KELLEN RIBEIRO DE OLIVEIRA

EFFECTS OF DIET DURING PREGNANCY ON PLACENTAL EFFICIENCY IN HOLSTEIN × GYR HEIFERS

Dissertation submitted to the Animal Science Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

Adviser: Polyana Pizzi Rotta

Co-advisers: Alex Lopes da Silva
Marcio de Souza Duarte
Yamê Fabres R. Sancler da Silva

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APPROVED: August 26, 2022.

Assent:

K Ribeiro Oliveira

Kellen Ribeiro de Oliveira
Author

Polyana Pizzi Rotta
Adviser

To my grandfather Recenvindo (*in memoriam*)
that I always loved and will love.

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I want to say thanks to my parents, Flaviana e Robson for all the support, to dream with me, to their constant lessons to make me a better person, and I need apologize for absences during these years. Thanks to my family for the emotional support and to believe in me.

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BIOGRAPHY

Kellen Ribeiro de Oliveira, daughter of Flaviana Fonseca Ribeiro de Oliveira and Robson Vander de Oliveira, was born in Curvelo, MG – Brazil on November 22, 1997. Granddaughter of dairy farmer, she always interested in animal production.

She started the Veterinary Medicine in 2016 and in 2021 become a veterinarian at Universidade Federal de Viçosa, Viçosa, MG – Brazil.

In March of 2021, she started her Master's degree in Animal Science with a major in ruminant nutrition, dairy cattle production and fetal programming at Universidade Federal de Viçosa, advised by Dr. Polyana Pizzi Rotta. She submitted her dissertation to the committee on August 05, 2022.

ABSTRACT

OLIVEIRA, Kellen Ribeiro M.Sc., Universidade Federal de Viçosa, August, 2022. **Effects of diet during pregnancy on placental efficiency in Holstein × Gyr heifers** Adviser: Polyana Pizzi Rotta. Co-advisers: Alex Lopes da Silva, Marcio de Souza Duarte and Yamê Fabres Robaina Sancler da Silva.

Placenta and fetal growth depend of umbilical and uterine blood flow, and adverse conditions may impact the animal's entire life. This study aimed to evaluate the effects of diet during pregnancy on the blood supply to the placenta in two nutritional regimes throughout the gestational period, colostrum production, the newborn parameters and uterine involution. A total of fourteen Holstein × Gyr heifers with an average body weight of 446 kg ± 46.7 kg and age of 25 ± 3.9 mo were randomly assigned to the following treatments: moderate body weight gain (**MOG**, n = 7), the usual at tropical systems, where heifers were fed to achieve 0.5 kg/d of average daily gain (**ADG**); and high body weight gain (**HIG**, n = 7), where heifers to achieve 0.75 of ADG. The heifers received the same diet with corn silage and a concentrate-based diet twice daily varying the dry matter (**DM**) according to ADG, adjusted each 28 d of each treatment until calving. The placentome vascularization was assessed by using a Color-Doppler ultrasound. After calving, cotyledon was sampled to analyze mRNA expression of genes involved in angiogenesis process in the placenta. After birth, calves were weighed, received colostrum, and had their efficiency of transfer of passive immunity (TPI) assessed. The MOG heifers had a greater number of cotyledons (81.5 ± 12.9 vs. 63.6 ± 10.5), and continuous growing in placentome vascularization compared to HIG heifers. Indeed, MOG heifers had a greater the mRNA expression of *VEGFB* and *IGFR1*, as well as estradiol concentration before calving compared to HIG heifers. On the other hand, a greater colostrum production was observed in HIG heifers (3.94 ± 1.05 vs. 2.18 ± 1.57), but with lower quality (25.2 ± 0.51 vs. 29.5 ± 0.65; Brix %). Further, postpartum serum calcium and glucose were greater on d 8 in MOG heifers, but an equal immune response and uterus involution. The calf from HIG heifers had a better vitality score and equal body weight (**BW**) at birth and efficiency in TPI. Collectively, our results show that moderate nutritional regimen during gestation of dairy heifers undergo maternal adaptations in the placenta to support gestation without damage to fetus and their postpartum life.

Keywords**:** Cotyledon. Doppler ultrasound. Fetal programming. Gene expression

RESUMO

OLIVEIRA, Kellen Ribeiro, M.Sc., Universidade Federal de Viçosa, agosto de 2022. **Efeitos da dieta materna durante a gestação na eficiência placentária em novilhas Holandês × Gir**. Orientador: Polyana Pizzi Rotta. Coorientadores: Alex Lopes da Silva, Marcio de Souza Duarte e Yamê Fabres Robaina Sancler da Silva.

O crescimento placentário e fetal é dependente do fluxo sanguíneo umbilical e uterino, condições adversas podem impactar em toda a vida do animal. Este estudo tem como objetivo avaliar os efeitos da dieta materna no suprimento sanguíneo placentário em dois regimes nutricionais durante a gestação, produção de colostro, parâmetros do recém-nascido e involução uterina. Quatorze novilhas Holandês × Gir com peso inicial de 446 ± 46,7 kg foram aleatoriamente destinadas a um dos seguintes tratamentos: ganho de peso moderado (**MOG**; n = 7), onde eram alimentadas para atingir 0,5 kg/dia e alto ganho de peso (**HIG**; n = 7), onde eram alimentadas para o ganho de 0,75 kg/dia. As novilhas recebiam duas vezes ao dia a mesma dieta composta por silagem de milho e concentrado variando a quantidade de MS fornecida de acordo com o GMD, ajustado a cada 28 dias de cada tratamento até o parto. A vascularização do placentoma foi avaliada utilizando um ultrassom com Color Doppler. Pós-parto, os cotilédones foram contados e amostrado para analisar a expressão de mRNA de genes relacionados com o processo de angiogênese na placenta. Após o nascimento, os bezerros foram pesados, receberam colostro e foi avaliada a eficiência na transferência de imunidade passiva (TIP). As novilhas de MOG tiveram maior número de cotilédones (81.5 ± 12.9 vs. 63.6 ± 10.5), e crescimento contínuo da vascularização de placentoma. Ainda, as novilhas de MOG tiveram maior expressão de mRNA de *VEGFB* (P = 0,0397) e *IGFR1* (P = 0,0470), assim como a concentração pré-parto de estradiol comparado com novilhas de HIG. Por outro lado, foi observada uma maior produção de colostro nas novilhas com HIG (3.94 ± 1.05 vs. 2.18 ± 1.57), porém com menor qualidade (25.2 ± 0.51 vs. 29.5 ± 0.65; Brix %). Ainda, cálcio e glicose séricos foram maiores no dia 8 para novilhas de MOG. Bezerros originados de novilhas HIG tiveram melhor escore de vitalidade, mas peso ao nascer e eficiência na transferência de imunidade passiva similar. Em conclusão, os resultados demonstram que um regime nutricional moderado durante a gestação de novilhas leiteiras promove adaptações na placenta para suportar a gestação sem danos ao feto e sua vida pós-parto.

Palavras-chave: Cotilédone. Expressão gênica. Programação fetal. Ultrassom Doppler.

