffected by androstenedione immunization (Martensz and Scaramuzzi, 1979).

**Circulating Concentrations of Ovarian Steroids.** Comparisons of plasma steroid concentrations between Twinner and Control cows administered PGF<sub>2 $\alpha$</sub>  on d 18 of the estrous cycle and daily blood samples collected at 0, 24, 48, and 72 h after PGF<sub>2 $\alpha$</sub>  revealed no difference ( $P > .1$ ) in plasma progesterone concentrations during the period of luteolysis (Figure 5, Panel A). In contrast, plasma estradiol concentrations (Figure 5, Panel B) were higher ( $P < .01$ ) in the Twinner cows at 0, 24, and 48 h after PGF<sub>2 $\alpha$</sub> , but several Twinner cows expressed estrus by 72 h after PGF<sub>2 $\alpha$</sub> , thus the significant decline in estradiol at 72 h (population  $\times$  time,  $P < .01$ ). The earlier expression of estrus in Twinner cows was also reflected in the elevated FSH ( $P < .05$ ) and LH ( $P < .05$ ) concentrations in Twinner cows at 72 h (Figure 5, Panel C and D, respectively). Otherwise, plasma FSH and LH concentrations were similar ( $P > .1$ ) between cattle populations when samples composing the preovulatory surge were excluded from the statistical analysis.

**Follicular Fluid Estradiol and Progesterone Concentrations.** Table 7 contains a comparison of estradiol concentrations in follicular fluid of follicles collected from cattle that produced single vs twin births. The ovaries were collected at slaughter 44 h after PGF<sub>2 $\alpha$</sub> . Based on diameter, individual follicles were assigned to one of four size categories:  $< 4$  mm (small follicle pool), 4 to 7.9 mm, 8 to 11.9 mm, or  $\geq 12$  mm. Follicular fluid estradiol concentrations were similar between the two cattle populations for the small follicle pool and for the large ovulatory follicles, whereas estradiol concentrations in the intermediate-sized follicles were more variable. For the single-birth cows, the 8- to 11.9-mm follicles were primarily subordinate follicles and, thus, were atretic, as indicated by the higher ( $P < .05$ ) follicular fluid progesterone concentrations (Table 7). Conversely, the 8- to 11.9-mm follicles from Twinner cows were primarily healthy follicles and had estradiol concentrations similar to those in the large follicles. Comparisons of ovarian follicular differentiation between high- (e.g., Finnsheep or Booroola) and low-prolificacy sheep breeds indicated an extended selection period for ovulatory follicles and ovulatory follicles of smaller or more variable size in the prolific ewes (Bindon and Piper, 1986; Driancourt et al., 1986). Frequently, the two ovulatory follicles in Twinner cows differ in their diameter.

In another study, ovaries were collected from Twinner and Control cows between d 3 and 7 of the estrous cycle and the three largest follicles (i.e., order 1 = the largest follicle) were measured and follicular fluid was collected and assayed individually (Table 8). Again, the second-largest follicle of Twinners (Table 8) was larger ( $P < .01$ ) in diameter and had greater ( $P < .01$ ) follicular fluid estradiol concentrations, suggesting the presence of two dominant follicles in cows from the Twinner population vs one dominant follicle in the contemporary Controls. Collection of the follicles during the

midluteal phase of the estrous cycle accounted for the lower follicular fluid estradiol concentrations in the latter study. Collectively, these results suggest that the greater circulating concentrations of estradiol in Twinners are influenced by the number of functional dominant or ovulatory follicles, but estradiol production by individual follicles is similar between cattle populations selected and unselected for twinning. Similarly, follicular fluid estradiol concentrations do not differ between low- and high-prolificacy populations of sheep, although there is a trend for ovulatory follicles of high-prolificacy ewes to have fewer granulosa cells and, thus, increased estrogen production per cell. Follicular cell numbers have not been compared between Twinner and Control cows. Follicular fluid progesterone concentrations (Table 7) for follicles in the same developmental status (i.e., estrogenic status) did not differ between the two cattle populations.

**Gonadotropin Response to Follicle Aspiration.** Removal of all ovarian follicles  $\geq 5$  mm in diameter within both ovaries will initiate a new follicular wave and the subsequent selection of a new dominant follicle(s). Thus, the transvaginal follicle aspiration experimental model was used to compare follicular recruitment and selection between cows from the Twinner vs contemporary beef cattle populations. Follicular response to follicle aspiration in individual cows from the Control and Twinner populations is illustrated in Figure 6. Emergence of a new follicular wave during a natural bovine estrous cycle is preceded by a transient increase in circulating FSH concentrations (Adams et al., 1992). Aspiration of all follicles  $\geq 5$  mm in diameter from both ovaries on d 7 or 8 of the estrous cycle (estrus = d 0) also resulted in a significant ( $P \leq .01$ ) increase in circulating FSH concentrations (Figure 7, Panel A) within 6 h after aspiration; the greatest mean for FSH concentration occurred at 30 h and then returned to baseline by 72 h after aspiration. As with previous FSH comparisons at proestrus and estrus (Figures 3 and 4), blood FSH concentrations did not differ ( $P > .1$ ) between cows in the Twinner and Control populations. In contrast to FSH, plasma LH concentrations (Figure 7, Panel B) were not influenced ( $P > .1$ ) by the aspiration procedure but did decrease ( $P < .01$ ) with time after aspiration. Likewise, plasma LH concentrations were not influenced ( $P > .1$ ) by twinning genetics. Similarly, plasma progesterone concentrations (Figure 8) did not differ ( $P > .1$ ) between the two populations. However, when cows were grouped by number of CL on the ovaries, plasma progesterone concentrations increased ( $P < .05$ ) with number of functional CL (Figure 8 insert). The follicular aspiration procedure had no effect ( $P > .1$ ) on plasma progesterone concentrations (Figure 8), although progesterone concentrations did increase ( $P < .05$ ) with advancement of the estrous cycle.

**IGF-I and GH Concentrations.** Comparisons of IGF-I concentrations between cattle selected and unselected for twinning (Echternkamp et al., 1990b) have indicated greater concentrations of IGF-I in both blood and follicular fluid of females from the Twinner population (Figure 9; Table 9). Greater ( $P \leq .01$ ) IGF-I concentrations were also found in the blood of the Twinner cows used in the aspiration study (Fig-

ure 7, Panel C). In addition, an increase ( $P \leq .05$ ) in plasma IGF-I concentrations occurred at 24 h and IGF-I remained elevated through 54 h after aspiration of the medium and large ovarian follicles.

The elevated IGF-I concentrations in cattle selected for dizygotic twins concur with results from in vitro studies reporting that the effects of IGF-I on ovarian function include stimulation of granulosa and thecal cell proliferation, increased numbers of FSH and LH receptors, and enhanced steroidogenesis by granulosa and thecal cells (Spicer and Echternkamp, 1995). In addition, the chronic treatment of swine (Spicer et al., 1992; Echternkamp et al., 1994), cattle (Gong et al., 1993), or sheep (Gong et al., 1996) with pST or bST increases the number of small and/or medium antral follicles. Also, there are several reports of a small increase in the incidence of dizygotic twins among dairy cows treated with bST to increase milk production (Cole et al., 1991).

