DIFFERENTIAL AVERAGE DAILY GAIN OF PREGNANT HOLSTEIN × GYR DAIRY HEIFERS CAUSES PLACENTAL ADAPTATIONS TO SUPPORT FETAL GROWTH AND DEVELOPMENT

Kellen R. Oliveira,1 Antônio P. O. Neto,1 Caio A. Diamantino,2 Isabela O. Eiterer,1 Renato D. Araújo,1 Yamê F. R. Sancler-Silva,1 Alex L. Silva,1 Marcio S. Duarte,3 and Poliana P. Rotta1*

1 Department of Animal Science, Universidade Federal de Viçosa, Viçosa, 36570-900, Brazil.
2 Department of Veterinary Medicine, Universidade Federal de Viçosa, Viçosa, 36571-000, Brazil.
3 Department of Animal Biosciences, University of Guelph, Guelph, N1G2W1, Canada.

ABSTRACT

This study aimed to evaluate the effects of differential average daily gain targets of dairy heifers throughout gestation on placental hemodynamics, uterine involution, colostrum production of the heifers, and impacts on newborn calf weight and immunity transfer. Fourteen Holstein × Gyr heifers with an average body weight of 446 ± 46.7 kg and age of 25 ± 3.9 mo were randomly assigned to the following treatments: moderate body weight gain (MOD, n = 7), where heifers were fed to achieve 0.50 kg/d; and high body weight gain (HIG, n = 7), where heifers were fed to achieve 0.75 kg/d. Target average daily gains were established based on common tropical dairy production systems. The heifers received a total mixed ration feed twice daily starting at 70 d of gestation. Placentome vascularization was assessed using a color Doppler ultrasound at 180, 210, and 240 d of gestation. After calving, cotyledons were counted and sampled to analyze the mRNA expression of placental angiogenesis markers. After birth, calves were weighed and fed colostrum, and transfer of passive immunity efficiency was assessed. A significant increase in cotyledons was detected for MOD placenta soon after expulsion (81.5 ± 12.91 vs. 63.6 ± 10.52). Placentome vascularization at the final third of gestation increased for MOD heifers compared with HIG. Greater mRNA expression after membrane expulsion of VEGFB and IGFR1 in cotyledons and a greater estradiol concentration in circulation 1 d before calving was found for MOD heifers compared with HIG heifers, however, uterine involution postpartum was not different between treatment groups. Greater colostrum production was observed in HIG heifers (3.9 ± 1.05 vs. 2.2 ± 1.57 L) but with lower quality (25.2 ± 0.51 vs. 29.5 ± 0.65 Brix). No differences were observed in birth weight or transfer of passive immunity efficiency between treatments; however, HIG calves had significantly greater vitality scores than MOD calves. The results of this study indicate that a moderate feeding regimen enhances placental blood flow by increasing angiogenesis, which suggests improved nutrient transfer to the fetus without major impacts on its development during the neonatal stage, colostrum production, or uterine involution in the heifers. Key words: angiogenesis, Doppler ultrasound, fetal programming, gene expression

INTRODUCTION

Nutrient supply in the bovine placenta occurs through the placentome, which is the union between the fetal cotyledon and maternal caruncle. The transport of nutrients between the cow and fetus depends on uterine and umbilical blood flow and fetal demands (Vonnahme and Lemley, 2012). Organogenesis occurs throughout embryonic development but only in the last trimester; when more than 60 percent of fetal growth occurs, is when fetal requirements increase. Recently, Sguizzato et al. (2020) demonstrated that a significant increase in fetal requirements for Holstein × Gyr cows begins at 70 d of gestation.

Maternal circumstances during conception and gestation are determinants of the neonatal phenotype (Laporta et al., 2020), originally named the “Barker hypothesis” (Barker, 1992). Challenging conditions to the mother (i.e., nutrient restriction or heat stress) during gestation may cause detrimental effects on her offspring (Recce et al., 2021), with effects on villus formation in the small intestine (Duarte et al., 2013; Gionbelli et al., 2017), the number of follicles in females (Mossa et al., 2013; Weller et al., 2016), reduction in fertility and milk yield (MY) during the first lactation (Monteiro et al., 2016). In addition, when the fetus is subjected to nutrient restriction, compensation in placental blood vascularization occurs as an adaptation to nutrient...
scarcity to meet fetal demands (Zhu et al., 2007; Rotta et al., 2015).

