

Ovarian follicular development in cattle selected for twin ovulations and births^{1,2}

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ABSTRACT: Comparisons of numbers of antral ovarian follicles and corpora lutea (CL), of blood hormone concentrations, and of follicular fluid steroid concentrations and IGFBP activity were conducted between cows selected (twinner) and unselected (control) for twin births to elucidate genetic differences in the regulation of ovarian follicular development. Ovarian follicular development was synchronized among cows by a single i.m. injection of PGF_{2α} on d 18 of the estrous cycle; six cows per population were slaughtered at 0, 24, 48, and 72 h after PGF_{2α}. Jugular vein blood was collected from each animal at PGF_{2α} injection and at 24-h intervals until slaughter. Ovaries of twinner cows contained more small (≤ 5 mm in diameter, $P < 0.05$), medium (5.1 to 9.9 mm, $P < 0.05$), and large (≥ 10.0 mm, $P < 0.01$) follicles and more ($P < 0.01$) CL than ovaries of controls. Follicular fluid concentrations of estradiol, androstenedione, testosterone, and progesterone reflected the stage of follicular development and were similar for twinner and control follicles at the same stage. Earlier initiation of follicular development and/or selection of twin-dominant follicles in some twinner cows resulted in greater concentrations of estradiol in plasma at 0,

24, and 48 h and of estradiol, androstenedione, and testosterone in follicular fluid of large follicles at 0 h after PGF_{2α} for twinner vs. control cows (follicular status \times time \times population, $P < 0.01$). Binding activities of IGFBP-5 and -4 were absent or reduced ($P < 0.01$) in follicular fluid of developing medium and large estrogen-active (estradiol:progesterone ratio > 1) follicles but increased with atresia. Only preovulatory Graafian follicles lacked IGFBP-2 binding, suggesting a possible role for IGFBP-2 in selection of the dominant follicle. Concentrations of IGF-I were twofold greater ($P < 0.01$), but GH ($P = 0.10$) and cholesterol ($P < 0.05$) were less in blood of twinners. Three generations of selection of cattle for twin ovulations and births enhanced ovarian follicular development as manifested by increased numbers of follicles within a follicular wave and subsequent selection of twin dominant follicles. Because gonadotropin secretion and ovarian steroidogenesis were similar for control and twinner cattle, enhanced follicular development in twinners may result from decreased inhibition by the dominant follicle(s), increased ovarian sensitivity to gonadotropins, and/or increased intragonadal stimulation, possibly by increased IGF-I.

Key Words: Cattle, Gonadotropins, Insulin-Like Growth Factor Binding Proteins, Ovarian Folliculogenesis, Steroids, Twins

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Introduction

Fraternal twins result from the ovulation and fertilization of two oocytes at approximately the same time. The two oocytes are produced by either the simultaneous (i.e., within a few hours) ovulation of two follicles on the same ovary or one follicle on each ovary. Production of the two oocytes by separate follicles is confirmed by the increased presence of two corpora lutea (CL) on the ovaries of cattle selected for the production of twins at the Roman L. Hruska U. S. Meat Animal Research Center (MARC; Echternkamp et al., 1990a). Results from a preliminary study to evaluate follicular development during the estrous cycle in twinner cattle indicated that natural twin or triplet ovulations resulted

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from the simultaneous formation of two or more dominant (ovulatory) follicles within a follicular wave rather than ovulation of single dominant follicles from two consecutive waves (Echternkamp, 2000). Objectives of the present study were 1) to determine whether long-term selection for twin ovulations and/or twin births enhanced recruitment of ovarian follicles and, thus, increased the probability for selection of twin or multiple ovulatory follicles and 2) to evaluate the relationship between circulating hormone concentrations and follicular development in cattle selected and unselected for twin births.

Experimental Procedure

Animals

The effect of genetic selection for twin ovulations and births in cattle on ovarian follicular development was evaluated in cyclic multiparous cows from the MARC twinner population (twinning; $n = 24$ cows) and from the MARC III composite population (control; $n = 24$ cows). The twinner population is a composite of nine breeds of cattle and is composed of females selected for the production of twin births since 1981. Since 1988, selection criteria included repeated measurements of ovulation rate (i.e., six estrous cycles) in all heifer progeny, twinning rate for retained females, and use of progeny-proven sires (Echternkamp et al., 1990a; Gregory et al., 1990; Van Vleck et al., 1991). The MARC III population is a composite of four cattle breeds ($\frac{1}{4}$ Pinzgauer, $\frac{1}{4}$ Red Poll, $\frac{1}{4}$ Angus, and $\frac{1}{4}$ Hereford) and unselected for twinning.

Ovarian follicular development was evaluated by serial slaughter and collection of ovaries from cows at 0, 24, 48, or 72 h after a single injection of 30 mg of PGF_{2 α} (Lutalyse; Pharmacia & Upjohn, Kalamazoo, MI) i.m. on d 18 of the estrous cycle. Six cows per population were slaughtered at each of the four time periods after PGF_{2 α} . A jugular vein blood sample (20 mL) was collected by venipuncture from each animal immediately before the PGF_{2 α} injection and at 24-h intervals thereafter until slaughter. Blood samples were refrigerated immediately and centrifuged; plasma was collected and stored at -20°C until assayed for FSH, LH, GH, cholesterol, estradiol, testosterone, and progesterone concentrations. Ovaries were collected immediately after slaughter, stored on ice, and evaluated for number of visible CL and follicles on the ovarian surface. Number and diameter of individual antral follicles were recorded; follicles were subsequently categorized as small (≤ 5 mm in diameter), medium (5.1 to 9.9 mm), or large (≥ 10.0 mm) follicles. Ovarian and CL weights were recorded, and follicular fluid was collected and stored individually for follicles >5 mm in diameter.

Hormone Analyses

LH. Plasma LH concentrations were determined by double-antibody RIA, as described previously (Echtern-

kamp, 1978). All plasma samples were assayed in a single assay. Duplicate samples with an intraassay CV $>10\%$ were reassayed.

FSH. Plasma FSH concentrations were determined in duplicate aliquots using a validated double-antibody heterologous RIA. Assay tubes containing 400 μL of plasma sample or 400 μL of FSH standard (USDA-bFSH-B-1) in 0.01 *M* PBS, 1% BSA, pH 7.5; 300 μL of ovine FSH antisera (NIDDK-anti-oFSH-1, 1:40,000 in 0.02 *M* PBS); and 100 μL of ¹²⁵I-labeled ovine FSH (NIDDK-oFSH-I-1; 20,000 cpm) were incubated for 48 h at 4°C. One hundred microliters of preprecipitated anti-rabbit gamma globulin in 0.1 *M* Tris buffer, pH 7.5, was added to each tube, and tubes were incubated for 48 h at 4°C. An aliquant of 1.5 mL of chilled wash buffer (0.005 *M* PBS, pH 7.5) was added to each tube, tubes were centrifuged for 20 min at $1,250 \times g$, supernatant was decanted, and radioactivity was measured in the precipitate. Parallelism was confirmed with serial dilutions of plasma from steers, which contained elevated concentrations of FSH. The intraassay CV for duplicate samples was $<10\%$.

GH. Plasma GH concentrations were measured in duplicate using a validated heterologous double-antibody RIA described previously (Klindt et al., 1985). The primary rabbit anti-oGH antisera (NIDDK-anti-oGH-2, AFP-Co123080) was diluted 1:10,000. The reference standard was USDA-bGH-B-1 (AFP-5200). Samples were assayed in one assay. Intraassay CV for duplicates was $<8\%$.

Cholesterol. Total plasma cholesterol was measured spectrophotometrically by the cholesterol oxidase procedure (Allien et al., 1974) in which cholesterol esterase and cholesterol oxidase produce hydrogen peroxide when incubated with cholesterol. Addition of P-hydroxybenzoate and 4-amino-antipyrine produced a red color proportional to the amount of cholesterol present; absorbance was measured at 500 nm.

