Effects of day of gestation and feeding regimen in Holstein × Gyr cows: III. Placental adaptations and placentome gene expression

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ABSTRACT

This study investigated the influence of day of gestation (DG) and feeding regimens (FR) on the expression of genes responsible for placenta development, nutrient transfer, and angiogenic factors in Holstein × Gyr cows. Forty pregnant multiparous Holstein × Gyr cows with an average initial body weight of 482 ± 10.8 kg and an initial age of 5 ± 0.8 yr were allocated to 1 of 2 FR: ad libitum (AL; $n = 20$) or maintenance level (ML; $n = 20$). Maintenance level was considered to be 1.15% of body weight (dry matter basis) and met 100% of the net energy requirements and AL provided 190% of the total net energy requirements. Cows were slaughtered at 4 DG: 139, 199, 241, and 268 d. After the cows were slaughtered, the placenta and uterus were separated and weighed. Caruncles and cotyledons were individually separated, counted, and weighed. Placenta expressed as kilograms and grams per kilogram of empty body weight (EBW) was heavier in ML- than in AL-fed cows at 268 d of gestation. Placenta expressed as kilograms and grams per kilogram of EBW was the lightest at 139 d of gestation, and the greatest mass was observed at 268 d in ML-fed cows. However, in AL-fed cows, the heaviest placenta expressed as grams per kilogram of EBW was observed from 199 d of gestation. Placentomes expressed as grams per kilogram of EBW were heavier in ML-fed cows during gestation, and the number of placentomes was greater in ML-fed cows at 268 d of gestation. We observed that *IGFR1* and *IGFR2* were involved in placenta adaptations when ML was provided, as their expression in placentome cells was greater in ML-fed cows at 268 d of gestation. The genes responsible for angiogenesis were also greater in ML-fed cows: *VEGFA*, *GUCY1B3*, *HIFA*, *FGF2*, and *NOS3* were altered by FR and DG interaction and they were greater in ML-fed cows at 268 d of gestation. In

addition, *VEGFB* and *ANGPT2* did not show interactions between FR and DG, but they were greater in ML-fed cows. Thus, we suggest that the placenta from an ML-fed cow develops adaptations to the reduced nutrient supply by altering its structure and gene expression, thereby developing mechanisms for potential increased nutrient transfer efficiency to the fetus.

Key words: gravid uterus, insulin-like growth factor receptor (IGFR), *IGFR1*, *IGFR2*, placentome

INTRODUCTION

The majority of mammalian livestock for animal production spend 35 to 40% of their life within the uterus being nourished solely by the placenta (Vonnahme et al., 2013). Thus, it is especially important to understand the influences of maternal nutrition on placental growth, development, and gene expression, because they directly affect fetal growth (Vonnahme et al., 2013). The relationship between maternal nutrient intake during pregnancy and growth of the fetus is extremely important for determining pregnancy success and the life-long health and productivity of an individual (Godfrey and Barker, 2000; Godfrey, 2002). Because profitability in the livestock industry depends upon efficiency of production characteristics such as growth and development after birth, the precursor of efficiency—fetal growth—must be optimal (Redmer et al., 2004; Zhu et al., 2014).

The size and nutrient transfer capacity of the placenta play a central role in determining the prenatal growth trajectory of the fetus, and hence, directly influences birth weight. Transplacental exchange is dependent upon uterine and umbilical blood flow, and these blood flows are in turn largely dependent on adequate vascularization of the placenta (Redmer et al., 2004). Ford (2000) stated that in sheep there are 70 to 120 placentomes, and each one is composed of fetal and maternal components. Cotyledons are tufts of chorionic villi that develop adjacent to the uterine caruncle on the uterine wall, and interdigitate with corresponding

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crypts of the uterine caruncle to form the placentome units (Ford, 2000). However, no study has been conducted to evaluate the effect of day of gestation (**DG**) and feeding regimen (**FR**) on placentome units in Holstein $\times$ Gyr cows.

Several studies (Vonnahme et al., 2003; Laviola et al., 2005; Zhu et al., 2007) have shown that maternal nutrient restriction alters placenta function, and hence, the fetal nutrient supply line, but little is known of the effects of maternal feed restriction on the expression of genes that regulate placenta growth and function in dairy cows. Both insulin-like growth factors have a key role in regulating feto-placental growth throughout gestation (Fowden, 2003). Thus, a difference in their gene expression receptors may significantly alter the regulation of placenta and fetal growth and development. Angiogenesis is the formation of new vascular beds, and it is a critical process for the growth and development of all tissues, including the placenta in ruminants (Reynolds and Redmer, 1992; Borowicz et al., 2007). The search for potential regulators of angiogenesis has led to the identification of the major angiogenic factors (Shalaby et al., 1995; Carmeliet et al., 1996; Ferrara et al., 1996). These major angiogenic growth factors in ruminants include the vascular endothelial growth factor family and its major transmembrane tyrosine kinase receptors (*FLT1* and *KDR*), *FGF2*, angiopoietins, and their tyrosine kinase receptor (*TEK*), *NOS3*, the NO receptor, *GUCY1B3*, and *HIF1A*.