Because nutrient delivery depends upon its availability, placental blood flow, and transport capacity (Edwards et al., 2020), these factors are determinants of the development of the fetus and may affect calf birth weight (Vonahme et al., 2007). However, if an insult occurs in early gestation, placental development may be impacted and may lead to altered fetal growth during late gestation (Camacho et al., 2018). The feeding regimen of the cow also influences placental and fetal development. Dairy production systems in tropical areas commonly raise heifers with moderate ADG targets, which may impair their ability to meet gestational nutritional requirements. According to NASEM (2021), primiparous cows must be at 91% of their mature BW immediately before the first calving and 82% postpartum to prevent compromise the future lactation and fetal growth. However, despite the current knowledge about the effects of maternal nutrition on fetal development, studies evaluating dietary regimens pertaining to ADG for dairy heifers that impact placental growth and metabolism are scarce.

It was hypothesized that heifers fed for moderate body weight gain (MOD) beginning at 70 d of the gestational period compared with high body weight gain (HIG) would have greater placental efficiency and blood supply without compromising the neonatal growth. Further, MOD cows will have no loss to uterine gain (HIG) would have greater placental efficiency and blood supply without compromising the neonatal growth. Further, MOD cows will have no loss to uterine gain than HIG cows. Based on this hypothesis, this study aimed to evaluate differential ADG regimens during pregnancy in Holstein × Gyr dairy heifers. Therefore, the objectives of this study were to evaluate the effects of maternal nutrition on fetal development, studies evaluating dietary regimens pertaining to ADG for dairy heifers that impact placental growth and metabolism are scarce.

**MATERIALS AND METHODS**

The experiment was conducted at the Dairy Research Facility of the Department of Animal Science of the Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil. All procedures were previously approved by the Animal Use Ethics Committee of the Department of Animal Science of the Universidade Federal de Viçosa, Minas Gerais, Brazil (protocol 015/2022).

**Animals and Management**

Fourteen crossbred 5/8 Holstein × Gyr pregnant heifers (BW 445.9 ± 46.7 kg) at 25 ± 3.9 mo of age were used in this study. The heifers were pregnant with embryos 3/4 Holstein × Gyr from the same farm. At 70 d of gestation, heifers were randomly assigned to one of 2 experimental treatments: MOD (0.50 kg/d ADG; n = 7) or HIG (0.75 kg/d ADG; n = 7), based on the ADG described by Azevedo et al. (2022) to tropical crossbreeds. Heifers were fed corn silage and a concentrate-based diet twice daily (0700 and 1600h; Table 1). To achieve moderate and high gain the heifers were fed 1.08% and 1.41% of their BW on a DM basis, respectively. Every 28 d, heifers were weighed before morning feeding, and their ration allotment was adjusted for the BW gain expected for each treatment. All heifers were individually housed in a tie stall system for 250 d and then moved to a Compost Barn system, where they remained until calving. After birth, calves were weighed using a mechanical scale and evaluated for vitality score based on a scale of 1–27, according to Murray-Kerr et al. (2018).

### Evaluation of Placentome Vascularization

Placentome vascularization was evaluated at 180, 210, and 240 d of gestation using an Ultrasound Z5VET® (Mindray Medical International Technology, Shenzhen, Guangdong, China) equipped with B and color Doppler modes linear probe (6–8 MHz). Images were acquired at a frequency of 7.5 MHz with 94% gain (grayscale) and 5.7 MHz with 60% gain (color mode) and a pulse repetition frequency of 1.7 kHz. In addition, 2 videos of each of the 5 evaluated placentomes were recorded for further laboratory analyses.

Images with greater vascularity were extracted from each video and recorded for further laboratory analyses. These images were processed and analyzed by Adobe Premiere Pro CC 2019 (Adobe Systems, San Jose, CA) to obtain the mean of the total placentome area (gray-scale pixels plus color pixels) and the vascularized area (color pixels). With these 2 values, the mean percentage of vascularity in the total area of each placentome scanned was calculated (vascularized area/total area of the placentome).

Additionally, the gravid uterus weight was estimated using calf body weight at birth according to NASEM (2021) at 180, 210, and 240 d of gestation, based on the following equations:

\[
Gr\text{U}t\text{er Wt}_\text{parturition} = \text{Calf birth weight} \times 1.825,
\]

\[
Gr\text{U}t\text{er wt} = (Gr\text{U}t\text{er Wt}_\text{parturition})^{(0.0243 \times (0.0000245 \times DayGest) + (280 – DayGest))},
\]

where DayGest = day of gestation, GrUterWt (parturition) = gravid uterine weight at parturition, and GrUter_wt = estimated gravid uterine weight on a...
specific day of gestation. This estimate generated 3 new parameters: the total placentome area/uterus weight, the vascularized area/uterus weight, and the percentage of vascularity/uterus weight.

