Large variation in steroid concentrations and insulin-like growth factor binding proteins exists among individual small antral follicles collected from within cows at random stages of the estrous cycle[1]

A. J. Roberts,*[2] M. J. Al-Hassan,†[3] P. M. Fricke,†[4] and S. E. Echternkamp†

*USDA-ARS, Fort Keogh Livestock and Range Research Laboratory, Miles City, MT 59301-4016;
†USDA-ARS, Roman L. Hruska US Meat Animal Research Center, Clay Center, NE 68933-0166

ABSTRACT: Variation in the biochemical status of individual small (≤5 mm diameter) antral follicles within the ovaries of a cow at any given time likely influences the capacity for undergoing recruitment, selection, and establishing dominance. The objectives of this study were to provide insight into the magnitude of variation in follicular fluid concentrations of steroids and activities of IGFBP that exists among individual small antral follicles within and between cows, and to determine the relationships between follicular fluid IGFBP and steroid concentrations in these follicles. A total of 108 small antral follicles were collected from 6 cows at random stages of the estrous cycle, with 10 to 26 follicles/cow. Concentrations of steroids (ng/mL of follicular fluid) in the overall population of follicles ranged from 0.1 (lowest detectable limit) to 51 for estradiol (E_2), 4 to 1,149 for progesterone (P_4), and 5 to 504 for androstenedione (A_4). Concentrations of E_2 and A_4 were associated positively (r = 0.2; $P < 0.02$), but E_2 (r = –0.4) and A_4 (r = –0.4) were associated negatively, with P_4. The proportion of variation in steroid concentrations accounted for by differences among animals ($P < 0.05$) was small for E_2 (12%), moderate for P_4 (43%), and greatest for A_4 (74%). Least differences between minimum and maximum concentrations of steroids observed in follicles from within a cow were 21-, 5.5-, and 3.5-fold for E_2, P_4, and A_4, respectively, whereas the greatest differences between minimum and maximum concentrations were 505-, 108-, and 26-fold for E_2, P_4, and A_4, respectively. Ranges of IGFBP concentrations (arbitrary densitometer units) detected in fluid from a subsample of 43 follicles were 1.18 to 4.50 for IGFBP-3, 0.54 to 4.68 for IGFBP-2, 0.07 to 2.56 for IGFBP-4, and 0.01 to 6.71 for IGFBP-5. Concentrations of E_2 were correlated negatively with each IGFBP (r = –0.4 to –0.8; $P < 0.05$) except IGFBP-3. In contrast, concentrations of A_4 were correlated positively with IGFBP-3 (r = 0.4; $P < 0.05$) but were not correlated with other IGFBP. Concentrations of P_4 were correlated positively (r > 0.4; $P < 0.05$) with IGFBP-4 and -5. The results indicate that steroid concentrations and IGFBP activities vary substantially among small antral follicles collected from within and among individual animals and that increasing production of E_2, the hallmark of a developing follicle, was associated with reduced activity of all IGFBP except IGFBP-3, thereby implicating these IGFBP in the regulation of follicular recruitment.

Key words: bovine, estradiol, follicle, insulin-like growth factor binding protein, ovary, progesterone

J. Anim. Sci. 2006. 84:2714–2724
doi:10.2527/jas.2006-077

INTRODUCTION

Characterization of changes in size and biochemical composition of follicles >5 mm in diameter has led to the theory that follicle growth occurs in recurrent waves of development (Ginther et al., 2001). Although research on smaller antral follicles is lacking, variations in developmental status of individual small antral follicles present on the ovaries of a cow at any given time may predetermine which follicles progress through the different stages of a wave of development (i.e., recruitment, selection, and establishment of dominance) when exposed to appropriate endocrine sig-

[1]Mention of a proprietary product does not constitute a guarantee or warranty of the product by USDA or the authors and does not imply its approval to the exclusion of other products that may be also suitable. Authors thank A. Paus and S. Schemm for assistance with laboratory procedures and S. Miles for clerical assistance in preparing the manuscript for publication.

[2]Corresponding author: andy@larrl.ars.usda.gov

[3]Current address: Department of Animal Production, College of Agriculture, King Saud University, Saudi Arabia.

[4]Current address: University of Wisconsin, Department of Dairy Science, 1675 Observatory Drive, Madison, WI 53706-1284.

Received February 13, 2006.
Accepted May 15, 2006.

Table 1. Estimated stage of the estrous cycle based on appearance and weight of corpus luteum (CL), circulating concentration of progesterone (P_4), and diameter (mm) and concentrations (ng/mL) of estradiol (E_2) and P_4 in follicular fluid from all individual follicles >5 mm in diameter from each of 6 cows

Cow ID:	279	276	281	282	278	277
Item	1 to 3 d after ovulation		Midluteal		Late-luteal	
Stage of cycle						
CL wt, g	0.2	0.3	7.0	3.4	3.0	3.2
Serum P_4, ng/mL	0.4	0.9	6.0	3.3	2.5	1.5
Follicle diameter: E_2: P_4	10: 0.5: 15	8: 0.1: 490	16: 60: 68	10: 0.1: 555	12: 486: 138	15: 647: 109
	8: 0.2: 1,005	6: 19: 36	8: 0.4: 323	>8: ND[1]: ND	7: 7.1: 628	12: 9.0: 896
	8: 0.1: 700	6: 17: 29	7: ND: ND	>8: ND: ND		8: 0.9: 189
	7: 0.1: 775	6: 0.6: 12				
	6: 0.3: 88	6: 2.2: 454				

[1]ND = Not determined, follicle ruptured during collection.

nals. Knowledge of the variation in biochemical characteristics existing among individual small antral follicles on the ovaries from within an animal at any given time is a prerequisite for determining if the aforementioned presumption is correct.

Follicular fluid steroid concentrations have been used to differentiate between healthy (i.e., estrogen-active) and atretic (i.e., estrogen-inactive) follicles (Moor et al., 1978; Carson et al., 1981; Ireland and Roche, 1982). Bioavailability of IGF-I, as regulated by IGFBP, has also been proposed to regulate progression of follicular development (Spicer and Echternkamp, 1995, Ginther et al., 2001). Data concerning steroid (Ireland et al., 1979; Henderson et al., 1982; Wise, 1987) and IGFBP (Echternkamp et al., 1994; Funston et al., 1996; Roberts and Echternkamp, 2003) content in small antral follicles have generally been obtained from fluid pooled from within or among cows. The extent of variation in steroid and IGFBP activity existing among individual small antral follicles within and between cows at various stages of the bovine estrous cycle remains to be determined. The current study investigated the magnitude of variation in follicular fluid concentrations of steroids and activities of IGFBP among individual small antral follicles collected from within and between cows and evaluated relationships between concentrations of steroids and IGFBP activities in these follicles.

