The influence of postnatal nutrition on reproductive tract and endometrial gland development in dairy calves

Meghan L. Wilson,* Sarah R. McCoski,* Adam J. Geiger,† R. Michael Akers,† Sally E. Johnson,* and Alan D. Ealy*¹

*Department of Animal and Poultry Sciences, and

†Department of Dairy Sciences, Virginia Tech, Blacksburg 24061

ABSTRACT

Uterine gland development occurs after birth in cattle and other mammals. The timeline of gland development has been described in various species, but little is known about how postnatal diet influences uterine gland development. This is especially concerning in dairy heifers, where a variety of milk replacer and whole milk nutrition options exist. Little work also exists in cattle to describe how early exposure to steroids influences reproductive tract and uterine gland development. The objective of this work was to determine the effects of early postnatal plane of nutrition and estrogen supplementation on uterine gland development in calves. In both studies, Holstein heifer calves were assigned to restricted milk replacer (R-MR) or enhanced milk replacer (EH-MR) diets. In study 1, calves (R-MR, $n = 6$; EH-MR, $n = 5$) were euthanized at 8 wk. In study 2, calves were weaned at 8 wk and administered estradiol (R-MR, $n = 6$; EH-MR, $n = 6$) or placebo (R-MR, $n = 6$; EH-MR, $n = 5$) for an additional 14 d before euthanasia. Average daily gain and final body weight was greater in both studies in heifers fed the enhanced diet. At 8 wk, EH-MR calves had a greater number of glands and a smaller average gland size, but total gland area was not different from the R-MR group. At 10 wk, uterine gland number and size were not affected by diet or estrogen. Expression profiles of several paracrine mediators of gland development were examined. Increases in transcript abundance for *IGF1* and *IGFBP3* and a decrease in abundance of *WNT7A* were detected in calves fed the enhanced diet at 8 wk of age. Plane of nutrition did not affect transcript profiles at 10 wk of age, but estradiol supplementation decreased *MET* and *WNT7A* transcript abundance. To conclude, heifer calves on a restricted diet exhibited a uterine morphology and transcript profile suggestive

of delayed uterine gland development. These changes appear to be corrected by wk 10 of life. Also, this work provides evidence supporting the contention that early estradiol exposure has detrimental effects on uterine gene expression.

Key words: uterus, reproductive tract, growth factors, milk replacers

INTRODUCTION

Mammalian uteri are devoid of endometrial glands at birth. The postnatal development of glands, or adenogenesis, begins immediately after birth and is normally complete by 8 wk of age in gilts and ewe lambs (Bartol et al., 1988b, 1999). The timeline for uterine gland development in cattle has not been described. This process involves a coordinated series of events wherein luminal epithelium differentiate into glandular epithelium located in the glandular buds present throughout regions of the uterus that do not contain caruncles. These invaginations form tubules that eventually coil and branch throughout the stroma to form an extensive network of uterine glands (Gray et al., 2001a).

The primary function of uterine glands is to produce and secrete epithelial products and selectively transport serum compounds into the uterine luminal fluid (Spencer, 2014). This mix of ions, AA, carbohydrates, proteins, lipids, and biologically active factors (e.g., growth factors) are essential for the continuation of early pregnancy. Ewes lacking uterine glands because of neonatal steroid hormone exposure undergo normal estrous cyclicity and produce viable embryos, but the embryos die around the time of maternal recognition of pregnancy (Gray et al., 2001b).

Avoiding steroid-containing implants or legumes that contain large quantities of phytoestrogens is recommended for cattle and other ruminants before puberty to prevent diminished reproductive potential in adulthood (Adams, 1995a; Becker et al., 2011). Heifers implanted with Compudose estradiol implant (Elanco Animal Health, Greenfield, IN), a nonsteroidal

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¹Corresponding author: ealy@vt.edu

estrogen agonist, have increased incidence of ovarian cysts (Deutscher et al., 1986). Uterine gland development also is compromised with early postnatal steroid exposure. The most severe reductions in uterine gland number and size occur in sheep, pigs, and cattle when estradiol or progesterone exposure begins at birth. Females display a permanent reduction or a total absence of uterine glands that persists throughout life (Bartol et al., 1988a, 1995; Spencer et al., 1993; Gray et al., 2001a). Endometrial gland density and uterine fluid content also is reduced by estrogen administration in 3- and 6-wk-old heifers (Bartol et al., 1995).

It is unclear how early postnatal nutrition affects endogenesis. This is especially concerning in dairy heifers, where a variety of milk replacer and whole milk nutrition options exist. A commonly used dairy calf milk replacer ration includes low amounts of protein and fat to promote early starter grain consumption, which hastens rumen development and, ultimately, reduces the overall feed costs (Brown et al., 2005; Hill et al., 2008). This scheme also eases the transition from a milk- to a grain-based diet at weaning. Other milk replacer rations contain greater protein and fat content, so they more closely approximate the composition and caloric density of normal cow milk. The high-protein, high-fat ration increases BW and ADG in the first several months of life (Hill et al., 2008; Daniels et al., 2009). Reductions in the age at puberty and first calving and increases in first-lactation milk production are reported in calves consuming high-protein, high-fat milk replacer rations when compared with calves consuming diets containing lower protein and fat (Davis Rincker et al., 2011; Soberon and Van Amburgh, 2013).