Testosterone. Concentrations of testosterone were measured in 10 μL of plasma or 1- μL equivalence of follicular fluid by RIA (Borg et al., 1993). Intraassay CV was 9.2%.

Androstenedione. Concentrations of androstenedione in 1 μL of follicular fluid were determined in one assay using a solid-phase RIA kit (ICN Biomedicals, Costa Mesa, CA). The intraassay CV was 2.0%.

Follicular Fluid Estradiol and Progesterone. Estradiol and progesterone were measured directly in an equivalence of 1 μL of follicular fluid by double-antibody RIA. Except for different primary antisera and radioactive ligands, the RIA protocol was the same for measurement of progesterone and estradiol in follicular fluid. The progesterone antiserum (catalog No. 07-170116) and ¹²⁵I-labeled progesterone tracer (catalog No. 07-170126) were purchased commercially (ICN Biomedicals, Inc.); progesterone antiserum was diluted 1:70,000. The estradiol antiserum (lot No. 037003) was acquired from Eli Lilly (Indianapolis, IN), and the radioactive tracer was ¹²⁵I-labeled estradiol (catalog No.

IM 135; Amersham Biosciences Corp., Piscataway, NJ); estradiol antiserum was diluted 1:3,000,000.

Aliquots of follicular fluid were diluted 1:20 in 0.01 M PBS, 0.15 M NaCl, pH 7.5, and 20 μ L of diluted follicular fluid or of steroid standard was added to the assay tube combined with 180 μ L of 0.01 M PBS, 0.1% gelatin, pH 7.5; 100 μ L of primary antisera; and 50 μ L (10,000 cpm) of radioactive tracer. Contents of tubes were mixed and incubated overnight at 4°C. The second antibody (100 μ L preprecipitated anti-rabbit gamma globulin in 0.1 M Tris buffer, pH 7.5) was added to the tubes and, again, contents of tubes were mixed and incubated overnight at 4°C. Chilled wash buffer (1.5 mL of 0.005 M PBS, pH 7.5, 4°C) was added to each tube and tubes were centrifuged at 4°C for 20 min at 1,250 \times g; supernatant was decanted and radioactivity was measured in the precipitate. Intra-assay CV for duplicate samples were 9.5% and 12.4%, respectively, for progesterone and estradiol assays.

Plasma Estradiol and Progesterone. For estradiol measurements in blood, duplicate 0.5-mL aliquots of plasma were extracted twice with 2 mL of diethyl ether. The combined ether extract was evaporated and the residue was resuspended in 200 μ L of 0.01 M PBS, 0.1% gelatin, 0.15 M NaCl, pH 7.4; contents of the tubes were mixed and stored overnight at 4°C. Amount of estradiol in tubes was measured by the RIA procedure described above for follicular fluid.

Progesterone was measured directly in duplicate aliquots of 0.05 mL of plasma without extraction. The RIA protocol for plasma was the same as for the measurement of progesterone in follicular fluid.

IGF-I. The acid-ethanol extraction (16 h at 4°C) and RIA procedures for quantifying free IGF-I in plasma have been described previously (Echternkamp et al., 1990b). The intraassay CV was 7.3%.

IGFBP. Follicular fluid IGFBP activity was analyzed by one-dimensional SDS-PAGE, Western blot analysis as described previously (Echternkamp et al., 1994a). Samples were heat-denatured and separated on 12% polyacrylamide gel via electrophoresis. Electrophoresed proteins were transferred from gels to nitrocellulose and ligand-blotted overnight with ¹²⁵I-IGF-I. After washing, nitrocellulose filters were exposed to x-ray film at -70°C for 48 h; band intensity on autoradiographs was determined using scanning densitometry. A reference sample of follicular fluid was included on each gel.

Statistical Analysis

Data were analyzed by mixed model analysis of SAS (Version 6.12, SAS Inst., Inc., Cary, NC). Data were transformed (square-root transformation) prior to analysis if variances among classes were determined to be heterogeneous by Hartley's test (Ott, 1984). The statistical model for plasma concentrations of estradiol, progesterone, testosterone, FSH, LH, GH, and cholesterol evaluated the fixed effect of cattle population, random

effect of time (h) after PGF_{2 α} , and the two-way interaction. Diameter and follicular fluid estradiol:progesterone ratio (follicular status) were used to categorize individual follicles as either medium (5.1 to 9.9 mm) or large (\geq 10.0 mm) and as estrogen-active (i.e., estradiol:progesterone >1) or estrogen-inactive (i.e., estradiol:progesterone \leq 1). The final ANOVA model for follicular fluid steroid concentrations and IGFBP binding activity evaluated fixed effect of cattle population, random effects of time (h) after PGF_{2 α} and follicular status, and statistically significant ($P < 0.05$) two-way and three-way interactions. Animal within population was the error term to test effect of population.

Results

Ovarian Characteristics

Comparisons of ovarian characteristics between control and twinner cattle are presented in Table 1. Ovaries of twinner cows contained more small ($P < 0.05$), medium ($P < 0.05$), and large ($P < 0.01$) antral follicles on their surface than ovaries of control cows. Twelve of the 24 twinners had two CL at time of PGF_{2 α} injection compared with two controls having two CL; thus, total luteal weight was heavier ($P < 0.05$) for twinner cows but the weight of individual CL did not differ ($P > 0.10$) between the two cattle populations. The PGF_{2 α} treatment induced luteolysis as exemplified by a reduction ($P < 0.01$) in total CL weight at 24, 48, and 72 h after injection (0 h, 4.9 g; 24 h, 4.3 g; 48 h, 2.6 g; and 72 h, 1.6 g; SEM = 0.2). Total ovarian weight (Table 1) was also heavier ($P < 0.01$) for twinner than for control cows with or without inclusion of CL.

Plasma Hormone Concentrations

Progesterone. The single injection of PGF_{2 α} on d 18 decreased ($P < 0.01$) plasma progesterone concentrations precipitously (Figure 1) within 24 h, followed by further decreases ($P < 0.01$) from 24 to 48 h after PGF_{2 α} in both cattle populations, and from 48 to 72 h in controls (cattle population \times time, $P < 0.01$). Means did not differ between the two cattle populations at 0 and 72 h after PGF_{2 α} , but means were greater at 24 and 48 h for controls compared with twinners (population \times time, $P < 0.01$). Low plasma progesterone concentrations, indicative of CL regression, were found in two control cows and four twinner cows at 0 h after PGF_{2 α} .

Estradiol. Means for plasma estradiol concentrations (Figure 1) were greater ($P < 0.01$) for twinners at 0, 24, and 48 h after PGF_{2 α} compared with controls. Means for plasma estradiol concentration increased between 0 and 24 h after PGF_{2 α} in both control and twinner cows, and between 24 and 72 h in controls vs. between 24 and 48 h in twinners (cattle population \times time, $P < 0.01$).

Testosterone. Plasma testosterone concentrations were low and did not differ ($P > 0.10$) between control

Table 1. Comparison of ovarian characteristics between cattle populations selected and unselected for twin ovulations and births

Variable	Control	Twinner	SEM	<i>P</i> <
No. of animals	24	24		
Total ovarian weight, g	19.2	26.6	1.3	0.01
No. of small follicles (≤ 5 mm)	43.5	60.8	5.1	0.05
No. of medium follicles (5.1 to 9.9 mm)	3.3	6.4	1.2	0.05
No. of large follicles (≥ 10 mm)	1.6	2.5	0.1	0.01
No. of corpora lutea	1.1	1.5	0.1	0.01
Corpus luteum weight, g	2.9	2.6	0.2	NS ^a
Total luteal weight, g	3.0	3.6	0.2	0.05

^aNS = means do not differ ($P > 0.10$).