Thus, our hypothesis is that placentas of cows fed a maintenance level (**ML**) diet are more efficient in transferring nutrients to the fetus than cows fed ad libitum (**AL**). This efficiency may be related to greater expression of genes that are involved in nutrient transfer and angiogenesis in the placenta. Our objectives in this study were to evaluate the placenta characteristics and expression of the genes *VEGFA*, *VEGFB*, *ANGPT2*, *FGF2*, *NOS3*, *IGFR1*, *IGFR2*, *GUCY1B3*, and *HIF1A* in placentome cells of dairy cows fed AL or ML at different DG.

MATERIALS AND METHODS

All animal care and handling procedures were approved by the Animal Care and Use Committee of the Department of Animal Science of the Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil, before initiation of the experiment.

Animals and Management

The animals used in this present experiment such as the management adopted is described in a companion paper (Rotta et al., 2015b). However, a brief descrip-

tion is provided as follows: 40 multiparous dry Holstein $\times$ Gyr cows with an average initial BW of 482 ± 10.8 kg and age of 5 ± 0.8 yr were allocated to 1 of 2 FR: AL ($n = 20$) or ML ($n = 20$). The average age of the cows used in this experiment was chosen because it represents the average age of cows raised in Brazil (IBGE, 2009). Maintenance level was considered to be 1.15% of BW (in DM basis). The average milk production of the last lactation was 12.5 ± 1.36 L/d for AL-fed cows and 13.4 ± 1.20 L/d for ML-fed cows. The average number of lactations was 2.9 ± 0.25 and 3.0 ± 0.25 for AL-fed and ML-fed cows, respectively. To evaluate the effects of different DG, pregnant cows were slaughtered at 4 DG. Each FR group was randomly divided into 4 groups, with 5 cows from AL and 5 cows from ML slaughtered at 139, 199, 241, and 268 d of gestation. Cows were fed a corn silage and concentrate-based diet at a ratio of 93:7 (on a DM basis) as a TMR twice daily, with 60% in the morning and 40% in the afternoon.

Slaughter and Laboratory Analyses

Preharvest handling was in accordance with good animal welfare practices, and slaughtering procedures followed the Sanitary and Industrial Inspection Regulation for Animal Origin Products (Brasil, 1997). The slaughters were performed on the same calendar day for each DG.

Before slaughter, feed was withheld overnight, but cows had ad libitum access to water. Cows were slaughtered at Universidade Federal de Viçosa by captive bolt stunning followed by exsanguination. After slaughter, the gravid uterus was removed and weighed; then, the surface area of 3 placentomes was measured and tissue from 1 placentome was sampled. Placentome tissue samples were collected in RNAHolder (BioAgency, São Paulo, SP, Brazil) and stored at -20°C until the RNA extraction procedure.

The placenta and uterus were separated and weighed. Amniotic liquid was also weighed. Caruncles and cotyledons were individually separated, counted, and weighed. The placenta and uterus were individually ground and a homogenized sample was created and maintained at -80°C until further analysis. Placenta and uterus were analyzed for DM and N concentrations (AOAC International, 2000; method 934.01 for DM and 981.10 for N).

Real-Time Transcription PCR Analysis

Total RNA was isolated from 100 mg of placentome tissue using the reagent Trizol (Invitrogen, Carlsbad, CA). The total RNA was resuspended in RNase-free ultrapure water and stored at -70°C until further use.

Concentrations of RNA were checked by NanoVue Plus Spectrophotometer (GE Healthcare, Piscataway, NJ) with an optimal optical density ratio at 260:280 nm between 1.8 and 2.1. Intact 28S and 18S rRNA subunit integrity was assessed by agarose gel electrophoresis to detect degradations of the RNA.

One microgram of total RNA was treated with RNase-free DNAase I (Invitrogen Life Technologies) to remove any contaminating DNA. Reverse transcription was performed using GoScript Reverse Transcription System (Promega, Madison, WI) and oligo (dT) primers (Invitrogen). Then, 80 µL of sterile autoclaved nuclease-free water (Invitrogen, Grand Island, NY) was added to the reverse transcription product. All reverse transcriptions and no-template controls were run at the same time.

Quantitative real-time PCR reactions were performed in triplicate using SYBR Green detection with GoTaq PCR Master Mix (Promega) and gene-specific primers. The gene target and reference sequence was recovered from nucleotide sequences obtained from the GenBank database. The following genes were evaluated: *VEGFA*, *VEGFB*, *FGF2*, *ANGPT2*, *NOS3*, *GUCY1B3*, *HIF1A*, *IGFR1*, and *IGFR2*. These sequences were used to construct primers by the PrimerQuest program provided by Integrated DNA Technologies Inc. (Coralville, IA) and are summarized in Table 1.