The current work was undertaken to obtain a more complete understanding of how early postnatal nutrition and estrogen supplementation at weaning influences reproductive tract morphology and uterine gland development in dairy heifers. Our hypothesis was that changes in preweaning nutrition (i.e., during the first 8 wk of life) influences reproductive tract development and uterine gland formation and that estradiol supplementation at weaning adversely affects these processes.

MATERIALS AND METHODS

Animal Handling and Experimental Design

Animal experiments were completed in accordance with and with the approval of the Virginia Tech Institutional Animal Care and Use Committee (#14-045-DASC). Two studies were initially conceived to examine how postnatal plane of nutrition and early estradiol exposure affects mammary gland develop-

ment. An initial publication of these findings has been completed (Geiger et al., 2016). The feeding scheme, time intervals, and use of heifer calves (expensive to use for terminal studies) provided the opportunity for companion studies examining the influences of early plane of nutrition and estradiol exposure on reproductive tract development.

The 2 studies used the same early postnatal feeding scheme. In both studies, Holstein heifer calves (*Bos taurus taurus*) were purchased from a commercial producer and maintained at the Virginia Tech Dairy Center (Blacksburg, VA). Calves were fed similarly for the first 5 to 8 d of life (Geiger et al., 2016). At 6 ± 2 d of age, calves were assigned randomly to restricted (R) or enhanced (EH) milk replacer (MR). The R diet (20.9% CP, 19.8% fat, DM basis; Southern States Inc., Richmond, VA) was fed at 0.44 kg of MR powder/animal per day. The EH diet (28.9% CP, 26.2% fat, DM basis; Land O'Lakes Animal Milk Products Co., Shoreview, MN) was fed at 1.08 kg of MR powder/animal per day. Both MR were reconstituted to 15% (wt/vol) water and fed in 2 equal portions at 0600 and 1700 h daily. At the beginning of wk 8, the amount of MR offered was reduced by half to prepare calves for weaning. Heifers were weaned at the end of wk 8. Calves were offered water ad libitum throughout the study. At the end of wk 4, calves were pair-fed starter grain to keep starter intakes similar between groups (25.6% CP, 4.0% fat, 19.8% NDF, DM basis; Southern States Cooperative). This was achieved by offering R-fed calves the average amount of starter grain consumed by ad libitum, EH-fed calves on the preceding day. Milk replacer and feed refusals, weekly growth measurements (BW, hip and withers height, heart girth), and daily fecal and respiration scores are described elsewhere (Geiger et al., 2016). On only a few occasions did calves not consume their entire allotment of milk replacer, and these occasions usually occurred during the first week on their diet.

The 2 studies differed in the duration on diet. In the first study, R- (n = 6) and EH-fed (n = 5) calves were euthanized at the end of wk 8 (56 ± 2 d of age). In the second study, R- and EH-fed calves (R-MR, n = 12; EH-MR, n = 11) were weaned and then maintained exclusively on starter grain (pair-wise fed) until the end of wk 10. An estradiol implant (R-MR, n = 6; EH-MR, n = 6) or a blank silicone implant (R-MR n = 6; EH-MR n = 5) was inserted into the ear of calves at the time of weaning, as described previously (25.7 mg of estradiol-17 β ; Compudose Implant; Elanco Animal Health, Greenfield, IN; Lammers et al., 1999). Circulating estradiol concentrations were not determined, but, according to manufacturer specifications, calves were

anticipated to contain approximately 120 pg/mL of circulating estradiol concentrations for approximately 36 h and then 70 to 80 pg/mL of estradiol thereafter. Calves were euthanized 14 d after implant or placebo insertion (70 ± 2 d of age).

Reproductive Tract Measurements

Calves were euthanized using a lethal dose of phenobarbital administered intravenously (Fatal-Plus, 10 mg/kg of BW, Vortech Pharmaceuticals, Dearborn, MI). A mid-ventral incision was made and the reproductive tract was removed. The broad ligament and vagina were trimmed away and the remainder of the tract was rinsed with water, dried, and weighed. Thereafter, ovaries, oviducts (trimmed of connective tissue), cervix, and uterus were dissected and weighed. Visible follicles were counted on each ovary.

Endometrial Gland Morphometrics

Uterine samples ($0.3\text{--}0.7\text{ cm}^3$) from the midpoint of each uterine horn were collected. Tissue was rinsed briefly in 0.01 M PBS (pH 7.4), fixed with 3.7% (wt/vol) formaldehyde, and infiltrated with paraffin (Histo-Scientific Research Laboratories, Mt. Jackson, VA). Four sections ($5\text{ }\mu\text{m}$) from each animal (2 sections for each uterine horn) were collected on glass slides, deparaffinized, rehydrated through a graded alcohol series, and stained with hematoxylin. The sections were further processed through a graded alcohol and xylene series and cover-slipped. Eight representative microscopic field images at 100-fold magnification were captured and digitized for each heifer using a Nikon Eclipse-Ti inverted microscope equipped with a charge-coupled camera (SPOT, Diagnostic Imaging, Sterling Heights, MI) and shutter control provided by NIS Elements software (Nikon Instruments Inc., Melville, NY). Examples of the uterine gland morphology are illustrated in Figure 1. Uterine glands containing fluid-filled lumens were readily detectable given the high contrast of the glandular epithelium (**GE**). This permitted quantification of gland numbers and size without using endometrial gland markers (e.g., *FOXA2*; Waters et al., 2014). Uterine glands not containing a lumen were not recorded. Only intercaruncular endometrium was analyzed (caruncular regions do not contain glands). Gland size was measured by tracing individual structures using NIS Elements software (Nikon Instruments Inc.). Percentage of cross sectional area containing glands was determined by dividing gland area with total cross sectional area. Total gland numbers were recorded for each image.