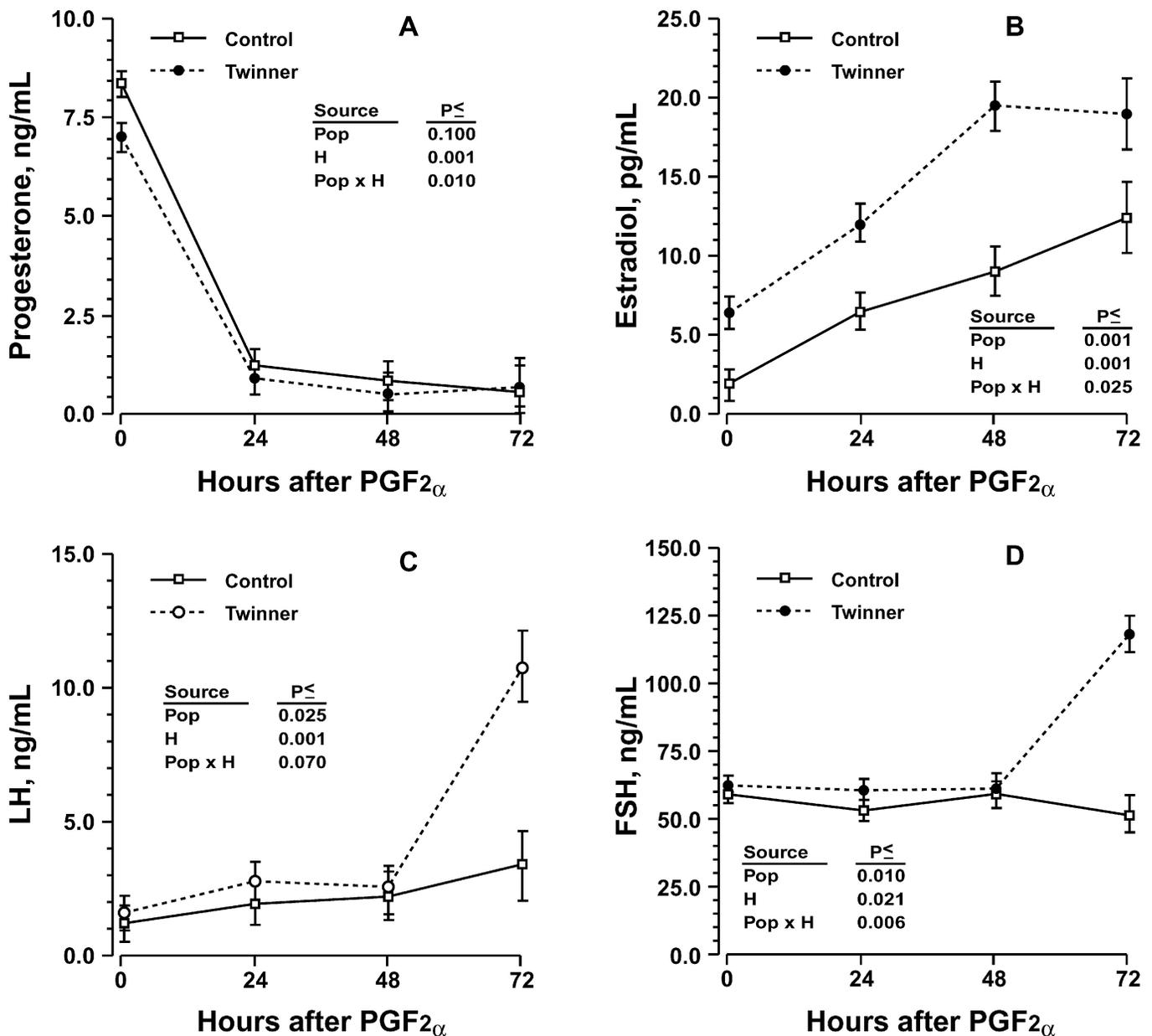


Figure 1. Comparisons of plasma progesterone (A), estradiol (B), LH (C), and FSH (D) concentrations (means \pm SEM) between twinner and control cows after a single injection of $\text{PGF}_{2\alpha}$ on d 18 of estrous cycle. (Pop = population, H = hour).

and twinner cows (0.11 vs. 0.10 ± 0.005 ng/mL, respectively). Means for plasma testosterone concentration decreased from 0 to 24 h (0.13 ± 0.003 vs. 0.11 ± 0.004 ng/mL; $P < 0.01$) and from 24 to 72 h (0.11 ± 0.004 vs. 0.09 ± 0.007 ng/mL; $P < 0.05$) after $\text{PGF}_{2\alpha}$ in both cattle populations.

LH. An increase in plasma LH concentration, indicative of a preovulatory LH surge, occurred in four of six twinner cows at 72 h after $\text{PGF}_{2\alpha}$, compared with one of six control cows. The increased number of twinner cows with elevated LH at 72 h (Figure 1) resulted in significant main effects of cattle population ($P < 0.05$) and time ($P < 0.01$) on LH.

FSH. Concurrent with LH, four of six twinner cows sampled at 72 h after $\text{PGF}_{2\alpha}$ had elevated plasma FSH concentration indicative of a preovulatory FSH surge. Thus, the increased mean for plasma FSH (Figure 1) in twinners at 72 h resulted in a significant ($P < 0.01$) cattle population \times time interaction, as well as significant main effects of population ($P < 0.01$) and time ($P < 0.05$).

IGF-I. Means for plasma IGF-I concentration (Figure 2) were twofold greater ($P < 0.01$) for twinner cows compared with control cows at all time intervals after $\text{PGF}_{2\alpha}$ injection. Also, an increase ($P < 0.05$) in the mean for plasma IGF-I concentration occurred between 48 and 72 h after $\text{PGF}_{2\alpha}$.

GH. Evaluation of GH in blood samples collected daily indicated a trend for plasma GH concentrations (Figure 2) to increase ($P < 0.05$) from 24 to 72 h after $\text{PGF}_{2\alpha}$ injection. Also, overall means for plasma GH concentration tended to be greater ($P = 0.10$) for control than for twinner cows (8.3 vs. 4.7 ± 1.1 ng/mL, respectively).

Cholesterol. Plasma concentrations of total cholesterol were greater for controls compared with twinners (108.5 vs. 92.6 ± 5.3 mg/dL, respectively; $P < 0.05$). In addition, cholesterol concentrations decreased ($P < 0.01$) between 0 and 24 h after $\text{PGF}_{2\alpha}$ (104.2 ± 3.7 vs. 99.8 ± 3.8 mg/dL, respectively) and then remained constant.

Follicular Fluid Hormones

Follicles categorized as estrogen-active had greater ($P < 0.01$) concentrations of estradiol, androstenedione, and testosterone, and less ($P < 0.01$) progesterone than estrogen-inactive follicles (Table 2). Furthermore, means for estradiol and androstenedione were greater ($P < 0.01$) for large than for medium estrogen-active follicles, whereas steroid concentrations did not differ ($P > 0.10$) between medium and large estrogen-inactive follicles. Comparisons of follicular fluid steroid concentrations among follicles from ovaries of twinner and control cows are presented in Figure 3 for estradiol, androstenedione, and testosterone. Coefficients for simple correlations (Table 3) between follicular fluid estradiol and androstenedione or testosterone, and between androstenedione and testosterone were positive and