The source of the increased circulating concentrations of IGF-I in Twinner cows has not been identified. Although ovarian follicular fluid of Twinner cows also had greater IGF-I concentrations (Echternkamp et al., 1990b), it is unlikely that the ovaries are a major contributor to systemic IGF-I concentrations relative to the mass of and potential production by liver and muscle. However, a comparison of IGF-I concentrations in blood collected from the abdominal aorta, portal vein, and hepatic vein of three ovariectomized ewes (Figure 10) revealed no differences in IGF-I concentrations among the vessels (Freetly and Echternkamp, unpublished data), suggesting that the liver was not a major contributor to basal IGF-I production in sheep. Profiles for measured metabolites confirmed normalcy of liver function in these ewes. Likewise, a comparison of IGF-I concentrations in plasma, liver, kidneys, and muscle of lambs revealed that only 4% of the total body IGF-I was in the liver, compared with 70% in muscle (Hua et al., 1993). The three ovariectomized ewes (Figure 10) were subsequently implanted with estradiol for 7 d and resampled. As previously observed in cattle (Simpson et al., 1997), estradiol increased IGF-I concentrations in two of the ewes. In cattle (Simpson et al., 1997), the estradiol-mediated increase in IGF-I was associated with an increase in GH.

In contrast to IGF-I, plasma GH concentrations (Figure 7, Panel D) did not differ between cattle populations; thus, the greater IGF-I concentrations in Twinner cows in the follicle aspiration study were not mediated by genetic differences in GH secretion. Similarly, genetic differences in plasma IGF-I concentrations between ovariectomized Angus and Brahman cows were not accompanied by differences in somatotropin (Simpson et al., 1997). Plasma GH concentrations increased within 12 h after aspiration of all medium and large follicles (Figure 7, Panel D), but results are unclear as to whether the increase in plasma GH concentrations was initiated by the experimental treatment or reflected diurnal fluctuations in GH. The follicular aspirations were always performed between 0800 and 1000, and blood samples were collected at 6-h intervals; thus, the low GH concentrations coincided with the morning blood collections. However, plasma IGF-I concentrations were also elevated during the same time period.

## Genomic Markers

Current results suggest that twinning rate in cattle is inherited as a quantitative trait (i.e., a relatively large number of genes are likely involved, with each gene having relatively small effects). In search of such genes, genomic DNA scans are being conducted to identify genomic markers, and subsequently candidate genes, linked to the observed changes in ovulation and twinning rate in the MARC twinning project. An initial genomic scan of the foundation sires and of their sons and daughters with approximately 300 equally spaced (i.e., approximately 10 cM apart) markers identified a region on chromosome 5 that contains a gene(s) involved in ovarian follicular recruitment and(or) development (Kappes et al., 2000). The relative position of the QTL is 40 cM from the centromeric end of the linkage group. Additional QTL for ovulation rate or twinning rate in the MARC population have been preliminarily identified on chromosomes 7, 9, 10, and 22 (Kappes, unpublished data). Interestingly, the genes for IGF-I and GDF-9 are on chromosomes 5 and 7, respectively. An earlier DNA analysis of the progeny from two sires used in the project identified regions on chromosomes 7 and 23 that were linked to ovulation rate (Blattman et al., 1996).

## Implications

Ovarian folliculogenesis is a complex system of morphological and biochemical events regulating growth and differentiation of follicles from the primordial to the ovulatory stage (single ovulatory follicle in cattle) and the release of a viable oocyte. Besides increased production of fraternal twins, long-term selection of cattle for twin ovulations and births has increased the number of developing preantral and antral follicles and of twin or multiple dominant follicles within a follicular wave. The increased folliculogenesis is associated with changes in the production of growth factors and ovarian hormones but not in gonadotropin secretion. Such cattle provide an animal model to identify extra- and intraovarian factors and genes regulating critical stages of ovarian follicular development.

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## Notes

- Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.
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**Table 1.** Distribution of ovulations between the left and right ovary in yearling Twinner heifers<sup>a</sup>

| Location of corpora lutea | Type of ovulation |      |                |      |
|---------------------------|-------------------|------|----------------|------|
|                           | Single            |      | Twin           |      |
|                           | n <sup>b</sup>    | %    | n <sup>b</sup> | %    |
| Left ovary                | 3,114             | 44.3 | 408            | 18.7 |
| Right ovary               | 3,920             | 55.7 | 541            | 24.8 |
| Both ovaries              | —                 | —    | 1,236          | 56.6 |
| $\chi^2$ value            | 92.36**           | —    | 53.64**        | —    |

<sup>a</sup>Number and location of corpora lutea were determined by rectal palpation or ultrasonography for six to eight estrous cycles/heifer.

<sup>b</sup>Number of estrous cycles.

\*\* $P < .01$ .

**Table 2.** Means for twinning rate by year of calving for females born in the project

| Year of calving    | Number of parturitions | Twinning rate |
|--------------------|------------------------|---------------|
| 1984               | 77                     | 1.04          |
| 1985               | 194                    | 1.06          |
| 1986               | 279                    | 1.08          |
| 1987               | 423                    | 1.09          |
| 1988               | 425                    | 1.12          |
| 1989               | 544                    | 1.15          |
| 1990               | 646                    | 1.20          |
| 1991               | 755                    | 1.23          |
| 1992               | 790                    | 1.24          |
| 1993               | 734                    | 1.28          |
| 1994               | 740                    | 1.34          |
| 1995               | 721                    | 1.31          |
| 1996               | 741                    | 1.35          |
| Regression on year | —                      | .027          |
| 1997 <sup>a</sup>  | 432                    | 1.35          |
| 1998               | 264                    | 1.51          |

<sup>a</sup>Regression on year was for 1984 through 1996.

<sup>b</sup>A reduction in herd size to 250 calving cows was initiated in 1997.

**Table 3.** Diameter of dominant and subordinate follicles in cattle selected for twin births<sup>a</sup>

| Ovulation | n <sup>b</sup> | Cycle pattern | Wave   | Interval, d <sup>c</sup> | Diameter, mm           |                        |
|-----------|----------------|---------------|--------|--------------------------|------------------------|------------------------|
|           |                |               |        |                          | Follicle 1             | Follicle 2             |
| Twin      | 4              | Two-wave      | First  | 8.5 ± .9                 | 12.8 ± .8              | 11.7 ± .8              |
|           |                |               | Second | 20.0 ± .9                | 15.3 ± .8              | 13.3 ± .8              |
| Twin      | 3              | Three-wave    | First  | 10.5 ± .9                | 15.0 ± .8              | 12.5 ± .8              |
|           |                |               | Second | 18.0 ± .9                | 14.0 ± .8              | 12.0 ± .8              |
|           |                |               | Third  | 22.0 ± .9                | 13.7 ± .8              | 13.0 ± .8              |
| Single    | 5              | Two-wave      | First  | 7.5 ± .8                 | 16.3 ± .7              | 14.5 ± .7              |
|           |                |               | Second | 20.3 ± .8                | 18.4 ± .7 <sup>d</sup> | 11.0 ± .7 <sup>e</sup> |

<sup>a</sup>Diameter of follicles and pattern of follicular development were evaluated for an estrous cycle by ultrasonography.