Endometrial Transcript Profiling

Multiple endometrial samples were collected from throughout each uterine horn and combined into pools (4–8 samples/pool) to ensure that pools contained samples from the entire endometrium, including both caruncular and intercaruncular regions (Ocón-Grove et al., 2008). Tissues were snap-frozen in liquid nitrogen. The RNA was extracted by homogenization and phase separation in TRIzol reagent (Thermo Fisher Scientific, Waltham, MA) and purified with the PureLink RNA Mini Kit (Thermo Fisher Scientific). Purity and quantity of RNA was determined using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). Only samples containing an absorbance ratio at 260 and 280 nm >1.8 were used.

Quantitative reverse-transcription (**RT**) PCR was completed as described previously (Yang et al., 2011) to examine how plane of nutrition and estradiol treatment influence transcript abundance for several putative mediators of adenogenesis. In brief, total RNA (10 ng/reaction) was treated with RNase-free DNase (New England Biolabs, Ipswich, M) and reverse transcribed (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Austin, TX). Then PCR was completed using SYBR Green Master Mix reagent (Applied Biosystems) and specific primer pairs (Table 1) using an Eppendorf Real-Plex Master Cycler (Eppendorf North America, Hauppauge, NY). Primer pairs were validated for efficiency before use (range = 89–103%; 60 to 300 bp amplification). Reactions were completed with 40 amplification cycles using 57 to 60°C annealing/synthesis (depending on the transcript) for 1 min and 95°C denaturation for 15 s. Amplification of a single PCR product was verified for each PCR run by dissociation curve analysis. Non-reverse transcribed RNA samples were included as negative controls. Ribosomal protein L19 (*RPL19*) served as the internal reference control; *RPL19* was an appropriate reference control for the bovine endometrium in previous work (McCarthy et al., 2012), and *RPL19* mRNA abundance did not differ based on diet or estrogen treatment in these studies (data not shown). The comparative threshold quantification cycle (C_T) method was used to quantify mRNA abundance (Cooke et al., 2009). The average ΔC_T value for each sample was calculated (C_T transcript of interest – C_T *RPL19*) and used to calculate the fold-change in relative abundance for each transcript.

Statistical Analyses

Data were analyzed by ANOVA using the general linear model (GLM) of the SAS (ver. 9.3, SAS Institute

Inc., Cary, NC) for the effects of diet, estradiol treatment, replicate, and their interactions. Three batches of calves were acquired for the work (Geiger et al., 2016), but no statistically significant effects of batch

were detected; thus, this term was not included in the final analysis. Covariates were included to normalize follicle numbers for ovarian weight and to adjust the uterine gland assessments based on total cross sectional

Figure 1. Representative uterine cross sections at 8 wk of age for heifer calves fed either a restricted (R) or enhanced (EH) milk replacer (MR) diet. Endometrial cross sections were harvested and processed for hematoxylin staining and image analysis. (A, B) Representative endometrial cross sections of the entire reproductive tract at 8 wk in heifers fed the R or EH diets (10× magnification; bar = 1 mm scale). (C, D) Representative endometrial cross sections of glandular epithelium images used to quantify numbers and sizes of endometrial glands (200× magnification). Labels indicate luminal epithelium (LE), glandular epithelium (GE), stroma (ST), and myometrium (Myo).

Table 1. Primer sequences

Transcript (NCBI Gene ID)	Primer	Sequence (5' to 3')	Reference ¹
<i>ESR1</i> (407238)	Forward	GAGGGCTGTAAGGCCTTCTT	—
	Reverse	ATCATCTCTCTGGCGCTTGT	—
<i>FGF10</i> (326285)	Forward	AGAGGACAGAAAACACGAAGGAAA	Cooke et al., 2009
	Reverse	GGTTATACTGCATCTGCAATCATTG	—
<i>FGFR2</i> ² (404193)	Forward	GACCTGGTGTCTGTACCTACCA	Ozawa et al., 2013
	Reverse	CTGGCAGCTAAATCTCGATGAA	—
<i>FGFR2IIIb</i> ³ (AJ419173)	Forward	CAAAGTGTACAGCGATGCCC	—
	Reverse	TTTATCCCCGAGTGCTTCAGA	—
<i>HGF</i> (282879)	Forward	TCTGAGTCGGAAGAGGGTTC	—
	Reverse	CATAGGGGATGGCGATGGGA	—
<i>IGF1</i> (281239)	Forward	ATGCCCAAGGCTCAGAAG	Moriel et al., 2014
	Reverse	GGTGGCATGTCATTCTTCACT	—
<i>IGFBP1</i> (282259)	Forward	AGTTGGTGGATGCTCTCCACT	Wathes et al., 2011
	Reverse	CACATCATCCACGATTCCTGTC	—
<i>IGFBP3</i> (282261)	Forward	ACAGACACCCAGAACTTCTCCTC	Moriel et al., 2014
	Reverse	GTTCAGGAACCTTGAGGTGGTTC	—
<i>MET</i> (280855)	Forward	AAACAGCCGTGGGTCAGTAG	—
	Reverse	GTGCCACCAGTTTTTGGACC	—
<i>RPL19</i> (510615)	Forward	GAAAGGCAGGCATATGGGTA	McCarthy et al., 2012
	Reverse	TCATCCTCCTCATCCAGGTT	—
<i>WNT5A</i> (530005)	Forward	GGCCAGAGTTGAGTTGTGGG	—
	Reverse	GACGGCGGTAATTAGGGCTT	—
<i>WNT7A</i> (533782)	Forward	AGGAGAAGGCTCGCAAATGG	—
	Reverse	GCGATGATGGCGTAGGTGAA	—