MATERIALS AND METHODS

Animals

Management of cows was in accordance with the local animal care and use committee. Six multiparous crossbred cyclic beef cows, ranging from 7 to 12 yr of age, were used for this study. Cows were reared and managed together at the US Meat Animal Research Center (USDA, ARS, Clay Center, NE) until slaughtered at random stages of the estrous cycle at the research center abattoir.

Both ovaries from each cow were collected immediately after slaughter and transported to the laboratory on ice. Corpora lutea (**CL**) were inspected visually and weighed, and stage of luteal development was estimated for each CL (Ireland et al., 1979). All visible antral follicles were dissected from the ovaries and classified as small (5.0 mm or less in diameter; n = 108), medium (between 5.0 and 8.0 mm; n = 8), or large (8.0 mm or greater; n = 13). Follicular fluid was aspirated from individual small antral follicles with a 25-μL Hamilton syringe. The syringe was washed several times with fresh water between follicles, which was determined to prevent cross-contamination between samples. Follicular fluid was aspirated from individual medium follicles with disposable 1-mL tuberculin syringes. Fluid was aspirated from large follicles with 3-mL disposable syringes. Fluid was stored at −20°C until assayed to determine concentrations of steroids and IGFBP activity. Jugular venous blood was collected from each cow at slaughter to determine circulating concentration of progesterone.

Analysis of Hormones and IGFBP

Previously validated RIA for androstenedione (**A_4**; Roberts and Skinner, 1990), estradiol-17β (**E_2**; Echternkamp et al., 2004), and progesterone (**P_4**; Echternkamp et al., 2004) were used to measure these steroids in unextracted aliquots of follicular fluid from individual follicles, and to measure P_4 in serum after extraction with heptane. All samples were run in a single assay for each steroid. Intraassay CV were 8.3, 12.5, and 15.1% for A_4, E_2, and P_4, respectively. Assay sensitivity (value at 90% binding) for A_4 and P_4 was 8 pg/tube and for E_2 was 0.31 pg/tube.

A subset of 43 follicular fluid samples from small antral follicles from 5 of the 6 individual animals was analyzed for IGFBP activity. The samples selected were representative of the spectrum of steroid concentrations measured in the small antral follicles. The number of samples analyzed was limited by the capacity of the gel and transfer apparatuses used for this procedure, thereby allowing for simultaneous analysis of all samples in 1 run. Follicular fluid IGFBP activity was determined by ligand blot analysis (Hossenlopp

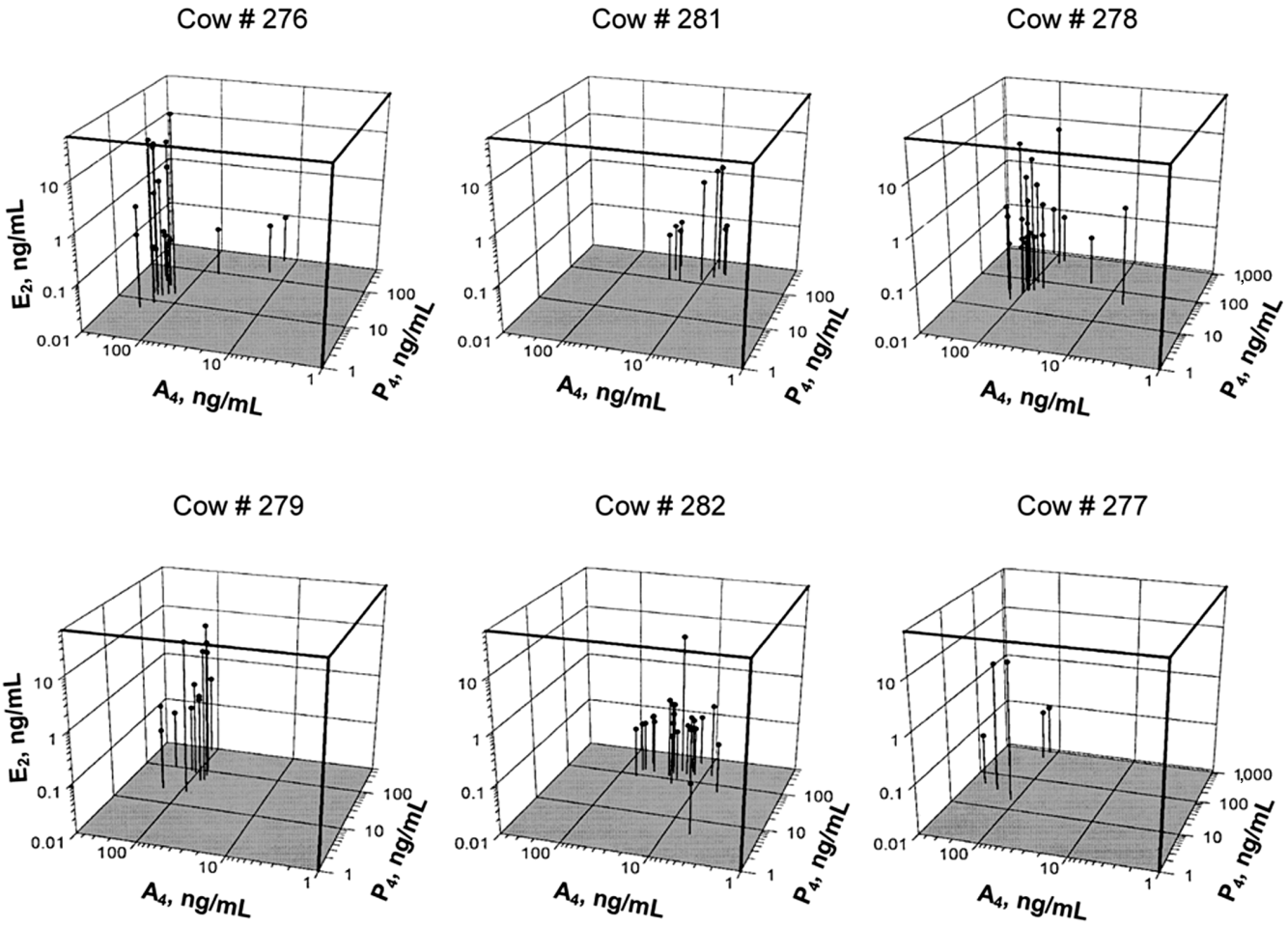

Figure 1. Concentrations of estradiol (E_2), progesterone (P_4), and androstenedione (A_4) in follicular fluid from individual small antral follicles collected from cows slaughtered at random stages of the estrous cycle. Data are shown for all follicles (n = 94) that had a sufficient volume of fluid to measure all 3 steroids. Mean concentrations of each steroid differed ($P < 0.05$) between cows.

et al., 1986) as described previously (Echternkamp et al., 1994, 2004). In brief, proteins in 1 μL of follicular fluid were separated under nonreducing conditions by 1-dimensional SDS-PAGE using a 4% stacking gel and a 12% polyacrylamide separating gel (Laemmli, 1970). Proteins were electrophoretically transferred from the gels to nitrocellulose membranes (BA-S 85, Schleicher and Schuell, Kane, NH). Membranes were incubated overnight with ^{125}I-labeled, human recombinant-IGF-I (DRG010, Bachem, Torrance, CA). The membranes were then rinsed and placed with x-ray film (XAR5 film, Eastman Kodak, Rochester, NY) for 3 and 7 d at −70°C to detect binding of IGF-I. Band intensity on autoradiographs was quantified by a scanning densitometer (LKB BrommaUltra Scan XL Laser Densitometer, Pharmacia LKB, Uppsalla, Sweden). Data from 7-d exposures are presented because the longer exposure resulted in detection of a greater number of bands without loss of detectable differences (i.e., without overexposure) among dark bands (results from analysis of 3-d exposure were similar to results from 7-d exposure for those bands detected at both times).

