POLYANA PIZZI ROTTA

COMPARISON OF PURINE BASES AND ^{15}N TO QUANTIFY MICROBIAL NITROGEN YIELD IN BEEF CATTLE AND THE EFFECTS OF DAY OF GESTATION AND FEEDING REGIMEN IN HOLSTEIN × GYR COWS

Thesis submitted to the Animal Science Graduate Program of the Universidade Federal de Viçosa as partial fulfillment of the requirements for the degree of Doctor Scientiae.

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BIOGRAPHY

Polyana Pizzi Rotta, daughter of Lazaro Joao Rotta and Jacira Pizzi Rotta, was born in Umuarama/PR-Brazil on March 17, 1987.

She started an undergraduate degree in Animal Science at the Universidade Estadual de Maringa in 2005 and obtained a Bachelor of Science in Animal Science in 2009. In 2010, she started the M.S. program with a major in ruminant nutrition and beef cattle production at the Universidade Federal de Vicosa. In February of 2012, she obtained a M.S. in Animal Science.

In the same year, she started her D.S. program in Animal Science with a major in ruminant nutrition and dairy cattle production. From August of 2013 to August of 2014, she was a visiting scholar at the Ruminant Nutrition Unit of Colorado State University, Fort Collins/CO – USA, where part of her research was developed.

On February 26th of 2015, she submitted her dissertation to the thesis committee to obtain the Doctor Scientiae degree in Animal Science.

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ABSTRACT

ROTTA, Polyana Pizzi, D.Sc., Universidade Federal de Viçosa, February of 2015. **Comparison of purine bases and ^{15}N to quantify microbial nitrogen yield in beef cattle and the effects of day of gestation and feeding regimen in Holstein × Gyr cows.** Adviser: Sebastião de Campos Valadares Filho. Co-Advisers: Marcos Inácio Marcondes and Fernanda Samarini Machado.