¹Primers not containing a reference were synthesized based on NCBI PCR primer software.

²Primers were chosen that would amplify all FGFR2 splice variant sequences.

³Primer pair derived from GenBank sequence AJ419173 to specifically amplify the IIIb variant for *FGFR2*.

area. Fold-change quantitative RT-PCR data were log-transformed before analysis. When needed, individual means were compared by pair-wise comparisons (PDIF analysis). Data presented as arithmetic means $\pm$ SEM. Differences were considered significant at $P \leq 0.05$.

RESULTS

Calf Performance on Diets and with Estrogen Treatment

Calf feed intake, growth performance, and health observations are reported in a separate manuscript (Geiger et al., 2016). Both BW and ADG data are presented to illustrate growth rate differences based on diet and estrogen treatment. Beginning BW was not different between treatment groups in the first or second study (average 40.4 ± 1.4 and 38.9 ± 1.8 kg, respectively); however, EH-fed calves were heavier ($P < 0.01$) than R-fed calves after 8 (Table 2) and 10 wk (Table 3). Estradiol treatment did not affect BW at the end of wk 10 in R-fed calves, but BW was greater ($P < 0.05$) in EH-fed calves containing an estradiol implant than EH calves without estrogen (Table 3).

The ADG was greater ($P < 0.01$) for EH- than R-fed calves on diets between wk 1 and 8 in both the

first (Figure 2A) and second (Figure 2B) study. In the second study, diet affected ADG at wk 9 and 10, with R-fed calves exhibiting greater ($P < 0.01$) ADG than EH-fed calves regardless of estradiol treatment (0.88 ± 0.06 vs. 0.61 ± 0.06 kg/d). Pair-wise comparisons

Table 2. Reproductive tract weights and follicle numbers of heifer calves fed either a restricted (R) or enhanced (EH) milk replacer (MR) diet for 8 wk ($\pm$ SEM)

Parameter ¹	R-MR (n = 6)	EH-MR (n = 5)
Final BW (kg)	52.3 $\pm$ 1.6 ^a	81.7 $\pm$ 4.2 ^b
Reproductive tract		
g	22.7 $\pm$ 1.9 ^a	36.8 $\pm$ 1.9 ^b
g/kg of BW	0.47 $\pm$ 0.03 ^a	0.48 $\pm$ 0.03 ^a
Uterus		
g	17.8 $\pm$ 1.8 ^a	25.6 $\pm$ 1.0 ^b
g/kg of BW	0.37 $\pm$ 0.03 ^a	0.33 $\pm$ 0.01 ^a
Cervix		
g	4.7 $\pm$ 0.3 ^a	7.2 $\pm$ 0.9 ^b
g/kg of BW	(0.09 $\pm$ 0.01) ^a	(0.09 $\pm$ 0.01) ^a
Ovaries		
g	2.1 $\pm$ 0.5 ^a	3.0 $\pm$ 0.6 ^a
g/kg of BW	0.05 $\pm$ 0.01 ^a	0.04 $\pm$ 0.01 ^a
Follicle numbers		
Total	30.7 $\pm$ 10.0 ^a	31.0 $\pm$ 12.1 ^a
Number/g of tissue	12.0 $\pm$ 2.8 ^a	9.1 $\pm$ 1.9 ^a

^{a,b}Different superscripts within row denote differences ($P < 0.05$).

¹Weight data are presented as total (g) and adjusted based on final BW (g/kg of BW).

determined that R-fed calves with estradiol implants exhibited greater ($P < 0.05$) ADG on wk 9 and 10 than both EH-fed treatment groups (Figure 2C). Also, R-fed calves lacking estradiol implants had a greater ($P < 0.05$) ADG than EH-fed calves lacking estradiol implants. Both diet and estradiol treatment altered ADG over the entire 10-wk period (Figure 2D), with EH-fed calves provided estradiol implants displaying the greatest ($P < 0.05$) ADG and EH-fed calves not provided estradiol performing better ($P < 0.05$) than either of the R-fed groups.

