

## Pregnancy, Bovine Somatotropin, and Dietary n-3 Fatty Acids in Lactating Dairy Cows: II. Endometrial Gene Expression Related to Maintenance of Pregnancy

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### ABSTRACT

The objectives were to examine the effects of bovine somatotropin (bST), pregnancy, and dietary fatty acids on expression of key endometrial genes and proteins regulating prostaglandin synthesis in lactating dairy cows. Two diets were fed, at about 17 d in milk (DIM), in which oil of whole cottonseed (control diet) was compared with calcium salts of fish oil-enriched lipid (FO). Ovulation was synchronized in cows with a presynchronization plus Ovsynch protocol and cows were inseminated artificially or not inseminated on d 0 (d 0 = time of synchronized ovulation;  $77 \pm 12$  DIM). On d 0 and 11, cows received bST (500 mg) or no bST, and were slaughtered on d 17 to recover uterine secretions and endometrial tissue. Number of cows in the control diet: 5 bST-treated cyclic (bST-C), 5 non-bST-treated cyclic (no bST-C), 4 bST-treated pregnant (bST-P), and 5 non-bST-treated pregnant (no bST-P) cows and in the FO diet: 4 bST-treated FO-cyclic (bST-FO-C) and 5 non-bST-treated cyclic (no bST-FO-C) cows. The FO diet increased progesterone receptor (PR) mRNA, and treatment with bST increased PR mRNA concentration in endometrium of no bST-C, but not in no bST-FO-C or no bST-P cows. Concentrations of estrogen receptor- $\alpha$  (ER $\alpha$ ) mRNA and protein, and oxytocin receptor (OTR) mRNA were decreased in no bST-P cows compared with no bST-C cows. Treatment with bST tended to increase OTR and ER $\alpha$  mRNA concentrations in cyclic cows fed control or FO diets. Immunohistochemistry demonstrated effects of bST, FO, and pregnancy on distributions of ER $\alpha$  and PR proteins in endometrium. Pregnancy and FO feeding decreased ER $\alpha$  abundance in luminal epithelium. Prostaglandin H synthase-2 (PGHS-2) protein was elevated in pregnant cows and localized to the luminal epithelium. Both FO and bST treatments reduced staining intensity of PGHS-2 pro-

tein. Concentrations of prostaglandin E synthase mRNA were elevated in either cyclic or pregnant cows in response to bST, whereas bST decreased prostaglandin F synthase mRNA in pregnant cows. Uterine lumen fluids had more PGF<sub>2 $\alpha$</sub>  and prostaglandin E<sub>2</sub> in pregnant than cyclic cows. Uterine lumen fluids of bST-P cows contained more prostaglandin E<sub>2</sub> than those from no bST-P cows. In summary, both pregnancy and bST altered endometrial gene expression, and cyclic cows responded differently to bST than pregnant cows. Feeding FO modulated PR, ER $\alpha$ , and PGHS-2 expression and distribution among endometrial cell types in a manner that may favor establishment and maintenance of pregnancy.

**Key words:** pregnancy, bovine somatotropin, fatty acid

### INTRODUCTION

Early pregnancy loss in lactating dairy cattle can have devastating effects on the economic success of dairy farms (Santos et al., 2004a). Nearly 40% of pregnancy losses occur in association with the period of 15 to 17 d following estrus. This is the critical period during which the conceptus must produce sufficient quantities of IFN- $\tau$  to prevent pulsatile prostaglandin (PG) secretion and maintain the corpus luteum (CL). Changing from a cyclic to a pregnant state not only depends on the production of antiluteolytic signals from the developing conceptus, but also on the capacity of the endometrium to respond to these signals, thus blocking pulsatile PGF<sub>2 $\alpha$</sub>  production. Such communications between the conceptus and maternal units are not always successful, thus leading to early embryonic loss.

The endometrium plays a critical role in regulating the estrous cycle and establishment of pregnancy. Elevated concentrations of plasma progesterone during the late luteal phase of the estrous cycle caused down regulation of progesterone receptors (PR) in the uterus (Wathes and Lamming, 1995). In pregnant ewes, the expression of estrogen receptor- $\alpha$  (ER $\alpha$ ) is suppressed during early pregnancy, and it has been hypothesized that IFN- $\tau$  inhibits oxytocin receptor (OTR) upregula-

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tion by inhibiting a preceding increase in  $ER\alpha$  expression (Spencer and Bazer, 1995). Although the role of PR and  $ER\alpha$  in regards to OTR regulation is obscure, OTR certainly are suppressed by  $IFN-\tau$  secreted from the conceptus (Wathes and Lamming, 1995). Intrauterine infusions of recombinant  $IFN-\tau$  in cyclic ewes from d 11 to 16 postestrus had no effect on prostaglandin H synthase-2 (**PGHS-2**) expression in the endometrial epithelium (Kim et al., 2003). In this latter study, it was suggested that antiluteolytic effects of  $IFN-\tau$  are to inhibit  $ER\alpha$  and OTR gene transcription, thereby preventing endometrial production of luteolytic pulses of  $PGF_{2\alpha}$ .

In the uterine luminal epithelium, arachidonic acid is released from phospholipids by hydrolysis and acted upon by PGHS-2 to form prostaglandin  $H_2$  (**PGH<sub>2</sub>**), which is converted to either  $PGF_{2\alpha}$  and (or) prostaglandin  $E_2$  (**PGE<sub>2</sub>**) through 2 reductases, prostaglandin F synthase (**PGFS**) and prostaglandin E synthase (**PGES**), respectively. Kim et al. (2003) reported that PGHS-2 mRNA concentrations were greater in endometrium from pregnant ewes than in that from cyclic ewes by d 16 postestrus. It is unknown whether relative expression of the 2 synthetic enzymes, PGFS and PGES, changes during the period of CL maintenance in pregnant lactating dairy cattle.

Programs to optimize reproductive performance in dairy cattle have received considerable attention. Recently, recombinant bST, a commercially available product used to increase milk production, was shown to increase pregnancy rates when given as part of a timed AI protocol in lactating dairy cows (Moreira et al., 2001). Santos et al. (2004a) showed that bST increased pregnancy rate through reducing pregnancy loss. Evidence exists for "cross-talk" between hormone signal-transduction systems such as  $ER\alpha$  with IGF-I (Lee et al., 1997; Klotz et al., 2002), or direct effects such as bST increasing  $IFN-\tau$  effectiveness (Badinga et al., 2002). Effects of bST on fertility may involve an interaction between bST and  $IFN-\tau$  signaling pathways to regulate PG secretion or other components of the PG cascade critical for maintenance of pregnancy. Little is known, however, about the molecular and cellular effects of bST on endometrial gene expression at the time of pregnancy recognition.