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SUMMARY

INTERPRETATIVE SUMMARY

Effects of diet during pregnancy on placental efficiency in Holstein × Gyr heifers. By *Oliveira et al.* This study evaluated the impact of diet during pregnancy on the uterine blood supply of Holstein × Gyr heifers with moderate (MOG) or high (HIG) BW gain. The MOG heifers had more cotyledons, continuous growth of vascularization in placentome, and mRNA expression of *IGFR1* and *VEGFB* in placental tissue. The HIG heifers produced more colostrum but with a lower quality. Calves of MOG heifers had similar BW at birth but the same efficiency in the transfer of passive immunity. MOG heifers undergo maternal adaptations in the placenta to support gestation without damage to fetus and their postpartum life.

Running head: Effects of diet during pregnancy on placental supply

EFFECTS OF DIET DURING PREGNANCY ON PLACENTAL EFFICIENCY IN HOLSTEIN × GYR HEIFERS

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 Polyana P. Rotta[1*]

[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.*

* Corresponding author: Polyana Pizzi Rotta. Mailing address: Av. PH Rolfs – Campus UFV. 36570-900. Viçosa/MG, Brazil. E-mail: polyana.rotta@ufv.br, phone: +55 (31) 99449-1703.

ABSTRACT

Placenta and fetal growth depend of umbilical and uterine blood flow, and adverse conditions may impact the animal's entire life. This study aimed to evaluate the effects of diet during pregnancy on the blood supply to the placenta in two nutritional regimes throughout the gestational period, colostrum production, the newborn parameters and uterine involution. A total of fourteen Holstein × Gyr heifers with an average body weight of 446 kg ± 46.7 kg and age of 25 ± 3.9 mo were randomly assigned to the following treatments: moderate body weight gain (**MOG**, n = 7), the usual at tropical systems, where heifers were fed to achieve 0.5 kg/d of average daily gain (**ADG**); and high body weight gain (**HIG**, n = 7), where heifers to achieve 0.75 of ADG. The heifers received the same diet with corn silage and a concentrate-based diet twice daily varying the dry matter (**DM**) according to ADG, adjusted each 28 d of each treatment until calving. The placentome vascularization was assessed by using a Color-Doppler ultrasound. After calving, cotyledon was sampled to analyze mRNA expression of genes involved in angiogenesis process in the placenta. After birth, calves were weighed, received colostrum, and had their efficiency of transfer of passive immunity (TPI) assessed. The MOG heifers had a greater number of cotyledons (81.5 ± 12.9 vs. 63.6 ± 10.5), and continuous growing in placentome vascularization compared to HIG heifers. Indeed, MOG heifers had a greater the mRNA expression of *VEGFB* and *IGFR1*, as well as estradiol concentration before calving compared to HIG heifers. On the other hand, a greater colostrum production was observed in HIG heifers (3.94 ± 1.05 vs. 2.18 ± 1.57), but with lower quality (25.2 ± 0.51 vs. 29.5 ± 0.65; Brix %). Further, postpartum serum calcium and glucose were greater on d 8 in MOG heifers, but an equal immune response and uterus involution. The calf from HIG heifers had a better vitality score and equal body weight (**BW**) at birth and efficiency in TPI. Collectively, our results show that moderate nutritional regimen during gestation of dairy

heifers undergo maternal adaptations in the placenta to support gestation without damage to fetus and their postpartum life.

Key words: cotyledon, Doppler ultrasound, fetal programming, gene expression

INTRODUCTION

Nutrient supply in the bovine placenta occurs by the placentome, represented by the union between the fetal cotyledon and maternal caruncle. The transport of nutrients between the heifer and fetus depends of uterine and umbilical blood flow, and changes occur when fetal demands increase (Vonnahme and Lemley, 2012). Quantitatively, fetal nutrient requirements become significant only in the last trimester when more than 60 percent of growth occurs. However, Sguizzato et al. (2020) demonstrated that the start of significant demand fetal in Holstein × Gyr occurs from 70 d of gestation.

Maternal circumstances during conception and gestation are determinants of the newborn's phenotype (Laporta et al., 2020). The exposure of the fetus to adverse conditions inside the uterus, like nutritional or heat stress, may generate permanent changes in the adult life because of utero environment and nutrient supply (Recce et al., 2021), with effects in the villi formation on small intestine (Gionbelli et al., 2015; Duarte et al., 2013) and number of follicles in female (Weller et al., 2016), for example. However, when the dam is submitted to nutrient restriction, compensation in the placenta blood vascularization occurs as an adaptation to nutrient scarcity to meet fetal demands (Zhu et al., 2007; Rotta et al., 2015).

The interaction between nutrient and gene has been considerable impacts on embryonic and fetal development (Costa et al., 2021), leading to gene transcription changes when intrauterine environment undergoes to hypoxia, and proinflammatory cytokines (Matouk and Marsden, 2008). Therefore, nutrient delivery depends upon its availability, placental metabolism, blood flow, and transport capacity (Edwards et al., 2020), and the placental size and morphology are determinants of the development of the fetus and may affect the body weight at birth (Vonnahme et al., 2007). However, if an insult occurs in the early gestation, the placenta development may be altered, and fetal growth during later pregnancy also may be altered (Camacho et al., 2018).

In dairy production systems in tropical areas heifers commonly gained moderate ADG which impairs the gestational nutritional requirements to be met. According to NASEM (2021), primiparous heifers must have 91% of the mature body weight immediately before the first calving, and to achieve this weight, an adequate gain is required to no compromising the future lactation and fetal growth. However, despite the current knowledge about maternal nutrition effects of fetus development, studies demonstrating the correlation between moderated and adequate dietary regimens for primiparous dairy heifers on placental growth and metabolism are scarce.

Thus, we hypothesized that heifers fed for moderate body weight gain (MOG) during the gestational period have greater placental efficiency and blood supply without compromising the growth of the newborn. Our objective was to evaluate the feeding regimens for dairy heifers on placenta hemodynamics and angiogenesis, and its effects on newborn.

MATERIAL AND METHODS

The experiment was carried out at 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 63% Holstein × 37% Gyr pregnant heifers with an average initial BW of 445.9 ± 46.65 kg and age of 25 ± 3.9 mo were used in this study. The heifers were pregnant with embryos ¾ Holstein × Gyr from the same farm in Minas Gerais, Brazil. At the 70th day of gestation, the heifers were randomly assigned into two experimental treatments:

moderate ADG (**MOG**) – 0.5 kg/d (n = 7) and high ADG (**HIG**) – 0.75 kg/d (n = 7). Heifers were fed corn silage and a concentrate-based diet twice daily (0700 h and 1600 h; Table 1). The animals were weighted every 28 d in the morning Thursday before feeding and had their feed intake adjusted for the BW gain expected to each treatment.

All animals were individually housed up to 250 d of gestation when they were moved to a collective Compost Barn system, where they stayed until calving. After birth, calves were weighed on a mechanical scale and evaluated for vitality score according to Murray-Kerr et al. (2018).