Statistical Analysis

All data were analyzed using SAS (SAS Inst. Inc., Cary, NC). Differences in concentrations of steroids and IGFBP activity among follicles from different animals were evaluated using the GLM procedure, with animal as a class variable. Because concentrations of E_2 in follicular fluid from many of the small antral follicles were below the sensitivity of the assay, a subsequent GLM procedure was performed in which follicles were classified into 2 groups, low E_2 (0.3 ng of E_2 or less/mL of follicular fluid) and high E_2 (>0.3 ng/mL). Differences in steroid concentrations and IGFBP activity were then evaluated using animal, follicle classification, and the interaction of animal × follicle classification. Where indicated by Hartley's test for homogeneity of variance, data were log transformed before analysis because of heterogeneous variances among the classifications.

When significant ($P < 0.05$) effects were detected in the GLM procedures, least squares means for classification variables were compared using the least sig-

Table 2. Concentrations and ratios of estradiol (E_2), progesterone (P_4), and androstenedione (A_4) in follicular fluid of small (5.0 mm or less in diameter) bovine follicles from individual animals, and the proportion of variation (R^2) in these measurements accounted for by differences among cows

	Cow ID						
Item	279	276	281	282	278	277	R^2
E_2, ng/mL	0.48[a] (0.1 to 12.3)[1]	1.42[a] (0.1 to 50.5)	0.28[ab] (0.1 to 2.1)	0.16[b] (0.1 to 3.9)	0.38[a] (0.1 to 13.3)	0.80[a] (0.1 to 10.1)	0.12
P_4, ng/mL	130[ab] (33 to 578)	19[d] (4 to 77)	221[a] (102 to 559)	156[ab] (4 to 432)	53[c] (19 to 1,148)	87[bc] (20 to 926)	0.43
A_4, ng/mL	97[b] (38 to 224)	200[a] (124 to 433)	13[d] (6 to 27)	22[c] (5 to 62)	84[b] (6 to 160)	202[a] (116 to 504)	0.74
P_4:E_2	1,172 ± 413[abc] (11 to 5,780)	159 ± 49[a] (0.1 to 774)	1,323 ± 316[cd] (81 to 2,946)	1,460 ± 203[d] (39 to 4,065)	1,192 ± 648[b] (2 to 11,486)	2,266 ± 1232[ab] (3 to 9,257)	0.41
P_4:A_4	2.5 ± 1.0[b] (0.3 to 14.4)	0.12 ± 0.02[a] (0.03 to 0.27)	20.2 ± 4.8[c] (5.4 to 40.9)	9.6 ± 1.6[c] (0.7 to 29.6)	2.1 ± 0.0.8[b] (0.1 to 15.7)	2.0 ± 1.3[b] (0.6 to 7.3)	0.72
A_4:E_2	434 ± 158 (6 to 2,240)	1,134 ± 289 (2.5 to 3,933)	117 ± 38 (3.8 to 267)	221 ± 39 (26 to 625)	592 ± 110 (6 to 1542)	1,016 ± 530 (20 to 3,348)	0.08
E_2 and P_4, No.	19	21	10	26	24	8	
A_4, No.	15	17	9	24	23	6	

[a–d]Values within a row without a common superscript differ ($P < 0.05$) when log base 10 transformed data were analyzed. Values are the antilog of the least squares means of steroid concentrations or the mean ± SE of the steroid ratios.
[1]Values inside parentheses are the range for the nontransformed steroid concentrations or the steroid ratios.

nificant differences procedure. For data that were log transformed before analysis, the antilog of the least squares means from the transformed data are presented. Simple correlations between follicular fluid steroid hormones and IGFBP were determined using the PROC CORR procedure of SAS. Linear and quadratic associations between each IGFBP and the 3 steroids within follicles were evaluated using an analysis of covariance, which included the fixed effect of cow and the linear and quadratic effect of each IGFBP on steroid concentration.

RESULTS

Classification of Cows by Stage of Cycle and by Large Follicle Steroid Concentrations

Visual appearance and weight of the CL, circulating concentrations of P_4, and steroid concentrations in fluid from all medium and large follicles were used to establish stage of the estrous cycle when follicles were collected from each animal (Table 1). Cows 279 and 276 were at the early postovulatory stage, and neither

Table 3. Concentrations (antilog of least squares means) of estradiol (E_2), androstenedione (A_4), and progesterone (P_4) in small antral follicles grouped by cow and E_2 category (0.31 ng/mL = 90% binding in the E_2 RIA)

Cow	E_2 class	No.	E_2	A_4	P_4
279	≥0.31	11	1.242[b]	92.90[b]	82.79[bc]
279	<0.31	8	0.133[c]	106.91	239.88[a]
276	≥0.31	10	4.819[a]	184.50[a]	13.37[e]
276	<0.31	11	0.122[c]	221.82	27.16[d]
281	≥0.31	4	1.18[b]	8.95[d]	276.06[a]
281	<0.31	6	0.109[c]	15.52	190.11[a]
282	≥0.31	4	0.736[b]	16.11[c]	182.81[ab]
282	<0.31	24	0.12[c]	25.82	152.05[a]
278	≥0.31	11	1.698[b]	71.94[b]	36.81[d]
278	<0.31	13	0.109[c]	94.19	71.12[c]
277	≥0.31	4	6.353[a]	230.67[a]	26.73[de]
277	<0.31	4	0.1[c]	178.24	286.42[a]
R^2			0.81	0.75	0.57
P for cow[1]			0.003	0.0001	0.0001
P for E_2 class			0.0001	0.18	0.0004
P for cow × E_2 class			0.0011	0.77	0.02

[a–e]Within a column, values without a common superscript letter differ ($P < 0.05$) when compared across cow by E_2 classifications (E_2 and P_4) or across cow classification (A_4).
[1]When using log-transformed data.