Microbial nitrogen yield (MN) is of great importance to protein metabolism in ruminants. The quantification of its flow to the small intestine is therefore important in calculating the amount of digestible MN available to the animal. Thus, an experiment was designed to evaluate the use of microbial markers (MM), sampling sites (SS), and marker systems (MS) to estimate MN in bulls and to develop equations to correct MN estimates. The MM systems that were evaluated were 1) purine bases (PB) and 2) ^{15}N labeling. The SS that were evaluated were 1) reticulum, 2) omasum, and 3) abomasum, and the studied MS were 1) single, 2) double, and 3) triple. Eight crossbred (Holstein × Zebu) bulls (353 ± 36.9 kg of BW; 24 ± 1 mo) with ruminal and abomasal cannulas were utilized in this experiment. For PB, the greatest ($P < 0.01$) values of MN were observed for the digesta that were sampled from the reticulum and abomasum. In contrast, for ^{15}N, the greatest ($P < 0.01$) values were observed for digesta that were sampled in the omasum and abomasum. Microbial nitrogen yield was only different ($P < 0.05$) from those estimated using abomasum and ^{15}N when using reticulum and ^{15}N. Thus, the equation that was developed to correct the MN value was as follows: MN (g/d) = $27.93 \pm 2.46 + 0.99 \pm 0.09 \times$ reticulum ^{15}N. The triple MS exhibited the greatest ($P < 0.01$) value of MN when compared to the single and double MS. No interactions ($P > 0.05$) were observed between MS and MM or SS; thus, the equation that was established to correct the MN value used only the MS. In conclusion, we have demonstrated that there is no difference when using ^{15}N to estimate MN yield if the omasum or abomasum are used. Therefore, the omasum can be used as an accurate SS to predict MN. The triple MS presented with greater values than the single and double MS. Thus, if single or double MS is used, the value must be corrected by the equation that was obtained by using the triple MS. Another study was conducted with the aim of investigating how the feeding regimen (FR) alters apparent total tract digestibility, performance, N balance, excretion of purine derivatives, fat deposition, maternal and fetal visceral organ mass, and the expression of genes that are responsible for placental development, nutrient transfer, and angiogenic factors in Holstein × Gyr cows at different days of gestation (DG). Forty-four pregnant multiparous Holstein × Gyr cows with an average initial body weight of 480 ± 10.1 kg and an initial age of 5 ± 0.5 years were allocated to 1 of 2 FR: ad libitum (AL; n = 20) and maintenance level (ML; n = 24). Maintenance level was considered to be 1.15% of body weight on a dry matter (DM) basis and met 100% of the energy requirements, while AL provided 190% of the total net energy requirements. Cows were individually fed a corn silage-concentrate based diet composed of 93% roughage and 7% concentrate (DM basis) as a total mixed ration. Pregnant cows were slaughtered at 4 DG: 139 (n = 11), 199 (n = 11), 241 (n = 11), and 268 d (n = 11). Overall, DM intake decreased as DG increased. The decrease that was observed in DM intake may be associated with the reduction in ruminal volume caused by the rapid increase in fetal size during late gestation. We observed an interaction for DM and organic matter apparent total tract digestibility between FR and DG; at 150, 178, and 206 d of gestation, ML-fed cows had greater DM and organic matter apparent total tract digestibility values than AL-fed cows. Rib fat thickness, mesentery, and kidney, pelvic, and heart fat were greater in AL- than in ML-fed cows at all DG, with the exception of rib fat thickness on d-139. Ad libitum-fed cows excreted more N in their feces and urine compared to ML-fed cows. Pregnant cows that were fed at maintenance had greater digestibility during some DG, excreted less N in feces and less N and urea in urine, and deposited less fat in the body. Mass of the heart, liver, and gastrointestinal tract were heavier in AL- than in ML-fed cows. Feeding regimen did not influence fetal body weight in this study. The majority of the visceral organ masses were similar in fetuses from cows that were fed AL and ML. This data indicates that maternal feed-restriction does not affect the development of most fetal organs and fetal development; however, some maternal organs are affected according to the FR that is provided. Mammary gland mass was heavier in AL- than in ML-fed cows, and the heaviest mass was observed at 268 d of gestation. The negative impact on mammary gland mass caused by the ML probably will not affect the subsequent lactation because the crude protein concentration in the mammary gland increased with this FR. However, we suggest that the AL diet should be provided in pregnant dry cows with caution since the amount of fat in the mammary gland increased at 268 d of gestation. Placenta was heavier in ML- than in AL-fed cows at 268 d of gestation; the lightest mass was observed at 139 d of gestation, and the heaviest mass was observed at 268 d in ML-fed cows. However, in AL-fed cows, the heaviest placenta was observed from 199 d of gestation. Placentomes 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 genes were involved in placental adaptations when ML was provided, as their expression in placentome cells were greater in ML-fed cows at 268 d of gestation. The genes that are responsible for angiogenesis were also greater in ML-fed cows. VEGFA, GUCYB3, HIFA, FGF2, and NOS3 were altered by FR and DG interaction and they were greater in ML-fed cows at 268 d of gestation. Also, VEGFB and ANGPT2 did not present an interaction between FR and DG, but they were greater in ML-fed cows. We recommend ML (1.15% of body weight with 93% of roughage) as a FR for pregnant dry cows; however, during the last month of gestation, AL seems to be the most appropriate FR to avoid loss of body

weight; however if AL diets in pregnant dry cows were to be provided, it had to be with caution since the amount of fat in the mammary gland increased at 268 d of gestation. Moreover, we suggest that the placenta from ML-fed cows develop adaptations to the reduced nutrient supply by altering its structure and gene expression, thereby developing mechanisms for a potential increase in the nutrient transfer efficiency to the fetus.

RESUMO

ROTTA, Polyana Pizzi, D.Sc., Universidade Federal de Viçosa, fevereiro de 2015. **Comparação entre bases purinas e ^{15}N para quantificar a produção de proteína microbiana em bovinos de corte e os efeitos do dia de gestação e do sistema de alimentação em vacas Holandês × Gir.** Orientador: Sebastião de Campos Valadares Filho. Coorientadores: Marcos Inácio Marcondes e Fernanda Samarini Machado.