Evaluation of placentome vascularization

After 180 d of gestation, placentome vascularization was evaluated in the times: 180 d, 210 d and 240 d by using the Ultrasound Z5VET® (Mindray Medical International Technology, 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), with a pulse repetition frequency of 1.7 kHz. Two videos of each five evaluated placentome were stored for posterior analyses.

The image with greater vascularity was extracted from each video and was 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 total area of the placentome and of color pixels area. With these two values, it was possible to obtain the percentage of pixels mean in the total area of each five placentome scanned.

The gravid uterus was estimated using calve body weight at birth according to NASEM (2021) (Equations 1 and 2) in each evaluation time at 180 d, 210 d and 240 d. That calculus was used to estimate the proportion of placentome area, pixels area, and vascularization in relationship to uterus weight.

$$\text{GrUter\_Wt}_{(\text{t=parturition})} = \text{Calf birthweight} \times 1.825$$

Equation 1

$$\text{GrUter\_wt} = (\text{GrUter\_Wt}_{(\text{t=parturition})} \times e^{(0.0243-(0.0000245\times\text{DayGest}))\times(280-\text{DayGest})}$$

Equation 2

Where DayGest = day of gestation, $\text{GrUter\_Wt}_{(t = \text{parturition})}$ = gravid uterine weight at parturition, GrUter_wt = estimated gravid uterine weight in specific day of gestation.

Placenta evaluation and sample collection

Was measured the elapsed time in minutes from placenta rupture to calf birth and the gestation length in days. After calving, the primiparous were monitored and the time for placenta expulsion and was counted. Retention of fetal membranes was considered when it was not expelled within 12 h after parturition (Magata et al., 2017; Takagi et al., 2002; Grunert et al.,1989).

Once expelled, the placenta was weighed using digital scale, the cotyledons were counted in morphometric analyses and the two cotyledons closest to the umbilical cord were measured, and the biggest of them was sampled. The samples were snap frozen in liquid nitrogen and stored at -80°C until the RNA extraction procedures. We calculated using the weight the placenta:calve ratio after the measures to evaluate the placenta efficiency (Camacho et al., 2018).

Total RNA extraction and gene expression analysis

Was used the GenBank database to obtain nucleotides sequences of target genes to make gene-specific primers according to Rotta et al. (2015). The genes evaluated was: *18S*, *VEGFA*,

VEBFG, *IGFR1*, *IGFR2*, *GUCY1B3*, *HIFA*, *SF3A1*, *FGF2*, *ANGPT2*, and *NOS3* and the sequence utilized to structure primers are summarized in Table 2.

Total RNA was isolated by using PureLink RNA Mini Kit (Invitrogen, Carlsbad, California). After the extraction, the concentration of total RNA was determined using NanoDrop Lite UV-Vis Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and RNA integrity was assessed by 1% agarose gel. The cDNA synthesis was performed by using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies Corporation, Carlsbad, CA) according to the manufacturer protocol.

Quantitative RT-PCR reactions were performed using SYBR-Green I detection with GoTaq qPCR Mix (Promega Madison, Wisconsin) and gene-specific primers (Table 2). The RT- qPCR was performed in MicroAmp Fast 96-Well Reaction Plate (0.1mL) (Applied Biosystems, Foster City, CA) following the cycle parameters: 95°C for 2 min, 40 cycles for 15 sec 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. Gene expression was calculated as $2^{-\Delta\Delta C}{}_{T}$ according to Livak and Schmittgen (2001).

Postpartum evaluation

After calving, the animals were milked, and the colostrum production was measured and evaluated quality using a Brix refractometer (Silva-Del-Rio et al., 2017).

The colostrum was provided to calves corresponded to 15% of BW at birth in the first two hours. After 24 h of the colostrum, the blood was collected in a vacuum tube with gel separator, centrifuged, and utilized one drop of the serum in the Brix refractometer to estimate plasmatic protein and obtain the transference of immunity passive efficiency.

All animals underwent uterine evaluation each 2 days during 20 d and at d 30 and 60 postpartum employing transrectal ultrasound using DP-20 Vet Power with linear transrectal

transductor (Mindray Animal Medical Technology, China) when uterus infection was diagnosed, and uterine wall thickness was measured at greater curvature of the pregnant horn. At 20 d postpartum the cows were submitted to a protocol to synchronize estrus and inseminated at 30 d postpartum by fixed-time artificial insemination protocol developed by Consentini et al.(2021; Figure 2). After 32 ± 4 d of insemination, the animals were diagnosed, and estrus was synchronized again if not pregnant.

The body condition score (BCS) was done for the same evaluator at the moment of calving and 30 d of lactation to analyze tissue mobilization in a transition period.

Blood sample collection

The blood sample was collected at prepartum and postpartum in the coccyges vein (Figure 1). When the animals had 272 ± 2.18 d of gestation, a blood sample was collected at 1800 h in a vacuum tube with gel separator, centrifuged, and sampled the serum stored at -20°C until calving. Was used the sample correspondent to one day before calving to analyses of estrogen and progesterone in one sample scheme using chemiluminescence kits Access Estradiol and Access Progesterone (Beckman Coulter, Ireland), respectively.

After calving, always before feeding, blood samples were collected at 2, 8, and 30 d after parturition for glucose analysis in a vacuum tube with sodium fluoride, while samples collected in a vacuum tube with lithium heparin at 2 and 8 days after parturition were used to determine serum calcium levels. Was used a Glucose Monoreagent kit and Calcium Arsenazo III kit (Quibasa-Bioclin, Brazil) to analyse of glucose and calcium respectively, using the biochemical analyzer BS-200 (Mindray Headquarters, China).

On day 8, a blood sample was collected in a vacuum tube with EDTA for hemogram and the leukogram. The blood sample was immediately centrifuged and analyzed in Hematoclin 2.8 VET (Quibasa-Bioclin, Brazil).

Statistical analyses

Firstly, data were analyzed for the residual distribution, the variables placentome morphometry and vascularization and postpartum uterine thickness did not follow a Gaussian distribution. Therefore, the most adequate distribution was verified using the package rriskDistributions of R (R Core Team, 2022) and a gamma distribution was chose for all variables. So, these models were adjusted using the function glmer of the package lme4 of R, being used the family Gama, link log function and the random effect of measurement day within to the animal.

The other variables were analyzed following a randomized block design where the animals were blocked by calving month. The data was submitted to an analysis of variance using the function lmer of the package lme4 of R, following the model:

$$Y_{ij} = \mu + T_i + B_j + \varepsilon_{ij}$$

Where: Yij = dependent variable; u = overall mean; Ti = fixed effect of treatment; Bj = random effect of blocking = eij = the random error.

The variables there were measured overtime (calcium, glucose and BCS) were submitted to analyze of variance including the effect of time as repeated effect in the model above. The following covariance matrices were tested: compound symmetry (CS), heterogeneous compound symmetry (CSH), autoregressive-order 1(AR1), heterogeneous autoregressive-order 1(ARH1), and variance components (VC). The best covariance matrix was choose based on the lower AIC, being CS for glucose and BCS and CSH for calcium.