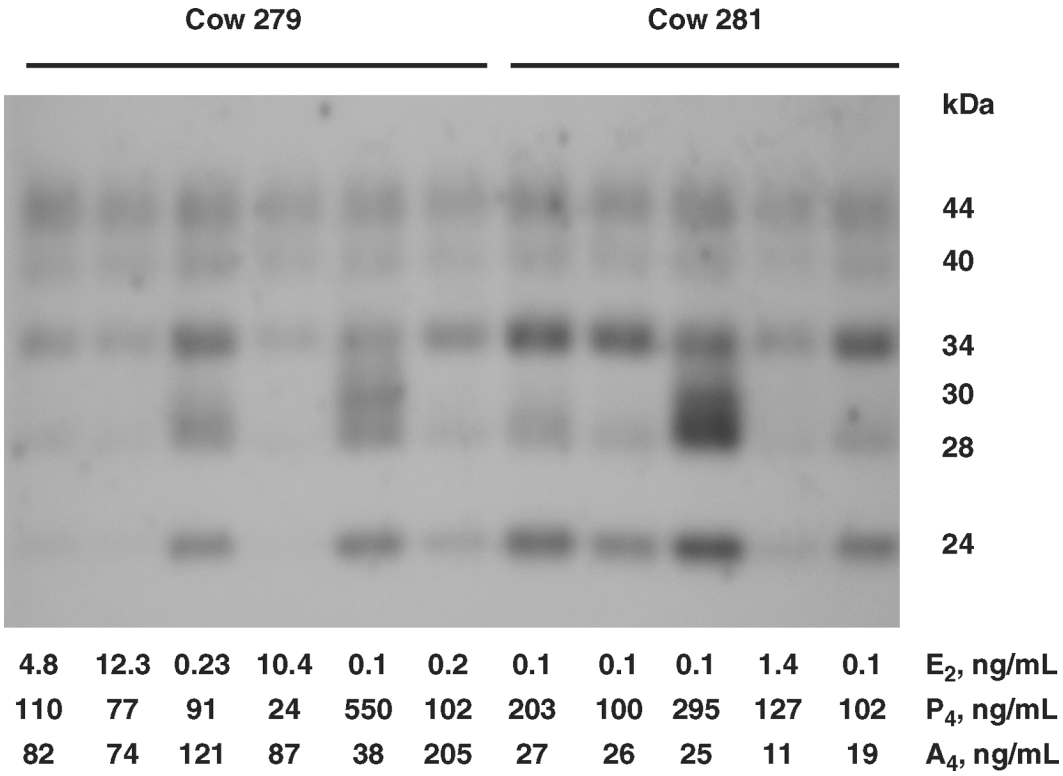

Figure 2. Representative ligand blot of IGFBP detected in follicular fluid from small antral follicles from 2 cows slaughtered at random stages of estrous cycle. Follicular fluid concentrations of estradiol (E_2), progesterone (P_4), and androstenedione (A_4) are shown at the bottom of the blot for each follicle.

cow had large estrogen-active follicles at time of slaughter. Cows 281 and 282 were at the midluteal stage of the estrous cycle. Follicular status of these 2 cows was not determined because fluid from 1 or 2 medium to large follicles was lost after accidental rupture during collection. Cows 277 and 278 were at the late luteal stage, and each cow had a large estrogen-active follicle.

Steroid Concentrations in Fluid from Small Antral Follicles

The number of small antral follicles collected from cows in this experiment varied from 10 to 26 per cow. A total of 108 follicles were analyzed for E_2 (mean = 2.0 ng/mL; SD = 5.7) and P_4 (mean = 158 ng/mL; SD = 208), but only 94 of the 108 follicles (87%) contained

Table 4. Relative amounts of IGFBP activity (arbitrary densitometry units) and concentrations of estradiol (E_2), androstenedione (A_4), and progesterone (P_4) in small bovine follicles (n = 41)

Item[1]	Mean ± SD	Minimum	Maximum	Fold difference between minimum and maximum
IGFBP-3	2.43 ± 0.77	1.18	4.50	3.8
IGFBP-2	1.79 ± 0.97	0.54	4.68	8.7
31 kDa IGFBP-5	0.89 ± 1.66	0.01[2]	6.71	671
28-29 kDa BP	1.57 ± 1.69	0.19	7.03	37
24 kDa IGFBP-4	0.99 ± 0.76	0.07	2.56	37
E_2, ng/mL	3.4 ± 8.7	0.1	50.5	505
P_4, ng/mL	185 ± 262	7	1149	170
A_4, ng/mL	90 ± 68	7	245	36

[1]Lowest detected activity of all IGFBP was 0.07. Lowest detected binding activity for the 31-kDa IGFBP-5 was 0.5 arbitrary densitometry units.
[2]This protein was not detected in 28 of the 41 samples evaluated, and a value of 0.01 was assigned to these samples.

Table 5. Simple correlations among IGFBP in follicular fluid from 41 small antral follicles

Item	28–29 kDa BP	31 kDa BP-5	IGFBP -2	IGFBP-3
24 kDa	0.75	0.69	0.78	0.57
P	0.001	0.001	0.001	0.001
28-29 kDa		0.62	0.61	0.53
P		0.001	0.001	0.001
31 kDa BP-5			0.28	0. 56
P			0.08	0.001
IGFBP-2				0.50
P				0.001

sufficient follicular fluid for analysis of A_4 (mean = 102 ng/mL; SD = 92). Of the 108 small antral follicles analyzed for E_2, 62 contained follicular fluid concentrations of E_2 that were <0.3 ng/mL, equivalent to 90% binding when 1 μL of fluid was evaluated in the RIA. Fluid remaining from these 62 follicles was insufficient to reanalyze a larger volume. Forty-three of these 62 follicles had concentrations at or below 0.1 ng/mL, the least detectable concentration when 1 μL of fluid was evaluated. Two follicles contained concentrations of A_4 below 5 ng/mL, the least detectable concentration when 1 μL of fluid was evaluated. Substantial variation existed between the minimum and maximum concentrations of steroids in the overall population of small antral follicles evaluated, including a >500-fold difference for E_2 (0.1 to 50.5 ng/mL), a 287-fold difference for P_4 (4 to 1,149 ng/mL), and a 100-fold difference for A_4 (5 to 504 ng/mL). A positive correlation existed between concentrations of E_2 and A_4 ($r = 0.23$; $P = 0.02$), but E_2 ($r = -0.39$; $P < 0.001$) and A_4 ($r = -0.43$; $P < 0.001$) were negatively correlated with P_4.

Steroid concentrations in all individual small antral follicles from each animal that contained sufficient volume to measure all 3 steroids are depicted in Figure 1. Mean steroid concentrations in small antral follicles differed among cows (Table 2). The proportion of variation in steroid concentrations accounted for by differences among animals was small for E_2 (12%), moderate for P_4 (43%), and greatest for A_4 (74%), as indicated by the coefficient of determination (R^2) shown in Table 2. The least differences between the minimum and maximum concentrations of steroids within a cow were 21-, 5.5-, and 3.5-fold for E_2, P_4, and A_4, respectively (Table 2). The greatest differences between the minimum and maximum steroid values from within a cow were 505-, 108-, and 26-fold for E_2, P_4, and A_4, respectively.