**Placental Evaluation and Sample Collection**

The elapsed time in minutes from placenta rupture to calf birth and the gestation length in days were recorded for each cow/calf pair. After calving, the cows were monitored, and the time for placenta expulsion was also recorded. Retention of fetal membranes was considered to have occurred when they were not expelled within 12 h after parturition (Grunert et al., 1989; Takagi et al., 2002; Magata et al., 2021). Once expelled, the placenta was weighed, and the number of cotyledons was counted. The closest and largest cotyledons to the umbilical cord were measured, and the 2 largest cotyledons were sampled. The samples were snap-frozen in liquid nitrogen and stored at −80°C until RNA was extracted. Additionally, the placental and calf weights were measured to calculate the placenta:calf ratio, aiming to evaluate the placental efficiency as described by Camacho et al. (2018).

**Total RNA Extraction and Gene Expression Analysis**

Total RNA was isolated using the PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA). After extraction, the concentration of total RNA was determined using a NanoDrop Lite UV–Vis Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE), and RNA integrity was assessed by 1% agarose gel electrophoresis. Next, complementary DNA (cDNA) synthesis was performed using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies Corporation, Carlsbad, CA) according to the manufacturer’s protocol. Quantitative RT-PCR was performed using SYBR-Green I detection with GoTaq qPCR Mix (Promega, Madison, WI) and gene-specific primers (doi:10.17632/c76pt4z68.2). Primer designs were obtained from Rotta et al. (2015) and GenBank for the following targets: 18S ribosomal RNA, vascular endothelial growth factor A (VEGFA), vascular endothelial growth factor B (VEGFB), insulin-like growth factor 1 receptor (IGF1R), insulin-like growth factor 2 receptor (IGF2R), guanulate cyclase 1 soluble subunit β (GUCY1B3), hypoxia-inducible factor 1-α (HIF1A), splicing factor 3a subunit 1 (SF3A1), fibroblast growth factor 2 (FGF2), angiopoietin 2 (ANGPT2), and nitric oxide synthase (NOS3).

Real-time qPCR was performed using QuantStudio 3 (Applied Biosystems, Foster City, CA) following the cycle parameters: 2 min at 95°C, 40 cycles of 15 s at 95°C, and 40 cycles of 1 min at 60°C. The expression of each target gene was normalized using the endogenous gene 18S ribosomal RNA for each sample. Calculation of gene expression was conducted using ∆CT according to Livak and Schmittgen (2001) for statistical analysis. The ∆CT values were then used to calculate the ∆∆CT.

**Calf Evaluation**

After calving, the cows were milked, colostrum production was measured, and colostrum quality was evaluated using a Brix refractometer (Silva-Del-Rio et al., 2017). The colostrum was provided to calves at 15% of birth weight within the first 2 h of life, with Brix% greater than 25. At 24 h after colostrum feeding, blood samples were collected via jugular venipuncture with vacutainers and red-top serum separator tubes. Blood samples were then centrifuged, and the separated serum was used to estimate serum IgG. The concentration of IgG was used as a marker of passive immunity transfer efficiency, as Lombard et al. (2020) described, where an excellent transfer is noted when serum IgG ≥25 g/L or equivalent Brix % was ≥9.4.

**Cows Postpartum Evaluation**

After calving, uterine evaluation was performed in all heifers. First, the uterus was assessed by transrectal ultrasound using a DP-20 Vet Power with a linear transrectal transducer (Mindray Animal Medical Technology, Shenzhen, Guangdong, China) to diagnose any cases of uterine infection and measure uterine wall thickness at the greater curvature of the pregnant horn. Uterine thickness was measured every 2 d during the first 20 d after calving, followed by another evaluation at 30 d and 60 d post-calving. At 20 d postpartum,
the cows were submitted to a protocol to synchronize estrus and inseminated at 30 d postpartum by a fixed-time artificial insemination protocol developed by Consentini et al. (2021). At 0 d, all cows received 100 µg of lucirelin (Tec-Relin, União Química, São Paulo, São Paulo, Brazil) and a 2.0 g vaginal progesterone implant (Repro Sync, Globalgen, Jabcoticabal, São Paulo, Brazil) was used for 8 d; at 7 and 8 d, 0.53 mg of cloprostenol sodium (PGF2α, Estron, União Química, Jabcoticabal, São Paulo, Brazil) was administered; at 8 d vaginal progesterone was removed, and was administered 1 mg of estradiol cypionate (Cipiotec, União Química, São Paulo, São Paulo, Brazil); after 48 h the cows were artificially inseminated. After 32 ± 4.0 d of insemination, the cows were evaluated, and estrus was synchronized again if the cow was not pregnant.

The same evaluator assessed the BCS at calving and 30 DIM. Cows were milked 3 times daily, and MY was recorded weekly using the DemaTron 70 control (GEA Group Aktiengesellschaft, Düsseldorf, Germany) for the first 6 weeks of lactation.