Reproductive Tract Metrics

Several gross anatomical assessments of reproductive tracts were made when calves were euthanized at wk 8 and 10 (Tables 2 and 3). At 8 wk of age, weights for the entire reproductive tract, uterus, and cervix were greater ($P < 0.05$) in EH- than R-fed calves. After adjusting for BW, none of these weights differed between diets. Neither unadjusted nor BW-adjusted ovarian weights differed based on diet at 8 wk.

In the second study, both diet and estradiol treatment on wk 9 and 10 influenced reproductive tract weights (Table 3). Both unadjusted and BW-adjusted reproductive tract weights were greater ($P < 0.05$) in EH-fed calves implanted with estradiol than all other treatment groups.

Uterine weights also differed at wk 10 depending on diet and estradiol treatment (Table 3), with unadjusted uterine weights being greatest ($P < 0.05$) in EH-fed

calves provided estradiol and least ($P < 0.05$) in R-fed calves provided a placebo. Unadjusted and BW-adjusted uterine weights were less ($P < 0.05$) in R-fed calves provided estradiol than R-fed calves lacking estradiol treatment, but the opposite effect was observed in the EH-fed calves, where EH-fed calves administered estradiol had greater ($P < 0.05$) unadjusted and BW-adjusted uterine weights than EH-fed calves not provided estradiol.

At 10 wk of age, cervix weight was greatest ($P < 0.05$) for estradiol-treated EH-fed calves and least ($P < 0.05$) for R-fed calves with no supplemental estradiol (Table 3). Estradiol treatment increased ($P < 0.05$) BW-adjusted cervix weights in both R- and EH-fed calves. Nonadjusted ovarian weights were greater ($P < 0.05$) in EH- than R-fed calves supplemented with estradiol, but no differences in ovarian weights were detected after adjusting for BW.

Assessments of follicle numbers were completed in both replicate studies. At 8 wk of age, neither total follicle numbers nor follicle numbers adjusted for ovarian weights were altered due to diet (Table 2). At 10 wk of age, estradiol-dependent effects on follicle numbers were detected (Table 3), where total follicle numbers were greater ($P < 0.05$) in EH-fed calves supplemented with estradiol than EH-fed calves not provided estradiol. Total follicle numbers for both R-fed groups were not different ($P > 0.05$) from each other or from the EH-fed groups; however, after adjusting for ovarian weight, both estradiol-treated EH- and R-fed calves contained more ($P < 0.05$) visible follicles than counterparts administered a placebo.

Table 3. Reproductive tract weights and follicle numbers of heifer calves fed either a restricted (R) or enhanced (EH) milk replacer (MR) diet with (+) or without (–) estradiol (E2) for 10 wk ($\pm$ SEM)

Parameter ¹	R-MR		EH-MR	
	–E2 (n = 6)	+E2 (n = 6)	–E2 (n = 5)	+E2 (n = 6)
Final BW (kg)	61.9 $\pm$ 1.5 ^a	62.7 $\pm$ 1.8 ^a	73.0 $\pm$ 5.6 ^b	83.1 $\pm$ 2.3 ^c
Reproductive tract				
g	31.9 $\pm$ 2.8 ^a	27.0 $\pm$ 1.0 ^a	26.9 $\pm$ 3.6 ^a	48.8 $\pm$ 3.2 ^b
g/kg of BW	0.56 $\pm$ 0.05 ^a	0.47 $\pm$ 0.02 ^{ab}	0.40 $\pm$ 0.03 ^b	0.66 $\pm$ 0.03 ^c
Uterus				
g	26.9 $\pm$ 2.3 ^a	18.5 $\pm$ 1.0 ^b	19.1 $\pm$ 1.9 ^b	34.9 $\pm$ 1.9 ^c
g/kg of BW	0.47 $\pm$ 0.04 ^a	0.32 $\pm$ 0.02 ^b	0.29 $\pm$ 0.01 ^b	0.47 $\pm$ 0.02 ^a
Cervix				
g	5.0 $\pm$ 0.8 ^a	8.5 $\pm$ 0.7 ^b	7.2 $\pm$ 1.4 ^{ab}	13.7 $\pm$ 1.8 ^c
g/kg of BW	0.10 $\pm$ 0.01 ^a	0.15 $\pm$ 0.01 ^b	0.11 $\pm$ 0.01 ^a	0.18 $\pm$ 0.02 ^b
Ovaries				
g	3.2 $\pm$ 0.4 ^{ab}	2.0 $\pm$ 0.5 ^a	2.9 $\pm$ 0.7 ^{ab}	4.5 $\pm$ 1.2 ^{bc}
g/kg of BW	0.06 $\pm$ 0.01 ^a	0.04 $\pm$ 0.01 ^a	0.04 $\pm$ 0.01 ^a	0.06 $\pm$ 0.01 ^a
Follicle numbers				
Total	37.2 $\pm$ 16.0 ^{ab}	50.3 $\pm$ 15.8 ^{ab}	20.2 $\pm$ 8.9 ^a	94.7 $\pm$ 38.4 ^b
Number/g of tissue	9.8 $\pm$ 3.0 ^a	22.9 $\pm$ 3.8 ^b	6.4 $\pm$ 1.8 ^a	16.7 $\pm$ 3.6 ^b

^{a–c}Different superscripts within row denote differences ($P < 0.05$).

¹Weight data are presented as total (g) and adjusted based on final BW (g/kg of BW).