To further explore potential developmental differences among follicles, follicles were classified into 2 categories based on concentration of E_2 (i.e., ≥0.31 ng/mL and <0.31 ng/mL). Mean concentrations of steroids for the follicles in the 2 E_2 categories from each animal are summarized in Table 3. As expected, including the E_2 category and the animal × E_2 category interaction in the statistical model resulted in a larger coefficient of determination for the analysis of E_2 ($R^2 = 0.81$) compared with when animal was the only source of variation accounted for ($R^2 = 0.12$; Table 2). Although concentrations of E_2 would be expected to differ due to E_2 category, the interaction of animal × E_2 category was also shown to be an important source of variation ($P < 0.002$). Mean concentrations of E_2 in follicles with ≥0.31 ng of E_2/mL differed among cows, but the differences among cows (i.e., mean separations) were not the same as that observed when all follicles were evaluated (Table 2).

Concentrations of P_4 in small antral follicles also varied due to animal, E_2 category, and the animal × E_2 category interaction (Table 3). This method of classifying follicles accounted for more of the variation in concentrations of P_4 ($R^2 = 0.57$) than when follicles were classified by cow only ($R^2 = 0.43$; Table 2). Mean concentrations of P_4 in small antral follicles from the 2 midluteal phase cows (#281 and 282) did not differ with regard to E_2 category. Mean concentrations of P_4 in small antral follicles from the other 4 cows were less in follicles with ≥0.31 ng of E_2/mL than in follicles with <0.31 ng of E_2/mL, when compared within animal.

In contrast to E_2 and P_4, concentrations of A_4 did not differ due to E_2 category ($P = 0.18$) or the cow × E_2 classification ($P = 0.87$). Thus, concentrations of A_4 in small antral follicles were primarily influenced by variation among cows. Mean concentrations of A_4 in small antral follicles from cows 276 and 277 (i.e., the 2 cows with high mean E_2 and low mean P_4 concentrations in small antral follicles from the ≥0.31 ng of E_2/mL grouping) were greater ($P = 0.05$) than mean concentrations of A_4 in follicles from other cows (Table 3). Mean concentrations of A_4 in small antral follicles from cows 281 and 282, the 2 cows with largest mean ratios of P_4:E_2 in fluid from small antral follicles, were less ($P = 0.05$) than those observed in follicles from cows 279 and 278.

Insulin-Like Growth Factor Binding Proteins in Follicular Fluid of Small Antral Follicles

A representative ligand blot of IGFBP in follicular fluid from small antral follicles is shown in Figure 2. Previous work demonstrated that the 40 and 44 kDa bands represent 2 different glycosylated forms of IGFBP-3; the 34 kDa band is IGFBP-2; IGFBP-5 migrates as a doublet at approximately 29 and 31 kDa; and IGFBP-4 exists as a deglycosylated and glycosylated protein of approximately 24 and 28 kDa, respectively (Funston et al., 1996). Because of the close proximity of the 28-kDa form of IGFBP-4 and the 29-kDa form of IGFBP-5, these 2 bands were not always distinguishable and were therefore analyzed as 1 band.

A summary of IGFBP activities and steroid concentrations observed in the subset of small antral follicles analyzed for IGFBP activity is shown in Table 4. Data from 2 follicles, both from cow 282, were omitted from Table 4 and subsequent analyses. Both follicles con-

Table 6. Mean ± SE concentrations of estradiol (E_2), androstenedione (A_4), and progesterone (P_4), and IGFBP activity[1] (densitometry units) in small antral follicles from individual cows, and probability (*P*) and proportion of variation (R^2) accounted for by differences among cows

Cow ID	279	276	281	282	278	*P* <	R^2
No. of cows	9	9	7	7	9		
E_2, ng/mL	2.2 ± 1.3 ^{ab}	10.4 ± 5.5^{a}	0.6 ± 0.3^{b}	0.2 ± 0.1^{b}	2.2 ± 1.5^{b}	0.03	0.25
P_4, ng/mL	195 ± 71 ^{a}	22 ± 5^{b}	190 ± 63^{a}	190 ± 90^{a}	334 ± 161^{a}	0.0001	0.47
A_4, ng/mL	92 ± 16 ^{b}	185 ± 14^{a}	17 ± 3^{c}	26 ± 6^{c}	98 ± 6^{b}	0.0001	0.85
24 kDa BP-4	0.5 ± 0.2^{b}	0.6 ± 0.2^{b}	1.1 ± 0.2^{ab}	1 ± 0.2^{b}	1.7 ± 0.2^{a}	0.002	0.36
28-29 kDa BP	0.7 ± 0.6	1.5 ± 0.6	1.3 ± 0.6	2.2 ± 0.6	2.3 ± 0.6	0.27	0.13
31 kDa BP-5	0.3 ± 0.6	0.7 ± 0.6	0.7 ± 0.6	1.0 ± 0.6	1.7 ± 0.6	0.48	0.09
IGFBP-2	1.0 ± 0.3^{c}	1.5 ± 0.3^{bc}	1.8 ± 0.3^{b}	1.8 ± 0.3^{b}	2.8 ± 0.3^{a}	0.0002	0.44
IGFBP-3	1.8 ± 0.2^{b}	2.9 ± 0 0.2^{a}	2.1 ± 0.2^{b}	2.3 ± 0.2^{ab}	3.0 ± 0.2^{a}	0.0003	0.35

$^{a–c}$Values within a row without a common superscript differ ($P < 0.05$).

[1]Follicles used to evaluate IGFBP activity represent a subsample of the total follicles collected from each cow, thus accounting for the differences in the number of cows and the mean steroid concentrations compared with those shown in Table 2.

tained nondetectable levels of E_2 (i.e., <0.1 ng of E_2/mL) and were the only 2 follicles that contained nondetectable levels of A_4 (<5 ng of A_4/mL). One of these follicles contained no detectable IGFBP activity and had less P_4 (4 ng/mL) than all other follicles. The other follicle was also devoid of IGFBP activity except IGFBP-2 (3.25 densitometry units) and contained 66 ng of P_4/mL. Positive correlations existed among all the different binding proteins ($P < 0.08$; Table 5).

Analysis of IGFBP activity in small antral follicles grouped by cow demonstrated that mean binding activities of IGFBP-4 (24 kDa form), IGFBP-2, and IGFBP-3 differed ($P = 0.05$) among cows (Table 6). Mean binding activities of the 28-29 kDa and 31 kDa proteins did not differ among cows. Steroid concentrations in this subset of follicles are also summarized in Table 6.