Before performing real-time PCR, part of the reverse transcription product was diluted, pooled, and then diluted serially to construct standard curves for optimal PCR conditions and to calculate PCR efficiency. For this purpose, 4 volumes of cDNA (0.5, 1.0, 2.0, and 4.0 nM) and 4 primer dilutions (100, 200, 400, and 800 nM) were tested. The following experimental protocol was used: quantification program consisting of 1 cycle of 95°C for 10 min, 40 cycles of 95°C for 10 s, and 15 s at 60°C. After 40 cycles of amplification, an additional step with a gradual increase in temperature of 60 to 95°C was used to obtain the dissociation curve. The amplification of target and reference genes was performed in different wells and in duplicate (Livak and Schmittgen, 2001). The PCR amplification efficiencies (*E*) were calculated for each reference gene assay using the formula $E = 10^{(-1/\text{slope})} \times 100$ (Pfaffl, 2001) and are presented in Table 2. The dissociation curves showed no peaks corresponding to primer dimers or nonspecific products for any of the target or reference genes, confirming that specific gene amplification was achieved.

After analysis of efficiency, the optimal annealing temperature and primer concentration were used to perform PCR. All reactions were performed on ABI Prism 7300 sequence detection system (Applied Biosystems, Foster City, CA) and cycle threshold values were exported into Excel files (Microsoft Corp., Redmond,

Table 1. Details of target and reference genes

Gene name	Gene ID	Forward sequence	Reverse sequence	Amplicon size, bp
<i>VEGFA</i>	NM_174216.1	ATGACGAAAGTCTGGAGTGTG	TCTCTATGTGCTGGCTTTG	94
<i>VEGFB</i>	NM_174487.2	AGAGTTGGATGAGGAGACCA	AGAGGAGCCAGCTGTTAGA	151
<i>ANGPT2</i>	NM_001098855.1	GCTGTACGACCACTTCTATCTC	GCTGGCTATGCTGCTTATTT	101
<i>FGF2</i>	NM_174056.3	CCTACTCCTAGGCAATATGGTAAAT	CAACCCACCTAGTCAGAGATTG	96
<i>NOS3</i>	NM_181037.3	CTGTCAATCCACTATGGCTCTAC	GTACAGGGAATCCACAGCTTC	109
<i>IGFR1</i>	NM_001077828.1	TCCCACTCCCTGGATTTCCT	GGGTTGGAAGACTGCTGATT	105
<i>IGFR2</i>	NM_174087.3	GGAAGTGCTCCAGCAAGATT	CGTCAATTTGGCTCTGATTTC	98
<i>GUCY1B3</i>	NM_174641.1	GGAAGGGTTGTTGGATGTAGAG	GCTTCGGGCAAGTAGATCAT	105
<i>HIF1A</i>	NM_174339.3	GAGGCTACCATCAGCTATT	GCAATTCATCTGTGCTTCATT	91
<i>SF3A1</i>	NM_001081510.1	ATGCCAACTCGTGGCTTAC	AGAGCAGGCTTCTCCTACTT	100
<i>BACTIN</i>	NM_001033618.1	ACTCCTGCTTGCTGATCCACATCT	AAGATCAAGATCATCGGCCTCCA	109
<i>GAPDH</i>	NM_001034034.1	GGCGTGAAACCAACGAGAAATATAA	CCCTCCACGATGCCAAAGT	99

Table 2. Polymerase chain reaction amplification efficiencies for each reference gene

Gene	Angular coefficient	R ²	Efficiency, %	Concentration, nM
<i>VEGFA</i>	-3.3136	0.9999	100.35	200
<i>VEGFB</i>	-3.2837	0.9987	101.62	200
<i>ANGPT2</i>	-3.4631	0.9891	95.43	800
<i>FGF2</i>	-3.3605	0.9995	98.42	200
<i>NOS3</i>	-3.2890	0.9933	101.39	800
<i>IGFR1</i>	-3.3086	0.9592	100.56	200
<i>IGFR2</i>	-3.2963	0.9995	101.08	400
<i>GUCY1B3</i>	-3.2156	0.9991	104.64	400
<i>HIFA</i>	-3.4744	0.9853	96.01	200
<i>SF3A1</i>	-3.2605	0.9964	102.63	200
<i>ACTB</i>	-3.3877	0.9958	97.33	100
<i>GAPDH</i>	-3.3538	0.9987	98.69	400

WA) for further analysis. The mean of the threshold cycle, defined as the fractional cycle number at which the fluorescence passes the fixed threshold, was determined using manual threshold settings. The level of expression of each target gene was normalized by dividing the relative quantity by averaged quantities of the 3 reference genes (Vandesompele et al., 2003). The normalization factor was calculated as the geometric mean of β -actin (*ACTB*), *GAPDH*, and splicing factor 3a, subunit 1 (*SF3A1*) for each sample.

Data Analysis

Before performing the statistical analysis, we tested the normality of variables and they were normally distributed. The experiment was analyzed as a 4×2 factorial design by using the MIXED procedure (SAS Inst. Inc., Cary, NC). Day of gestation, maternal FR, fetal sex, and their interaction were included as fixed effects in the model. Means were separated using the LSMEANS option of SAS and were considered significant when $P \leq 0.10$. In the absence of maternal FR $\times$ DG interaction, main effects are reported; otherwise, interaction means are discussed.