Another commercially available product that may benefit fertility of lactating dairy cows is a calcium salt of lipid-enriched fish oil (**FO**) supplement containing n-3 fatty acids such as eicosapentaenoic acid (**EPA**) and docosahexaenoic acid (**DHA**). Previous studies indicated that n-3 fatty acids could decrease  $PGF_{2\alpha}$  secretion by bovine endometrial cells in vitro (Mattos et al., 2003).

Little is known about the effects of a FO-enriched supplement and its interaction with bST treatment on endometrial function at the molecular level. The objectives of the present study were to examine the effects of pregnancy, bST treatment, and a FO-supplemented diet on the regulatory enzymes of the PG cascade and how they may regulate genes and proteins in the uterine environment that are known to influence pregnancy recognition.

## MATERIALS AND METHODS

### Materials

Gonadotropin-releasing hormone (Fertagyl; Intervet Inc., Millsboro, DE),  $PGF_{2\alpha}$  (Lutalyse; Pfizer Animal Health, Kalamazoo, MI), and bST (Posilac; Monsanto Co., St. Louis, MO) were used for experimental treatments of cows. Other purchased materials included Trizol, cDNA Cycle kit, TOPO vector (TOPO TA Cloning Kits), and Random Primers DNA Labeling System (Invitrogen Corp., Carlsbad, CA); Taq polymerase (M166A; Promega, Madison, WI). Also purchased was biotin-conjugated antirabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA), normal horse serum, biotinylated horse antimouse IgG, horseradish peroxidase-avidin-biotin complex, 3, 3'-diaminobenzidille (DAB kit; Vectastain; Vector Laboratories, Burlingame, CA); enhanced chemiluminescence (ECL) kit (Renaissance Western Blot Chemiluminescence Reagent Plus; NEN Life Science Products, Boston, MA); ultrasensitive hybridization buffer (ULTRAhyb; Cat # 8670; Ambion Inc., Austin, TX); dCTP  $\alpha$ - $^{32}P$  and Biotrans nylon membrane (MP Biomedicals, Irvine, CA); isotopically labeled [5, 6, 8, 11, 12, 14, 15- $^3H$ ]- $PGF_{2\alpha}$  and  $PGE_2$ , nitrocellulose membranes (Hybond-ECL), horseradish peroxidase-linked antimouse IgG (NA931V), and antirabbit IgG (NA934V; Amersham Biosciences Corp., Piscataway, NJ). All other laboratory materials were from Fisher Scientific (Pittsburgh, PA) and Sigma Chemical Co. (St. Louis, MO).

### Cows and Experimental Diets

A more detailed description of cows, management, and collection of samples is given in a companion article (Bilby et al., 2006b). Briefly, 40 multiparous Holstein cows in late gestation were fed diets formulated to contain 1.51 Mcal of  $NE_I$ /kg, 13.1% CP, and a cation-anion difference of -90 mEq/kg (DM basis) beginning approximately 3 wk before expected calving. Upon calving, cows were fed 2 dietary treatments containing none or 1.9% calcium salt of a fish oil-enriched lipid product (EnerG-II Reproduction formula, Virtus Nutrition, Fairlawn, OH). The fatty acid profile of the fat source as given by

the manufacturer was 2.2% C14:0, 41.0% C16:0, 4.2% C18:0, 30.9% C18:1, 0.2% C18:1 *trans*, 8.0% C18:2, 0.5% C18:3, 0.4% C20:4, 2.0% C20:5, 2.3% C22:6, and 2.7% unknown. The control diet contained a greater concentration of whole cottonseed, and therefore, was similar in concentration of ether extract and NE<sub>L</sub> to that containing FO (Bilby et al., 2006b). The control diet was fed to all cows during the first 9 DIM. From 10 to 16 DIM, 10 cows were assigned to consume an FO diet containing half the final concentration of the fat product (0.95% of dietary DM) to adjust the cows to a new fat source. Starting at 17 DIM, these cows were switched to the 1.9% FO diet and continued on that diet until the end of the study. Cows fed the ruminally protected FO consumed approximately 14.8 g/cow per day of EPA and DHA. Thirty cows were assigned to the control diet for the duration of the study. Cows were milked thrice daily and calibrated electronic milk meters recorded milk weights at each milking. Body weights were measured and BCS assigned weekly by the same 2 individuals.

### ***Estrus Synchronization and Tissue Collection***

Detailed description of the estrus synchronization is described in Bilby et al. (2006b). The number of cows used for analyses on d 17 in the control diet was 5 bST-treated cyclic (**bST-C**), 5 non-bST-treated cyclic (**no bST-C**), 4 bST-treated pregnant (**bST-P**), and 5 non-bST-treated pregnant (**no bST-P**) cows; and in the FO diet: 4 bST-treated FO-cyclic (**bST-FO-C**) and 5 non-bST-treated cyclic (**no bST-FO-C**) cows. Conceptuses and uterine secretions were recovered and endometrial tissue collected as described by Bilby et al. (2004).

### ***RNA Isolation and Northern Blotting***

Total RNA was isolated from endometrial tissues (n = 28) and the Northern blotting procedure was performed as described by Bilby et al. (2004). The specific bovine cDNA used were ER $\alpha$ , OTR, PR, PGHS-2, PGES, PGFS, or glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**). Probes were obtained using a reverse transcription PCR procedure as described in Guzeloglu et al. (2004a). Complementary DNA was reverse-transcribed from total RNA (5  $\mu$ g) prepared from the bovine CL following the manufacturer's protocol with the cDNA Cycle kit, utilizing established primer sets for bovine OTR, PR, and PGES (Guzeloglu et al., 2004a).

The ER $\alpha$  cDNA was a gift from N. H. Ing (Texas A&M University, College Station, TX). The PGFS (Xiao et al., 1998) and PGHS-2 (Liu et al., 2001) cDNA were a gift from J. Sirois (University of Montreal, St. Hyacinthe, Canada).