**Blood Sample Collection**

Blood samples were collected from the coccygeal vein or artery at −1, 2, 8 and 30 d in relation to calving. When heifers reached 272 ± 2.18 d of gestation, one day before parturition, a blood sample was collected at 1800 h into a serum separator tube and then centrifuged; serum was separated and stored at −20°C until calving. Samples collected at −1 d relative to calving were used to analyze estradiol and progesterone levels using the chemiluminescence kits Access Estradiol (B84493) and Access Progesterone (33550) (Beckman Coulter, Clare, Ireland), respectively. The sample chosen for analysis was determined due to previous literature of the peak in these levels (Ryan, 2002) and the importance of this timing regarding parturition evolution (i.e., time to delivery, placenta expulsion).

Before feeding, blood samples were collected in a vacuum tube with sodium fluoride at 2, 8, and 30 d after parturition for glucose measurement to observe the energy homeostasis recovery, and samples were collected in a vacuum tube with lithium heparin at 2 and 8 d after parturition to analyze serum calcium homeostasis after parturition at the onset of lactation (Hernández-Castellano et al., 2019). Glucose Monoreagent kit and Calcium Arsenazo III kit (Quibasa-Bioclin, Belo Horizonte, Minas Gerais, Brazil) were used for glucose and calcium analyses, respectively, and assays were read on a biochemical analyzer BS-200 (Mindray Headquarters, Shenzhen, Guangdong, China). At 8 d, a blood sample was collected in a vacuum tube with EDTA for hemogram and leukogram tests. The blood sample was immediately centrifuged and analyzed in a Hematoclin 2.8 VET (Quibasa-Bioclin, Belo Horizonte, Minas Gerais, Brazil).

**Statistical Analyses**

Data were initially analyzed for residual distribution; the variables of placentome morphometry and vascularization, and postpartum uterine thickness did not follow a Gaussian distribution. Therefore, the most adequate distribution was verified using the package riskDistributions of R (R Core Team, 2022), and a gamma distribution was chosen for all variables. Therefore, these models were adjusted using the function glmer of the package lme4 of R, using the family Gamma, link log function, and the random effect of measurement day within the animal.

The experiment was designed in a randomized block design where the animals were blocked by calving month. Only one male calf was born in this study in each treatment; although placental data was kept and used in the analysis. One cow in the MOD group experienced retention of fetal membranes; therefore, placental weight was not obtained. ANOVA was performed using the function lmer of the package lme4 of R, following the model:

\[ Y_{ij} = \mu + T_i + B_j + \varepsilon_{ij}, \]

where \( Y_{ij} \) = dependent variable; \( \mu \) = overall mean; \( T_i \) = fixed effect of treatment; \( B_j \) = random effect of blocking, and \( \varepsilon_{ij} \) = random error.

The variables measured over time (calcium, glucose, BCS, and MY) were submitted for ANOVA, including the effect of time as a repeated measure in the model above. The following covariance matrices were tested: compound symmetry, heterogeneous compound symmetry, autoregressive-order 1, heterogeneous autoregressive-order 1, and variance components. The best covariance matrix was chosen based on the lower AIC, compound symmetry for glucose and BCS and heterogeneous compound symmetry for calcium.

Outlier detection was considered when the internal student residual was greater than |2.5|. When necessary, means were tested using the Tukey test at a significance level of 0.05, and tendency was declared at 0.05 < \( P \) ≤ 0.10.

**RESULTS**

A similar BW at 70 d of gestation was observed between treatments (\( P = 0.82 \)). However, heifers in the HIG treatment had greater ADG (0.74 ± 0.086 vs. 0.49
± 0.090; *P* < 0.01), allowing them to have a greater BW at 270 d of gestation (582 ± 49.3 vs. 534 ± 49.6; *P* = 0.01; Table 2).

**Placental Morphometry and Vascularization**

The uterine size increased over the days of gestation (*P* < 0.01) but did not differ between treatments (*P* = 0.91; Figure 1). An interaction between days of gestation and ADG was observed for the placentome area (*P* = 0.02) and the placentome area relative to the gravid uterus from 180 to 240 d of gestation (*P* = 0.03). The placentome area for both treatments increased from 210 to 240 d of gestation (*P* = 0.02).

The vascularized area of the placentome showed an interaction (*P* < 0.01; Figure 2) between days of gestation and ADG. Indeed, the ratio of the vascularized area represented in pixels to the gravid uterus weight differed by days of gestation and ADG from 180 to 240 d (*P* = 0.03). Similarly, there was an interaction (*P* < 0.01) between days of gestation and ADG on the vascularization percentage and for the ratio of the vascularization percentage to the gravid uterus weight (*P* < 0.01). The percentage of placentome vascularization of MOD heifers increased from 180 to 240 d and was similar to HIG heifers at 240 d of gestation. The placentome area relative to the gravid uterus and vascularized area of the placentome relative to the gravid uterus decreased from 180 to 240 d because the increase in uterus weight was more significant than the increase in the placentome, vascularized area, and vascularization percentage.