RESULTS

Utero and Placenta Characteristics

We did not observe a difference ($P = 0.31$) in the mass of gravid uterus between AL- and ML-fed cows (Table 3). However, when expressed as grams per kilogram of empty body weight (EBW), a difference ($P = 0.01$) was observed for the gravid uterus according to the different FR that were studied. Maintenance-fed cows had a heavier ($P = 0.01$) gravid uterus expressed as grams per kilogram of EBW than AL-fed cows. Considering that fetal weight was similar between FR (AL:

17.0 ± 0.75 and ML: 15.9 ± 0.69 kg), the components responsible for this increase were the uterus, placenta, and fetal fluids, which were heavier ($P = 0.04$) in ML-fed cows.

As expected, DG affected uterine mass (Table 3). Uterine mass increased ($P < 0.01$) as gestation progressed from 139 to 268 d of gestation. No difference ($P = 0.34$) was observed in uterine mass between AL- and ML-fed cows (Table 3). Figure 1 illustrates the relationship between the mass of uterus and fetus during gestation. We observed that during gestation, the uterine mass in AL- and ML-fed cows was similar ($P = 0.34$). Thus, no alteration occurred in the uterine mass based on FR evaluated in this study. However, placenta mass was altered ($P < 0.01$) by FR. Figure 2 shows the difference in placenta mass at 268 d of gestation between AL- and ML-fed cows. At 268 d of gestation, placenta mass was greater in ML- than in AL-fed cows; it was 1.63 times greater in ML-fed cows. Thus, we can assume that because fetal mass was not altered by FR, ML-fed cows may have had placental adaptations to be more efficient in transferring nutrients to the fetus.

Another important change caused by FR concerned fetal liquids expressed as grams per kilogram of EBW. Greater ($P = 0.04$) fetal liquid mass was found in ML- than AL-fed cows. Fetal liquid mass was similar ($P = 0.77$) at 139, 199, and 241 d of gestation. However, at d 268, we observed the greatest mass, being 1.88 times greater than the fetal liquid mass from d 241.

The mass of caruncles and cotyledons were similar ($P = 0.20$) according to the evaluated FR (Table 4). However, when expressed as grams per kilogram of EBW, both caruncles and cotyledons were heavier ($P = 0.07$) in ML-fed cows. Because placentomes are the sum of caruncles and cotyledons, the same result was observed for placentomes. Maintenance-fed cows had greater ($P = 0.02$) placentome mass expressed as grams per kilogram of EBW than AL-fed cows.

Figure 1. Relation between mass of uterus and fetus during gestation in Holstein × Gyr cows. Results are means ± SEM. Different lowercase letters are different at $P \leq 0.10$ for ad libitum diets during gestation; different uppercase letters are different at $P \leq 0.10$ for maintenance diets during gestation.

The mass of caruncles, cotyledons, and placentomes were different ($P = 0.07$) according to DG (Table 4). For caruncles and placentomes, the greatest masses occurred at 241 and 268 d of gestation, and the lowest mass was at d 139. However, when considering cotyledons, the heaviest mass was at d 268.

Placentome units presented with an interaction ($P < 0.01$) between FR and DG (Figure 3). At 139 and 199 d of gestation, no difference ($P = 0.69$) was observed between FR and placentome units. However, at 241 and 268 d of gestation, the greatest ($P < 0.01$) values were observed in ML-fed cows. Moreover, placentome units of AL-fed cows differed by DG; at d 139, the lowest value was observed, whereas no difference ($P = 0.66$) occurred at 199, 241, and 268 d of gestation (Figure 3).

Figure 2. Relation between mass of placenta and fetus during gestation in Holstein × Gyr cows. Results are means ± SEM. Different lowercase letters are different at $P \leq 0.10$ for ad libitum diets during gestation; different uppercase letters are different at $P \leq 0.10$ for maintenance diets during gestation. *Difference between ad libitum and maintenance diets at $P \leq 0.10$.

Table 3. Gestational characteristics (means) of Holstein × Gyr cows fed ad libitum or maintenance at different days of gestation

Item	Feeding regimen				Day of gestation						P-value ¹			
	Ad libitum	Maintenance	SEM		139	199	241	268	SEM		FR	DG	FR × DG	Sex
Gravid uterus, kg	35.5	32.9	1.71		10.3 ^d	23.6 ^c	39.6 ^b	63.1 ^a	2.47		0.31	<0.01	0.85	0.66
g/kg of EBW ²	61.2	72.1	3.00		21.4 ^d	46.4 ^c	73.7 ^b	116 ^a	4.35		0.01	<0.01	0.47	0.48
Uterus, g/kg of EBW	11.2	13.4	0.42		5.00 ^d	9.96 ^c	15.2 ^b	17.4 ^a	0.610		<0.01	<0.01	0.34	0.29
Placenta, g/kg of EBW	3.31	4.87	0.214		1.47	3.65	4.30	6.20	0.313		<0.01	<0.01	<0.01	0.87
Ad libitum	—	—	—		1.42 ^{b,A}	3.42 ^{a,A}	4.06 ^{a,A}	4.19 ^{a,B}	0.456		—	—	—	—
Maintenance	—	—	—		1.51 ^{c,A}	4.08 ^{b,A}	4.62 ^{b,A}	8.99 ^{a,A}	0.456		—	—	—	—
P-value	—	—	—		0.68	0.54	0.56	<0.01	—		—	—	—	—
Amniotic liquid, kg	8.16	8.05	0.570		5.43 ^b	6.52 ^b	7.34 ^b	13.1 ^a	0.767		0.89	<0.01	0.70	0.90
g/kg of EBW	14.1	17.7	1.21		11.3 ^b	12.8 ^b	13.7 ^b	24.0 ^a	1.71		0.04	<0.01	0.96	0.81

^{a-d}Means within a row with different superscripts differ ($P \leq 0.10$).