Associations Among Insulin-Like Growth Factor Binding Proteins and Concentrations of Steroids

Correlations between IGFBP and concentrations of steroids in individual follicles are summarized in Table 7. Concentrations of E_2 were negatively correlated ($P < 0.01$) with each IGFBP except IGFBP-3. Regression analysis indicated that the relationship of each individual IGFBP, including IGFBP-3, with the log concentration of E_2 was curvilinear ($P < 0.05$ for quadratic effect, data not shown). Binding activity of IGFBP-3, but not other IGFBP, was associated positively ($P < 0.05$ for linear effect) with concentrations of A_4. Binding activity of the 24-kDa form of IGFBP-4 and the 31-kDa form of IGFBP-5 were correlated positively ($P < 0.01$) with concentrations of P_4. Positive correlations ($P < 0.01$) existed among the log base 10 transformation of the P_4:E_2 ratio and all binding proteins except IGFBP-3 (Table 7). Positive correlations ($P < 0.02$) existed among the log base 10 transformation of the A_4:E_2 ratio and all the different binding proteins (Table 7). The log base 10 transformation of the P_4:A_4 ratio was correlated ($P < 0.06$) with 31 kDa IGFBP-5 (r = 0.34) and 24 kDa IGFBP-4 (r = 0.31), but not other IGFBP.

To further evaluate the relationship among individual binding proteins and concentrations of steroids in small antral follicles, analyses of covariance were per-

Table 7. Correlations among IGFBP activities, concentrations of estradiol (Log E_2), progesterone (P_4), and androstenedione (A_4), and ratios of P_4 to E_2 (Log PE) and A_4 to E_2 (Log AE) in fluid from 41 small antral follicles

Item	24 kDa BP-4	28-29 kDa BP	31 kDa BP-5	IGFBP -2	IGFBP-3
Log[1] E_2	–0.76	–0.56	–0.44	–0.59	–0.18
P	0.001	0.001	0.001	0.001	0.25
A_4	–0.14	0.04	–0.07	0.01	0.33
P	0.37	0.82	0.69	0.95	0.04
P_4	0.51	0.21	0.58	0.03	0.36
P	0.001	0.18	0.001	0.84	0.02
Log PE	0.72	0.48	0.51	0.40	0.11
P	0.001	0.002	0.01	0.001	0.49
Log AE	0.75	0.62	0.40	0.66	0.38
P	0.001	0.001	0.01	0.001	0.02

[1]Log base 10-transformed values.

Table 8. Summary of covariate analysis of log-transformed concentrations of steroids in small antral follicles (n = 41), fitting cow as a class variable, with linear and quadratic effects and individual IGFBP

Dependent variable[1]	IGFBP fit in model[2]	R_2	*P* for model	*P* for cow	*P* for BP	*P* for BP × BP
Log E_2	24 kDa BP-4	0.83	0.001	0.001	0.001	0.07
	28-29 kDa	0.7	0.001	0.001	0.001	0.001
	31 kDa BP-5	0.49	0.005	0.007	0.003	0.03
	IGFBP-2	0.71	0.001	0.001	0.001	0.004
	IGFBP-3	0.4	0.005	0.002	0.09	0.25
Log A_4	24 kDa BP-4	0.85	0.001	0.001	0.56	0.7
	28-29 kDa	0.87	0.001	0.001	0.33	0.13
	31 kDa BP-5	0.86	0.001	0.001	0.36	0.7
	IGFBP-2	0.88	0.001	0.001	0.06	0.24
	IGFBP-3	0.86	0.001	0.001	0.15	0.2
Log P_4	24 kDa BP-4	0.61	0.004	0.001	0.55	0.13
	28-29 kDa	0.54	0.001	0.001	0.05	0.12
	31 kDa BP-5	0.7	0.001	0.001	0.001	0.04
	IGFBP-2	0.53	0.001	0.001	0.16	0.08
	IGFBP-3	0.63	0.001	0.001	0.03	0.006
Log PE	24 kDa BP-4	0.86	0.001	0.001	0.003	0.81
	28-29 kDa	0.76	0.001	0.001	0.001	0.001
	31 kDa BP-5	0.73	0.001	0.001	0.001	0.003
	IGFBP-2	0.64	0.001	0.001	0.001	0.007
	IGFBP-3	0.56	0.001	0.001	0.69	0.77

[1]Log E_2 = Log base 10 transformation of estradiol concentration; Log A_4 = Log base 10 transformation of androstenedione concentration; Log P_4 = Log base 10 transformation of progesterone concentration; and Log PE = Log base 10 transformation of ratio of estradiol to progesterone concentration.

[2]24 kDa BP-4 = 24 kDa form of IGFBP-4; 28-29 kDa = binding activity of 28 kDa form of IGFBP-4 and 29 kDa form of IGFBP-5, or both; and 31 kDa BP-5 = 31 kDa form of IGFBP-5.

formed, fitting cow as a classification variable and each binding protein as a continuous variable. Based on a visual appraisal of data, the quadratic effect of each binding protein was fit into each model. Each individual binding protein accounted for significant variation in concentrations of E_2 and P_4 (Table 8). Changes in IGFBP-2 tended ($P < 0.06$) to influence concentrations of A_4. However, other IGFBP did not account for variation in concentrations of A_4 (Table 8).

DISCUSSION

Results from this study demonstrate that concentrations of steroids and activities of IGFBP in fluid of small antral follicles vary substantially within and between cows. A 500-fold difference existed between the lowest and greatest concentration of E_2 measured in small antral follicles. However, many small antral follicles had E_2 concentration below the sensitivity of the assay (0.1 ng/mL when 1 μL fluid was evaluated), and insufficient sample remained for evaluation at greater volumes. Because of these limitations, the magnitude of variation in E_2 concentrations among small antral follicles likely exceeds that determined in this study. Although the sensitivity of the E_2 RIA was at least 10-fold greater than the RIA for P_4 and A_4, concentrations of P_4 were detectable in all follicles and A_4 was below the level of detection (5 ng/mL) in only 2 follicles. In addition, concentrations of P_4 and A_4 in fluid from most small antral follicles were greater than concentrations of E_2, when expressed on a nanogram per milliliter basis. Therefore, estimations of variation in concentrations of P_4 (287-fold difference between minimum and maximum) and A_4 (100-fold difference) may be more accurate than that determined for E_2, albeit magnitude of variation in P_4 and A_4 was less than that observed for E_2. It is expected that these variations reflect large differences in developmental status among follicles; as such, the destiny of many of these small antral follicles may be established at or before the small antral (<5.0 mm diameter) stage. A comparison of means for each of the steroids across Tables 2, 3, and 6 provides evidence as to how results from pooling could vary due to sampling (i.e., Table 2 represents all follicles, Table 3 represents all follicles split into 2 groups based on E_2 concentration, Table 6 represents a subsample of follicles selected to represent the entire population). These results provided evidence that small antral follicles should not be indiscriminately pooled when conducting research on processes regulating follicular development.