<sup>b</sup>Number of animals.

<sup>c</sup>Interval from estrus to maximal diameter.

<sup>d,e</sup> $P \leq .01$ .

**Table 4.** Comparison of number of microscopic follicles between cattle unselected and selected for twinning<sup>a</sup>

| Population | n <sup>c</sup> | Microscopic follicle classes <sup>b</sup> |         |                   |          |
|------------|----------------|---|---------|-------------------|----------|
|            |                | Primordial                                | Primary | Secondary         | Tertiary |
| Control    | 7              | 104.7                                     | 58.7    | 6.3 <sup>d</sup>  | 2.0      |
| Twiner     | 7              | 153.7                                     | 84.3    | 12.9 <sup>e</sup> | 2.0      |
| SEM        | —              | 27.4                                      | 17.3    | 2.0               | .5       |

<sup>a</sup>Mean number of follicles per 200 fields (2 × 2 mm)/animal. Ovaries were collected on d 6 or 7 of the estrous cycle (estrus = d 0).

<sup>b</sup>Preantral follicles were classified as either primordial (oocyte surrounded by a single layer of pregranulosa cells), primary (oocyte surrounded by a single layer of cuboidal granulosa cells), secondary (multiple layers of granulosa cells), or tertiary follicles (multiple layers of granulosa cells and antrum ≤ 1 mm).

<sup>c</sup>Number of animals.

<sup>d,e</sup>Means differ within a column ( $P \leq .05$ ).

**Table 5.** Comparison of number of surface follicles between cattle unselected and selected for twinning<sup>a</sup>

| Population | n <sup>b</sup> | Surface follicle classes |                         |                   | Total             | Corpora lutea    |
|------------|----------------|--------------------------|-------------------------|-------------------|-------------------|------------------|
|            |                | Small<br>(1 to 3.9 mm)   | Medium<br>(4 to 7.9 mm) | Large<br>(≥ 8 mm) |                   |                  |
| Control    | 7              | 35.4 <sup>c</sup>        | 2.9                     | 2.4               | 40.7 <sup>c</sup> | 1.1 <sup>c</sup> |
| Twiner     | 7              | 49.0 <sup>d</sup>        | 3.3                     | 2.6               | 54.9 <sup>d</sup> | 1.6 <sup>d</sup> |
| SEM        | —              | 5.6                      | .9                      | .4                | 5.8               | .2               |

<sup>a</sup>Mean number of surface follicles and corpora lutea on both ovaries of animals reported in Table 4. Ovaries were collected on d 6 or 7 of the estrous cycle (estrus = d 0).

<sup>b</sup>Number of animals.

<sup>c,d</sup>Means differ within a column ( $P \leq .1$ ).

**Table 6.** Pretreatment concentrations of FSH, LH, and estradiol (E<sub>2</sub>) in control and twinner cows<sup>a</sup>

| Population | FSH, ng/mL | LH, ng/mL | E <sub>2</sub> , ng/mL |
|------------|------------|-----------|------------------------|
| Control    | 23.9       | 1.9       | 7.7                    |
| Twiner     | 23.4       | 2.5       | 10.8                   |
| SEM        | 1.3        | .2        | 3.3                    |

<sup>a</sup>Means for hormone concentrations before GnRH injection; n = 40 cows per population.

**Table 7.** Effect of follicle size on estradiol and progesterone concentrations in ovarian follicular fluid of control and twin-producing cattle<sup>a</sup>

| Follicle diameter, mm | Control        |                            |                           | Twiner         |                            |                           |
|-----------------------|----------------|----------------------------|---------------------------|----------------|----------------------------|---------------------------|
|                       | n <sup>b</sup> | Estradiol, ng/mL           | Progesterone, ng/mL       | n <sup>b</sup> | Estradiol, ng/mL           | Progesterone, ng/mL       |
| < 4.0                 | 13             | 12.7 ± 84.8 <sup>c</sup>   | 42.2 ± 33.1 <sup>c</sup>  | 14             | 16.7 ± 81.7 <sup>c</sup>   | 65.0 ± 31.8 <sup>c</sup>  |
| 4.0–7.9               | 5              | 296.2 ± 155.6 <sup>c</sup> | 111.5 ± 60.7 <sup>c</sup> | 12             | 41.4 ± 90.0 <sup>c</sup>   | 95.9 ± 37.4               |
| 8.0–11.9              | 6              | 121.6 ± 124.8 <sup>c</sup> | 222.8 ± 48.7 <sup>d</sup> | 9              | 704.2 ± 106.9 <sup>d</sup> | 90.1 ± 41.7               |
| ≥ 12                  | 12             | 764.2 ± 90.0 <sup>d</sup>  | 37.9 ± 35.1 <sup>c</sup>  | 15             | 684.5 ± 78.9 <sup>d</sup>  | 127.4 ± 33.1 <sup>d</sup> |
| Overall               | 36             | 298.7 ± 58.7               | 103.6 ± 22.9              | 50             | 361.7 ± 46.5               | 94.6 ± 18.1               |

<sup>a</sup>Least squares means ± SEM.

<sup>b</sup>Number of follicles.

<sup>c,d</sup>Means differ among size categories × population ( $P < .01$ ).

**Table 8.** Comparison of follicle diameter<sup>a</sup> and estradiol concentrations<sup>a</sup> by declining follicle size (order 1 to 3) between cows unselected and selected for twin births

| Order | Control        |                        |                           | Twiner         |                        |                           |
|-------|----------------|------------------------|---------------------------|----------------|------------------------|---------------------------|
|       | n <sup>b</sup> | Diameter, mm           | Estradiol, ng/mL          | n <sup>b</sup> | Diameter, mm           | Estradiol, ng/mL          |
| 1     | 11             | 12.2 ± .2 <sup>c</sup> | 175.8 ± 21.0 <sup>c</sup> | 18             | 13.7 ± .2 <sup>c</sup> | 178.6 ± 16.4 <sup>c</sup> |
| 2     | 12             | 8.4 ± .2 <sup>d</sup>  | 38.9 ± 20.1 <sup>d</sup>  | 17             | 11.5 ± .2 <sup>c</sup> | 150.3 ± 16.9 <sup>c</sup> |
| 3     | 11             | 7.6 ± .2 <sup>d</sup>  | 9.7 ± 21.0 <sup>d</sup>   | 14             | 9.4 ± .3 <sup>d</sup>  | 60.5 ± 18.6 <sup>d</sup>  |

<sup>a</sup>Population × order ( $P \leq .01$ ).