### ***Immunohistochemical Analyses***

Paraffin sections (5  $\mu$ m) from the antimesometrial border of the uterus from 27 cows (5 no bST-C, 5 bST-C, 4 no bST-FO-C, 4 bST-FO-C, 4 no bST-P, and 5 bST-P) were prepared. After deparaffinization, an antigen retrieval procedure was performed by heating sections in a microwave oven at high power for 5 min in 0.01 M sodium citrate buffer (pH 6.0). Sections were allowed to cool in microwave for 28 min, and then washed in distilled water and in PBS (0.01 M, pH 7.2). Nonspecific endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide in methanol for 10 min at room temperature. After a 10-min wash in PBS, nonspecific binding was blocked using 2% BSA in PBS for ER $\alpha$ , 5% (vol/vol) normal horse serum in PBS for PR, and 5% normal goat serum in PBS for PGHS-2 in a humidified chamber at room temperature for 1 h. Tissue sections were then incubated in the dark at room temperature with the primary antibody: 1) monoclonal anti-ER $\alpha$  (NeoMarkers, Medicorp, Montreal, QC, Canada) or the negative-control mouse IgG at an equivalent concentration diluted 1:500 in 2% BSA, and incubated overnight; 2) monoclonal anti-PR (NeoMarkers, Lab Vision, Fremont, CA) or the negative-control mouse IgG at equivalent concentration diluted 1:500 in 5% horse serum and incubated for 2 h; 3) polyclonal anti-PGHS-2 (Cayman Chemical, Cedarlane Lab., Hornby, ON, Canada) or the negative control, anti-PGHS-2 preincubated with PGHS-2 blocking peptide (1:5 vol/vol ratio) for 1 h, diluted 1:200 in PBS, and incubated for 2 h. After three 5-min washes in PBS, the sections were incubated in the dark for 1 h at room temperature with a biotinylated horse antimouse IgG for ER $\alpha$  (diluted 1:200 in 2% BSA) and PR (diluted 1:800 in 5% horse serum) antibodies, or biotinylated goat antirabbit IgG for the PGHS-2 antibody (diluted 1:200 in 5% goat serum). Thereafter, three 5-min washes in PBS were performed and tissue sections were incubated for 30 min at room temperature with horseradish avidin-biotin-peroxidase complex. Site of the bound enzyme was visualized by the application of 3,3'-diaminobenzidine in H<sub>2</sub>O<sub>2</sub>. Sections were counterstained with hematoxylin and dehydrated before they were mounted with Permount.

### ***Microscopic Image Analysis***

Subjective image analysis was performed to estimate the relative abundance of ER $\alpha$ , PR, and PGHS-2 staining in different cell types. One evaluator assessed immunostaining on 10 randomly selected fields of intercaruncular endometrium in 3 pieces of endometrium from each cow. Caruncular endometrium was not evident in all cows. Five uterine compartments were evaluated:

luminal epithelium (**LE**), superficial glandular epithelium (**SGE**; close to the uterine lumen), deep glandular epithelium (**DGE**; close to the myometrium), superficial intercaruncular stroma (**SS**; just beneath the luminal epithelium layer), and deep intercaruncular stroma (**DS**; between superficial stroma and the myometrium). Because specific staining for PGHS-2 protein was detectable exclusively in the cytoplasm of endometrial luminal epithelial cells, only those cells were scored. Intensity of staining was scored on a 4-point scale, where 0 = no staining (no brown), 1 = less (light brown), 2 = moderate (brown), and 3 = heavy (dark brown), and the staining intensities were expressed as percentage of positively stained cells for each point in the scale (Guzeloglu et al., 2004a).

### Western Blotting for ER $\alpha$ and PGHS-2 Proteins

Endometrial tissues (300 mg) from 27 cows were used for Western blotting of ER and PGHS proteins, as described by (Guzeloglu et al., 2004a).

### Radioimmunoassay

Concentrations of PGF $_{2\alpha}$  and PGE $_2$  were measured in uterine flushings by direct radioimmunoassay (**RIA**) as described by Danet-Desnoyers et al. (1994) and Gross et al. (1988), respectively. The anti-PGF $_{2\alpha}$  antiserum was diluted 1:5,000 and the anti-PGE $_2$  antiserum was diluted 1:1000 in Tris buffer. Intraassay coefficients of variation for PGF $_{2\alpha}$  and PGE $_2$  assay were 11.8 and 8.7%, respectively.

### Statistical Analyses

Abundances of ER $\alpha$  and PGHS-2 proteins in Western blots as well as ER $\alpha$ , PR, OTR, PGFS, PGES, and PGHS-2 mRNA in Northern blots were analyzed using the least squares ANOVA, GLM procedure of SAS (SAS Institute, Inc., Cary, NC). Main effects of treatment (no bST-C, no bST-P, no bST-FO-C, bST-C, bST-FO-C, bST-P), gel, and treatment  $\times$  gel interaction were examined, and for mRNA responses, band intensities of GAPDH mRNA were used as a covariate to adjust for loading differences. If treatment  $\times$  gel effects were not significant they were removed from the model. Predesigned orthogonal contrasts were used to compare treatment means for: bST, pregnancy status and bST  $\times$  pregnancy status; bST, FO, and bST  $\times$  FO.

Total contents of PGF $_{2\alpha}$  and PGE $_2$  in uterine flushings were analyzed using the GLM procedure of SAS. The model included the effect of treatment (no bST-C, no bST-P, no bST-FO-C, bST-C, bST-FO-C, and bST-P), and orthogonal contrasts were constructed among

treatments to examine effects of: bST, pregnancy status and bST  $\times$  pregnancy status; bST, FO, and bST  $\times$  FO.

Data generated from immunohistochemistry of ER $\alpha$ , PR, and PGHS-2 were analyzed by the Mixed model procedure of SAS (SAS Inst. Inc.) for each type of cell. The model included treatment (no bST-C, no bST-P, no bST-FO-C, bST-C, bST-FO-C, and bST-P), class (none, less, moderate, and heavy staining intensity), and treatment  $\times$  class interaction. Cow within treatment was the error term used to test for treatment effects. A series of orthogonal contrasts were constructed to test treatment effects (bST, pregnancy status and bST  $\times$  pregnancy status; bST, FO, and bST  $\times$  FO), class (none, less, moderate, and heavy staining intensity), and treatment  $\times$  class interactions.

## RESULTS

### Endometrial PR Expression

Injections of bST increased ( $P < 0.05$ ; bST  $\times$  FO interaction) steady-state concentrations of PR mRNA in cyclic cows only when FO was absent from the diet, because cows fed FO had similar elevated concentrations to control cows given bST (Table 1). In addition, bST increased ( $P < 0.01$ ; interaction) steady state concentrations of PR mRNA in cyclic but not pregnant cows (Table 1).

Immunohistochemical staining of PR was exclusively in the nuclei of the SGE and DGE, with little or no staining in the LE, SS, and DS. Within the SGE, FO increased ( $P < 0.05$ ) the amount of moderate and heavy staining (Figure 1C). The bST, as a main effect, reduced ( $P < 0.01$ ; Figure 2) moderate and heavy staining in the DGE from cyclic and pregnant cows. In the endometrium of cyclic cows, however, no bST-FO-C alone reduced (bST  $\times$  FO interaction;  $P < 0.01$ ) the moderate and heavy staining and there was not a further reduction with bST (Figure 3).

### Endometrial ER $\alpha$ Expression

Steady state concentrations of ER $\alpha$  mRNA in endometrial tissues tended ( $P < 0.10$ ) to be reduced in pregnant cows compared with cyclic cows fed a control diet (Table 1). Pregnancy decreased ( $P < 0.05$ ) abundance of ER $\alpha$  protein in the endometrium (Table 1) as detected by Western blotting.