A produção de proteína microbiana (PM) é de grande importância para o metabolismo proteico em animais ruminantes. Assim, um experimento foi conduzido para avaliar o uso de marcadores microbianos (MM), locais de coleta (LC) e sistemas de marcadores (SM) para estimar a PM em bovinos não castrados e desenvolver equações para corrigir as estimativas de PM. Os MM utilizados foram 1) bases purinas (BP) e 2) ^{15}N. Os LC avaliados foram: 1) retículo, 2) omaso e 3) abomaso e os SM estudados foram: 1) simples, 2) duplo e 3) triplo. Oito bovinos cruzados (Holandês × Zebu) não castrados (353 ± 36.9 kg de PC; 24 ± 1 meses) com fístulas ruminal e abomasal foram uilizados. Quando as BP foram utilizadas, os maiores ($P < 0,01$) valores de PM foram observados para a digesta coletada no retículo e no abomaso. Por outro lado, utiliando o ^{15}N, os maiores ($P < 0,01$) valores foram observados para a digesta coletada no omaso e abomaso. A PM foi apenas diferente ($P < 0,05$) quando utilizou-se a digesta coletada no retículo e o ^{15}N em relação à digesta coletada no abomaso e o ^{15}N. Assim, a equação desenvolvida para corrigir a PM foi: $PM (g/d) = 27,93 \pm 2,46 + 0,99 \pm 0,09 \times$ retículo ^{15}N. O SM triplo apresentou maior ($P < 0,01$) valor para a PM comparado aos SM simples e duplo. Não houve interação ($P > 0,05$) entre os SM e MM ou LC; assim, a equação recomendada para corrigir os valores de PM utiliza apenas a variável SM. Em suma, nós concluimos que não há diferença ao utilizar ^{15}N para estimar a PM desde que o local de coleta seja o omaso ou o abomaso. Dessa forma, o omaso pode ser utilizado como um acurado LC para estimar a PM. O SM triplo apresentou os maiores valores em relação aos sistemas simples e duplo. Assim, caso os sistemas simples ou duplo sejam utilizados, o valor da PM deverá ser corrigida pela equação obtida utilizando os valores do SM triplo. Um outro estudo foi conduzido objetivando investigar como o sistema de alimentação (SA) e os dias de gestação (DG) alteram a digestibilidade aparente, o desempenho, o balanço de nitrogênio, a excreção de derivados de purina, a deposição de gordura, a massa de órgaos maternos e fetais e a expressão de genes responsáveis pelo desenvolvimento, transferência de nutrientes e fatores angiogênicos na placenta em vacas leiteiras Holandes × Gir. Quarenta e quarto vacas múltiparas Holandês × Gir com um PC inicial de 480 ± 10.1 kg e com idade inicial de 5 ± 0.5 anos foram distribuídas em 1 dos 2 SA: ad libitum (AL; n = 20) e nível de mantenca (NM; n = 24). O nível de mantença foi considerado sendo 1,15% do PC (% MS) e supriu 100% da exigência de energia, enquanto que a dieta AL forneceu 190% da exigência de energia. As vacas foram individualmente alimentas com uma dieta a base de silagem de milho e concentrado na relação 93:7 (% MS). As vacas gestantes foram abatidas em 4 diferentes DG: 139 (n = 11), 199 (n = 11), 241 (n = 11) e 268 d (n = 11). De maneira geral, o consumo de MS diminuiu conforme os DG aumentaram. A diminuição que foi observada para o consumo de MS pode estar associada com a diminuição do volume ruminal causado pelo rápido aumento no tamanho do feto durante o final da gestação. Nós observamos uma interação entre a digestibilidade da MS e da matéria orgânica entre SA e DG; aos 150, 178 e 206 DG, as vacas alimentadas a nível de mantença apresentaram um maior valor para a digestibilidade aparente da MS e da matéria orgânica em relação às vacas que foram alimentadas AL. A espessura de gordura de cobertura, o mesenterio e a gordura interna foram maiores para as vacas alimentadas AL em relação às vacas alimentadas a NM, sendo a única exceção, a espessura de gordura de coberta aos 139 dias, que foi similar para os dois SA. As vacas alimentadas AL apresentaram maior excreção de N nas fezes em relação às vacas alimentadas a NM. A glândula mamária foi mais pesada em vacas alimentadas AL em relação às vacas alimentadas a NM, e o maior valor foi observado aos 268 dias de gestação. O SA não influenciou o peso fetal. A maior parte dos órgãos fetais tiveram pesos similares para as vacas alimentadas AL e a NM. Estes dados indicam que a restrição alimentar não afeta o desenvolvimento da maior parte dos órgãos fetais e o próprio desenvolimento fetal. Entretanto, o peso de alguns órgãos maternos foram afetados de acordo com o SA oferecido. Além disso, o efeito negativo sobre a glândula mamária causado pelo SA a NM provavelmente não afetará as subsequentes lactações, porque a concentração de proteína bruta na glândula mamária aumentou com o fornecimento da dieta a NM. Nós sugerimos que a dieta AL para vacas leiteiras deve ser fornecida com cuidado devido ao aumento de gordura observado nessa vacas a 268 dias de gestação. A placenta foi mais pesada em vacas alimentadas a NM em relação às vacas alimentadas AL aos 268 dias de gestação. Ainda, a placenta foi mais leve aos 139 dias e mais pesada aos 268 dias de gestação em vacas alimentadas a NM. Entretanto, para as vacas alimentadas AL, a placenta foi mais pesada a partir de 199 dias de gestação. Os placentomas foram mais pesados em vacas alimentadas a NM durante a gestação e o número de placentomas foi maior em vacas alimentadas a NM aos 268 dias de gestação. Nós observamos que os genes IGFR1 e IGFR2 foram responsáveis por adaptações placentárias quando a dieta a NM foi fornecida, pois as suas expressões em células de placentomas foram maiores em vacas alimentadas a NM aos 268 dias de gestação. Os genes reponsáveis pela angiogênese também tiveram maior expressão em vacas alimentadas a NM. A expressão dos genes VEGFA, GUCYB3, HIFA, FGF2 e NOS3 foi alterada pela interação entre SA e DG sendo maior em vacas alimentadas a NM aos 268 dias de gestação. Ainda, VEGFB e ANGPT2 não apresentaram interação entre SA e DG, mas tiveram maior expressão em vacas alimentadas a NM. Assim, nós recomendamos o NM (1,15 % do PC - dieta composta por 93% de volumoso)