Endometrial Gland Morphometrics

Diet affected uterine gland measurements after 8 wk (Figure 3A-C). The EH-fed calves contained greater ($P < 0.05$) numbers of uterine glands (Figure 3A) that were smaller ($P < 0.05$) in size (Figure 3B) than R-fed calves. No diet-dependent difference in the percentage of total endometrial area occupied by glands was observed (Figure 3C). No diet or estradiol effects were detected at 10 wk of age (Figure 4). No differences in uterine gland morphologies were detected between

uterine horns or among sections within individuals (data not shown).

Endometrial Transcript Profiles

At 8 wk of age, EH-fed calves contained greater ($P < 0.05$) mRNA transcript abundance for *IGF1* and *IGFBP1* (Figure 5A). The EH-fed calves also contained reduced ($P < 0.05$) amounts of *WNT7A* (Figure 5A). No differences in transcript abundances for *ESR1*, *FGF10*, *FGFR2*, *FGFR2IIIb*, *HGF*, *MET*, *IGFBP3*,

Figure 2. Average daily gain for heifer calves. Calves ($n = 5-6$ /treatment) fed either a restricted (R) or enhanced (EH) milk replacer (MR) diet for 8 wk. In the first study, calves were euthanized at 8 wk of age. (A) The ADG of calves in study 1. In the second study, calves were exposed to estradiol or blank implants from wk 8 to 10 and then euthanized. (B) The ADG of heifers for the first 8 wk; (C) ADG between wk 8 and 10; (D) ADG for the entire 10-wk period. Different letters (a–c) within each panel denote differences ($P < 0.05$). Error bars indicate SEM.

Figure 3. Morphometric analysis of endometrial glands at 8 wk of age. Calves ($n = 5-6/\text{treatment}$) were fed either a restricted (R) or enhanced (EH) milk replacer (MR) diet for 8 wk, and then were euthanized. Endometrial cross sections were harvested and processed for hematoxylin staining and image analysis. (A) Gland number (adjusted for total area evaluated); (B) average gland size; (C) percentage of the total endometrial area evaluated occupied by glandular epithelia and lumen. Different letters (a,b) within each panel represent differences ($P < 0.05$). Error bars indicate SEM.

Figure 4. Morphometric analysis of endometrial glands at 10 wk of age. Calves ($n = 5-6/\text{treatment}$) were fed either a restricted (R) or enhanced (EH) milk replacer (MR) diet and then were exposed to estradiol or blank implants from wk 8 to 10. Calves were euthanized at 10 wk of age. Endometrial cross sections were harvested and processed for hematoxylin staining and image analysis. (A) Gland number (adjusted for total area evaluated); (B) average gland size; (C) percentage of the total endometrial area evaluated occupied by glandular epithelia and lumen. No differences between treatments within each figure were detected ($P > 0.05$), indicated by common letter (a). Error bars indicate SEM.

and *WNT5A* were detected at wk 8. Diet did not affect transcript abundance at 10 wk of age (Figure 5B); however, *MET* and *WNT7A* mRNA abundance was reduced ($P < 0.05$) in endometria from groups not provided estrogen. No other estradiol or estradiol by diet interaction was detected.

DISCUSSION

Two milk replacers with different compositions were fed at different levels from wk 1 to 8 of life to create the R- and EH-MR diets. Similar to other studies, the 2 diets produced differences in BW and ADG (Hill et al., 2008; Daniels et al., 2009). This marked difference in growth performance was exacerbated beyond what would normally occur in a commercial setting by limit-feeding starter grain consumption in the R-fed calves to levels consumed by the EH-fed group. Grain consumption by the EH-fed group is reduced due the excessive CP and fat in MR (Sweeney et al., 2010; de Passillé et al., 2011; Geiger et al., 2016). Thus, the R- and EH-MR diets provided a suitable deviation in growth performance to serve as a model for examining how plane of nutrition affects postnatal reproductive tract development. The R-fed group likely does not mirror commercial calves; their growth rates were well below industry standards for growing dairy calves during milk replacer feeding (Sweeney et al., 2010; de Passillé et al., 2011; Geiger et al., 2016).

Both EH feeding and estradiol supplementation improved postweaning performance in calves. The greater ADG observed in R-fed calves after weaning is especially interesting when considering that starter grain consumption in the R-fed calves was restricted to the same level consumed by calves on the EH diet (via employing a pair-wise feeding strategy). This likely represents the heightened preparedness of the rumen and intestines in R-fed calves for grain consumption (Brown et al., 2005; Hill et al., 2008). Estradiol supplementation minimized the reduction in growth performance observed in EH-fed calves. Estradiol-containing implants are commonly used to improve pre- and postweaning beef heifer growth performance (Sawyer et al., 1987; Moran et al., 1991; Hardt et al., 1995; Cleale et al., 2013).

The primary goal of our work was to examine how postnatal diet and estrogen exposure affects uterine gland development. The timing of adenogenesis remains largely unexplored in early postnatal calf development. In the ewe lamb (Bartol et al., 1988b; Taylor et al., 2000), shallow luminal epithelial crypts appear in intercaruncular regions between postnatal d (PD) 0 and 7. Glandular epithelial buds invaginate into the stroma by PD14, and tubular structures with minimal branching exist by PD21. After PD21, coining and

branching morphogenesis of the tubular glands occurs and the depth of the glands continues until it reaches the deep stratum spongiosum stroma. By PD56 (8 wk of age), endometrial glands are histologically similar to the adult uterus. The outcomes presented here suggest that a similar timeline exists for heifer calves, although diet affected the progression of this process.