Our variables were tested to outlier detection and were considered outliers when the internal student residuals were greater than |2.5|. When necessary, means were separated using the Tukey test at a level of significance of 0.10.

RESULTS

A similar initial body weight was observed between treatments (P = 0.823). However, the heifers in HIG treatment had greater ADG (P < 0.001) allowing them to have a greater BW at the end of gestation (P = 0.01; Table 3). Collectively, these results demonstrate the effectiveness of experimental treatments.

Placentome Morphometry and Vascularization

The uterine mass increased over the days of gestation (P = 0.0002) but did not differ between treatments (P = 0.907; Table 3). However, the placentome area and placentome area relative to the gravid uterus presented interaction between days of gestation (**DG**) and treatment (**T**) (P = 0.017; P = 0.027). The placentome area of MOG heifers continued to increase at 240 d and equals to HIG.

Pixels area reflect the vascularization in the placentome, and it had interaction between DG and T (P < 0.01) as pixels area compared to the gravid uterus (P < 0.01). In the same way, there was interaction between DG and T in the percentage of vascularization (P = 0.004) and vascularization relative to the gravid uterus (P < 0.01; Table 3). Both parameters continued to increase to MOG heifers and equals to HIG heifers at 240 d, while relative to gravid uterus, pixels and vascularization remained the same in the evaluated period.

Placental Gene Expression

The mRNA expression of endothelial growth factor A (*VEGFA*) did not differ (P = 0.75) between treatments. However, the mRNA expression endothelial growth factor B (*VEGFB*) was greater in the placenta of MOG compared to HIG heifers (P = 0.039; Table 4).

We observed a greater mRNA expression of insulin-like growth factor receptor 1 (*IGFR1*; P = 0.047) in MOG compared to HIG heifers, while expression of insulin-like growth factor receptor 2 (*IGFR2*) did not differ between treatments (P = 0.378; Table 4).

The mRNA expression of soluble guanylate cyclase (*GUCY1B3;* P = 0.326), hypoxia-inducible factor 1 (*HIFA1A*; P = 0.410), splicing factor 3A subunit 1 (*SF3A1*; P = 0.726), fibroblast growth factor 2 (*FGF2;* P = 0.504), and angiopoietin 2 (*ANGPT2*; P = 0.521) were similar in both treatments. Indeed, the mRNA expression of nitric oxide synthase (*NOS3*) did not differ among treatments (P = 0.491; Table 4).

Parturition and Postpartum Evaluation

Estradiol concentration was greater in HIG (P = 0.02), but progesterone concentration did not differ (P = 0.713) between treatments before calving. Despite that, the calving parameters did not differ, with the same gestation length for both treatments (P = 0.853), no differences in calving time (P = 0.697), and placenta expulsion time (P = 0.359; Table 3) were observed. Born in each treatment only one male calf.

Placenta weight did not differ between treatments (P = 0.336), neither the calf birth weight (P = 0.329; Table 3). However, MOG had most cotyledons in the placenta than HIG (P = 0.024). One animal in the MOG group had placenta retention, so we did not obtain the weight of this placenta.

A greater colostrum production was observed in HIG heifers (P = 0.090), but lower Brix % compared to MOG (P = 0.001) likely due to a dilution effect. The calf from HIG heifers had a better vitality score (P = 0.085), but despite that, efficiency in the transfer of passive immunity was equal to both treatments (P = 0.900; Table 3).

On d 8 postpartum, we did a hemogram and leukogram of all the primiparous and did not find differences between treatments in all parameters evaluated (Table 6).

We observed interaction between day and treatment between treatments in serum calcium ($P = 0.040$) and glucose ($P = 0.082$). The MOG primiparous had the equal serum calcium at d 2 but increased its concentration more on d 8(Figure 3). MOG and HIG primiparous also had similar concentration of glucose on d 2, however, MOG presented greater values at d 8 and this equals at d 30 (Figure 3).

The BCS decreased from parturition to the 30th day of lactation ($P < 0.0001$) but did not differ ($P = 0.803$) between treatments (Figure 4).

The thickness of the pregnant horn in the uterus postpartum decreased over the days ($P = 0.001$) due to postpartum uterine involution. However, in both treatments the uterus was equally involuted ($P = 0.826$), as shown in Figure 5 and all animals presented clinical metritis. No animal had estrus before the fixed time artificial insemination protocol.

DISCUSSION

The effectiveness of experimental treatments was observed which heifers fed for high weight gain during pregnancy had greater BW at calving.

The area of the placentomes and the vascularization of the placentomes concerning the gravid uterus continue increased in MOG heifers at final of gestation, which indicate an increase of the active surface for nutrient transport to meet fetal requirements. However, the proportion of placentomes and vascularization regarding the uterine mass is similar to both treatments. Moreover, the heifers from MOG present a greater number of cotyledons demonstrating greater area to nutrient transport. Collectively, our findings suggest a compensation mechanism is the increased number of cotyledons and active surface to nutrient transport to the fetus. This

continuous growth had objective meet the increasing fetal exigences in the final of pregnancy in an attempt to increase nutrient transport.

Supporting the findings in Collor Doppler, heifers in MOG had most expression of two important angiogenesis genes: *IGFR1* and *VEGFB*. The greater expression of *IGFR1* in MOG group, the main IGF-1 flag is responsible for somatic growth. Which may indicate better cell proliferation and differentiation, survival, and migration in the placenta (Hernández et al., 2020) beyond regulating their function (Sferruzzi-Perri et al., 2017) and increase fetal and maternal binucleate cell numbers, crucial to maintain pregnancy (Ravelish et al., 2004; Palmieri et al., 2008). Thus, MOG animals had greater signalizing to placental angiogenesis and manutention, expressed in most vascularization and, consequently, greater efficiency in nutrient transport to fetus.

Greater mRNA expression of *VEGFB* in the placenta of MOG heifers is related to vascular cell survival due to anti-apoptotic factors (Lal et al., 2018). These placentas had superior endothelial resistance to injuries, making them less fragile and better able to support the fetus during gestation. Nitric oxide (NOS3) is affected by placental oxygen pressure and is a mediator of *VEGF*- and *ANG*- (LeGallo, 2014), this promotes tissue perfusion by the relaxation of vascular smooth muscle, but we did not find differences between treatments.

These genes more expressed added to most cotyledons in MOG placenta, and continuous growth of placentomes and vascularization demonstrate a higher resistance of the placenta to injuries, providing greater vascularization to meet fetus requirements.

Previous studies have highlighted the compensation potential of pregnant cows when passed for nutritional restrictions (Zhu et al., 2007; Vonnahme et al., 2007; Rotta et al., 2015). Our data indicate that heifers fed to moderate gain, even without greater expression of *NOS3,* which indicates a shortage of oxygen supply, compensate for a lower nutritional apport of nutrients comparing to HIG, as do cows in restriction.