<sup>b</sup>Number of follicles.

<sup>c,d</sup>Means differ ( $P < .01$ ).

**Table 9.** Comparison of IGF-I concentrations in serum and ovarian follicular fluid between cattle unselected and selected for twin births<sup>a</sup>

| Population | n  | IGF-I concentration, ng/mL |                               |
|------------|----|----------------------------|-------------------------------|
|            |    | Serum                      | Follicular fluid <sup>c</sup> |
| Control    | 13 | 306.3 ± 48.9 <sup>d</sup>  | 281.1 ± 48.9 <sup>f</sup>     |
| Twinner    | 13 | 468.5 ± 48.9 <sup>e</sup>  | 368.0 ± 48.9 <sup>g</sup>     |
| Overall    | 26 | 387.4 ± 34.6               | 323.6 ± 34.6                  |

r = .88<sup>h</sup>

<sup>a</sup>Means ± SEM.

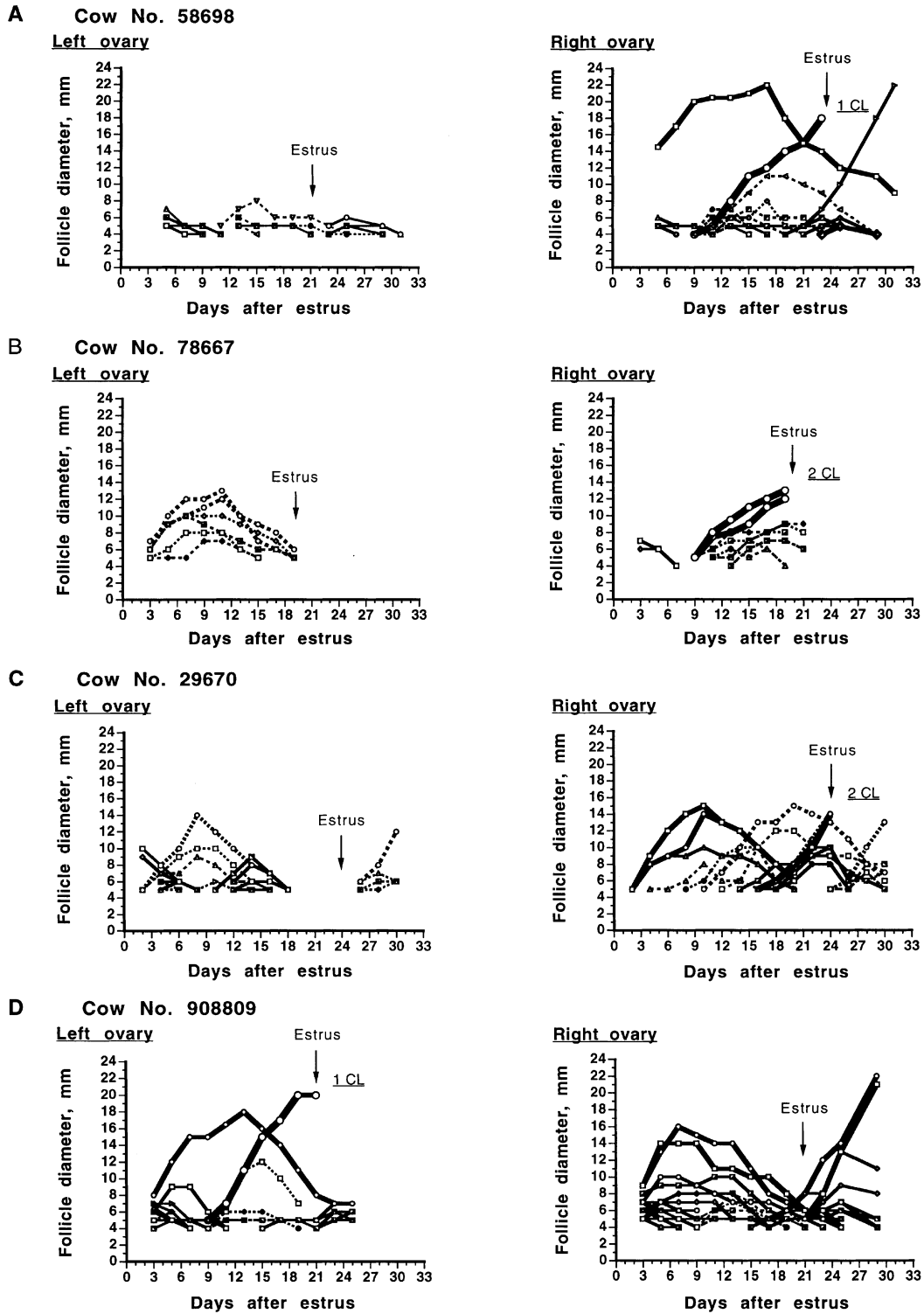
<sup>b</sup>Number of animals.

<sup>c</sup>Within animal means were calculated for follicles > 4 mm in diameter.

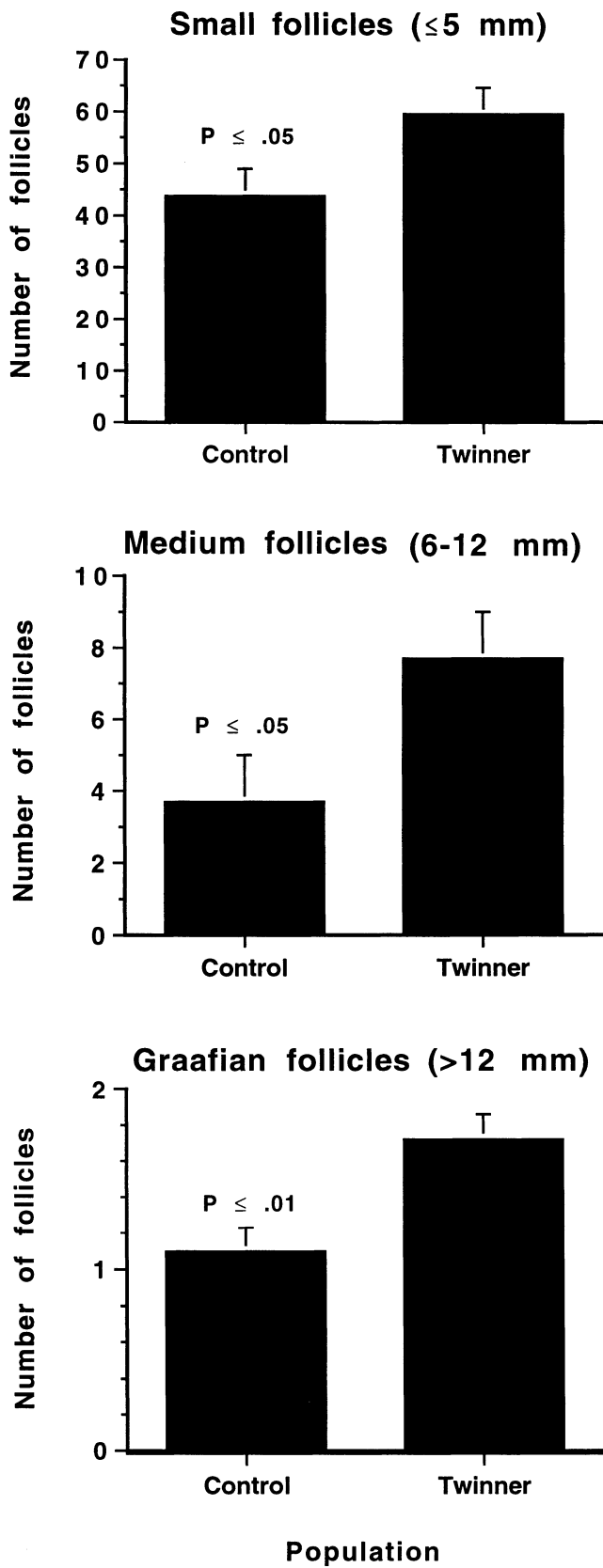
<sup>d,e</sup>Means differ between populations ( $P < .05$ ).