^{A,B}Means within a column with different superscripts differ ($P \leq 0.10$).

¹FR = feeding regimen main effects; DG = day of gestation main effects; FR × DG = interaction between feeding regimen and day of gestation; sex = sex of fetus main effects. No interaction was observed between feeding regimen and sex ($P \geq 0.10$), day of gestation and sex ($P \geq 0.10$), and feeding regimen, day of gestation, and sex ($P \geq 0.10$).

²Empty body weight.

Table 4. Placental characteristics (means) of Holstein × Gyr cows fed ad libitum or maintenance level at different days of gestation

Items	Feeding regimen		SEM	Day of gestation				SEM	P-value ¹			
	Ad libitum	Maintenance		139	199	241	268		FR	DG	FR × DG	Sex
Caruncles, kg	2.12	2.01	0.130	0.49 ^c	1.76 ^b	2.98 ^a	3.02 ^a	0.181	0.57	<0.01	0.46	0.58
g/kg of EBW ²	3.66	4.41	0.284	1.02 ^c	3.46 ^b	5.55 ^a	5.54 ^a	0.410	0.07	<0.01	0.18	0.51
Cotyledons, kg	0.92	1.17	0.115	0.26 ^c	0.98 ^b	1.15 ^b	1.82 ^a	0.170	0.10	<0.01	0.46	0.69
g/kg of EBW	1.59	2.57	0.234	0.54 ^c	2.02 ^b	2.14 ^b	3.54 ^a	0.342	0.01	<0.01	0.14	0.48
Placentome, kg	3.04	3.18	0.107	0.75 ^d	2.74 ^c	4.13 ^b	4.84 ^a	0.195	0.83	<0.01	0.38	0.90
g/kg of EBW	5.24	6.97	0.434	1.56 ^c	5.38 ^b	7.69 ^a	8.88 ^a	0.636	0.02	<0.01	0.11	0.87
Placentome, cm ²	92.7	100	2.20	47.6 ^c	105 ^b	122 ^a	125 ^a	3.20	0.02	<0.01	0.11	0.56

^{a-d}Means within a row with different superscripts differ ($P \leq 0.10$).

¹FR = feeding regimen main effects; DG = day of gestation main effects; FR × DG = interaction between feeding regimen and day of gestation; sex = sex of fetus main effects. No interaction was observed between feeding regimen and sex ($P \geq 0.10$), day of gestation and sex ($P \geq 0.10$), and feeding regimen, day of gestation, and sex ($P \geq 0.10$).

²Empty body weight.

Figure 3. Placentome units (means ± SEM) during gestation in Holstein × Gyr cows. *Difference between ad libitum and maintenance diets at $P \leq 0.10$.

The pattern was different for ML-fed cows in regards to the number of placentomes, because at d 139 the lowest value was observed, and at 199 and 241 d of gestation they were similar ($P = 0.58$), with greater values at 268 d of gestation.

Figure 4 illustrates the percent of CP in the placenta and uterus. No difference ($P = 0.54$) was observed for percent of CP in the uterus based on the different FR and DG. However, we observed a difference ($P < 0.01$) in percent of CP based on placenta. Both AL- and ML-fed cows had greater percent of CP at 199, 241, and 268 d than at 139 d of gestation.

Placentome area was greater ($P = 0.02$) in ML-fed cows (Table 4). The DG influenced ($P < 0.01$) the placentome area. The greatest values were observed at 241 and 268 d of gestation, and the lowest value was at d 139. The placentome area increased 2.6 times between d 139 and 241. This indicates that the placentome is adapted to increase in area, because as the DG increases, the nutrient requirements also increase; thus, a greater amount of nutrients may be transferred to the fetus.

Placental Gene Expression

Gene expression expressed as relative abundance of insulin-like growth factor receptors (*IGFR1* and *IGFR2*) is shown in Figures 5 and 6, respectively. We observed that *IGFR1* and *IGFR2* were constant in AL-fed cows throughout gestation, whereas in ML-fed cows, the greatest ($P < 0.01$) values were observed at 241 and 268 d of gestation for *IGFR1*, and from 199 d for *IGFR2*.

In AL-fed cows, no difference ($P = 0.97$) was found in *VEGFA* abundance throughout gestation. However, in placentomes of ML-fed cows, a difference occurred in its expression; greater ($P < 0.01$) values were observed at d 241 and later than at d 139 and 199 of gestation.

Figure 4. Crude protein concentration (means $\pm$ SEM) in placenta (A) and uterus (B) in Holstein $\times$ Gyr cows at different days of gestation. Different lowercase letters are different at $P \leq 0.10$ for ad libitum diets during gestation; different uppercase letters are different at $P \leq 0.10$ for maintenance diets during gestation. No difference ($P \geq 0.10$) was observed for protein concentration in the uterus.