We found a higher serum concentration of estradiol-17β in HIG animals before calving comparing to MOG, without a difference in concentration of progesterone. These hormones can be produced by trophoblast giant cells at cattle placenta are the related to calving, unleash delivery, neutrophils activation, and placental maturation, essential to their expulsion postpartum (Beagley et al., 2010). A higher concentration of estradiol-17β may be associated to a greater availability of cholesterol molecules, the basis for the synthesis of steroid hormones, and final maturation of trophoblast giant cells at placenta maturation to expulsion (Schuler et al., 2008). However, we did not find any differences in gestation length, calving time or placenta delivery, indicating that higher concentration of estradiol-17β was not enough to change calving parameters.

The same body weight of the newborn and placenta to MOG and HIG indicates the success of compensating nutrients for their growth due increased blood supply. Even though calves of MOG were born with a low vitality score, but this had no impact on passive transfer of immunity above the considered excellent of 9.4 Brix% (Lombardi et al., 2020). In the same way, the colostrum production was greater to HIG primiparous, but with quality inferior to MOG due dilution effect.

Primiparous in both treatments presented hematocrit, total leukocytes, segmented neutrophils, rods neutrophils, platelets, and total plasma protein inside the parameters according to Divers and Peek (2018). However, they had a lymphocytosis, commonly occurred postpartum, especially in primiparous (Jonsson et al, 2013). Prepartum nutrition did not interfere with the immune response and the blood homeostasis postpartum, indicating that primiparous had the same health status and the lower ADG was not systemic damage.

Primiparous' serum calcium and glucose postpartum were greater in MOG. Despite that, both treatments had subclinical hypocalcemia at d 2 and d 8 postpartum, with calcium

concentration below 8.59 mg/dL (Martinez et al., 2012). Hypocalcemia be most present in multiparous cows, but in primiparous calcium metabolism commonly is most active.

That lower concentration of serum calcium can impact in the immune response increasing the risk of disease and in uterus involution (McArt and Neves, 2019), as we observe the presence of metritis in all animals and lymphocytosis.

The uterine wall thickness may have been affected by hypocalcemia, metritis, and lymphocytosis. However, the diet during pregnancy had no influence the immune response required to better uterine postpartum recovery.

Serum glucose concentration was greater in MOG animals at d 8 evidencing an energy metabolism postpartum faster but that equals at 30d. The BCS demonstrating simile body fat mobilization for both from calving to thirty days postpartum. However, the faster response in MOG cows increasing glucose suggest that fat mobilization starts most closely to calving in these animals, return to homeostasis earlier.

CONCLUSIONS

Heifers feeding regimen with moderate body weight gain during gestation undergo maternal adaptations in their placenta to support gestation without damage to fetus and their postpartum life. So, they had greater blood supply and cotyledon number, providing an efficient nutrient transport. MOG primiparous had better calcium and energy metabolism, however most studies needed to evaluate the impacts in their milk production in early lactation.

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Table 1. Ingredients and chemical composition of diets offered to the treatments MOG and HIG during gestation.

Item	Treatments[2]	
	MOG	HOG
Ingredient (%DM)		
Corn silage	71.93	71.85
Soybean meal	24.68	24.76
Dicalcium phosphate	2.31	2.25
Mineral mix[1]	1.15	1.12
Chemical composition (%)		
Dry matter	38.06	38.03
Crude protein	17.2	17.2
Ether extract	2.72	2.68
Neutral detergent fiber	43.0	42.9
Rumen degradable protein	12.0	12.0
Rumen undegradable protein	5.2	5.2
Metabolizable protein: metabolizable energy	45.1	45.2
Calcium	1.0	0.94
Phosphor	0.54	0.49
Sulfur	0.17	0.14

[1]Mineral mix composition= 170 g/kg calcium, 60 g/kg phosphor, 15 g/kg magnesium, 45 g/kg sulfur, 70 g/kg sodium, 0.1 g/kg cobalt

[2] MOG = moderate body weight gain (0.5 kg/d); HIG = high body weight gain (0.75 kg/d).

Table 2. Details of primers for target and reference genes analyzed by qPCR

Gene name	Accession number	Gene sequence of nucleotide[1]	Product size, bp
VEGFA	NM_174216.1	For ATGACGAAAGTCTGGAGTGTG'	94
		Rev TCTCCTATGTGCTGGCTTTG'	
VEGFB	NM_174487.2	For AGAGTTGGATGAGGAGACCA'	151
		Rev AGAGGAGCCAGCTGTTAGA	
ANGPT2	NM_001098855.1	For GCTGTACGACCACTTCTATCTC	101
		Rev GCTGGCTTATGCTGCTTATTT	
FGF2	NM_174056.3	For CCTACTCCTAGGCAATATGGTAAAT	96
		Rev CAACCCACCTAGTCAGAGATTG	
NOS3	NM_181037.3	For CTGTCATTCCACTATGGCTCTAC	109
		Rev GTACAGGGAATCCAACAGTCTC	
IGFR1	NM_001077828.1	For TCCCATCTCCCTGGATTTCT	105
		Rev GGGTTGGAAGACTGCTGATT	
IGFR2	NM_174087.3	For GGAAGTGGTCCAGCAAGATT	98
		Rev CGTCAATTTGGGCTCTGATTTC	
GUCY1B3	NM_174641.1	For GGAAGGGTTGTTGGATGTAGAG	105
		Rev GCTTCGGGCAAGTAGATCAT	
HIF1A	NM_174339.3	For GAGGCTCACCATCAGCTATTT	91
		Rev GCAATTCATCTGTGCCTTCATT	
SF3A1	NM_001081510.1	For ATGCCAACTCGCTGGCTTAC	100
		Rev AGAGCAGGCTTCTCCTACTT	
BACTIN	NM_001033618.1	For ACTCCTGCTTGCTGATCCACATCT	109
		Rev AAGATCAAGATCATCGCGCCTCCA	
18S	NM_001033614	For CCTGCGGCTTAATTTGACTC	99
		Rev AACTAAGAACGGCCATGCAC	

[1]For = forward; Rev = reverse

Table 3. Means and standard errors of the means of initial and final body weight of the heifers, calving characteristics and colostrum parameters of calves from moderate or high body weight gain Holstein × Gyr heifers.

	Treatments[1]		
Item	MOG	HIG	P-value[3]
Initial body weight, kg	446 ± 37.7	449 ± 37.4	0.823
Final body weight, kg	534 ± 49.6	582 ± 49.3	0.014
Average daily gain	0.489 ± 0.0864	0.738 ± 0.0858	<0.001
Calving parameters			
Estrogen, pg/mL	155 ± 25.8	238 ±21.4	0.020
Progesterone, ng/mL	0.617 ± 0.0885	0.574 ± 0.0918	0.713
Gestation length, days	276 ± 2.18	277 ± 2.18	0.856
Calving time, min	65.2 ± 20.10	58.7 ± 15.00	0.697
Placenta expulsion time, min	400 ± 55.0	345 ± 47.6	0.339
Placenta weight, kg	5.24 ± 0.806	5.90 ± 0.567	0.447
Calf weight, kg	39.7 ± 2.04	37.2 ± 1.80	0.329
Placenta weight: Calf weight, %	12.9 ± 1.70	15.8 ± 1.33	0.132
Total number of cotyledons	81.5 ± 12.9	63.6 ± 10.5	0.024
Colostrum parameters			
Colostrum production, kg	2.18 ± 1.57	3.94 ± 1.05	0.090
Colostrum quality, Brix %	29.5 ± 0.65	25.2 ± 0.51	0.001
Calf serum, Brix %	10.5 ± 1.15	10.6 ± 0.93	0.900
Vitality score	23.2 ± 2.08	24.8 ± 1.88	0.085

[1] MOG = moderate body weight gain (0.5 kg/d); HIG = high body weight gain (0.75 kg/d).
[2]Significant ($P \leq 0.10$).