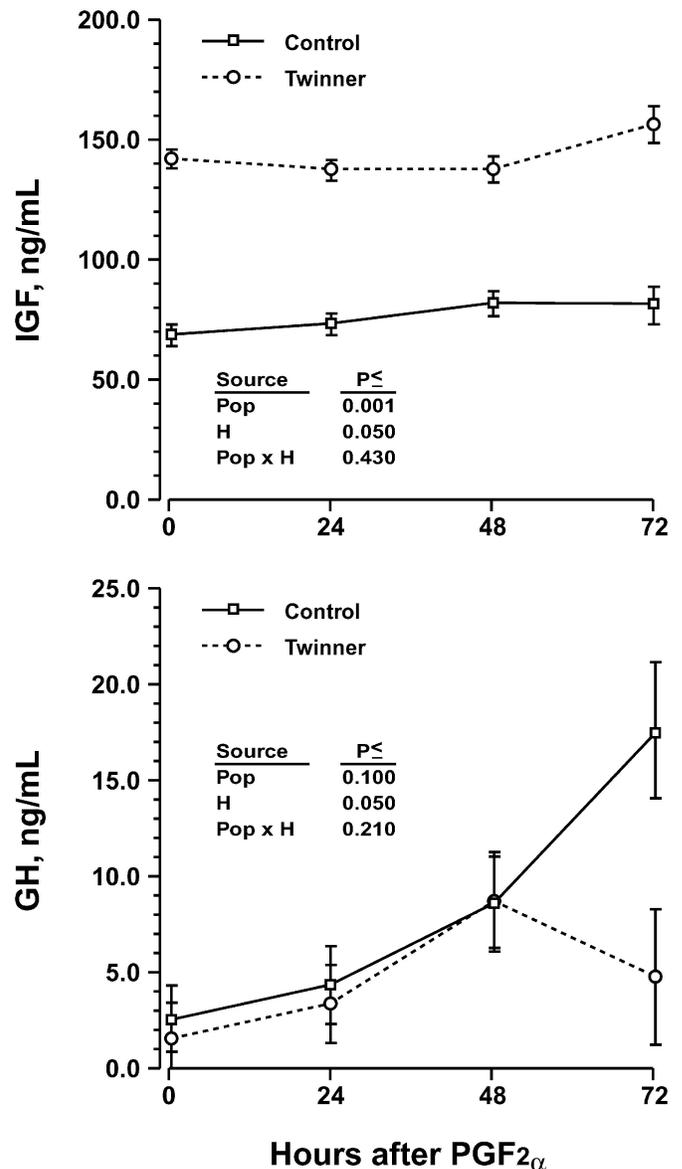


Figure 2. Comparisons of plasma IGF-I and GH concentrations (means \pm SEM) between twinner and control cows after a single injection of $\text{PGF}_{2\alpha}$ on d 18. (Pop = population, H = hour).

significant ($P < 0.01$), but correlations with follicular fluid progesterone concentrations were negative ($P < 0.01$).

Progesterone. Follicular fluid progesterone concentrations were less ($P < 0.01$) in estrogen-active than in estrogen-inactive follicles (Table 2) but, within the estrogen-active or estrogen-inactive categories, concentrations did not differ ($P > 0.10$) between medium and large follicles. Also, overall means did not differ ($P > 0.10$) between the two cattle populations or among time intervals after $\text{PGF}_{2\alpha}$ injection.

Estradiol. Means for the follicular status \times time \times cattle population interaction ($P < 0.01$) are illustrated in Figure 3. Estradiol concentrations were low ($P < 0.01$) in large estrogen-active follicles of control cows at 0 h

Table 2. Comparison of follicular fluid estradiol, progesterone, androstenedione, and testosterone concentrations among follicular status categories

Follicular status	n	Estradiol, ng/mL	Progesterone, ng/mL	Androstenedione, ng/mL	Testosterone, ng/mL
Large estrogen-active	40	553.80 ± 25.62 ^a	57.75 ± 47.18 ^a	55.01 ± 3.56 ^a	87.26 ± 10.05 ^a
Medium estrogen-active	46	97.16 ± 26.70 ^b	18.37 ± 49.17 ^a	29.82 ± 3.71 ^b	70.66 ± 10.47 ^a
Large estrogen-inactive	50	5.22 ± 25.62 ^c	416.00 ± 47.18 ^b	14.58 ± 3.56 ^c	17.15 ± 10.05 ^b
Medium estrogen-inactive	46	3.15 ± 26.70 ^c	381.25 ± 49.17 ^b	17.01 ± 3.71 ^c	29.08 ± 10.47 ^b

^{a,b,c}Means (± SEM) with different superscripts differ within a column ($P < 0.01$).

compared with the subsequent times and compared with large estrogen-active follicles of twinner cows. Means for estradiol concentration in follicular fluid of large estrogen-active follicles increased between 0 and 24 ($P < 0.01$) and between 24 and 72 ($P < 0.01$) h after PGF_{2α} in controls vs. between 24 and 48 h ($P < 0.01$) in twinnings. Estradiol concentrations in follicular fluid of medium estrogen-active follicles from control cows were unaffected by time, whereas estradiol was elevated ($P < 0.01$) in the medium estrogen-active follicles from twinner cows (i.e., primarily one cow) at 48 h after PGF_{2α}. In addition, means for estradiol were greater ($P < 0.05$) in follicular fluid of medium estrogen-active follicles of twinner cows at 48 h, but less at 72 h, after PGF_{2α} compared with controls. Means did not differ ($P > 0.10$) between medium and large estrogen-inactive follicles. Correlations among follicular fluid concentrations of the measured steroids are presented in Table 3.

Androstenedione. Changes in androstenedione concentrations (Figure 3) with time after PGF_{2α} varied among follicular status-size categories and between controls and twinnings (follicular status × time × cattle population, $P < 0.01$). Like estradiol, androstenedione concentrations were very low ($P < 0.01$) in large estrogen-active follicles of control cows at 0 h but increased ($P < 0.01$) between 0 and 24 h to concentrations comparable to those of large estrogen-active follicles of twinner cows. Means for androstenedione concentration in the fluid of large estrogen-active follicles from twinner cows increased between 0 and 72 h after PGF_{2α}, resulting in the mean being greater ($P < 0.01$) at 48 and 72 h after PGF_{2α} for large estrogen-active follicles of twinner cows compared with control cows (follicular status × time × cattle population, $P < 0.01$). The mean for androstenedione was elevated in medium estrogen-active follicles of twinnings ($P < 0.01$) at 48 h after PGF_{2α} compared with the other time periods within twinnings or with medium estrogen-active follicles of control cows. Means for the lower ($P < 0.01$) androstenedione concentrations (Table 2) in the follicular fluid of medium or large estrogen-inactive follicles did not differ ($P > 0.10$) among time periods after PGF_{2α}.

Testosterone. Testosterone concentration (Figure 3) in follicular fluid of large estrogen-active follicles of twinner cows increased ($P < 0.01$) quadratically with time after PGF_{2α}, increases for means at 48 ($P < 0.05$) and 72 h ($P < 0.01$) were significant, compared with

transient elevations ($P < 0.01$) for means of controls at 24 and 72 h after PGF_{2α} (follicular status × time × cattle population, $P < 0.01$). Means for large estrogen-active follicles of controls were greater ($P < 0.05$) at 24 h but less ($P < 0.05$) at 72 h compared with means for large estrogen-active follicles of twinnings. Like estradiol and androstenedione, medium estrogen-active follicles of twinnings had elevated ($P < 0.01$) testosterone concentrations at 48 h after PGF_{2α} compared with other time periods or with controls. Low ($P < 0.01$) concentrations of testosterone (Table 2) in the fluid of medium and large estrogen-inactive follicles were unaffected ($P > 0.10$) by time after PGF_{2α}.

Follicular Fluid IGFBP

Stage of development and steroid production had major effects on the amount of IGFBP binding activity detected in the follicular fluid of individual follicles (Figure 4). Binding activity for IGFBP-5 was absent and for IGFBP-4 was lower or absent from follicular fluid of large estrogen-active follicles and of growing medium estrogen-active follicles, whereas IGFBP-2 was only absent from the fluid of dominant (preovulatory) follicles. Low IGFBP-4 binding activity was also found in follicles with steroid profiles and reduced vascularity indicative of early atresia, whereas binding activity for IGFBP-5 was found only in the follicular fluid of follicles with elevated progesterone concentrations (Figure 4). Binding activity for IGFBP-3 was less ($P < 0.01$) in the follicular fluid of medium and large estrogen-active follicles than in medium and large estrogen-inactive follicles. Thus, binding activities in follicular fluid were correlated positively with progesterone and negatively with estradiol concentrations in follicular fluid (Table 3).