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**Placental mRNA Expression**

The mRNA expression of endothelial growth factor A (VEGFA) did not differ. (*P* = 0.75; Figure 3) between treatments. However, the mRNA expression of endothelial growth factor B (VEGFB) was greater (*P* = 0.04) in the placenta of MOD heifers than in HIG heifers. Greater (*P* = 0.05) mRNA expression of insulin-like growth factor receptor 1 (IGFR1) in MOD heifers than in HIG heifers was observed, while the expression of insulin-like growth factor receptor 2 (IGFR2) did not differ (*P* = 0.38) between treatments.

The mRNA expression levels of soluble guanylate cyclase (GU-CY1B3), hypoxia-inducible factor 1 (HIFA1A), splicing factor 3A subunit 1 (SF3A1), fibroblast growth factor 2 (FGF2), angiopoietin 2 (ANGPT2), and nitric oxide synthase (NOS3) did not differ between treatments (*P* > 0.10).

**Calf and Parturition Evaluation**

Heifer estrogen concentration was greater (*P* = 0.02; Table 2) in HIG than in MOD, while the progesterone concentration did not differ (*P* = 0.71) between treatments before calving. The ADG was significantly different between the 2 treatments, as was intended for the study (*P* < 0.01). No differences between treatments were observed for

### Table 2. Means and standard errors of the means of heifer initial and final body weight during gestational time, hormones related to calving, elapsed time of calving, placenta expulsion, weight, number of cotyledons, calf weight at birth and vitality, and colostrum parameters

<table>
<thead>
<tr>
<th>Item</th>
<th>MOD</th>
<th>HIG</th>
<th>P-value³</th>
</tr>
</thead>
<tbody>
<tr>
<td>N of animals per group</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Heifer body weight on 70 d of gestation, kg</td>
<td>446 ± 37.7</td>
<td>449 ± 37.4</td>
<td>0.82</td>
</tr>
<tr>
<td>Heifer body weight on 270 d of gestation, kg</td>
<td>534 ± 49.6</td>
<td>582 ± 49.3</td>
<td>0.01</td>
</tr>
<tr>
<td>Average daily gain, kg</td>
<td>0.49 ± 0.086</td>
<td>0.74 ± 0.086</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Parameters related to calving</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estrogen, pg/mL</td>
<td>155 ± 25.8</td>
<td>238 ± 21.4</td>
<td>0.02</td>
</tr>
<tr>
<td>Progesterone, ng/mL</td>
<td>0.62 ± 0.089</td>
<td>0.57 ± 0.092</td>
<td>0.71</td>
</tr>
<tr>
<td>Gestation length, d</td>
<td>276 ± 2.18</td>
<td>277 ± 2.18</td>
<td>0.86</td>
</tr>
<tr>
<td>Elapsed time of calving, min</td>
<td>65.2 ± 20.10</td>
<td>58.7 ± 15.00</td>
<td>0.70</td>
</tr>
<tr>
<td>Placenta expulsion time, min</td>
<td>400 ± 55.0</td>
<td>345 ± 47.6</td>
<td>0.34</td>
</tr>
<tr>
<td>Placenta weight, kg</td>
<td>5.24 ± 0.806</td>
<td>5.90 ± 0.567</td>
<td>0.45</td>
</tr>
<tr>
<td>Calf weight at birth, kg</td>
<td>39.7 ± 2.04</td>
<td>37.2 ± 1.80</td>
<td>0.33</td>
</tr>
<tr>
<td>Placenta weight: Calf weight, %</td>
<td>12.9 ± 1.70</td>
<td>15.8 ± 1.33</td>
<td>0.13</td>
</tr>
<tr>
<td>Total number of cotyledons</td>
<td>81.5 ± 12.9</td>
<td>63.6 ± 10.5</td>
<td>0.02</td>
</tr>
<tr>
<td>Colostrum parameters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colostrum production, kg</td>
<td>2.18 ± 1.57</td>
<td>3.94 ± 1.05</td>
<td>0.09</td>
</tr>
<tr>
<td>Colostrum quality, Brix %</td>
<td>29.5 ± 0.65</td>
<td>25.2 ± 0.51</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Calf serum, Brix %</td>
<td>10.5 ± 1.15</td>
<td>10.6 ± 0.93</td>
<td>0.90</td>
</tr>
<tr>
<td>Vitality score, points³</td>
<td>23.2 ± 2.08</td>
<td>24.8 ± 1.88</td>
<td>0.09</td>
</tr>
</tbody>
</table>