Table 4. Means and standard error of means of gravid uterus estimated and their comparations with placentome area, pixels area and vascularization at 180 d, 210 d and 240 d of pregnancy analyzed through Collor Doppler ultrasound.

	MOG[1]			HIG[2]			P-value[3]		
Item	180 d	210 d	240 d	180 d	210 d	240 d	T	TG	T:TG
Gravid uterus, kg	13.3 ± 2.07^{Cc}	25.6 ± 3.98^{Bb}	45.5 ± 7.08^{Aa}	13.7 ± 1.90^{Cc}	26.1 ± 3.63^{Bb}	46.6 ± 6.47^{Aa}	0.907	0.0002	0.49
Placentome area, cm²	758 ± 87.2^{Ab}	850 ± 96.7^{Ab}	1074 ± 121.9^{Aa}	904 ± 106.2^{Aa}	835 ± 98.4^{Aa}	906 ± 102.6^{Aa}	0.897	0.004	0.017
Placentome area/Gravid uterine	56.7 ± 9.21^{Aa}	33.3 ± 5.36^{Ab}	23.5 ± 3.78^{Ac}	64.6 ± 10.50^{Aa}	31.4 ± 5.12^{Ab}	19.2 ± 3.05^{Ac}	0.776	<0.0001	0.027
Pixels area, cm²	123 ± 22.0^{Bb}	140 ± 25.1^{Ab}	219 ± 39.2^{Aab}	212 ± 38.4^{Aa}	135 ± 24.1^{Aa}	174 ± 30.2^{Aa}	0.755	0.0004	0.0001
Pixels area/Gravid uterine	9.38 ± 2.069^{Aa}	5.61 ± 1.237^{Ab}	4.87 ± 1.074^{Ac}	15.07 ± 3.307^{Aa}	5.07 ± 1.104^{Ab}	3.68 ± 0.784^{Ac}	0.961	<0.0001	0.0001
Vascularization, %	16.9 ± 1.69^{Bb}	17.5 ± 1.80^{Ab}	21.9 ± 2.23^{Aa}	23.3 ± 2.50^{Aa}	17.1 ± 1.79^{Ab}	18.7 ± 1.93^{Aab}	0.669	0.069	0.004
Vascularization/Gravid uterus	1.25 ± 0.242^{Aa}	0.68 ± 0.131^{Ab}	0.48 ± 0.093^{Ac}	1.71 ± 0.318^{Aa}	0.65 ± 0.122^{Ab}	0.39 ± 0.072^{Ac}	0.947	<0.0001	0.002

[1]MOG = Moderate ADG;
[2]HIG = High ADG;
[3]Significant ($P \leq 0.10$); T = treatment; TG = Time of gestation; T:TG = interaction between treatment and time of gestation. Lowercase letters compare results inside the same treatment; uppercase letters compare the same time in different treatments.

Table 5. Reference gene 18S and target genes normalized using the reference gene and gene expression calculated as 2-ΔΔCT in the cotyledon closest of umbilical cord of moderate or high body weight gain of heifers' placenta.

Target gene	MOG[1]	HIG[2]	P-value[3]
18S	16.0 ± 2.83	16.1 ± 2.61	0.9814
VEGFA	27.3 ± 1.04	28.6 ± 2.99	0.7503
VEGFB	18.1 ± 1.06	15.5 ± 0.83	0.0397
IGFR1	13.8 ± 2.97	9.3 ± 2.85	0.0470
IGFR2	6.1 ± 2.19	7.63 ± 1.96	0.3787
GUCY1B3	6.48 ± 1.78	8.33 ± 1.52	0.3256
HIFA	7.1 ± 2.12	8.58 ± 1.77	0.4107
SF3A1	8.87 ± 3.78	10.29 ± 2.95	0.7263
FGF2	8.96 ± 2.90	10.73 ± 2.63	0.5039
ANGPT2	15.9 ± 3.51	18.3 ± 2.98	0.5218
NOS3	10.5 ± 2.53	12.0 ± 2.35	0.4919

[1]MOG = Moderate ADG;
[2]HIG = High ADG;
[3]Significant ($P \leq 0.10$).

Table 6. Hemogram on day eight postpartum of primiparous fed to moderate or high body wight gain during gestation.

Item	Mean		P-value[3]
	MOG[1]	HIG[2]	
Hematocrit, %	28.0 ± 2.15	29.5 ± 2.00	0.413
Total leukocytes ($\times 10^6/\mu L$)	11.55 ± 2.382	11.09 ± 2.229	0.820
Segmented neutrophils ($\times 10^6/\mu L$)	3.56 ± 0.618	3.19 ± 0.544	0.621
Rods neutrophils($\times 10^6/\mu L$)	8.70 ± 0.598	9.51 ± 0.527	0.910
Lymphocytes($\times 10^6/\mu L$)	5.44 ±0.886	6.36 ± 0.787	0.394
Platelet count ($\times 10^6/\mu L$)t	355.26 ± 93.422	347.02 ± 90.179	0.909
Total plasma protein (g/dL)	7.07 ± 0.208	7.13 ± 0.191	0.775

MOG = Moderate ADG;
[2]HIG = High ADG;
[3]Significant ($P \leq 0.10$).

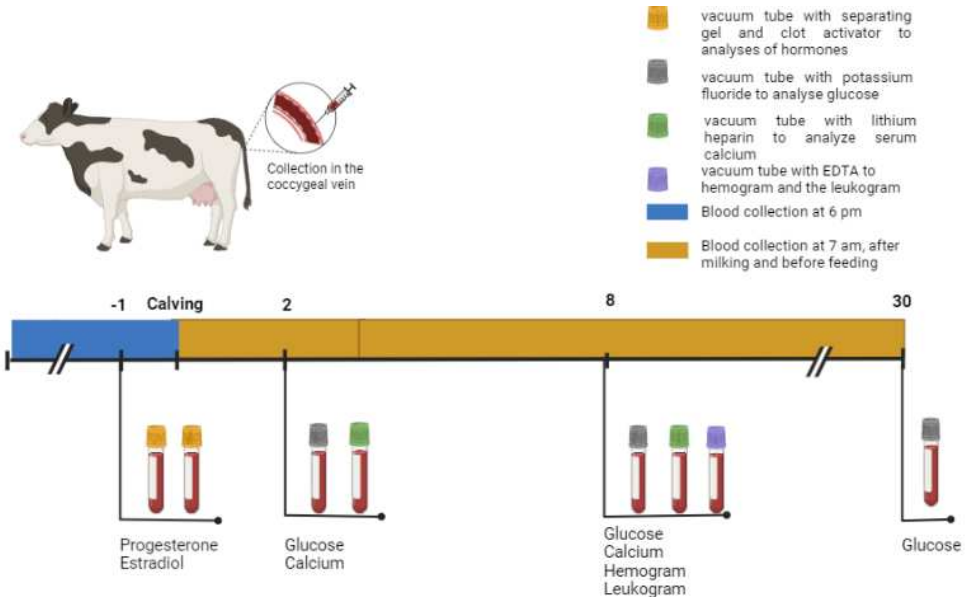

Figure 1. Prepartum and postpartum blood collection scheme in the heifers.