<sup>f,g</sup>Means differ between populations ( $P < .01$ ).

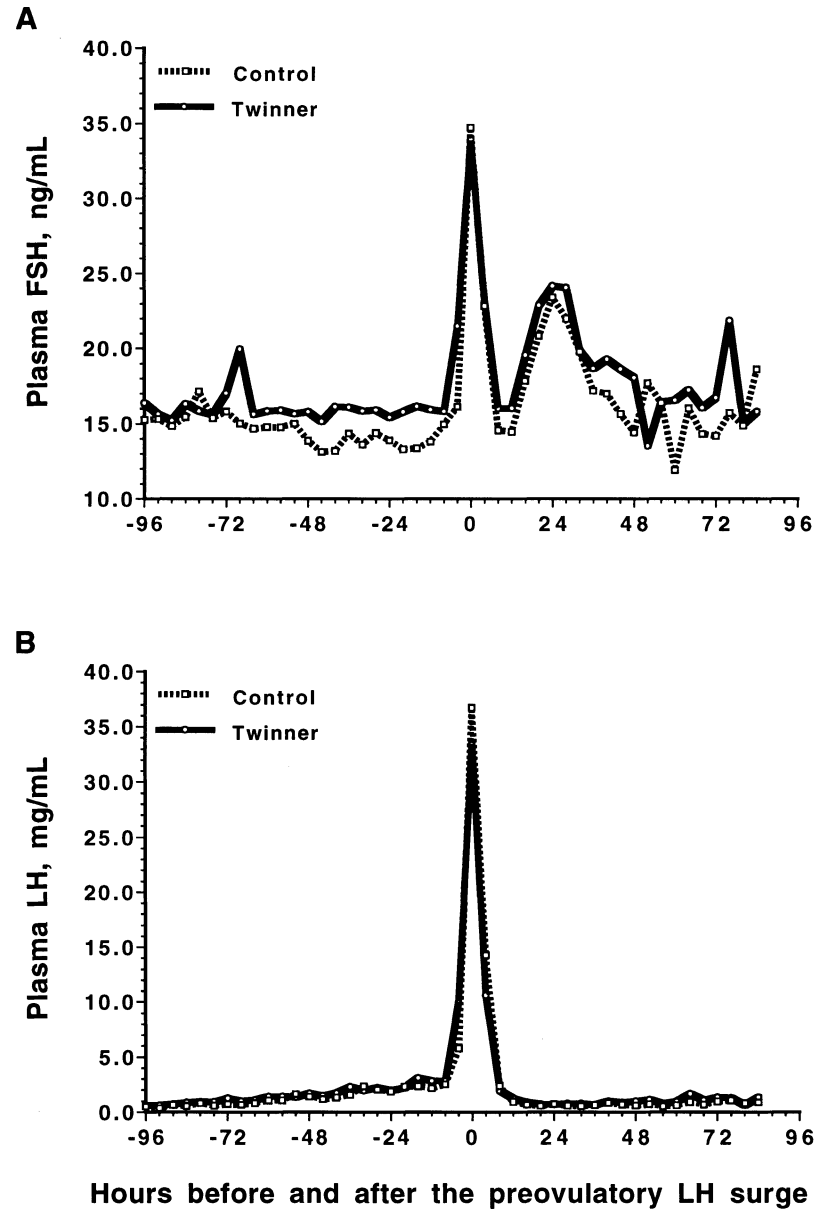
<sup>h</sup> $P < .01$ .



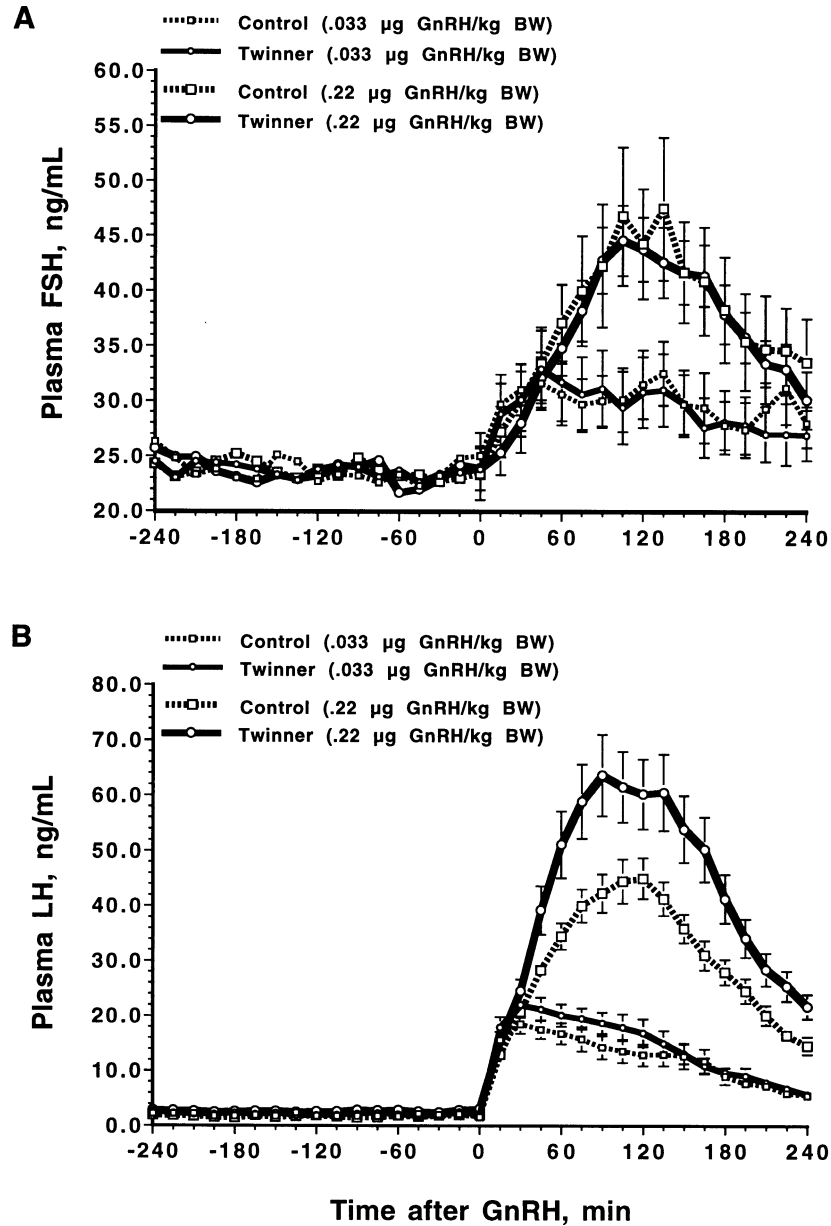
**Figure 1.** Dynamics of individual ovarian follicles for a two-wave bovine estrous cycle in a Control cow (Panel A) and a Twinner cow (expressing twin ovulations, Panel B), a three-wave bovine estrous cycle in a Twinner cow (expressing twin ovulations, Panel C), and a two-wave cycle in a Twinner cow with a single ovulatory follicle (Panel D). Each line represents an individual follicle.