Immunohistochemistry was used to localize ER $\alpha$  in the endometrium, and staining was detected exclusively in the nuclei of LE, SGE, DGE, and SS (Figure 1E). Pregnancy ( $P < 0.01$ ; Figure 1F) and no bST-FO-C decreased ( $P < 0.01$ ; Figure 4) ER $\alpha$  abundance in LE, and pregnancy decreased ( $P < 0.05$ ) ER $\alpha$  abundance in

**Table 1.** Least squares means and pooled SE for uterine endometrial mRNA and protein, and uterine luminal flushings (ULF) protein expression at d 17 after a synchronized estrus (d 0) in lactating cyclic (C) cows fed a control diet, pregnant (P) cows fed a control diet, and cyclic cows fed a fish oil-enriched lipid (FO) diet and injected with or without bST on d 0 and 11 (n = 28)

Response <sup>1</sup>	Treatment <sup>2</sup>							Contrast <sup>3</sup> cyclic			Contrast <sup>3</sup> pregnant		
	No bST-C		bST-C		No bST-FO-C		SE	FO	bST	bST × FO	P	bST	bST × P
	No bST-C	bST-C	No bST-FO-C	bST-FO-C	No bST-P	bST-P							
PR mRNA, AU	60	69	68	67	61	59	2.1	NS	†	*	*	NS	**
ER $\alpha$ mRNA, AU	11	15	12	16	11	8	2.1	NS	†	NS	†	NS	NS
ER $\alpha$ protein, AU	70	71	70	73	67	70	1.2	NS	NS	NS	*	NS	NS
OTR mRNA, AU	45	50	49	52	39	41	1.3	NS	†	NS	**	NS	NS
PGHS-2 mRNA, AU	84	83	85	83	78	82	3.3	NS	NS	NS	NS	NS	NS
PGHS-2 protein, AU	39	48	70	70	91	80	1.8	NS	NS	NS	*	NS	NS
PGFS mRNA, AU	27	28	28	28	27	25	0.6	NS	†	NS	*	NS	**
PGF <sub>2<math>\alpha</math></sub> , ng/ULF	57	60	130	50	618	672	92	NS	NS	NS	**	NS	NS
PGES mRNA, AU	45	48	46	47	43	46	0.9	NS	*	NS	†	**	NS
PGE <sub>2</sub> , ng/ULF	39	39	82	15	250	357	38	NS	NS	NS	**	NS	NS

<sup>1</sup>PR = Progesterone receptor; ER $\alpha$  = estradiol receptor  $\alpha$ ; OTR = Oxytocin receptor; PGHS-2 = prostaglandin H synthase-2; PGFS = prostaglandin F synthase; PGES = prostaglandin E synthase. Arbitrary units (AU) were generated by densitometry and mRNA results were adjusted using glyceraldehyde-3-phosphate dehydrogenase as a covariate.

<sup>2</sup>No bST-C = No bST cyclic, bST-C = bST-cyclic, No bST-FO-C = No bST-fish oil-cyclic, bST-FO-C = bST-fish oil cyclic, No bST-P = No bST-Pregnant, bST-P = bST-pregnant.

<sup>3</sup>Contrasts for cyclic cows were: FO = all FO-fed cows vs. all cyclic-control fed cows, bST = all cyclic cows injected with bST vs. all cyclic cows not given bST, and bST × FO interaction. Contrasts for pregnant cows were: P = all pregnant cows vs. cyclic control-fed cows, and bST = all pregnant and cyclic cows fed the control diet and injected with bST vs. all pregnant and cyclic cows fed the control diet without bST, and bST × P interaction.

† $P \leq 0.10$ , \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , NS = nonsignificant.

the SGE compared with cyclic control fed cows (Figure 1F).

### Endometrial OTR Expression

Among cyclic control and FO-fed cows, bST tended ( $P < 0.10$ ; Table 1) to increase OTR mRNA. Steady-state concentrations of OTR mRNA in pregnancy were decreased ( $P < 0.01$ ) compared with cyclic control fed cows (Table 1).

### Endometrial PGHS-2 Expression

No differences were detected in steady-state concentrations of PGHS-2 mRNA due to treatments (Table 1). In contrast, PGHS-2 protein in pregnant endometrial tissue was increased ( $P < 0.05$ ) 2-fold compared with cyclic cows as detected by Western blotting (Table 1).

Staining for PGHS-2 protein was localized specifically to the cytoplasm of endometrial LE cells as detected by immunohistochemistry. When primary antibody was first incubated with PGHS-2 blocking peptide, the absence of staining demonstrated the specificity for PGHS-2 (Figure 1G). Some light staining also was detected in SGE, DGE, SS, and DS. Staining in these endometrial cell types, however, was inconsistent and not evaluated. Within the LE, pregnancy increased ( $P < 0.01$ ) percentage of heavy staining (Figure 1H) and bST treatment blocked this response in pregnant cows (no-bST-P = 72% > bST-P = 39%; 7% SE). Feeding FO

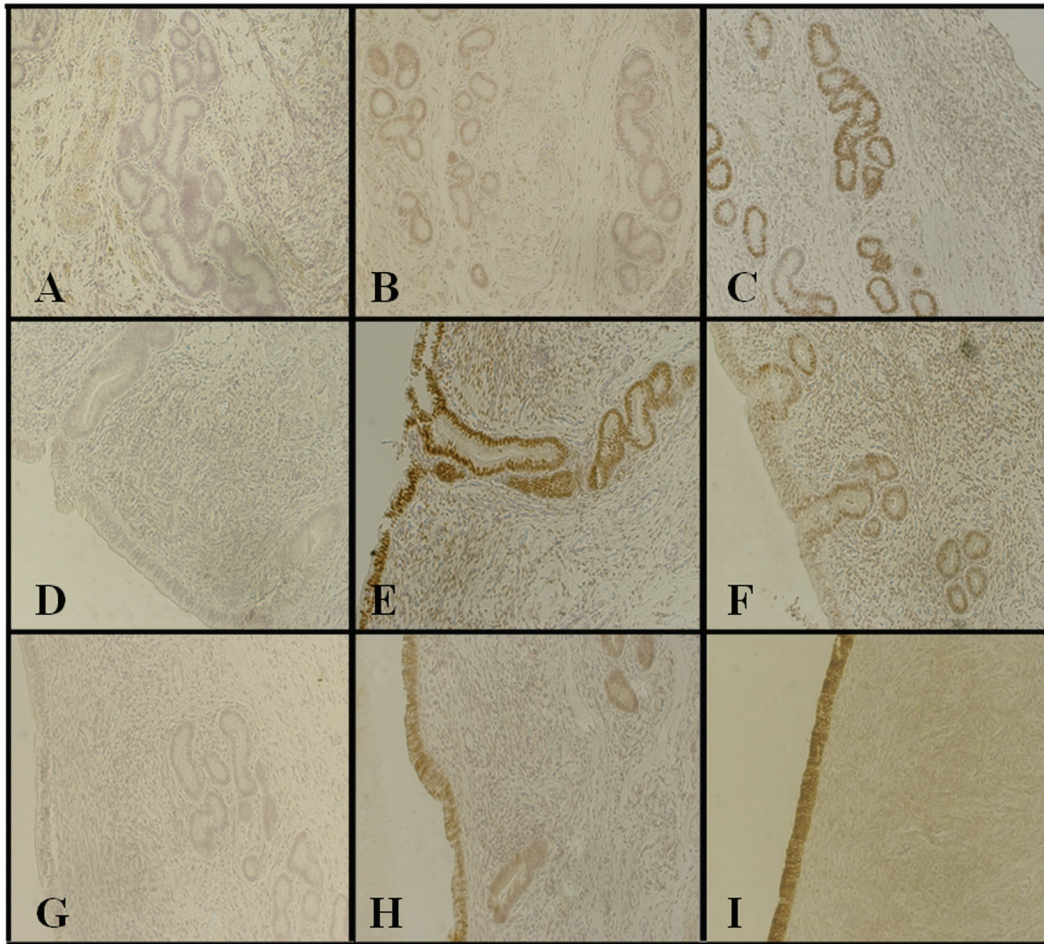
decreased ( $P < 0.01$ ) the percentage of PGHS-2 heavy staining in cyclic cows (no bST-FO-C/bST-FO-C = 33.5% < no bST-C/bST-C = 49.5%; 7% SE).