The proportion of variation in steroid concentrations accounted for by differences among animals was greatest for A_4, intermediate for P_4, and lowest for E_2 (see R^2 values in Table 2). Although data for E_2 concentrations were not distributed normally due to many follicles being assigned a value of 0.1 ng of E_2/mL, it appeared that variation of E_2 within a cow was equal to or greater than variation between cows. Because of the nonlinear nature of the E_2 data and because only a

small proportion of the variation in E_2 concentrations was accounted for by differences among cows, it was of interest to subclassify follicles by whether they were above or below the detection limit of the E_2 assay (90% binding), as shown in Table 3. As expected, this method of classification accounted for a much greater proportion of the variation observed in E_2 concentrations (an increase from 12% shown in Table 2 to 81% shown in Table 3), but it also accounted for 13% more of the variation in P_4. In contrast, classifying follicles within cows by E_2 had little effect on A_4.

As was observed for the steroid concentrations, binding activities of IGFBP in individual follicles also varied greatly among and within cows. Binding by IGFBP-3 exhibited the least variation, followed by IGFBP-2, IGFBP-4, and IGFBP-5. Differences in the amount of variation observed for each IGFBP were due to large variations among minimum values detected for each IGFBP rather than wide differences among maximum values detected for each IGFBP. Studies on large bovine follicles also demonstrate that binding activities of IGFBP-3 and -2 fluctuate less across follicles than IGFBP-4 and -5, with the exception being that large estrogen active follicles contain little or no IGFBP-2 activity (Echternkamp et al., 1994, 2004; Roberts and Echternkamp, 2003).

Activities of individual IGFBP were negatively associated with concentrations of E_2. Previous research also demonstrated negative association among individual IGFBP and E_2 concentrations in individual follicles >5mm in diameter, with IGFBP being most markedly reduced or undetectable in fluid of preovulatory (Echternkamp et al., 1994, 2004) or first wave dominant follicles (Austin et al., 2001; Beg et al., 2001). Of the low molecular weight IGFBP evaluated (those excluding IGFBP-3), magnitude of the negative association with E_2 concentration was least for 31 kDa IGFBP-5, which exhibited more of a threshold pattern of association with detection of 31 kDa IGFBP-5 activity being almost exclusively limited to follicles with nondetectable concentrations of E_2. Inverse associations among IGFBP activity and E_2 were not evident in fluid from within animal pools of small antral follicles (Funston et al., 1996), and associations among follicular fluid E_2 concentrations with activity or mRNA of IGFBP in follicular cells were much less prevalent in pools from small antral follicles than when evaluated in individual large follicles (Roberts and Echternkamp, 2003), providing further evidence of the limitations to the approach of pooling small antral follicles. The current study expands the scope of the inverse associations among concentrations of E_2 and activities of the individual IGFBP to include small antral follicles.

In the current study, positive associations were observed between P_4 and IGFBP-3, -4, and -5. Association between IGFBP-5 and P_4 appeared to be more of a continuous response than the association between IGFBP-5 and E_2, which as discussed above, exhibited more of a threshold response because only 2 follicles with detectable levels of E_2 contained detectable IGFPB-5 activity. This observation is consistent with recent findings in large follicles in which progression into atresia was accompanied first by increases in IGFBP-2, followed by increases in IGFBP-4, and last by increases in IGFBP-5 (Echternkamp et al., 2004). A positive association was also observed between P_4 and IGFBP-5 in pools of small antral follicles (Funston et al., 1996), and the magnitude of the association was similar ($r = 0.6$) as the correlation observed in the current study. In medium and large follicles, activities of IGFBP –2, -3, -4, and -5 were all associated positively with concentrations of P_4 (Echternkamp et al., 2004). Variations in IGFBP activities and mRNA within granulosa and thecal cell components of follicles at different stages of antral development have also been shown to be associated with the levels of E_2 and P_4 in follicular fluid (Roberts and Echternkamp, 2003).

Although the cause and effects of the associations among steroid concentrations and IGFBP remain to be established (i.e., its not clear whether steroids regulate IGFBP or visa verse), the preponderance of research supports an inhibitory role of IGFBP on IGF-stimulated actions (proliferation, differentiation, and antiapoptotic) on follicular cells (Monget et al., 1996; Poretsky et al., 1999). However, evidence of E_2 regulation of IGFBP production by bovine follicular cells does exist (Spicer and Chamberlain, 2002; Voge et al., 2004) and may provide for a feedback regulatory system. In the current study, the statistical models fitting IGFBP as independent variables accounted for much more of the variation in concentrations of E_2 and P_4 observed among the individual small antral follicles than statistical models that only accounted for differences among animals. This was especially true for E_2 when IGFBP-2 and -4 were considered and for P_4 when IGFBP-4 and -5 were considered (compare R^2 values in Tables 6 and 7). Because steroidogenesis in the bovine follicle occurs through the delta 5-pathway, increases in developmental status would be reflected by increases in E_2 and subsequent atresia would be characterized by decreases in E_2 in the face of increasing P_4 accumulation. Thus the associations between steroid concentrations and IGFBP activities observed in this study on small antral follicles and previous studies on medium to large follicles (reviewed in Monget et al., 2002; Mihm and Austin, 2002) support the hypothesis that recruitment and selection of follicles occurs through a process that involves an advantage for continued development through decreased levels of IGFBP, providing for increased intrafollicular availability of IGF. Whereas atresia of nonrecruited, subordinate, and dominant follicles occurs in response to increases in intrafollicular levels of IGFBP, which may act to inhibit IGF-I stimulated proliferation and E_2 production (Spicer et al., 1997; Mason et al., 1998; Spicer and Chamberlain, 1999) or act independent of IGF-I (Wright et al., 2002) to bring about atresia. Although the hypothesis is sup-

ported by research on medium to large follicles, the challenge remains to determine how variations in biochemical characteristics of the population of small antral follicles on the ovaries of an animal at any given time are associated with follicular waves.

In summary, concentrations of E_2 and P_4, and IGFBP activities in follicular fluid from small antral follicles vary greatly within a cow, and variations within animals may be as great as the variation among animals. Variations in concentration of E_2 in fluid from small antral follicles were associated inversely with activity the lower molecular weight IGFBP, as has been observed at later stages of follicular development. This finding expands the potential roles that these IGFBP may have in regulating follicular development to include processes involved in controlling recruitment. Because these biochemical factors are known to play a role in regulating development of large antral follicles, the practice of indiscriminately pooling cells or fluid from small antral follicles within or across animals is not appropriate for research to identify mechanisms regulating development of small antral follicles. To establish whether variations in steroid concentrations and IGFBP activities in small antral follicles are determining factors associated with follicular recruitment during waves of development, it may be necessary to examine small antral follicles on an individual basis, as has been applied in studies on medium and large follicles, or utilize morphological or biochemical characteristics to classify follicles into different health status groups within animals. Although the ratio of E_2:P_4 is used as an indicator of health status for larger follicles, the inverse of this ratio was not much better at accounting for variation in IGFBP in small antral follicles than concentrations of E_2 (as determined by comparison of correlation coefficients in Table 7) and the large variations in concentrations of P_4 and E_2 results in similar P_4:E_2 ratios for follicles with large differences in steroid concentrations (i.e., P_4/E_2 of 177/1.4 = 126 and 29/0.23 = 126). Thus, ratios of P_4 and E_2 may not be as useful for classifying small antral follicles as larger follicles. The observation that concentration of A_4 in fluid from small antral follicles varied substantially between cows requires further study to determine if this finding extends from differences due to stage of follicular waves. If so, the ratio of A_4:E_2 may provide a useful method to classify small antral follicles in cattle.