In the present studies, dietary interventions were not initiated until 6 ± 2 d of age. Earlier initiation of these diets was not possible because the calves were not born on-site. Thus, uterine gland development was already underway as the dietary treatments were initiated (Bartol et al., 1988b; Taylor et al., 2000). Even so, differences in rate of uterine gland formation could be detected at 8 wk of age. Based on the outcomes, we proposed that the alterations in gland size and number at 8 wk of age between calves on the 2 diets reflect changes in the progression of adenogenesis. The larger size and lower numbers of glands in R-fed calves strongly suggest that these calves had progressed through the initial stages of adenogenesis but contained glands that were less coiled and branched than the EH-fed calves. The size and shape of glandular structures support this contention. The lack of differences in uterine gland morphology or gland numbers and size between diets at 10 wk of age suggests that R-fed calves have the capacity to complete adenogenesis within this 2-wk window. Alternatively, as heifers were placed on the same diets between wk 8 and 10 (weaned from MR and fed starter grain exclusively), it is possible that feeding a common diet over this short period may have influenced uterine gland status at the end of wk 10.

The implications of the altered ontogeny of uterine development remain speculative. The compensatory gland development between 8 and 10 wk may prevent long-lasting adverse consequences on fecundity in the R-fed calves. However, as will be discussed, endometrial gene expression was altered at 8 and 10 wk of age based on plane of nutrition; thus, long-lasting effects of early nutrition may indeed exist.

Uterine expression profiling work suggests that the local *IGF1* system plays a part in mediating the progression of adenogenesis based on postnatal nutritional status. Locally derived *IGF1* is an essential mesenchymal regulator of postnatal uterine gland development in mammals. In mice, uterine glands are absent in *IGF1* null females (Baker et al., 1996). In the sheep, *IGF1* and several *IGFBP* are expressed in the endometrial stroma, and *IGF1* receptor is expressed in glandular epithelium (Taylor et al., 2001). In our study, endometrial *IGF1* mRNA abundance was greater in EH- than R-fed calves at 8 wk, but not 10 wk of age. Several *IGFBP* also are expressed in the postnatal ovine endometrium (Hayashi et al., 2005), and in calves *IGFBP1* mRNA

Figure 5. Selective transcript profiles for endometrial gland regulatory genes. Calves ($n = 5-6$ /treatment) were fed either a restricted (R) or enhanced (EH) milk replacer (MR) diet. (A) Calves were euthanized at 8 wk of age and endometrial samples were collected and processed for RNA extraction and quantitative reverse-transcription PCR. (B) Calves were exposed to estradiol (E2) or blank implants from wk 8 to 10. Calves then were euthanized and endometrial samples were collected and processed. Data are depicted as transcript abundance relative to the reference control (*RPL19*). The asterisk (*) indicates differences between diet (A) or estradiol treatment (B) ($P < 0.05$). Error bars indicate SEM.

was greater in EH-fed calves at 8 wk of age whereas *IGFBP3* mRNA abundance was not different between groups at either time point.

Transcript abundance for *WNT5a* and *WNT7a* was examined in the current work because each patterning factor is involved with endometrial epithelium proliferation and uterine gland formation. Gene *WNT7a* was of primary interest because ablation of *WNT7A* gene function prevents uterine gland formation in mice (Dunlap et al., 2011). Also, steroid-induced disruption in gland formation reduces *WNT7A* expression in sheep (Hayashi and Spencer, 2006; Spencer et al., 2012). Ovine *WNT7A* is expressed solely by endometrial epithelium (Hayashi and Spencer, 2006; Hayashi et al., 2007). If the premise that endometria in R-fed calves contain less developed glands, and thus fewer glandular epithelia, then one would expect reduced endometrial *WNT7A* abundance. However, the opposite scenario occurred, wherein the R-fed calves contained a greater abundance of *WNT7A* mRNA than the EH-fed calves at wk 8. Perhaps the increased expression of *WNT7A* reflects a compensatory mechanism for overcoming the adverse effects of reduced nutrition on uterine gland morphogenesis. The relative abundance of *WNT7A* was not different between dietary groups at 10 wk of age, when uterine gland development in the R-fed calves had reached the same developmental status as those found in the EH-fed calves.

The remaining paracrine and endocrine regulators of endometrial adenogenesis examined herein were not affected by diet at wk 8 or 10. Of particular interest was examining *FGF10* and *HGF*; these stroma-derived factors interact with their luminal and glandular epithelial-localized receptors (*FGFR2IIIb* and *MET*, respectively) expressed throughout early postnatal life (Chen et al., 2000a,b; Satterfield et al., 2008). Their key morphogenetic roles in other epithelial tissues, such as lung and intestinal epithelium, implicate them as mediators of similar activities in the uterus (Sekine et al., 1999; Shin et al., 2006). Expression of *ESR1*, although unchanged, was examined based on its importance for normal gland branching and coiling in ewes (Carpenter et al., 2003a). Efforts also were made to examine *PRLR* abundance. Prolactin is needed for normal early gland development and glandular epithelial proliferation in sheep (Taylor et al., 2000; Carpenter et al., 2003b). Both the long and short *PRLR* isoforms have been detected in ovine glandular epithelium, with greatest concentrations of each within proliferating glandular regions (Taylor et al., 2000). Previously validated bovine *PRLR* primers (Tao et al., 2013) failed to detect either isoform.