¹MOD = moderate body weight gain (0.50 kg/d); HIG = high body weight gain (0.75 kg/d).
²Significant was declared at *P* ≤ 0.05, and trends when 0.10 ≤ *P* > 0.05.
³Vitality score on a scale from 1 to 27.
gestation length, calving time, placenta expulsion time, or placenta weight (P ≥ 0.34; Table 2). However, MOD heifers had a greater (P = 0.02) number of cotyledons in the placenta than HIG heifers. A tendency of greater volume of colostrum was produced by HIG heifers (P = 0.09) but this colostrum was of lower Brix % compared with MOD heifers (P < 0.01). Calf birth weight and efficiency in passive immunity transfer were similar (P > 0.10) in both treatments; however, calves born to HIG heifers tended to have a better (P = 0.09) vitality score.

**Cows Postpartum Evaluation** Hemogram and leukogram tests were conducted to evaluate immune status and recovery of the cows and found no differences (P > 0.10; Table 3) between treatments for all parameters evaluated.

An interaction was observed between days of gestation and ADG on serum calcium (P = 0.04; Figure 4). Moderate body weight gain and HIG cows had similar (P > 0.10) serum calcium levels at 2 d, but in MOD cows, its concentration increased at 8 d (Figure 4). Moderate body weight and HIG cows also had similar (P > 0.10) concentrations of glucose at 2 d; however, MOD cows tended to present greater values at 8 d (P = 0.08), and there was no difference (P > 0.10) at 30 d.

The BCS decreased (P < 0.01) from parturition until 30 d of DIM but did not differ (P = 0.80) between treatments. The thickness of the pregnant horn in the postpartum uterus decreased (P < 0.01; Figure 5) from parturition to 60 d due to postpartum uterine involution. However, in both treatments, the uterus was equally (P = 0.83) thick. No animal expressed heat before the timed artificial insemination.

Milk yield increased (P < 0.01; Figure 6) from the first to the sixth week of lactation, but it was similar (P = 0.31) for both treatments, and we did not find an interaction (P = 0.20) between feeding regimen and DIM.

**DISCUSSION**

The effectiveness of experimental diets was observed, in which heifers fed for high ADG during pregnancy had greater BW at the time of calving.

The area of the placentomes and percentage of vascularization of the gravid uterus was similar in MOD heifers and HIG heifers at the end of gestation, which likely indicates an increase in the active surface for nutrient transport overtime for MOD. This is reflected in the increase at placentome vascularity at 210 d of gestation when experiencing similar vascularization of
HIG heifers. However, the ratio of placentomes and vascularization to the uterine weight was similar for both treatments evaluated. Moreover, MOD heifers had more cotyledons, demonstrating a greater area for nutrient transport. Collectively, our findings suggest that a compensation mechanism for lower weight gain is an increased number of cotyledons and an increased active surface for nutrient transport to the fetus, which may reflect in an increase in proliferative cells of the cotyledon (Vonnahme et al., 2007; Camacho et al., 2018). Additionally, previous studies have found an increased vascular density at the cotyledon (Zhu et al., 2007), which may explain the high percentage of vascularization in MOD heifers, resulting in this continuous growth to meet the increasing fetal requirements in late gestation by increasing nutrient transport. Micke et al. (2015) demonstrated that the umbilical cord diameter is reduced in the first trimester when undernutrition occurs. Umbilical blood flow was not measured in this study; however, it is a variable to consider in the future as it may confound the response variables.

Supporting the color Doppler findings, heifers fed MOD diet had a greater mRNA expression of 2 important angiogenesis markers: *IGFR1* and *VEGFB*. The greatest expression of *IGFR1*, the main IGF-1 receptor responsible for somatic growth in the placental tissue of MOD heifers may indicate more cell proliferation, differentiation, survival, and migration in the placenta (Hernández et al., 2020). Additionally, *IGFR1* regulates placental function (Sferruzzi-Perri et al., 2017) and increases fetal and maternal binucleate cell numbers, which is crucial to maintaining pregnancy (Palmieri et al., 2008). Thus, MOD heifers may have enhanced placental angiogenesis, leading to greater efficiency in nutrient transport to the fetus.

Greater mRNA expression of *VEGFB* in the placenta in MOD heifers may be related to vascular cell survival due to anti-apoptotic factors (Lal et al., 2018). Although nitric oxide is a mediator of VEGF and ANG, which are affected by placental oxygen pressure (Legallo, 2014), it promotes tissue perfusion by inducing the relaxation of vascular smooth muscle. However, differences in nitric oxide synthase between MOD and HIG heifers were not observed due to chronic hypoxia throughout gestation.