como o SA para vacas gestantes secas. No entanto, durante o último mês de gestação, o SA AL parece ser o mais apropriado para evitar a perda de peso. No entanto, caso a dieta AL seja fornecida a vacas secas gestantes, isso deverá ser feito com cautela porque há um aumento na gordura da glândula mamária aos 268 dias de gestação. Ainda, nós sugerimos que a placenta de vacas alimentadas a NM desenvolve adaptações ao reduzido suprimento de nutrientes alterando sua estrutura e a expressão genética; desenvolvendo assim, mecanismos para um potencial aumento na transferência de nutrientes ao feto.

INTRODUCTION

Understanding the changes in nutrient digestion that occur in different compartments of the ruminant digestive tract may help to explain differences that occur in animal performance (Titgemeyer, 1997). However, nutritional studies face some obstacles, including the determination of the most appropriate digesta sampling site for the estimation of ruminal outflow and the best marker method (single, double, or triple) that should be used for analysis.

The decision regarding the use of the most appropriate marker method is also controversial. In Brazil, most studies use the single marker method (1 outflow phase) using abomasal or duodenal sampling. Faichney (1993) emphasized the necessity of using the double marker method (2 outflow phases), and recent studies have used the triple marker method (Hristov, 2007; Krizsan et al., 2010; Rotta et al., 2014).

The quantification of microbial nitrogen yield (MN) flow to the small intestine is important in calculating the amount of digestible MN available to the animal. Among the techniques that are used, ^{15}N, purine bases (PB), and urinary excretion of purine derivatives are the most common (Blummel and Lebzien, 2001; Belenguer et al., 2002; Ma et al., 2014) microbial markers (MM) which can be used to estimate MN flow.

According to Reynal et al. (2005) and Ipharraguerre et al. (2007), when omasal sampling of digesta and bacteria are performed to calculate MN flow, the use of ^{15}N is recommended. However, these authors did not observe differences between ^{15}N and PB when the samples were taken from the duodenum. Using ^{15}N, Krizsan et al. (2010) suggested that digesta sampled from the reticulum could be used, as opposed to digesta sampled from the omasum. However, no study with other MM using the digesta sampled in the reticulum was conducted. In addition, studies whose objectives were to evaluate the flow of MN used the triple marker system (MS) (Reynal et al., 2005; Krizsan et al., 2010) without reference to single and double MS.

Collection from the abomasum sampling site is confounded by the incomplete diversion of digesta outflow and the metabolic secretions that occur in this region (Harmon and Richards, 1997). Furthermore, animals with abomasal or duodenal cannulas require special care. These types of fistulas introduce further complications and often are only functional for a short period of time (Harmon and Richards, 1997). Therefore, studies of sites that may replace the abomasum or duodenum for digesta sampling are necessary. Recent studies have been conducted to sample reticular digesta for the estimation of ruminal outflow (Hristov, 2007; Krizsan et al., 2010; Rotta et al., 2014).