### Endometrial PGFS and PGES mRNA Expression

An interaction was detected ( $P < 0.01$ ) between cyclic and pregnant control-fed cows with bST slightly increasing steady-state concentrations of endometrial PGFS mRNA in cyclic (bST-C > No bST-C), but decreasing concentrations in pregnant (bST-P < no bST-P) control-fed cows (Table 1). Relative steady-state concentrations of PGES mRNA in endometrium were increased in response to bST for cyclic control and FO-fed cows ( $P < 0.05$ ; Table 1) as well as for cyclic and pregnant cows ( $P < 0.01$ ; Table 1).

### Total Contents of PGF<sub>2 $\alpha$</sub> and PGE<sub>2</sub> in Uterine Luminal Flushings

Volumes of recovered flushing fluids (36.2 mL) and percentage recovered (90.4%) did not differ among treatments. Total PGF<sub>2 $\alpha$</sub>  (645 ± 93 vs. 58 ± 87 ng;  $P < 0.01$ ) and PGE<sub>2</sub> (303 ± 37 vs. 39 ± 35 ng; Table 1) contents were substantially greater ( $P < 0.01$ ) in uterine luminal flushings of pregnant cows (no bST-P/bST-P) compared with cyclic control fed cows (no bST-C/bST-C), respectively. Treatment with bST or FO had no effect on uterine flushing PG content in cyclic cows. In addition, statistical analyses restricted to pregnant



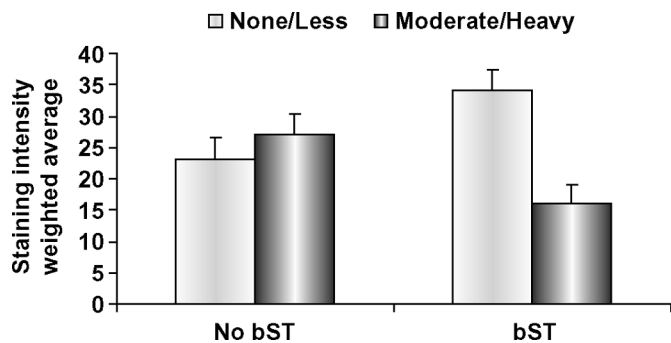
**Figure 1.** Expression of progesterone receptor (PR; panels A, B, C), estrogen receptor- $\alpha$  (ER $\alpha$ ; panels D, E, F), and prostaglandin H synthase-2 (PGHS-2; panels G, H, I) in bovine endometrium at d 17 following an induced ovulation. No immunopositive staining was detected when primary antibody was replaced by mouse IgG (panel A for PR and panel D for ER $\alpha$ ) or when primary antibody was first incubated with PGHS-2-blocking peptide (panel G for PGHS-2). Staining for ER $\alpha$  and PR was detected exclusively in the nuclei of the epithelial and stromal cells. Fish oil (panel C) increased ( $P < 0.05$ ) abundance of PR in the superficial glandular epithelium compared with the cyclic control-fed cows (panel B). Pregnancy decreased ( $P < 0.05$ ) ER $\alpha$  staining in the luminal and superficial glandular epithelium (panel F) compared with the cyclic control-fed cows (panel E). Staining for PGHS-2 was observed in the cytoplasm of endometrial luminal epithelial cells. Pregnancy (panel I) increased ( $P < 0.05$ ) the intensity of staining for PGHS-2 protein in the luminal epithelial cells compared with the cyclic control-fed cows (panel H). Magnification =  $\times 20$ .

cows ( $\pm$  bST), bST increased ( $P < 0.05$ ) PGE<sub>2</sub> ( $250 \pm 39$  vs.  $357 \pm 35$  ng;  $P \leq 0.05$ ), but not PGF<sub>2 $\alpha$</sub> .

#### **Simple and Partial Correlations for the Prostaglandin Cascade at d 17**

A series of simple and partial correlations were detected in the uterus at d 17 (Table 2). Although correlations are not proof of causative effects, significant associations were detected for endometrial gene expression, protein abundance, and PG within the uterine flushings at d 17. Endometrial PR mRNA concentration was correlated negatively with ER $\alpha$  protein, PGHS-2 protein, and correlated positively with PGES mRNA and PGFS

mRNA. These associations were detected both as simple and partial correlations indicating an inherent association after adjustment for treatment effects (Table 2). Endometrial ER $\alpha$  mRNA expression was correlated positively with OTR mRNA, PGHS-2 mRNA, and negatively correlated with PGF<sub>2 $\alpha$</sub>  in the ULF; however, these associations were not detected after adjustment for treatments. In contrast, ER $\alpha$  protein abundance was correlated positively with PGHS-2 protein and negatively with both PGES and PGFS mRNA. The OTR mRNA expression was correlated positively with PGHS-2 mRNA and PGFS mRNA, and negatively correlated with both PGE<sub>2</sub> and PGF<sub>2 $\alpha$</sub>  in the ULF. However, after adjustment for treatments (e.g., pregnancy

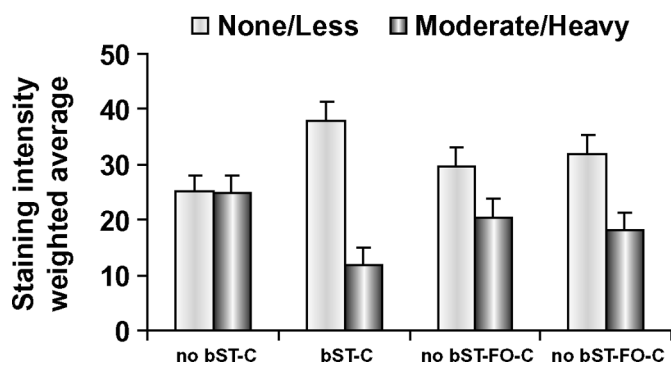


**Figure 2.** Expression of progesterone receptor (PR) in bovine endometrium at d 17 following an induced ovulation. The bST injections decreased ( $P < 0.01$ ) abundance of PR in the deep glandular epithelium of both cyclic and pregnant cows compared with cyclic and pregnant cows not injected with bST. Staining intensity weighted average (none = 0, less = 1 vs. moderate = 2, heavy staining = 3); no bST = cyclic and pregnant control-fed cows; bST = bST-cyclic and bST-pregnant control-fed cows.