Placental compensation for lower maternal nutritional supply hypothesis may support greater mRNA expression of *IGFR1* and *VEGFB*, and the greater number of cotyledons in the placenta of MOD heifers, together increasing the area of nutrient transport. In addition, the growth of placentomes and vascularization percentage of these heifer placentas in the final

![Figure 2](image-url)

**Figure 2.** Means and standard error of means of (A) vascularization area (cm<sup>2</sup>) and (B) vascularization area related to the gravid uterus (%) analyzed in the placentome through Collor Doppler ultrasound at 180, 210, and 240 d of gestation of Holstein × Gyr pregnant heifers fed to moderate gain (MOG; 0.5 kg/d) or high gain (HIG; 0.75 kg/d) during all gestation. Pixels % was used to generate this data related to the vascularized area and yielded the same results. Significant was declared at *P* ≤ 0.05, and trends when 0.10 ≤ *P* ≥ 0.05; lowercase letters indicate results inside the same treatment; uppercase letters indicate the same time in different treatments. The green bar represented HIG average daily gain, and the white bar represented heifers fed to MOG.
third of gestation demonstrate greater resistance to placental injuries, providing more vascularization to meet fetal requirements. Micke et al. (2011) found more expression of IGFR1 in perirenal tissue in calves from undernourished dams, the largest storage site of brown adipose tissue, fundamental for thermogenesis and storage capacity. This greater expression in the placenta may suggest a fetal tissue adaptation to scarcity of nutrients.

Previous studies have highlighted the compensation potential of pregnant cows under nutritional restrictions to support fetal growth without differences in calf birth weight (Vonnahme et al., 2007; Zhu et al., 2007; Rotta et al., 2015). Our data indicate the same occurs to heifers fed to MOD, which compensated for lower nutritional supply, with similar calf birth weight compared with that born from HIG heifers.

Table 3. Hemogram and leukogram count at 8 d postpartum of cows fed to moderate or high body weight gain from 70 d of gestation until calving

<table>
<thead>
<tr>
<th>Item</th>
<th>MOD</th>
<th>HIG</th>
<th>P-value$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematocrit, %</td>
<td>28.0 ± 2.15</td>
<td>29.5 ± 2.00</td>
<td>0.41</td>
</tr>
<tr>
<td>Total leukocytes, $\times 10^6/\mu$L</td>
<td>11.6 ± 2.38</td>
<td>11.1 ± 2.23</td>
<td>0.82</td>
</tr>
<tr>
<td>Segmented neutrophils, $\times 10^6/\mu$L</td>
<td>3.6 ± 0.62</td>
<td>3.12 ± 0.54</td>
<td>0.62</td>
</tr>
<tr>
<td>Rods neutrophils, $\times 10^6/\mu$L</td>
<td>8.7 ± 0.60</td>
<td>9.5 ± 0.53</td>
<td>0.91</td>
</tr>
<tr>
<td>Lymphocytes, $\times 10^6/\mu$L</td>
<td>5.4 ± 0.89</td>
<td>6.4 ± 0.79</td>
<td>0.39</td>
</tr>
<tr>
<td>Platelet count, $\times 10^6/\mu$L</td>
<td>355 ± 93.4</td>
<td>347 ± 90.2</td>
<td>0.91</td>
</tr>
<tr>
<td>Total plasma protein, g/dL</td>
<td>7.1 ± 0.21</td>
<td>7.1 ± 0.19</td>
<td>0.78</td>
</tr>
</tbody>
</table>

$^1$MOD = moderate body weight gain (0.50 kg/d); HIG = high body weight gain (0.75 kg/d).
Significant was declared at $P \leq 0.05$, and trends when $0.10 \leq P \geq 0.05$.

Figure 3. Target genes relative expression normalized using the endogenous gene 18S ribosomal RNA calculated as $\Delta \Delta CT$. Negative $\Delta \Delta CT$ indicates genes upregulated in the MOG treatment, and positive $\Delta \Delta CT$ indicates genes upregulated in the HIG treatment. $^*$ = significant was declared at $P \leq 0.05$, and trends when $0.10 \leq P \geq 0.05$. $^1$MOD = Moderate average daily gain during all gestation; $^2$HIG = High average daily gain during gestation.
MOD heifers, without a difference in the progesterone concentration. These hormones are produced by trophoblast giant cells at the cattle placenta and are related to calving, delivery, neutrophil activation, and placental maturation, which are essential to their expulsion postpartum (Beagley et al., 2010). However, we did not find any differences in gestation length, calving time, or placenta delivery between days of gestation, indicating that a greater concentration of estradiol-17β was insufficient to change calving parameters in this study.