IGFBP-3. Binding by IGFBP-3 (Table 4) was identified in follicular fluid by two bands of binding activity, 44- and 40-kDa forms. Similarity in binding patterns between the 44- and 40-kDa forms was reflected by an overall correlation coefficient of 0.93 ($P < 0.01$). The general trend was for estrogen-active follicles to have less IGFBP-3 binding activity than estrogen-inactive follicles (follicular status; $P < 0.01$). Specifically, medium and large estrogen-active follicles had less ($P < 0.01$) 44-kDa activity than large estrogen-inactive follicles (Table 4). Estrogen-active follicles also had less 40-kDa activity compared with medium ($P < 0.05$) and

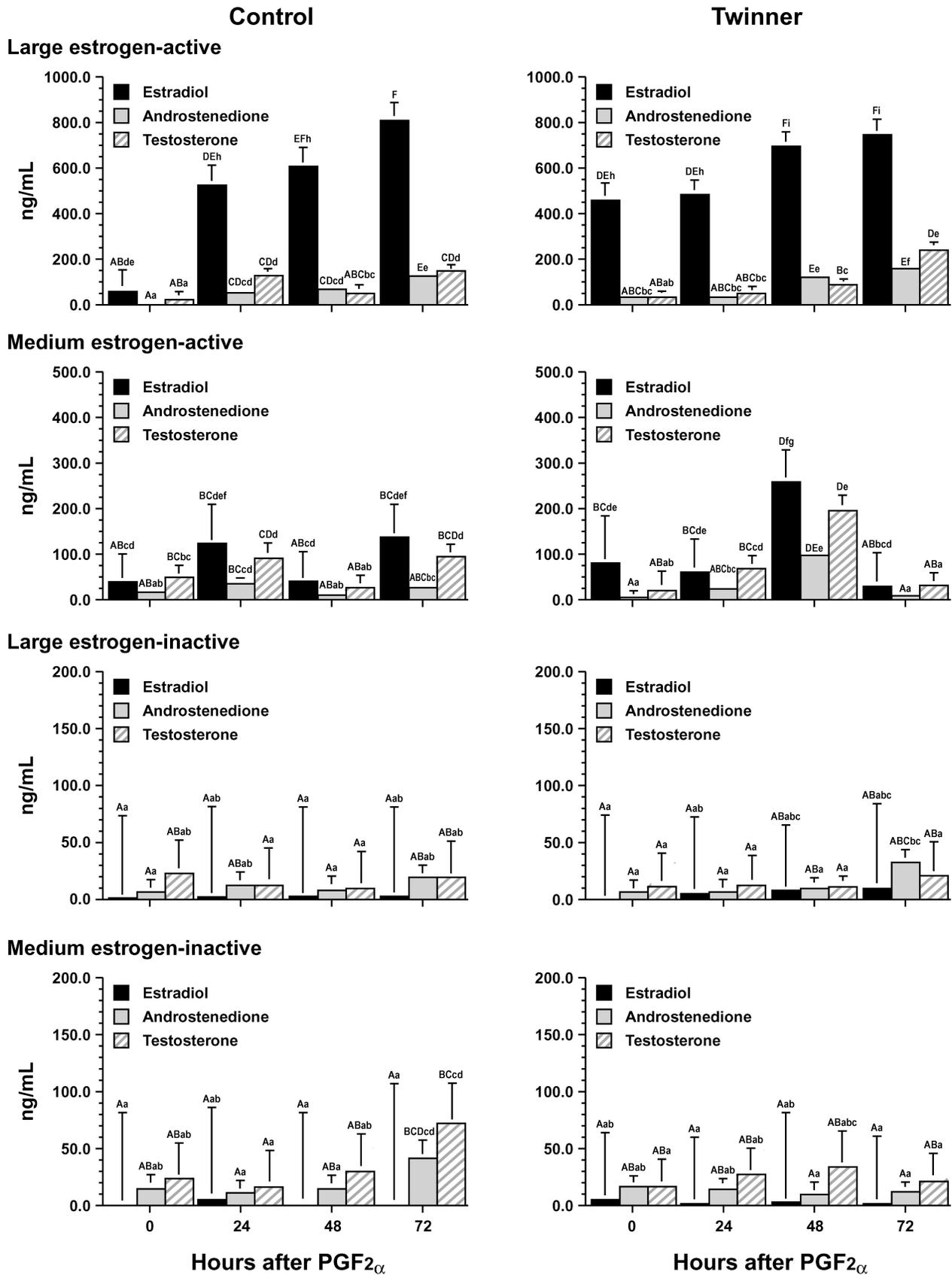


Figure 3. Comparisons of follicular fluid estradiol, androstenedione, and testosterone concentrations (means \pm SEM) in large (>10 mm) and medium (5.0–9.9 mm) estrogen-active and estrogen-inactive follicles between twinner and control cows after a single injection of PGF_{2 α} on d 18. ^{A,B,C,D,E,F,a,b,c,d,e,f,g,h,i,j} Means with different superscripts differ; ^{A,B,C,D,E,F} *p* < 0.01, ^{a,b,c,d,e,f,g,h,i,j} *p* < 0.05.

Table 3. Simple correlations among follicular fluid steroid concentrations and IGFBP activity^a

Variable	Correlation coefficients (r) ^b										
	Estr	Prog	Andr	Tes	44 kDa	40 kDa	34 kDa	32 kDa	30 kDa	28 kDa	24 kDa
Estr		-0.26	0.77	0.52	-0.17	-0.26	-0.47	-0.31	-0.25	-0.22	-0.36
Prog	-0.26		-0.28	-0.23	0.19	0.36	0.20	0.48	0.27	0.32	0.45
Andr	0.77	-0.28		0.64	-0.09	-0.17	-0.28	-0.26	-0.14	-0.18	-0.28
Tes	0.52	-0.23	0.64		-0.17	-0.24	-0.21	-0.22	-0.13	-0.13	-0.21
44 kDa	-0.17	0.19	-0.09	-0.17		0.93	0.33	0.41	0.28	0.11	0.38
40 kDa	-0.26	0.36	-0.17	-0.24	0.93		0.56	0.58	0.47	0.24	0.59
34 kDa	-0.47	0.20	-0.28	-0.21	0.33	0.56		0.44	0.62	0.36	0.66
32 kDa	-0.31	0.48	-0.26	-0.22	0.41	0.58	0.44		0.53	0.51	0.68
30 kDa	-0.25	0.27	-0.14	-0.13	0.28	0.47	0.62	0.53		0.42	0.76
28 kDa	-0.22	0.32	-0.18	-0.13	0.11	0.24	0.36	0.05	0.42		0.57
24 kDa	-0.36	0.45	-0.28	-0.21	0.38	0.59	0.66	0.68	0.76	0.57	

^aSteroids and IGFBP compared were estradiol (Estr), progesterone (Prog), androstenedione (Andr), testosterone (Tes), 44- and 40-kDa IGFBP-3, 34-kDa IGFBP-2, 32- and 30-kDa IGFBP-5, and 28- and 24-kDa IGFBP-4.

^bPositive or negative coefficients ≥ 0.19 are significant ($P \leq 0.01$); $n = 191$ follicles.

large ($P < 0.01$) estrogen-inactive follicles, and medium estrogen-inactive follicles had less ($P < 0.05$) 40-kDa activity than large estrogen-inactive follicles. Total activity of IGFBP-3 (i.e., 40 kDa + 44 kDa) was also less in medium and large estrogen-active follicles compared with medium ($P < 0.05$) or large ($P < 0.01$) estrogen-inactive follicles. Also, overall binding activity for the individual forms of IGFBP-3 and for total IGFBP-3 activity were greater ($P < 0.01$) in the follicular fluid of follicles from twinner cows compared with control cows (Table 4).