Moreover, greater values ($P < 0.01$) of *VEGFA* were observed in ML-fed cows than AL-fed cows at 241 and 268 d of gestation (Table 5).

In contrast, *VEGFB* was not influenced ($P = 0.49$) by DG, and similar values were found throughout gestation. However, we observed that FR affected ($P < 0.01$) its expression. The greatest ($P < 0.01$) value was found in ML-fed cows.

As observed for *VEGFB*, no difference ($P = 0.12$) was detected for *ANGPT2* in the studied DG. However, we observed that ML-fed cows had greater ($P = 0.06$) values for *ANGPT2* than AL-fed cows. For *FGF2* and *NOS3*, similar ($P < 0.01$) values were observed during gestation in AL-fed cows, but for ML-fed cows, DG affected their expression, and the lowest value was found at d 139 of gestation. Ad libitum and maintenance-fed cows differed ($P < 0.01$) in *FGF2* and *NOS3* at 199,

241, and 268 d of gestation, with the greatest values observed in ML-fed cows.

Soluble guanylate cyclase (*GUCY1B3*) was constant ($P = 0.65$) throughout gestation in AL-fed cows (Table 5). However, in ML-fed cows, the greatest ($P < 0.01$) values were observed at 241 and 268 d of gestation, but no difference ($P = 0.39$) was observed between 199 and 268 d of gestation. At 241 and 268 d of gestation, ML-fed cows had greater ($P < 0.01$) values for *GUCY1B3* than did AL-fed cows.

Hypoxia inducible factor-1 (*HIF1A*) was similar ($P = 0.40$) in AL-fed cows throughout gestation, but for ML-fed cows, it differed ($P < 0.01$) among DG. At 241 and 268 d of gestation, the greatest ($P < 0.01$) values were observed for *HIF1A*. Moreover, FR differed at 241 and 268 d of gestation, with ML-fed cows presenting the greatest values for *HIFA*.

Figure 5. Relative abundance (means $\pm$ SEM) of insulin-like growth factor receptor 1 (*IGFR1*) during gestation in Holstein $\times$ Gyr cows. *Different at $P \leq 0.10$. Different lowercase letters are different at $P \leq 0.10$ for ad libitum diets during gestation; different uppercase letters are different at $P \leq 0.10$ for maintenance diets during gestation.

Figure 6. Relative abundance (means $\pm$ SEM) of insulin-like growth factor receptor 2 (*IGFR2*) during gestation in Holstein $\times$ Gyr cows. *Different at $P \leq 0.10$. Different lowercase letters are different at $P \leq 0.10$ for ad libitum diets during gestation; different uppercase letters are different at $P \leq 0.10$ for maintenance diets during gestation.

Table 5. Gene expression (relative abundance) of Holstein $\times$ Gyr cows fed ad libitum or maintenance at different days of gestation

Gene	Feeding regimen		SEM	Day of gestation				SEM	<i>P</i> -value ¹			Sex
	Ad libitum	Maintenance		139	199	241	268		FR	DG	FR $\times$ DG	
<i>ANGPT2</i>	0.004	0.006	0.0001	0.003	0.005	0.006	0.004	0.0010	0.06	0.12	0.64	0.87
<i>FGF2</i>	0.010	0.023	0.0015	0.008	0.017	0.023	0.020	0.0010	<0.01	<0.01	<0.01	0.64
Ad libitum	—	—	—	0.009 ^{a,A}	0.011 ^{a,B}	0.012 ^{a,B}	0.010 ^{a,B}	0.0034	—	—	—	—
Maintenance	—	—	—	0.006 ^{b,A}	0.023 ^{a,A}	0.034 ^{a,A}	0.031 ^{a,A}	0.0034	—	—	—	—
<i>P</i> -value	—	—	—	0.44	0.02	<0.01	<0.01	—	—	—	—	—
<i>NOS3</i>	0.004	0.009	0.0006	0.004	0.007	0.008	0.008	0.0009	<0.01	<0.01	0.08	0.36
Ad libitum	—	—	—	0.004 ^{a,A}	0.004 ^{a,B}	0.005 ^{a,B}	0.005 ^{a,B}	0.0012	—	—	—	—
Maintenance	—	—	—	0.004 ^{b,A}	0.010 ^{a,A}	0.011 ^{a,A}	0.011 ^{a,A}	0.0012	—	—	—	—
<i>P</i> -value	—	—	—	0.89	0.06	0.04	0.05	—	—	—	—	—
<i>VEGFA</i>	0.091	0.180	0.0085	0.052	0.098	0.204	0.188	0.0133	<0.01	<0.01	<0.01	0.56
Ad libitum	—	—	—	0.057 ^{a,A}	0.126 ^{a,A}	0.088 ^{a,B}	0.093 ^{a,B}	0.0120	—	—	—	—
Maintenance	—	—	—	0.046 ^{b,A}	0.069 ^{b,A}	0.320 ^{a,A}	0.283 ^{a,A}	0.0162	—	—	—	—
<i>P</i> -value	—	—	—	0.80	0.12	<0.01	<0.01	—	—	—	—	—
<i>VEGFB</i>	0.263	0.404	0.0266	0.292	0.351	0.368	0.321	0.0395	<0.01	0.49	0.33	0.45
<i>GUCY1B3</i>	0.003	0.005	0.0003	0.003	0.004	0.005	0.004	0.0005	<0.01	0.01	<0.01	0.59
Ad libitum	—	—	—	0.003 ^{a,A}	0.005 ^{a,A}	0.002 ^{a,B}	0.003 ^{a,B}	0.0007	—	—	—	—
Maintenance	—	—	—	0.003 ^{c,A}	0.004 ^{bc,A}	0.008 ^{a,A}	0.006 ^{ab,A}	0.0007	—	—	—	—
<i>P</i> -value	—	—	—	0.99	0.98	0.07	0.07	—	—	—	—	—
<i>HIF1A</i>	0.003	0.009	0.001	0.004	0.004	0.009	0.008	0.0014	<0.01	0.03	<0.01	0.85
Ad libitum	—	—	—	0.004 ^{a,A}	0.004 ^{a,A}	0.003 ^{a,B}	0.003 ^{a,B}	0.0019	—	—	—	—
Maintenance	—	—	—	0.003 ^{b,A}	0.004 ^{b,A}	0.015 ^{a,A}	0.012 ^{a,A}	0.0019	—	—	—	—
<i>P</i> -value	—	—	—	0.89	0.92	0.02	0.02	—	—	—	—	—