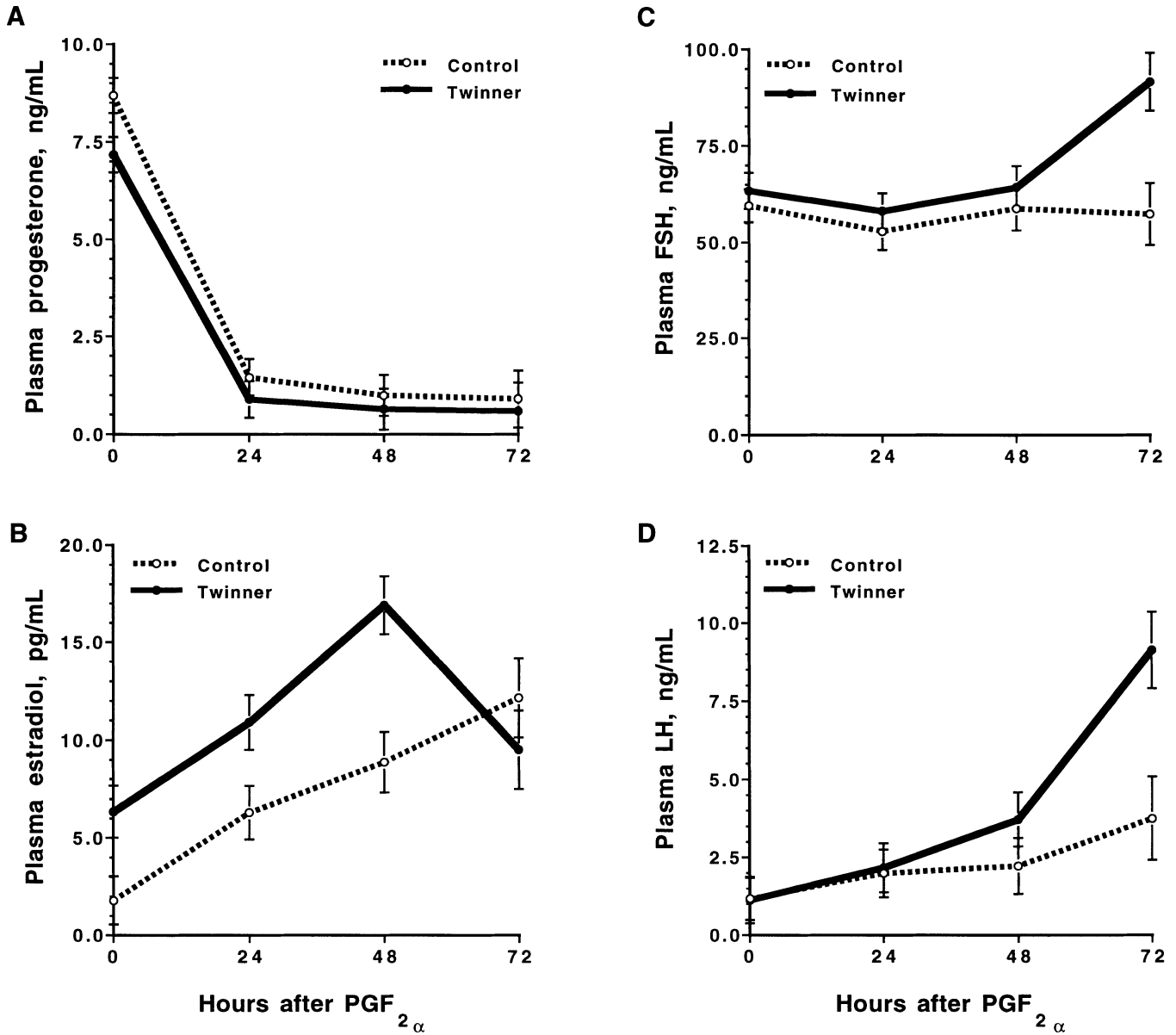
**Figure 2.** Distribution of ovarian follicles by size during proestrus between cattle populations selected (Twiner) and unselected (Control) for twin births: small follicle pool ( $\leq 5$  mm in diameter), medium follicle (6 to 12 mm), and graafian follicle ( $> 12$  mm).



**Figure 3.** Comparison of circulating concentrations of FSH (Panel A) and LH (Panel B) during proestrus and estrus between cattle populations selected (Twinner) and unselected (Control) for twin births. Blood samples were collected at 4-h intervals starting on d 18 of the estrous cycle and continuing until d 3 after onset of estrus. Data were standardized among cows to the peak LH concentration.



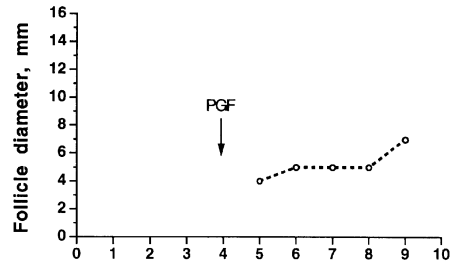
**Figure 4.** Comparison of FSH (Panel A) and LH (Panel B) response to GnRH between cattle populations selected (Twinner) and unselected (Control) for twin births. A single injection of GnRH was given 44 h after PGF<sub>2 $\alpha$</sub> , and blood samples were collected at 15-min intervals for 4 h before and after the GnRH injection.