IGFBP-2. Binding activity for IGFBP-2 was detected in follicular fluid as a 34-kDa form (band). Binding by IGFBP-2 (Figure 4) was absent from the fluid of the large preovulatory follicles with elevated estradiol concentrations (i.e., estradiol:progesterone > 10), low in estrogen-active follicles with intermediate estradiol concentrations, and greatest ($P < 0.01$) in estrogen-inactive follicles. Thus, IGFBP-2 binding activity was correlated negatively ($r = -0.47$; $P < 0.01$) with follicular fluid estradiol concentrations (Table 3). Similarly, comparisons of IGFBP-2 binding activity (Table 5) among the four follicular status groups revealed lower ($P < 0.01$) intensity in the fluid of large estrogen-active follicles compared with the other three groups, and lower ($P < 0.01$) activity in medium estrogen-active follicles than in estrogen-inactive follicles; binding activity did not differ ($P > 0.10$) between medium and large estrogen-inactive follicles. An overall reduction in follicular fluid IGFBP-2 binding activity occurred ($P < 0.01$) between 0 and 24 h after PGF_{2 α} (Table 5); activity did not change ($P > 0.10$) between 24 and 72 h. The IGFBP-2 binding did not differ ($P > 0.10$) between cattle populations.

IGFBP-5. Binding by IGFBP-5 (Table 5) was detected as 30- and 32-kDa bands, and binding activity for the two forms was correlated positively overall ($r = 0.53$; $P < 0.01$). Neither form of IGFBP-5 was detectable in the follicular fluid of large estrogen-active follicles from either twinner or control cows. In addition, the 32-kDa

band was not detected in medium estrogen-active follicles, whereas the 30-kDa band was detected in a few medium estrogen-active follicles of control cows. The IGFBP-5 binding activity was intermediate ($P < 0.01$) in large estrogen-inactive follicles and greatest ($P < 0.01$) in medium estrogen-inactive follicles. Thus, activities of both 30- and 32-kDa IGFBP-5 were correlated ($P < 0.01$) positively with follicular fluid progesterone concentrations and negatively with estradiol concentrations (Table 3). Overall binding activity for the 32-kDa IGFBP-5 was also greater ($P < 0.05$) in the fluid of medium and large estrogen-inactive follicles from twinner vs. control cows (Table 5).

In contrast with 32-kDa, 30-kDa binding activity (Table 5) was elevated ($P < 0.05$) in the fluid of medium and large estrogen-inactive follicles at 72 h compared with 0, 24, and 48 h. The increase in 30-kDa binding at 72 h occurred in both cattle populations.

IGFBP-4. Binding activity of IGFBP-4 (Table 5) was also present in follicular fluid in two forms, 24-kDa and 28-kDa, and their binding activities were correlated positively overall ($r = 0.57$; $P < 0.01$). Binding activities (Table 5) for both the 24- and 28-kDa forms of IGFBP-4 were affected ($P < 0.01$) by the steroidogenic status of the follicle, being low or undetectable in the fluid of medium or large estrogen-active follicles, intermediate in large estrogen-inactive follicles, and greatest in medium estrogen-inactive follicles. Thus, IGFBP-4 activities were correlated ($P < 0.01$) positively with follicular fluid progesterone concentrations and negatively with estradiol concentrations (Table 3). Furthermore, binding to 24- and/or 28-kDa IGFBP-4 in medium and large estrogen-active follicles was frequently associated with the early onset of atresia and reduced vascularity as assessed visually.

Binding activity of 28-kDa IGFBP-4 was also affected ($P < 0.05$) by time and cattle population (Table 5). Overall binding activity for the 28-kDa IGFBP-4 showed a significant ($P < 0.01$) increase in both cattle populations

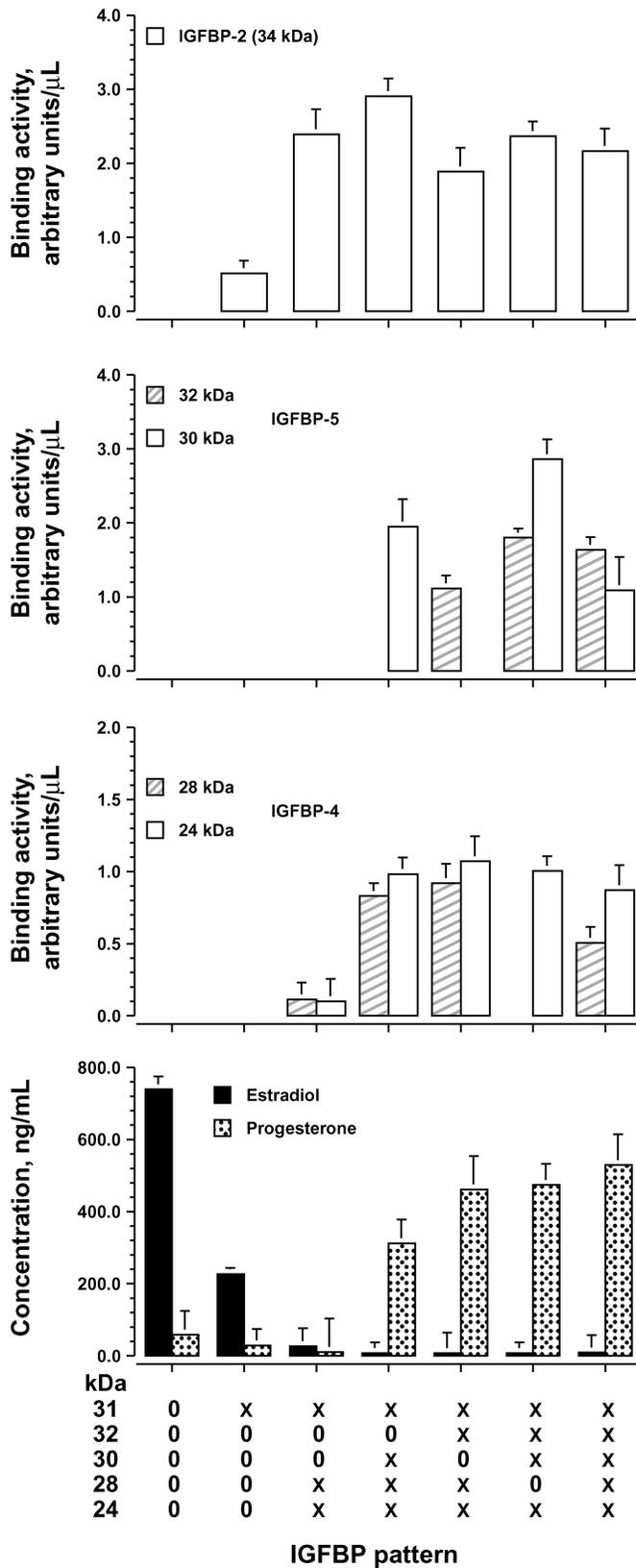


Figure 4. Patterns of IGFBP-2, -5, and -4 binding activity and estradiol and progesterone concentrations (means \pm SEM) in follicular fluid from medium and large antral follicles. Individual follicles were categorized by the absence (o) or presence (x) of individual IGFBP bands in their follicular fluid.

at 72 h after PGF_{2 α} and was greater ($P < 0.01$) in the fluid of estrogen-inactive follicles from control vs. twinner cows (Table 5).