Figure 2. Hormonal treatment of timed artificial insemination (TAI) utilized in primiparous postpartum. Gonadotropin-releasing hormone (GnRH), prostaglandin F2α (PGF), progesterone (P4), estradiol cypionate (EC).

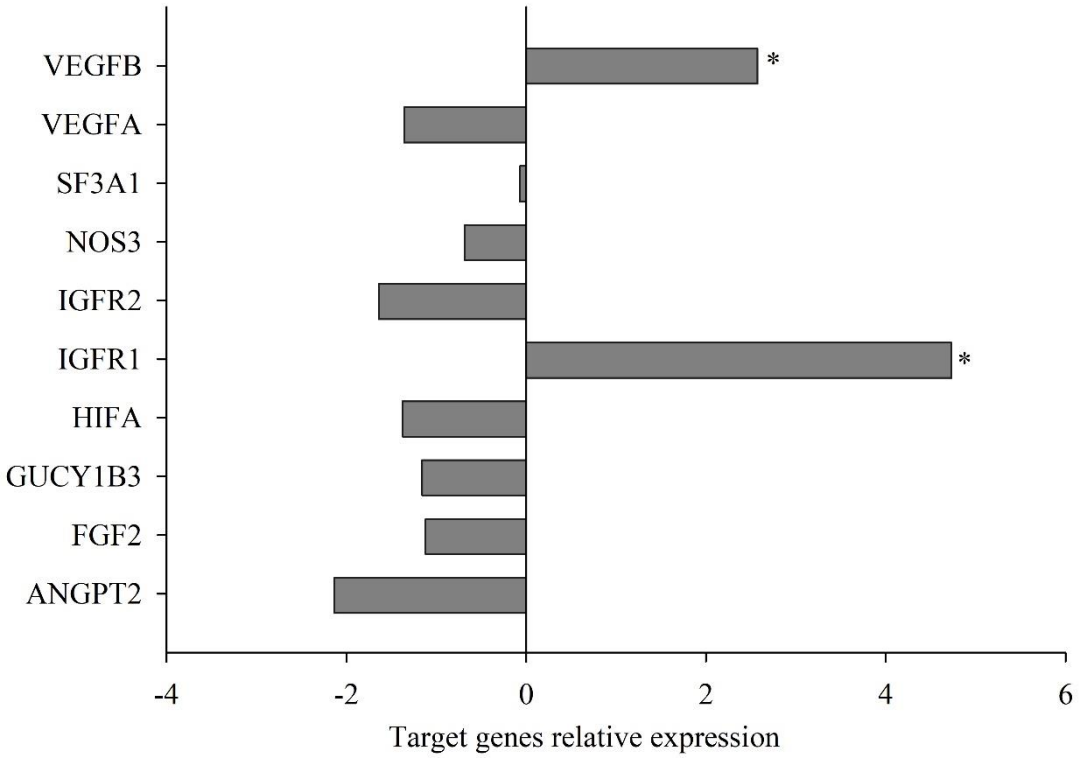

Figure 3. Target genes relative expression (MOG[1] – HIG[2]) normalized using the endogenous gene *18S* ribosomal RNA calculated as $2^{-\Delta\Delta C_T}$. On the right are the genes most expressed in the MOG treatment, and on the left are the genes most expressed in the HIG treatment.

* = significant with P < 0.10.

[1]MOG = Moderate ADG;

[2]HIG = High ADG;

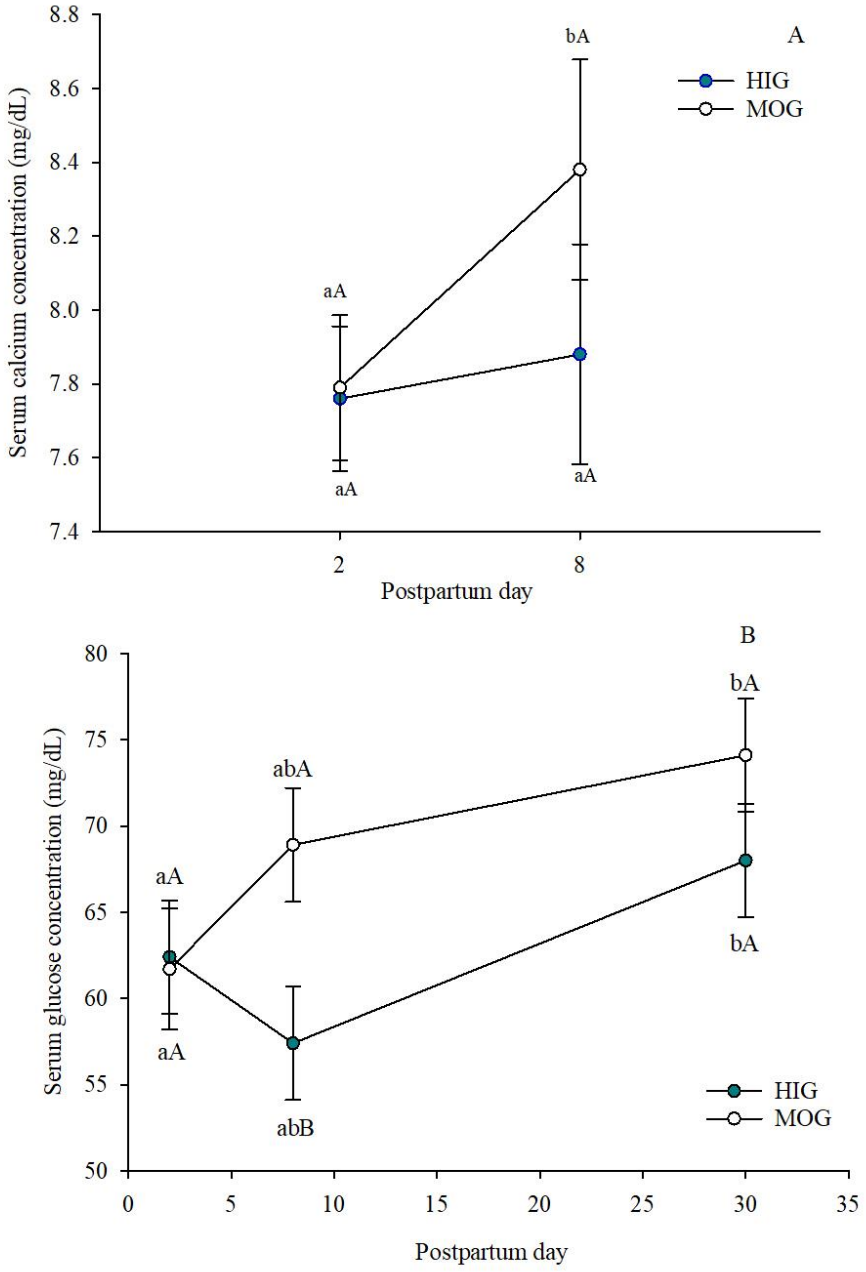

Figure 4. Means and standard error of means in (A) serum calcium concentration on days 2 and 8 postpartum and (B) serum glucose concentration on days 2, 8 and 30 postpartum in primiparous fed to moderate (MOG) or high (HIG)body weight gain during pregnancy; lowercase letters compare results inside the same treatment; uppercase letters compare the same time in different treatments.