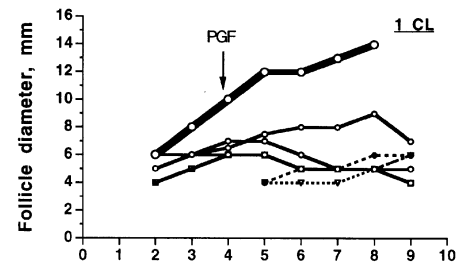
**Figure 5.** Comparison of circulating concentrations of progesterone (Panel A), estradiol (Panel B), FSH (Panel C), and LH (Panel D) between cattle populations selected (Twin) and unselected (Control) for twin births. Blood samples were collected at 0, 24, 48, and 72 h after PGF<sub>2α</sub> administered on d 18 (estrus = d 0).

**A Cow No. 900626**

Left ovary

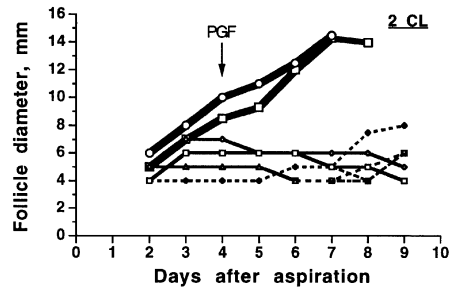


Right ovary

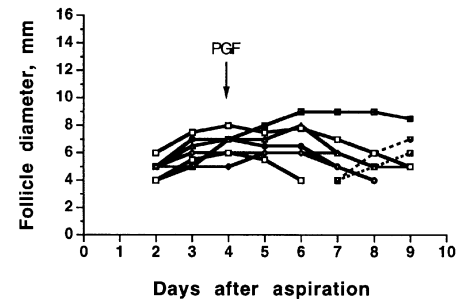


**B Cow No. 948525**

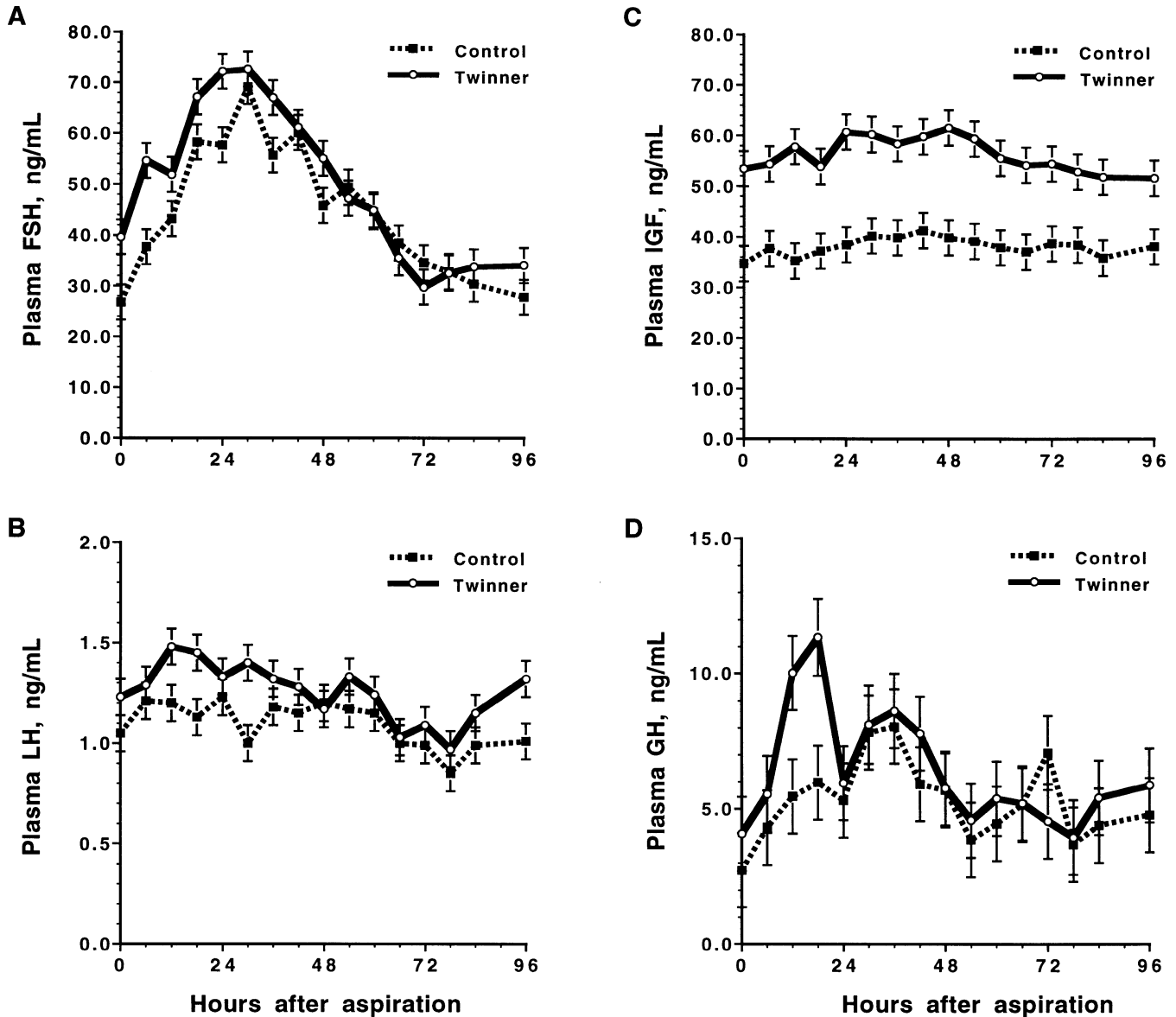
Left ovary



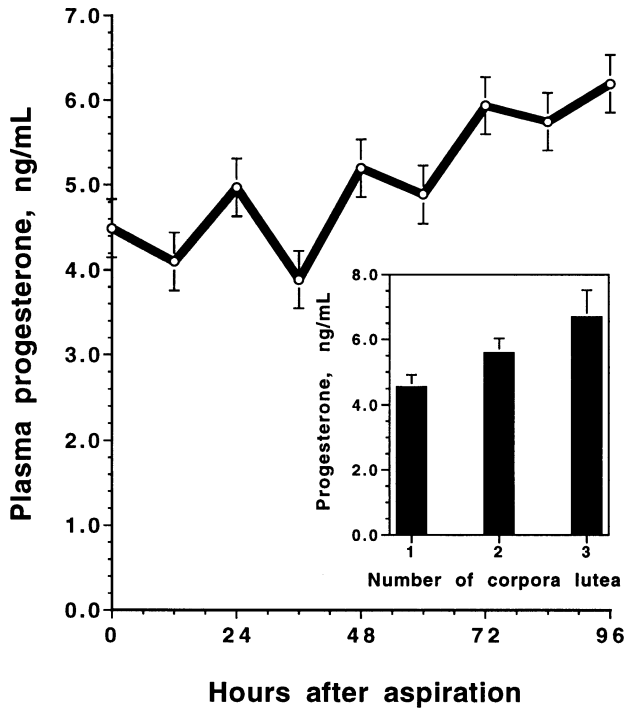
Right ovary



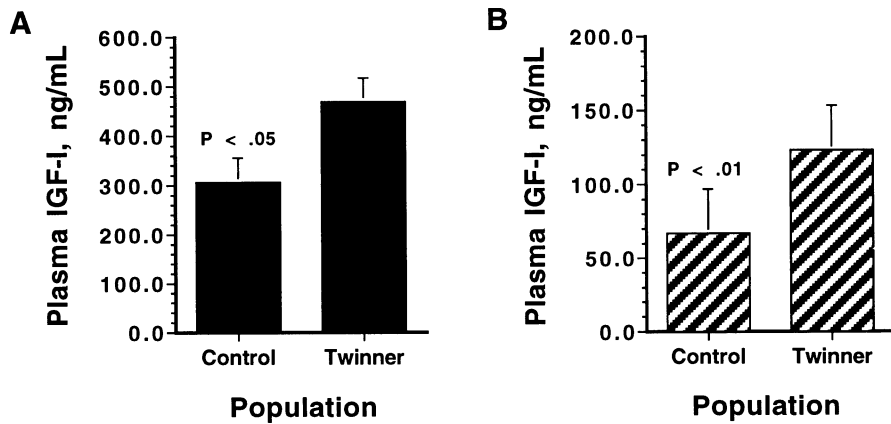
**Figure 6.** Dynamics of ovarian follicular development after follicle aspiration for a Control cow (Panel A) and a Twinner cow (twin ovulation, Panel B).



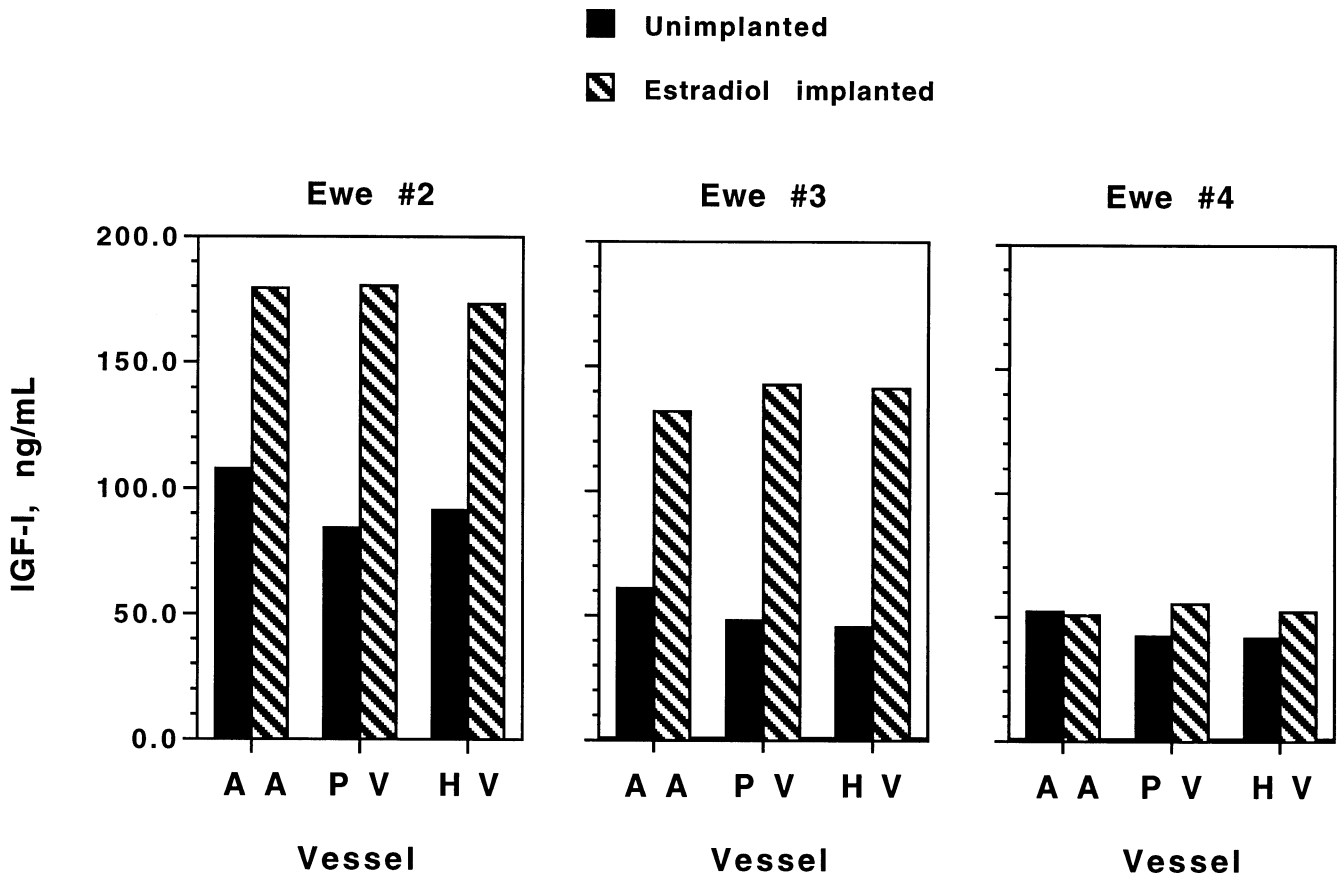
**Figure 7.** Comparison of FSH, LH, IGF-I, and GH response to aspiration of all ovarian follicles  $\geq 5$  mm on d 7 or 8 (estrus = d 0) between cattle populations selected (Twinner) and unselected (Control) for twin births. Plasma FSH concentrations were elevated ( $P \leq .01$ ) within 6 h after aspiration, whereas LH was