Discussion

Long-term selection of cattle for dizygotic (fraternal) twin births, initially for twin births and subsequently a combination of yearling ovulation rate and life-time twinning rate (Echternkamp et al., 1990a; Gregory et al., 1990; Van Vleck et al., 1991), has increased twinning rate by about 3%/yr to an annual frequency of 50 to 55% twinning (Echternkamp and Gregory, 2002) in the MARC twinning herd. Although some evidence exists for a superior maternal uterine environment in these females and a QTL (Thallman et al., 1999) has been linked to twinning rate separate from ovulation rate, the primary selection response has been an increased frequency of twin and triplet ovulations (Echternkamp et al., 2002). Collectively, results from the present and previous studies indicate that selection for natural production of twin (multiple) ovulations has modified the regulatory system of ovarian folliculogenesis to enable the activation and development of more preantral follicles (Cushman et al., 2000), the maintenance of a larger pool of small antral follicles, the recruitment of more follicles within the cohort of developing follicles, and the selection of two or more dominant (ovulatory) follicles within a follicular wave (Echternkamp, 2000).

The physiological basis for this genetic modification of ovarian folliculogenesis in twinner females is unknown, but a consistent finding has been greater blood and follicular fluid concentrations of IGF-I in twinner cows compared with unselected cattle (Echternkamp et al., 1990b; Echternkamp et al., 1999). Insulin-like growth factor-I stimulates proliferation, differentiation, and steroidogenesis (Spicer et al., 1993; Spicer and Echternkamp, 1995) and inhibits apoptosis (Chun et al., 1996; Guthrie et al., 1998) in ovarian follicular cells. Thus, increased IGF-I in twinner females may enhance follicular development by recruiting more follicles and/or by reducing atresia. Studies with IGF-I-null mice further substantiate that IGF-I is essential for granulosa cell proliferation and follicular growth (Kadokia et al., 2001). Furthermore, sustained treatment of cattle (Gong et al., 1993) or swine (Echternkamp et al., 1994b) with exogenous recombinant bovine or porcine GH, respectively, increased blood IGF-I concentrations and numbers of small antral follicles within the ovaries, but an increase in ovulation rate only occurred if exogenous FSH was subsequently administered (Gong et al., 1993). Because the treatment of ovariectomized sheep (Echternkamp, 1999) or cattle (Richards et al., 1991; Simpson et al., 1997) with estradiol increased circulating concentrations of IGF-I and GH, increases in plasma concentrations of IGF-I and GH with time after PGF_{2 α} injection were likely stimulated by concurrent increases in estradiol secretion. Thus, increased num-

Table 4. Effect of follicular status and population on IGFBP-3 binding activity in follicular fluid

Source of variation	n	IGFBP binding activity, arbitrary units/ μ L		
		44 kDa	40 kDa	BP-3
Follicular status		<0.01	<0.01	<0.01
Large estrogen-active	50	2.80 \pm 0.19 ^a	0.74 \pm 0.07 ^a	3.54 \pm 0.27 ^{a,c}
Medium estrogen-active	46	2.86 \pm 0.19 ^a	0.73 \pm 0.08 ^a	3.60 \pm 0.28 ^{a,c}
Large estrogen-inactive	50	3.66 \pm 0.19 ^b	1.33 \pm 0.07 ^{b,c}	4.99 \pm 0.27 ^b
Medium estrogen-inactive	46	3.35 \pm 0.19 ^{a,b}	1.10 \pm 0.08 ^{b,d}	4.45 \pm 0.28 ^d
Population				
Control	81	2.61 \pm 0.13 ^a	0.79 \pm 0.05 ^a	3.40 \pm 0.18 ^a
Twiner	111	3.73 \pm 0.13 ^b	1.17 \pm 0.05 ^b	4.91 \pm 0.18 ^b

^{a,b,c,d}Means (\pm SEM) with different superscripts differ within follicular status or population by column; ^{a,b} $P < 0.01$; ^{c,d} $P < 0.05$.

bers of developing and dominant follicles and increased plasma estradiol concentrations in twinner females may contribute to the increased IGF-I concentrations. However, overall means for plasma GH concentration tended to be lower in twinner cows compared with control cows, but the daily blood collections in the present study have limited interpretative value for pulsatile GH secretion.

Plasma cholesterol concentrations were lower in twinner vs. control cows, which may have been either a direct or indirect response to selection for ovulation rate. Comparisons of reproductive and endocrine traits and of organ weights between lines of swine selected for high or low serum cholesterol revealed that the low cholesterol line had increased ovulation rate and litter size, increased serum FSH (but decreased inhibin) concentrations, and increased liver weight compared with the high line (Wise et al., 1993); serum GH concentrations did not differ between the two lines of swine. Some of the foundation animals in the twinner population contributed genetics of several dairy or dual-purpose cattle breeds (e.g., Holstein, Swedish Friesen, Norwe-

gian Red, and Swedish Red and White) not present in the control population. Thus, lower cholesterol in twinner cows may reflect metabolic differences between the two cattle populations. For example, plasma cholesterol concentrations have been reported to be correlated positively ($r = 0.41$ to 0.44) with energy balance in lactating dairy cows (Spicer et al., 1993; Francisco et al., 2002). Because differences in plasma FSH concentrations or release have not been found between twinner cattle and cattle unselected for ovulation rate (Echternkamp et al., 1999; Echternkamp, 2000), increased ovulation rate in twinner cows does not seem to result from increased FSH secretion.

Alternatively, the emergence of multiple dominant follicles in twinner females is the consequence of their dominant follicles, producing less of the inhibitory factor that suppresses the development of the subordinate follicle(s); thus, the subordinate follicle(s) continues to grow, mature, and become ovulatory. As reported previously (Ginther et al., 2001), comparisons of profiles for follicular fluid estradiol concentrations and IGFBP-2, -4, and -5 binding activities among cohorts of growing

Table 5. Effect of follicular status, cattle populations, and time after PGF on IGFBP-2, -5, and -4 binding activity in follicular fluid

Source of variation	n	IGFBP binding activity, arbitrary units/ μ L				
		BP-2 (34 kDa)	BP-5 (32 kDa)	BP-5 (30 kDa)	BP-4 (28 kDa)	BP-4 (24 kDa)
Follicular status						
Large estrogen-active	50	0.35 \pm 0.17 ^a	0.00 \pm 0.06 ^a	0.00 \pm 0.12 ^a	0.00 \pm 0.06 ^a	0.01 \pm 0.08 ^a
Medium estrogen-active	46	0.67 \pm 0.18 ^b	0.00 \pm 0.07 ^a	0.01 \pm 0.12 ^a	0.02 \pm 0.07 ^a	0.02 \pm 0.09 ^a
Large estrogen-inactive	50	2.53 \pm 0.17 ^c	0.45 \pm 0.06 ^b	0.52 \pm 0.12 ^b	0.30 \pm 0.06 ^b	0.69 \pm 0.08 ^b
Medium estrogen-inactive	46	2.70 \pm 0.18 ^c	0.69 \pm 0.07 ^c	1.45 \pm 0.12 ^c	0.60 \pm 0.07 ^c	1.28 \pm 0.09 ^c
Population						
Control	81		0.18 \pm 0.05 ^d		0.27 \pm 0.05 ^a	
Twiner	111		0.36 \pm 0.04 ^e		0.18 \pm 0.04 ^b	
Time after PGF, h						
0	39	1.92 \pm 0.22 ^a		0.38 \pm 0.14 ^a	0.12 \pm 0.08 ^a	
24	49	1.47 \pm 0.17 ^b		0.36 \pm 0.12 ^a	0.20 \pm 0.07 ^a	
48	58	1.48 \pm 0.16 ^b		0.28 \pm 0.12 ^a	0.18 \pm 0.06 ^a	
72	46	1.38 \pm 0.18 ^b		0.72 \pm 0.12 ^b	0.37 \pm 0.07 ^b	

^{a,b,c,d,e}Means (\pm SEM) that do not have a common superscript differ within source of variation by column; ^{a,b,c} $P < 0.01$; ^{d,e} $P < 0.05$.

follicles suggested that the emergence of the dominant follicle began in control cows when the largest developing follicle was about 8 mm in diameter. However, selection of the dominant follicle(s) in twinners appeared to be more variable and the dominant follicle(s) was not clearly identifiable in some cows at 10 to 10.5 mm in diameter. This possible anatomical difference in follicle selection between cattle populations warrants further investigation.