Another goal of this work was to examine how estradiol exposure affects uterine gland development and gene expression. Similar amounts of estradiol were

provided in this work that were provided in previous studies in ewe lambs (Hayashi et al., 2004), although studies in sheep provided daily estradiol injections whereas implants were used for the present work. The lack of estradiol effects on adenogenesis likely occurred because the estradiol treatment did not begin until wk 9. Inhibitory effects of estradiol are more readily apparent during the initial periods of adenogenesis (Hayashi et al., 2004). The estradiol implant decreased *MET* and *WNT7A* expression in both dietary groups; both factors are estrogen-sensitive (Hayashi and Spencer, 2005, 2006). In previous work, the most severe reduction in uterine gland development in beef heifers was observed when progesterone and estradiol implants were provided at birth, but reductions in uterine gland development were also detected when implants were provided at 21 or 45 d of age (Bartol et al., 1995). In Bartol et al. (1995), implanted heifers contained lower uterine luminal proteins than nonimplanted controls regardless of when the implants were first administered.

Additional endpoint analyses were made to explore how diet and estrogen supplementation affects gross reproductive tract anatomy. In the present studies, changes in uterine weight were the primary determinant of the observed changes in overall reproductive tract weight. For the calves not supplemented with estradiol, it is unclear why EH-fed calves had lighter uteri than R-fed calves; perhaps this outcome occurred because of the poor postweaning performance of this group. Uterine weights were improved in EH-fed calves supplemented with estradiol, which is consistent with the expected uterotrophic effects of estrogen supplementation observed in multiple species, including cattle (Prichard et al., 1989; Burton and Wells, 2002). However, estradiol supplementation suppressed uterine weights in the R-fed calves, which suggests that nutritional status interferes with the positive effects of estradiol on the uterus.

Weight of the cervix was not affected by diet at wk 10, but both dietary groups possessed the same estradiol-dependent increase in cervix weight. It is not clear if the increases in cervical weight represent a positive influence of estradiol on cervix development or if it indicates the beginning of cervical malformations that may reduce fertility. In sheep, long-term exposure to estradiol compromises cervical morphology and specifically increases the incidence of cervical fold fusion and glandular cyst formation and reduces mucus cell numbers (Adams, 1995b). Changes in mucus composition following estrogen exposure are implicated for reducing sperm transport and subsequent fertilization rates (Adams, 1995a,b).

Estradiol-dependent ovarian responses were evident in the calves examined at 10 wk of age. Visible follicle

numbers were increased in both estradiol-treated EH- and R-fed calves; it is uncertain whether this is a positive or negative outcome. Follicle numbers of calves are directly related to subsequent ovarian function and fertility (Ireland et al., 2011; Mossa et al., 2013); however, general consensus is that growth-promoting implants containing estradiol can have detrimental effects on fertility when administered to heifers (Deutscher et al., 1986; Bartol et al., 1995; Lamb, 2013). The ovary is one likely target for these negative consequences. Providing an estradiol agonist at 1, 6, or 9 mo of age increased the incidence of ovarian cysts and estrus activity not associated with ovulation in heifers (Deutscher et al., 1986). Neither BW-adjusted ovarian weights nor follicle numbers were altered by calf diet. Failure to detect changes in follicle numbers may have been due to the short duration on the diet or conducting follicle counts too soon after beginning estradiol treatment. Visible follicle numbers are an indicator of ovarian follicle reserve status in cattle (Ireland et al., 2009), and links between gestational nutrition and ovarian follicle reserve status are evident in sheep and cattle (Evans et al., 2012; Viñoles et al., 2013). However, no evidence exists to indicate that visible follicle numbers or the underlying ovarian follicle reserve can be adversely affected in the early postnatal period; no such detrimental effects could be detected in this work.

CONCLUSIONS

The timeline of adenogenesis in calves appears similar to that of sheep, where most branching morphogenesis occurs by 8 wk of age. However, restricted feeding appears to delay the normal progression of adenogenesis, as seen by the reduction in gland cross-section numbers and uterine wet weights at 8 wk of age. These diet-dependent delays in uterine development are corrected 2 wk after weaning. The work also provides additional evidence that early postnatal exposure to estradiol negatively affects reproductive tract development. No detectable alterations in uterine histoarchitecture were evident when estradiol treatment was provided near the completion of adenogenesis; however, a subset of local adenogenesis regulators was altered by estradiol treatment and visible follicle numbers were excessively great in these calves. These observations support the contention that early estradiol exposure is detrimental to uterine activity, ovarian activity, and fertility in adults. To conclude, early plane of nutrition may dictate the rate of uterine gland development in cattle, and early estradiol exposure has detrimental effects on uterine gene expression and ovarian physiology.

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