LITERATURE CITED

Austin, E. J., M. Mihm, A. C. Evans, P. G. Knight, J. L. Ireland, J. J. Ireland, and J. F. Roche. 2001. Alterations in intrafollicular regulatory factors and apoptosis during selection of follicles in the first follicular wave of the bovine estrous cycle. Biol. Reprod. 64:839–848.

Beg, M. A., D. R. Bergfelt, K. Kot, M. C. Wiltbank, and O. J. Ginther. 2001. Follicular-fluid factors and granulosa-cell gene expression associated with follicle deviation in cattle. Biol. Reprod. 64:432–441.

Carson, R. S., J. K. Findlay, I. J. Clarke, and H. G. Burger. 1981. Estradiol, testosterone, and androstenedione in ovine follicular fluid during growth and atresia of ovarian follicles. Biol. Reprod. 24:105–113.

Echternkamp, S. E., H. J. Howard, A. J. Roberts, J. M. Grizzle, and T. Wise. 1994. Relationships among concentrations of steroids, insulin-like growth factor-1, and insulin-like factor binding proteins in ovarian follicular fluid of beef cattle. Biol. Reprod. 51:971–981.

Echternkamp, S. E., A. J. Roberts, D. D. Lunstra, T. Wise, and L. J. Spicer. 2004. Ovarian follicular development in cattle selected for twin ovulations and births. J. Anim. Sci. 82:459–471.

Funston, R. N., G. E. Seidel, Jr., J. Klindt, and A. J. Roberts. 1996. Insulin-like growth factor I and insulin-like growth factor-binding proteins in bovine serum and follicular fluid before and after the preovulatory surge of luteinizing hormone. Biol. Reprod. 55:1390–1396.

Ginther, O. J., M. A. Beg, D. R. Bergfelt, F. X. Donadeu, and K. Kot. 2001. Follicle selection in monovular species. Biol. Reprod. 65:638–647.

Henderson, K. M., A. S. McNeilly, and I. A. Swanston. 1982. Gonadotrophin and steroid concentrations in bovine follicular fluid and their relationship to follicle size. J. Reprod. Fertil. 65:467–473.

Hossenlopp, P., D. Seurin, B. Segovia-Quinson, S. Hardouin, and M. Binoux. 1986. Analysis of serum insulin-like growth factor binding proteins using Western blotting: Use of the method for titration of the binding proteins and competitive binding studies. Anal. Biochem. 154:138–143.

Ireland, J. J., P. B. Coulson, and R. L. Murphree. 1979. Follicular development during four stages of the estrous cycle of beef cattle. J. Anim. Sci. 49:1261–1269.

Ireland, J. J., and J. F. Roche. 1982. Development of antral follicles in cattle after prostaglandin-induced luteolysis: Changes in serum hormone, steroids in follicular fluid and gonadotropin receptors. Endocrinology 111:2077–2086.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227:680–685.

Mason, H. D., S. Cwyfan-Hughes, J. M. Holly, and S. Franks. 1998. Potent inhibition of human ovarian steroidogenesis by insulin-like growth factor binding protein-4 (IGFBP-4). J. Clin. Endocrinol. Metab. 83:284–287.

Mihm, M., and E. J. Austin. 2002. The final stages of dominant follicle selection in cattle. Domest. Anim. Endocrinol. 23:155–166.

Monget, P., N. Besnard, C. Huet, C. Pisselet, and D. Monniaux. 1996. Insulin-like growth factor-binding proteins and ovarian folliculogenesis. Horm. Res. 45:211–217.

Monget, P., S. Fabre, P. Mulsant, F. Lecerf, J.-M. Elsen, S. Mazerbourg, C. Pisselet, and D. Monniaux. 2002. Regulation of ovarian folliculogenesis by IGF and BMP system in domestic animals. Domest. Anim. Endocrinol. 23:139–154.

Moor, R. M., M. F. Hay, H. M. Dott, and D. G. Cran. 1978. Macroscopic identification and steroidogenic function of atretic follicles in sheep. J. Endocrinol. 77:309–318.

Poretsky, L., N. A. Cataldo, Z. Rosenwaks, and L. C. Giudice. 1999. The insulin-related ovarian regulatory system in health and disease. Endocr. Rev. 20:535–582.

Roberts, A. J., and S. E. Echternkamp. 2003. Insulin-like growth factor binding proteins in granulosa and thecal cells from bovine ovarian follicles at different stages of development. J. Anim. Sci. 81:2826–2839.

Roberts, A. J., and M. K. Skinner. 1990. Hormonal regulation of thecal cell function during antral follicle development in bovine ovaries. Endocrinology 127:2907–2917.

Spicer, L. J., and C. S. Chamberlain. 1999. Insulin-like growth factor binding protein-3: Its biological effect on bovine granulosa cells. Domest. Anim. Endocrinol. 16:19–29.

Spicer, L. J., and C. S. Chamberlain. 2002. Estradiol and luteinizing hormone regulation of insulin-like growth factor binding protein by bovine granulosa and thecal cells. Endocrine 17:161–168.

Spicer, L. J., and S. E. Echternkamp. 1995. The ovarian insulin and insulin-like growth factor system with an emphasis on domestic animals. Domest. Anim. Endocrinol. 12:223–245.

Spicer, L. J., R. E. Stewart, P. Alvarez, C. C. Francisco, and B. E. Keefer. 1997. Insulin-like growth factor-binding protein-2 and -3: Their biological effects in bovine thecal cells. Biol. Reprod. 56:1458–1465.

Voge, J. L., C. A. T. Santiago, P. Y. Aad, D. W. Goad, J. R. Malayer, and L. J. Spicer. 2004. Quantification of insulin-like growth factor binding protein mRNA using real-time PCR in bovine granulosa and theca cells: Effect of estradiol, insulin and gonadotropins. Domest. Anim. Endocrinol. 26:241–258.

Wise, T. 1987. Biochemical analysis of bovine follicular fluid: Albumin, total protein, lysosomal enzymes, ions, steroids and ascorbic acid content in relation to follicular size, rank, atresia classification and day of estrous cycle. J. Anim. Sci. 64:1153–1169.

Wright, R. J., M. P. J. Holly, R. Galea, M. Brincat, and H. D. Mason. 2002. Insulin-like growth factor (IGF)-independent effects of IGF binding protein-4 on human granulose cell steroidogenesis. Biol. Reprod. 67:776–781.