^{a-c}Means within a row with different superscripts differ ($P \leq 0.10$).^{A,B}Means within a column with different superscripts differ ($P \leq 0.10$).¹FR = feeding regimen main effects; DG = day of gestation main effects; FR $\times$ DG = interaction between feeding regimen and day of gestation; sex = sex of fetus main effects. No interaction was observed between feeding regimen and sex ($P \geq 0.10$), day of gestation and sex ($P \geq 0.10$), and feeding regimen, day of gestation, and sex ($P \geq 0.10$).

DISCUSSION

Utero and Placental Characteristics

Wallace et al. (2001) evaluated adolescent ewes that were fed moderate or high amount of feed and observed that placenta mass was heavier in those that were fed moderate amounts. Although the mass of placenta and fetus were positively correlated in both groups, the placenta was more affected than the fetus in the overnourished group, thereby leading to a significant difference in the fetal:placenta mass ratio. Although fetal organogenesis is largely complete during early gestation, exponential growth of the fetus is limited to the last third of pregnancy (Redmer et al., 2004), beginning around d 200 and ending around d 280 in Holstein $\times$ Gyr dairy cows (Rotta et al., 2015a). Thus, approximately 90% of fetal growth occurs during the last third of pregnancy. However, in this study, we observed that in AL-fed cows, the majority of placenta growth occurred at 199 d of gestation, at which point the placenta achieved its maximum weight, which is based on the fact that the mass of placenta at d 241 and 268 was similar to that at d 199 of gestation. Thus, the fetus has obtained about 10% of its eventual birth weight by the time the placenta has reached its maximal size (Redmer et al., 2004). However, when considering ML-fed cows, the growth pattern of placenta was different because it continued to grow until d 268. Thus, feeding cows the ML diet resulted in a different growth pattern of placenta, which coincides with the greater nutrient requirements of the gravid uterus (Ferrel et al., 1976).

The number of placentomes varies widely between individual cows. Mossman (1987) reported that the number of placentomes varied from around 50 to 175 per uterus, whereas Laven and Peters (2001) reported values ranging from 40 to 120 placentomes. The latter authors reported that the mean number of placentomes ranged from just over 50 per uterus in pregnancies of less than 71 d to more than 70 in pregnancies of more than 191 d of gestation. Data from other ruminants are inconclusive as to whether this is a real effect or not. Abdel Raouf and Badawi (1966), reported that in water buffalo, mean placentome number increased from early to late gestation but in sheep and yak, day of gestation appeared to have no significant effect on placentome number (Redmer et al., 2009; Liu et al., 2010). This is the first study evaluating the effect of DG and FR in Holstein $\times$ Gyr cows, and our results suggest that the Gyr breed may increase the number of placentomes. Previous studies with Holstein cattle noted that the placentome number is about 100 units. However, our study found values about 2- to 3-fold greater for placentome units in Holstein $\times$ Gyr. Even though we did

not test the influence of breed on placentome number, our results give us reason to suggest that use of the Gyr breed in crossbreeding with Holstein animals may explain the increased placentome number.

The heavier placentome expressed as grams per kilogram of EBW in ML-fed cows may have resulted in a greater uterine blood flow and umbilical blood flow to the fetus. Absolute blood flow to the myometrium increases in proportion to placentome mass, whereas relative uterine blood flow may fluctuate and decrease somewhat (Rosenfeld et al., 1974; Bjellin et al., 1975) or remain constant (Ford et al., 1984; Dowell and Kauer, 1997) during pregnancy. Wallace et al. (2002, 2003) observed that a heavier placentome is responsible for an increase in both uterine and umbilical blood flow in sheep and adult ewes. Thus, the greater uterine and umbilical blood flow results in greater nutrient transfer to the fetus, thereby avoiding fetal injury in ML-fed cows. Those authors also reported that animals that are fed a moderate amount of feed presented with heavier placentome than those fed a high amount of feed. Thus, the results obtained in the present experiment agree with those reported by Wallace et al. (2002, 2003), because we can associate results based on a moderate amount of feed with data from ML-fed cows (approximately 0.30 kg/d ADG), and results from cows fed high amounts would associate with those from AL-fed cows (approximately 1.10 kg/d ADG).