Similar birth weight of the newborn and placenta in MOD and HIG groups indicates the success of MOD heifers in compensating for lower nutrient provision for their growth by generating increased blood supply to the fetus. Although calves of MOD heifers were born with a low vitality score, this had no impact on the passive transfer of immunity, which was considered excellent (9.4 Brix%; Lombard et al., 2020). Monteiro et al. (2014) demonstrated that calves born to cows suffering heat stress during late gestation had compromised passive IgG transfer. Therefore, the nutrient supply to the fetus in utero did not impact immunoglobulin absorption in the intestine of the fetus with success in transfer immunity, despite the smaller villi size found by Duarte et al. (2013). The tendency of greater colostrum volume production in HIG heifers alongside lower quality, when compared with MOD heifers, is likely attributed to a dilution effect. The water dilution increases colostrum volume, lowering IgG concentration; this is related to the increases in lactose synthesis (Dunn et al., 2017; Soufleri et al., 2021). Guy et al. (1994) determined that decreased IgG concentration is related to non-IgG compounds of colostrum.

Cow serum calcium postpartum was greater in the MOD group. Nevertheless, both groups had subclinical hypocalcemia at 2 and 8 d postpartum, with calcium concentrations below 8.59 mg/dL (Martinez et al., 2012). Hypocalcemia is more common in multiparous cows because, in primiparous cows, calcium metabolism is more active (Lean et al., 2019). The lower serum calcium concentration may impact the immune response, increasing the risk of disease and retarding uterus involution (McArt and Neves, 2019). The cows presented normal hematocrit, total leukocytes, segmented neutrophils, rod neutrophils, platelets, and total plasma protein levels, and no differences were seen between treatments, which was expected based on a report by Divers and Peek (2018). Prepartum ADG did not influence the postpartum immune response or blood homeostasis, indicating that the cows had the same health status. In this study, we observed a high concentration

![Figure 4](image-url)
of lymphocytes in all primiparous cows, with a value greater than $13.8 \times 10^3/\mu L$ (Bradford, 2022), indicating lymphocytosis, which commonly occurs postpartum in primiparous cows (Jonsson et al., 2013). This inflammatory response could impact the recovery of cows, but the uterine wall thickness was similar in both groups, with no impact on uterine involution, despite the greater concentration of calcium in MOD heifers. However, the feeding regimen during pregnancy had no influence on the immune response required to improve uterine postpartum recovery.

Serum glucose concentration tends to be greater in MOD cows at 8 d, indicating a faster energy metabolism postpartum in MOD cows than in HIG cows, but serum glucose was similar at 30 d for both. This better energy metabolism was not reflected in MY, suggesting that different feeding regimens do not cause negative effects during pregnancy until the sixth week of lactation. Therefore, the faster recovery in MOD cows postpartum, indicated by an increased glucose level associated with the same BCS and MY, suggests that fat mobilization starts closest to calving in these cows. Furthermore, the initial lower serum calcium concentration in HIG cows than in MOD cows did not associate with the initial MY.

The findings of this study to crossbred Holstein × Gyr heifers would not apply to pure Holstein because Weller et al. (2016) demonstrated prejudice to crossbred mammary gland development when ADG was greater than 0.7 kg/d, the recommended to Holstein heifers. Also, Holstein × Gyr has a lower maturity weight, requiring less ADG than Holstein heifers.

**CONCLUSIONS**

Heifers fed for moderate body weight gain starting at 70 d of gestation undergo maternal adaptations in their placenta to support gestation without negatively affecting the developing fetus and their postpartum life. In addition, they had greater placental blood supply and cotyledon number, indicating provided efficient nutrient transport. Moderate-gain cows had higher calcium and glucose concentration at 2 d, but this did not affect uterine recovery or milk production in early lactation.

![Figure 5](image.png)

Figure 5. Pregnant horn thickness postpartum (mm) measured at the greater curvature of the pregnant horn from 2 to 60 d postpartum of Holstein × Gyr cows fed to moderate (MOD; 0.5 kg/d) or high (HIG; 0.75 kg/d) body weight gain during all pregnancy. Significant was declared at $P \leq 0.05$, and trends when $0.10 \leq P \geq 0.05$. Green points represented heifers treated to HIG average daily gain, and white points represented heifers fed to MOG.
ACKNOWLEDGMENTS

We are grateful to the following Brazilian foundations for their help with this study: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES; Brasilia, DF, Brazil), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNpq; Brasilia, DF, Brazil), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG; Belo Horizonte, MG, Brazil), and Instituto de Ciência e Tecnologia de Ciência Animal (INCT-CA; Viçosa, MG, Brazil).

REFERENCES


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Figure 6. Milk yield in the first 6 weeks of lactation of cows fed during gestation to moderate (MOD; 0.5 kg/d) or high (HIG; 0.75 kg/d) body weight gain during pregnancy. Significant was declared at $P \leq 0.05$, and trends when $0.10 \leq P \geq 0.05$. Green points represented heifers treated to HIG average daily gain, and white points represented heifers fed to MOG.
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