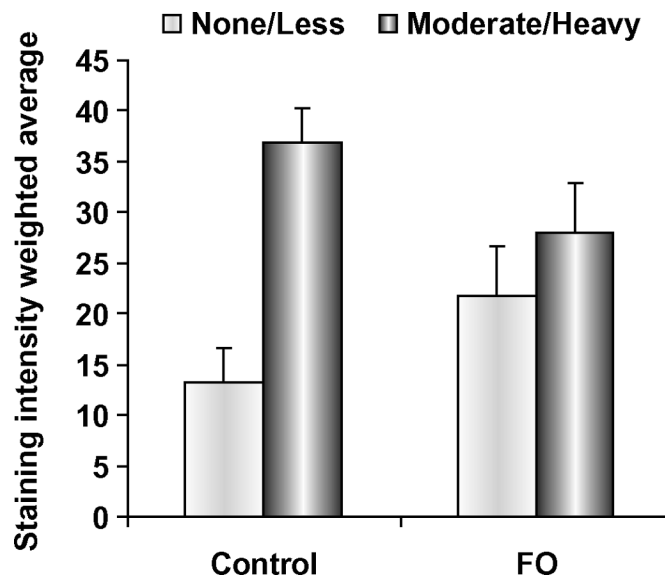
status) these associations were not detected. The PGHS-2 mRNA was correlated positively with PGHS-2 protein. Abundance of PGHS-2 protein was correlated negatively with both PGES and PGFS mRNA. All simple correlations involving uterine luminal concentrations of PGE<sub>2</sub> and PGF<sub>2 $\alpha$</sub>  were not detected when adjusted for treatments (e.g., high concentrations found in pregnancy but not during the cycle).

## DISCUSSION

An understanding of the differential regulation of PG secretion in cyclic and pregnant animals is pivotal to



**Figure 3.** Expression of progesterone receptor (PR) in bovine endometrium at d 17 following an induced ovulation. The bST reduced moderate and heavy staining in the deep glandular epithelium of cyclic control-fed cows; however, FO alone reduced the moderate and heavy staining and there was not a further reduction with bST (bST  $\times$  FO interaction;  $P < 0.01$ ). Staining intensity weighted average (none = 0, less = 1 vs. moderate = 2, heavy staining = 3); no bST-C = cyclic control-fed cows, bST-C = bST-treated, cyclic control-fed cows, no bST-FO-C = cyclic FO-fed cows, bST-FO-C = bST-treated, cyclic FO-fed cows.



**Figure 4.** Expression of estrogen receptor- $\alpha$  (ER $\alpha$ ) in bovine endometrium at d 17 following an induced ovulation. The FO reduced moderate and heavy staining in the luminal epithelium compared with cyclic control-fed cows ( $P < 0.01$ ). Staining intensity weighted average (none = 0, less = 1 vs. moderate = 2, heavy staining = 3); control = cyclic control and bST cyclic control-fed cows; FO = cyclic-FO and bST-treated, cyclic FO-fed cows.

our understanding of pregnancy establishment and the endometrial response to factors such as bST and FO. In this study, bST tended to increase pregnancy rates at d 17 (Bilby et al., 2006b), consistent with previous studies utilizing large numbers of lactating dairy cows (Moreira et al., 2001; Santos et al., 2004a).

Effects of pregnancy, bST, and FO on steady-state concentrations of mRNA (ER $\alpha$ , PR, and PGHS-2) and their respective proteins were examined in uterine endometrium. A 4.3-kb PR mRNA transcript was detected in the endometrium at d 17 following an induced ovulation, consistent with published reports of PR transcript size (Meikle et al., 2001). Progesterone receptor mRNA was elevated in FO-fed cows and bST did not further stimulate PR mRNA expression compared with cyclic control-fed cows. In contrast, bST stimulated PR mRNA in cyclic control-fed cows, but had no effect in pregnant cows.

Consistent with Kimmins and MacLaren (2001), immunohistochemistry indicated that the PR was localized mainly in the stroma and the glandular epithelium. More specifically, immunohistochemistry showed that the heaviest PR staining was in the SGE and DGE. In the SGE, FO appeared to increase PR abundance, which would agree with PR mRNA expression. Progesterone not only prepares the uterus for implantation of the embryo but also helps maintain pregnancy by stimulating uterine secretions for nourishment to the developing

**Table 2.** Simple and partial correlations<sup>1</sup> of uterine endometrial and uterine luminal flushing (ULF) variables<sup>2</sup> at d 17 after a synchronized estrus (d 0) in lactating cyclic cows fed a control diet, pregnant cows fed a control diet, cyclic cows fed a fish oil–enriched lipid diet and injected with or without bST on d 0 and 11

	PR mRNA	ER mRNA	ER protein	OTR mRNA	PGHS-2 mRNA	PGHS-2 protein	PGES mRNA	PGFS mRNA	PGE <sub>2</sub> ULF	PGF <sub>2α</sub> ULF
PR mRNA			-0.71**			-0.35†	0.74**	0.58**		
ER mRNA				0.41*	0.44*	-0.34†				-0.44*
ER protein	-0.80**				0.34†	0.42*	-0.58**	-0.45**		
OTR mRNA					0.34†			0.37*	-0.49**	-0.52**
PGHS-2 mRNA						0.41*				
PGHS-2 protein	-0.56**		0.54**		0.57**		-0.45**	-0.32†		
PGES mRNA			-0.69**			-0.51**		0.35†		
PGFS mRNA	0.74**		-0.73**			-0.42*	0.41†		-0.40*	-0.41*
PGE <sub>2</sub> ULF										0.77**
PGF <sub>2α</sub> ULF										

<sup>1</sup>Simple correlations above the diagonal; partial correlations adjusted for treatments below the diagonal.

<sup>2</sup>PR = Progesterone receptor; ER = estradiol receptor; OTR = oxytocin receptor; PGHS-2 = prostaglandin H synthase-2; PGES = prostaglandin E synthase; PGFS = prostaglandin F synthase; PGE<sub>2</sub> = prostaglandin E<sub>2</sub>.