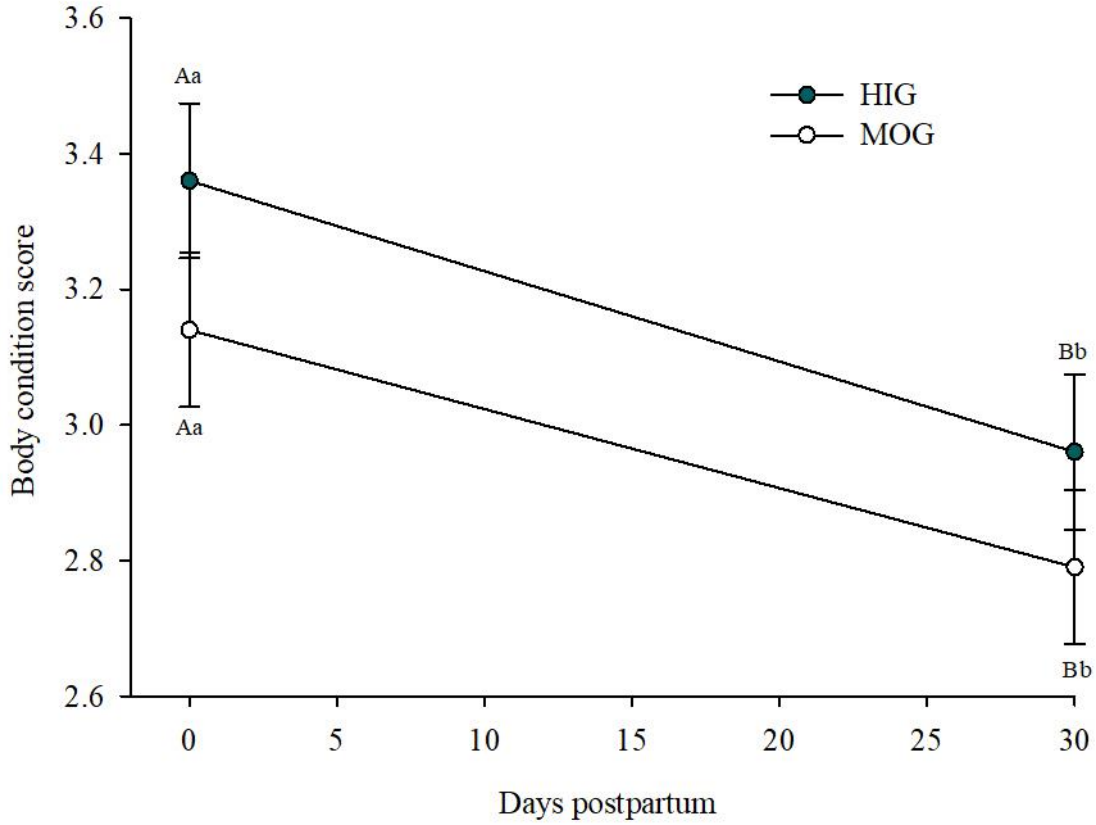

Figure 5. Means and standard error of means of body condition score on day of calving and 30 days postpartum of primiparous fed to moderate (MOG) or high (HIG) body weight gain during pregnancy. lowercase letters compare results inside the same treatment; uppercase letters compare the same time in different treatments.

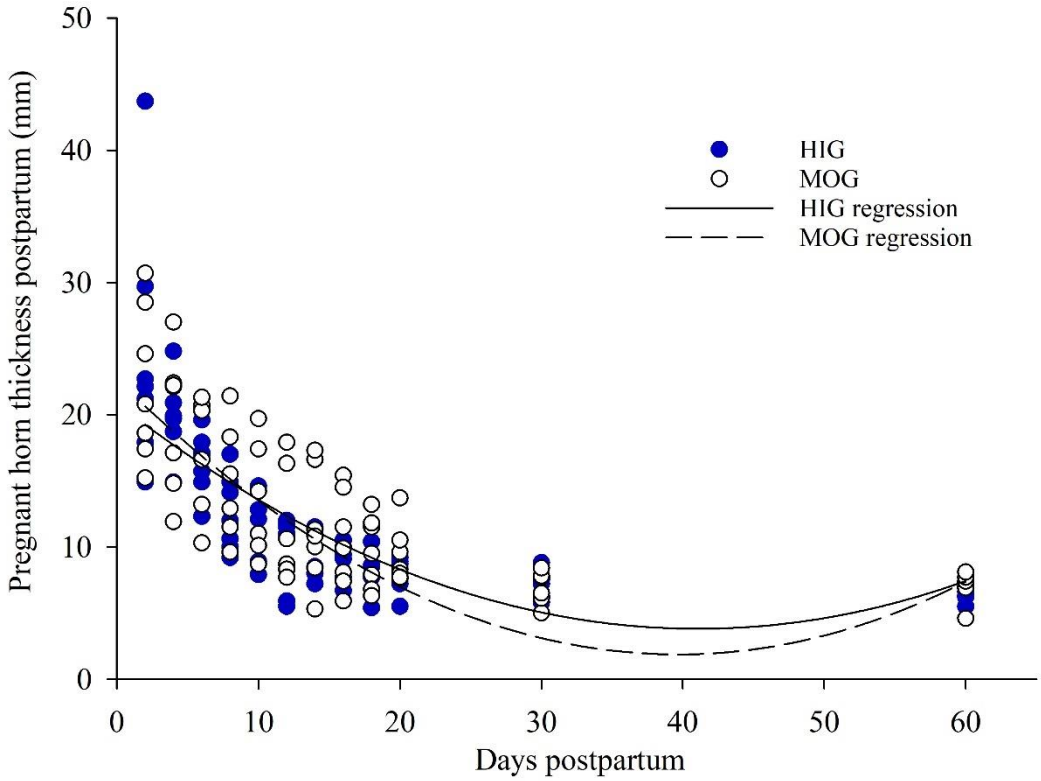

Figure 6. Uterine wall thickness (mm) measured at greater curvature of the pregnant horn from d 2 to 60 d postpartum.