unaffected ( $P \geq .1$ ) by follicle aspiration. Neither plasma FSH nor LH concentrations differ ( $P \geq .1$ ) between cattle populations. Blood of Twinners had greater ( $P \leq .01$ ) IGF-I concentrations and plasma IGF-I concentrations were enhanced ( $P \leq .05$ ) by follicle aspiration. Circulating concentrations of GH were elevated ( $P \leq .01$ ) at 12, 18, 30, 36, 42, and 48 h after follicle aspiration, but GH did not differ ( $P \geq .1$ ) between cattle populations.



**Figure 8.** Plasma progesterone concentrations after aspiration of all ovarian follicles  $\geq 5$  mm on d 7 or 8 (estrus = d 0). Progesterone concentrations did not differ ( $P \geq .1$ ) between populations but increased ( $P \leq .01$ ) with time after aspiration and with number of CL on the ovaries (insert).



**Figure 9.** Comparison of blood IGF-I concentrations between cattle populations selected (Twinner) and unselected for twin births (Panel A) or between Twinner and MARC III composite cows (Panel B).



**Figure 10.** Comparison of IGF-I concentrations among the abdominal aorta (AA), portal vein (PV), and hepatic vein (HV) of three ovariectomized ewes before and after implantation with estradiol for 7 d.