For ML-fed cows, a different placentome adaptation occurred. This may be related to the greater nutritional transfer capacity of these cows to avoid fetal growth retardation. The greater placentome units found at 268 d of gestation in ML-fed cows may be explained by the higher nutrient requirements for gestation during this period (NRC, 2001). Maternal overfeeding is associated with altered fetal development and long-term adverse consequences on the progeny (George et al., 2010; Reynolds and Caton, 2012).

Placental Gene Expression

The increase in the gene expression of *IGFR1* and *IGFR2* occurred when the nutritional requirements were greater (NRC, 2001). We suggest that this is evidence that *IGFR1* and *IGFR2* are sensitive in placentome alterations in the maternal metabolic environment and hypothesize that an alternative endocrine factor may regulate the growth of fetuses from feed-restricted cows. To provide the fetus and newborn with sufficient energy and nutrients, the pregnant dam undergoes several physiological, metabolic, and endocrine adaptations (Prentice et al., 1995; Olausson and Sohlstrom, 2003). Changes in the insulin-like growth factor receptor (IGFR) system are one type of adaptation. Several

studies have demonstrated the importance of *IGFR1* and *IGFR2* for gestation (DeChiara, et al., 1990; Constância et al., 2002; Fowden, 2003), but none of these studies examined effects in dairy cattle.

Vascular endothelial growth factor A acts locally in the bovine placenta to modulate steroidogenesis during gestation and can be considered an important regulator of placenta development and function (Sousa et al., 2012). Moreover, it is responsible for regulating cell growth and differentiation, as well as the control of angiogenesis and neovascularization in organs and tissues (Pfarrer et al., 2006). Thus, the greater its concentration in an animal, the greater the potential for vascularization; therefore, we can assume that more nutrients are transferred from the cow to the fetus.

The greatest values of *VEGFA* at 241 d of gestation observed in this study may be explained, in part, by the greater nutrient requirements during the final phase of gestation, and because ML-fed cows had a lower DMI (Rotta et al., 2015b) than AL-fed cows. Thus, ML-fed cows may have improved their vascular efficiency in transferring more nutrients to the fetus. When cells are deprived of oxygen, they increase their production of *VEGF-A5*; *VEGF-A* mediates the growth of new blood vessels from pre-existing vessels by binding to the cell surface receptors *VEGFR1* and *VEGFR2*, 2 tyrosine kinases located in endothelial cells of the cardiovascular system. These 2 receptors act through different pathways to contribute to endothelial cell proliferation and migration, and formation of tubular structures (Huusko et al., 2010).

Vascular endothelial growth factor B is related to embryonic angiogenesis (Claesson-Welsh, 2008) and endothelial cell growth, particularly in muscle (Olofsson et al., 1996). Thus, the greater values of *VEGFB* expression in ML-fed cows suggest greater angiogenesis and cell growth in the fetus. In contrast to *VEGFA*, *VEGFB* plays a less pronounced role in the vascular system: whereas *VEGFA* is important for the formation of blood vessels, such as during development or in pathological conditions, *VEGFB* seems to play a role only in the maintenance of newly formed blood vessels during pathological conditions (Zhang et al., 2009).

Expression of *ANGPT2* is associated with angiogenesis and vessel maturation (Li et al., 2013). Nitric oxide has also been implicated as a mediator of *VEGF*- (Ziche et al., 1997; Parenti et al., 1998) and *FGF2*- (Babaei et al., 1998) induced angiogenesis; *VEGF* is a major regulator of placental angiogenesis and may play a significant role in the upregulation of growth signaling pathways in cotyledons under maternal nutrient restriction (Reynolds et al., 2005). Other growth factors may also be responsible for the upregulation of these growth signaling pathways.

The protein encoded by *HIF1* is a bHLH-PAS transcription factor found in mammalian cells growing at low oxygen concentrations. It plays an essential role in cellular and systemic responses to hypoxia (Ratcliffe, 2002). Thus, the greater expression of *HIF1* observed in ML-fed animals during the final gestation period may suggest that a decrease in oxygen concentration is necessary to increase the expression of *HIF1*.

CONCLUSIONS

Feeding regimen alters growth of placenta, cotyledon, caruncle, and placentome in Holstein × Gyr cows. Greater expression of *IGFR1* and *IGFR2* was observed in ML-fed cows, and the reduced nutrient intake of those cows did not affect the fetal weight, probably because genes related to the angiogenic process were more expressed in ML-fed cows than AL-fed cows, suggesting that ML-fed cows had more placenta vascularity. This finding suggests that ML-fed cows have physiological ways to compensate low levels of nutrients that otherwise could impair the development of their fetuses.

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