† $P < 0.10$ ; \* $P < 0.05$ ; \*\* $P < 0.01$ .

conceptus. By FO elevating endometrial PR mRNA and PR protein in the SGE, progesterone may have a more profound effect on the uterus of cows fed FO.

The PR protein also was detected in the deep glands on d 16 in the bovine endometrium of pregnant cows (Robinson et al., 1999). Previous studies showed that GH treatment in ovariectomized ewes receiving ovarian steroid replacement therapy did not alter expression of PR in the uterus (Spencer et al., 1999). However, in the present study bST appeared to have differential effects on PR protein localization staining intensity depending on the pregnancy status and diet (Figures 2 and 3, respectively).

As observed in cattle (Meikle et al., 2001), a 6.8-kb ER $\alpha$  mRNA transcript was detected in the endometrium at d 17 following an induced ovulation. On d 17 after GnRH, ER $\alpha$  mRNA and protein concentration were reduced in pregnant compared with cyclic cows, regardless of whether cows received bST or not. Immunohistochemistry indicated that ER $\alpha$  receptor of the LE, SGE, and DGE of the endometrium of cyclic cows was greater than that of pregnant cows. Similarly, an upregulation of ER $\alpha$  mRNA and protein in the luminal epithelium was detected around d 14 to 16 of the estrous cycle in cyclic cows and expression was very low in pregnant cows at the same stage (Kimmins and MacLaren, 2001).

Pulsatile secretion of PGF<sub>2α</sub> from endometrial tissue, that initiates luteolysis, is thought to be dependent upon an increase in OTR concentration within the luminal epithelium. Four transcripts of OTR (5.6, 3.3, 2.1, and 1.5 kb) were detected in endometrial tissue from a cow in estrus (Guzeloglu et al., 2004a). All 4 transcripts were present at relatively low levels in endometrial tissue at d 17, and the most prominent (5.6 kb) transcript was quantified. In the present study, pregnancy

decreased steady-state concentrations of OTR mRNA, and bST stimulated OTR mRNA in both cyclic control and FO-fed cows. The decrease in the OTR mRNA concentration in pregnant cows treated with or without bST may be due to a concurrent decrease in ER $\alpha$  mRNA and protein expression in pregnant cows, as reported in sheep (Spencer et al., 1995). Indeed, OTR mRNA was correlated with ER $\alpha$  mRNA. The bST stimulation in OTR mRNA was associated with a concurrent increase in ER $\alpha$  mRNA stimulation in cyclic control and FO-fed cows. The OTR is undetectable in the bovine endometrium during much of the luteal phase, but increases on or after d 15 of the estrous cycle (Robinson et al., 1999). On d 17, at the time of expected initiation of luteolysis, OTR concentrations were about 10% of what was seen during estrus (Fuchs et al., 1990). Injection of oxytocin into nonpregnant cows induced the release of PGF<sub>2α</sub> on d 16 (Lamming and Mann, 1995). These findings indicate that only subtle increases of OTR concentrations are needed to induce a luteolytic release of PGF<sub>2α</sub>. Conversely, small but significant reductions in OTR mRNA due to pregnancy, as detected in the present study, may have profound inhibitory effects on pulsatile secretion of PGF<sub>2α</sub>.

In the endometrium, PGHS-2 protein was localized to the luminal epithelium. A PGHS-2 transcript of 4.4 kb, as shown by Liu et al. (2001), and a specific 72-kDa PGHS-2 protein band were detected in all samples of endometrium at d 17 following an induced ovulation. Endometrial expression of PGHS-2 mRNA did not differ in response to pregnancy, bST treatments, or FO. However, PGHS-2 protein was increased in endometrium of pregnant cows. The increase in pregnancy was detected both by Western blotting and immunohistochemistry. Immunohistochemistry responses revealed an interaction between bST and pregnancy with bST injections



attenuating the pregnancy increase in PGHS-2 protein in the LE. This attenuation was not statistically significant when quantified with Western blotting although there was a 22% reduction. Immunohistochemistry allows for a spatial evaluation of PGHS-2 protein that was present mainly in the LE and showed inhibitory effects in response to bST in pregnant cows and to FO feeding in cyclic cows. Such differences cannot be detected in an endometrial protein homogenate comprised of all cell types. Emond et al. (2004) also reported that endometrial expression of PGHS-2 protein was increased during early pregnancy and in response to intrauterine infusions of IFN- $\tau$ . Arosh et al. (2002) showed that the PGHS-2 protein concentrations peaked around d 16 to 18 of the cycle without any changes in PGHS-2 mRNA concentrations. In sheep, PGHS-2 mRNA levels were similar in cyclic and pregnant ewes, although PGHS-2 protein expression was maintained at greater levels in pregnant rather than cyclic ewes after about d 15 (Charpigny et al., 1997). The expression of PGHS-2 mRNA was found to be greater in pregnant ewes between d 10 and 18, with concentrations decreasing by d 16 in cyclic ewes (Kim et al., 2003). The present study detected no changes in PGHS-2 mRNA; collectively, these findings are consistent with an upregulation of endometrial PGHS-2 protein during pregnancy, and support the hypothesis of Charpigny et al. (1997) that pregnancy may be associated with increased translation efficiency and (or) increased stability of PGHS-2 protein in the absence of changes in steady-state concentrations of PGHS-2 mRNA. Interestingly, this increased expression of PGHS-2 protein in the endometrium of early pregnancy could explain the greater basal concentrations of 13,14-dihydro 15-keto PGF $_{2\alpha}$  (PGFM) reported in pregnant cows (Williams et al., 1983). However, increased basal concentrations of PGFM reflect a secretion pattern that is not luteolytic or pulsatile in nature. An absence of luteolytic pulses, but greater basal concentrations, of plasma PGFM indicate that pregnancy does not suppress completely the endometria's ability to synthesize PG, but alters the pattern of secretions. Pregnancy-induced expression of PGHS-2 protein in the uterus might indicate that the antiluteolytic action of IFN- $\tau$  to suppress pulsatile secretion of PGF $_{2\alpha}$  is not likely at the level of PGHS-2 expression. A decline in the concentrations of ER $\alpha$  mRNA, ER $\alpha$  protein, and OTR mRNA of pregnant cows treated with or without bST may have caused a suppressive effect on pulsatile secretion of PGF $_{2\alpha}$  by endometrium that would normally initiate luteolysis. In early pregnancy, constant increases in production of IFN- $\tau$  by well-developed embryos would inhibit the luteolytic pulse generator for secretion of PGF $_{2\alpha}$ . The increase in PGHS-2 protein of pregnancy could contribute to the greater basal

concentrations of PGFM detected in the circulation during early pregnancy (Williams et al., 1983). When poorly developed embryos produce small amounts of IFN- $\tau$  at the time of pregnancy recognition, pulsatile secretion of PGF $_{2\alpha}$  may occur, leading eventually to luteolysis as a consequence of a lack in attenuation of oxytocin receptor and estrogen receptor expressions.