The objective of the present study was to compare the physiological development of individual antral follicles and the selection of the dominant follicle(s) between cows selected and unselected for the production of twin ovulations. Even though all of the cows received the injection of PGF_{2 α} on d 18 of the estrous cycle, the status of follicular development varied among cows, which is the consequence of variation within and among cows in the number and duration of follicular waves within an estrous cycle and in the emergence and selection of the dominant follicle(s) within a follicular wave. The follicular phase of the estrous cycle was advanced in some of the twinner cows as noted by earlier onset of luteolysis and of preovulatory gonadotropin release. This advancement in twinners contributed to the significant cattle population \times time interactions for plasma concentrations of estradiol, LH, and FSH and to the follicular status \times time \times cattle population interactions for follicular fluid concentrations of estradiol, testosterone, and androstenedione. Thus, detected differences in estradiol concentrations in the fluid of large estrogen-active follicles between controls and twinners likely resulted from timing differences in follicular development rather than genetic differences in steroidogenesis or numbers of follicular cells per follicle found in ewes with the fecundity Booroola gene (Montgomery et al., 2001). Also, greater estradiol concentrations at 24 and 48 h after injection of PGF_{2 α} in the blood of twinner cows may have resulted from the increased numbers of large and medium antral follicles within their ovaries. This variation in timing or speed of follicular development after injection of PGF_{2 α} among the 48 cows emphasizes the challenge of achieving precisely regulated ovulation and timed insemination.

Binding of IGF-I to IGFBP-2, -4, and -5 in follicular fluid was influenced negatively by estradiol concentrations in both medium and large follicles with binding activity for IGFBP-4 and -5 disappearing earlier in follicular maturation than for IGFBP-2. Similar negative relationships between estradiol and IGFBP-2, -4, or -5 binding have been reported previously for cattle (Echternkamp et al., 1994a; Stewart et al., 1996), sheep (Monget et al., 1993; Spicer et al., 1995), and swine (Echternkamp et al., 1994b). All of the IGFBP are high-affinity carrier proteins that bind both IGF-I and -II, prolong their half-life, and, in most situations, inhibit the biological actions of IGF-I and -II. Consequently, effects of IGF-I and -II on follicular development and atresia may be more associated with changes in follicular fluid IGFBP activity than with changes in follicular

fluid IGF-I and -II concentrations. Thus, the absence of IGFBP-4 and -5 binding activity in medium and large estrogen-active follicles may facilitate the stimulatory effects of IGF-I and -II on follicular cell mitogenesis and steroidogenesis during follicular recruitment and development. However, concentrations of IGFBP-2 and -3 in bovine follicular fluid are several times greater than for IGFBP-4 and -5 (Stewart et al., 1996); thus, changes in follicular fluid IGFBP-2 would likely have a greater effect on the bioavailability of IGF-I and -II. Although correlated negatively with estradiol concentration, total suppression of IGFBP-2 activity was observed only in mature preovulatory follicles and appeared to be associated with selection of the dominant or preovulatory follicle(s). Perhaps the extended presence of IGFBP-2 in the dominant follicle(s) extends availability of IGF-I and/or IGF-II to the follicle by extending their half-lives in follicular fluid.

The later disappearance of IGFBP-2 may indicate that the synthesis and/or degradation of IGFBP-2 are regulated differently from IGFBP-4 and -5. The decrease in follicular fluid IGFBP-2 and -5 binding activities in cattle has been reported to coincide with a decrease in IGFBP-2 and -5 mRNA and protein levels in the accompanying granulosa and thecal cells (Roberts and Echternkamp, 2003). Conversely, IGFBP-4 mRNA and protein levels were elevated in both the granulosa and thecal cells of large estrogen-active, healthy follicles, and levels were similar to those in granulosa cells of small and large atretic follicles but greater than those in thecal cells of small and large atretic follicles (Roberts and Echternkamp, 2003). Likewise, *in vitro* production of IGFBP-4 by bovine granulosa cells of small and large follicles was inhibited by estradiol and LH separately, but estradiol had no effect on IGFBP-2, -3, or -5 production by either thecal or granulosa (Spicer and Chamberlain, 2002). Proteolytic degradation of both IGFBP-4 (Mazerbourg et al., 2000; Spicer et al., 2001) and IGFBP-5 (Spicer et al., 2001; Bridges et al., 2002), and to a limited extent IGFBP-2, in follicular fluid has been confirmed in several species and has been attributed to the presence of pregnancy-associated plasma protein-A in the follicular fluid of preovulatory follicles (Monget et al., 2003).

The onset of atresia in individual follicles coincided with the reappearance of first IGFBP-2, then IGFBP-4, and finally IGFBP-5. The only genetic difference in IGFBP binding activity was greater IGFBP-3 binding in the follicular fluid of follicles from twinner cows, which may have been the consequence of greater circulating IGF-I and, thus, increased diffusion of the IGFBP-3-IGF-I complex from the ovarian vasculature into the antral follicular fluid. As with IGFBP-2, -4, and -5, IGFBP-3 binding was less in estrogen-active than estrogen-inactive follicles.

In summary, the selection of cattle for the natural production of twin (multiple) ovulations, and resulting twin births, has modified the regulatory system of ovarian folliculogenesis to enable the activation and devel-

opment of more preantral follicles, the maintenance of larger pools of small antral follicles, the recruitment of more follicles within the cohort of developing follicles, the selection of two or more dominant (ovulatory) follicles within a follicular wave, and/or a reduced atresia of preantral and antral follicles. Because of reported stimulatory effects of IGF-I on proliferation and differentiation, and reduced apoptosis, of ovarian follicular cells, it is speculated that the greater IGF-I in blood and follicular fluid of twinner females, compared with genetically unselected females, contribute to their enhanced follicular development. Differences in estradiol concentrations in the fluid of large estrogen-active follicles between controls and twinningers at time of PGF_{2 α} injection (follicular status \times time \times population) likely resulted from timing differences in follicular development rather than genetic differences in steroidogenesis or numbers of follicular cells per follicle. The greater estradiol concentrations at 24 and 48 h after injection of PGF_{2 α} in the blood of twinner cows likely resulted from increased numbers of large and medium antral follicles within their ovaries. The binding of IGF-I to IGFBP-2, -4, and -5 was influenced negatively by estradiol concentrations in the follicular fluid of both medium and large follicles; the total suppression of IGFBP-2 activity occurred only in mature preovulatory follicles. The only consistent genetic difference in IGFBP binding activity was increased binding by IGFBP-3 in the follicular fluid of follicles from twinner cows, which may have resulted from increased circulating IGF-I and, consequently, increased diffusion of the IGFBP-3-IGF-I complex into the follicles.

Implications

Long-term selection of cattle for ovulation rate and twinning rate has modified the regulatory system of ovarian folliculogenesis to enhance follicular development and to induce selection of two ovulatory follicles within a follicular wave; consequently, the frequency of twin births has increased to >50%. The increased follicular development may result from greater blood and follicular concentrations of IGF-I in twinningers vs. control cows. Gonadotropin and steroid secretion did not differ between the two cattle populations studied. The absence of IGFBP-2, -4, and -5 in developing preovulatory follicles implies roles for these binding proteins in the recruitment and selection of the dominant follicle(s), possibly by regulating IGF-I and -II availability to the follicular cells. The disappearance of IGFBP-2 binding activity occurred only in advanced maturational stages of development of the preovulatory follicle(s), which is further indication of a role for IGFBP-2 in the selection of ovulatory follicles.

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