Furthermore, pregnancy-associated events such as regulation of localized immune function, angiogenesis, regulation of blood flow, and development of implantation sites require presence of PGHS-2 protein (Matsumoto et al., 2002). Increased PGHS-2 protein in pregnancy may support these localized responses but the afferent regulators of PGHS-2 for pulsatile secretion of PGF $_{2\alpha}$  have been suppressed. Low concentrations of IFN- $\tau$  have been shown to be inhibitory to PGF $_{2\alpha}$  secretion and PGHS-2 mRNA expression by endometrial cells *in vitro* (Guzeloglu et al., 2004b). Therefore, underdeveloped embryos, which would produce reduced amounts of IFN- $\tau$ , may not survive the process of implantation due to a possible negative effect of reduced IFN- $\tau$  concentrations on PGHS-2 mRNA. However, these responses would contribute to extended interestrus intervals for cows losing embryos.

It is important to determine the responses of PGFS and PGES mRNA (i.e., relative gene expression that may or may not be related to enzymatic protein or activity) regarding potential downstream metabolism of PGH $_2$ . It is hypothesized that downstream metabolism of PGH $_2$  to PGF $_{2\alpha}$  could be decreased or conversion of PGH $_2$  into PGE $_2$  increased, because luteolytic peaks of PGF $_{2\alpha}$  are reduced in pregnancy. Transcripts of 1.3 kb for PGES (Arosh et al., 2002) and 1.4 kb for PGFS (Xiao et al., 1998) were detected in the endometrium at d 17 following an induced ovulation. An interaction between bST and pregnancy occurred in which bST increased PGFS mRNA expression in cyclic control-fed cows but bST decreased PGFS mRNA expression in pregnant cows. Also, bST increased PGES mRNA expression in pregnant, cyclic control- and FO-fed cows. Because bST decreased PGFS mRNA and increased PGES mRNA in pregnant cows, this may be potentially associated with more PGE $_2$  and less PGF $_{2\alpha}$  secretions creating a stronger antiluteolytic signal to maintain pregnancy. This may be another explanation for the bST-induced increase in pregnancy rate, beyond that of increased conceptus development, that contributes to a decrease in early embryonic loss as previously reported (Moreira et al., 2001; Santos et al., 2004a). Also the reduction in PGFS and PGES mRNA due to pregnancy may be a consequence of the overall reduction in the hormonal afferent components regulating the PG cascade (i.e., ER $\alpha$  mRNA and protein, and OTR mRNA). However, presence of the conceptus differentially modulates en-

ometrial responsiveness to bST in a manner to reduce PGFS mRNA and increase PGES mRNA to support pregnancy. When the conceptus was not present, bST stimulated ER $\alpha$  and OTR mRNA, and this was associated with an increase in PGFS and PGES. Indeed, the amount of PGE<sub>2</sub> in uterine flushings was elevated in pregnant cows treated with bST. In our study, pregnant cows had far greater concentrations of PGF<sub>2 $\alpha$</sub>  and PGE<sub>2</sub> in uterine flushings than cyclic animals. Bovine conceptuses were able to convert radiolabeled arachidonic acid into PG (Lewis et al., 1982). Thus, high luminal content of PG in pregnant ruminants may be due, at least in part, to PG synthesis and release by the conceptus. Also, bST increased endometrial PGES mRNA expression in pregnant cows, possibly accounting for some of the PGE<sub>2</sub> that was increased in bST-treated pregnant cows compared with non-bST-treated pregnant cows.

Effect of bST treatment on uterine OTR, ER $\alpha$ , and PR genes and ER $\alpha$  and PGHS-2 proteins of lactating Holstein cows was significant and diverse. Critical regulatory components such as mRNA for OTR, ER $\alpha$ , PGES, and PGFS were stimulated in cyclic cows due to bST. However, different responses occurred in pregnant cows, which may reflect direct conceptus-induced alterations in regulation of gene expression due to such agents as IFN- $\tau$ , or indirect conceptus-induced alterations within the endometrium, that effect responsiveness to bST. Spencer et al. (1999) demonstrated that pretreatment of ewes with IFN- $\tau$  was necessary to induce endometrial responsiveness to placental lactogen and GH. Badinga et al. (2002) demonstrated, in a bovine endometrial cell line, that both bovine GH and IFN- $\tau$  suppressed phorbol 12,13-dibutyrate-induced PGF<sub>2 $\alpha$</sub>  production. When added in combination there was an additive effect in reducing PGF<sub>2 $\alpha$</sub>  secretion. The need for IFN- $\tau$  priming for a GH response also appears to be true for responses of the GH-IGF family (Bilby et al., 2006b).

The FO had little effect on the endometrial components that directly regulate the PG cascade. This was not unexpected because EPA and DHA exert their regulatory effects as alternative substrates that reduce the lipid pool of arachidonic acid in the endometrium (Bilby et al., 2006a). Such a displacement reduces the overall amount of arachidonic acid precursor for PG of the 2 series and increases the precursor pools of EPA and DHA for biosynthesis of 3-series PG (Mattos et al., 2003).

## CONCLUSIONS

At d 17 of the cycle, the potential luteolytic cascade was affected as pregnancy decreased steady-state concentrations of ER $\alpha$  mRNA, ER $\alpha$  protein, OTR mRNA,

and increased PGHS-2 protein. A decline in the concentrations of ER $\alpha$  and OTR would contribute to attenuation in the endometrial pulsatile secretion of PGF<sub>2 $\alpha$</sub>  that would initiate luteolysis in cyclic lactating cows. Increased PGHS-2 protein in early pregnancy could contribute to the greater basal concentrations of PGFM detected in the circulation and support developmental processes of the conceptus–endometrial unit.

Collectively, this study showed that the differential responses (i.e., PGFS) to bST depend on pregnancy status. The bST may regulate enzymes (i.e., PGES) downstream of PGHS-2 to decrease PGF<sub>2 $\alpha$</sub>  and increase PGE<sub>2</sub>, thereby maintaining pregnancy. Pregnancy seems to attenuate gene expression and protein secretion of hormonal components regulating the PG cascade thereby reducing pulsatile release of PGF<sub>2 $\alpha$</sub> . Feeding FO not only increased PR mRNA expression, but also had differential cellular responses with FO increasing PR in the SGE that may be beneficial for preparation of the uterus to maintain pregnancy. In addition, feeding FO reduced localization of ER $\alpha$  (i.e., LE, DGE, and SS) and PGHS-2 (i.e., LE), and altered amount of substrate precursor available for PG synthesis (Bilby et al., 2006a) that would contribute to a reduction in pulsatile release of PGF<sub>2 $\alpha$</sub> . Development of pharmaceutical (e.g., bST) and nutraceutical (e.g., selective fatty acids) management schemes that are integrated with reproductive management may increase pregnancy rates and warrant further investigation.

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