Most of the studies that evaluate MN yield have used digesta sampled from the abomasum or duodenum (Gonzalez-Ronquillo et al., 2004; Ipharraguerre et al., 2007). However, fitting animals with abomasal and/or duodenal cannulas is costly, and the cannulas can be difficult to maintain (Harmon and Richards, 1997). If the digesta sampling could be performed in the omasum or reticulum through a ruminal cannula, it would be easier to obtain the samples and manage the animals. Additionally, due to the high cost and labor-intensive nature of performing triple MS experiments, it would be beneficial to be able to use a single or double MS and make adjustments with equations derived from triple markers.

According to the National Research Council (2001), DMI is an important factor in nutrition because it establishes the amount of nutrients that are available for maintenance and production. Underfeeding nutrients can restrict production and may affect the pregnancy (Vonnahme et al., 2007). Moreover, feeding in excess of requirements increases feed costs (Herd et al., 2003), and may result in increased excretion of nutrients into the environment (Nennich et al., 2006) and fat deposition (Rincker et al., 2008; Duarte et al., 2013).

Some studies have demonstrated that DMI decreases during late gestation (Dorshorst and Grummer, 2002; Hayirli and Grummer, 2004; French, 2006); however, most of these experiments have used Holstein, and thus, information for Holstein × Gyr cows is lacking. The Gyr cattle breed (Bos indicus) is important because of its tolerance to heat and parasites, its rusticity, and its adaptation to the tropics (Silva et al., 2011; Santana et al., 2014). Limit-feeding has been shown to increase diet digestibility when compared to animals with ad libitum (AL) access to feed (Galyean et al., 1979; Murphy et al., 1994; Clark et al., 2007). However, no study has been conducted using pregnant Holstein × Gyr cows to evaluate diet digestibility during early, mid, and late-gestation of cows that are fed at maintenance level (ML) or with AL access to feed.

According to Ferrell (1988), visceral tissues are responsible for making up about 41% of the total energy expenditure even though they only represent 6% of the empty body weight (EBW) in beef cows. In non-lactating dairy cows, Smith and Baldwin (1973) found that the liver accounts for 22.5% of the total animal energy expenditure. Thus, a feeding regimen (FR) that supplies nutritional requirements without compromising fetal development may provide an alternative that increases savings in nutritional costs for dairy cow production.

There are few studies (He et al., 2013; Drackley et al., 2014) that have been conducted to evaluate the effects of FR on maternal and fetal organ mass in dairy cows. Some studies have demonstrated that underfeeding during gestation can cause intrauterine growth restriction, which results in impaired development and potential long-term consequences (He et al., 2013). Growth of the mammary gland is significantly retarded by overfeeding during the later stages of the pre-pubertal period, thus resulting in reduced milk yield (Tucker, 1981; Sejrsen et al., 1983). However, overfeeding during post puberty appears to have no ill effect on mammary growth (Tucker, 1981). However, no study has been conducted with pregnant Holstein × Gyr cows fed at different levels in order to evaluate the mammary gland composition and growth.

The majority of mammalian livestock in 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 impact fetal growth (Vonnahme et al., 2013). The relationship between maternal nutrient intake during pregnancy and the 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). Since profitability in the livestock industry is dependent upon efficiency of production characteristics such as growth and development after birth, the precursor of efficiency, namely fetal growth, must be optimal (Redmer et al., 2004; Zhu et al., 2013).

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 which develop adjacent to uterine caruncles on the uterine wall, and interdigitate with corresponding crypts of the uterine caruncle to form the placentome units (Ford, 2000).

Several studies (Vonnahme et al., 2003; Laviola et al., 2005; Zhu et al., 2007) have shown that maternal nutrient restriction alters placental 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 placental growth and function in dairy cows. Both insulin-like growth factors have a key role in regulating feto-placental growth throughout gestation (Fowden, 2013). Thus, a difference in their gene expression receptors may significantly alter the regulation of placental 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, the aims of this study were:

1) Estimate MN yield and its calculated efficiency by using PB and ^{15}N, with data obtained from three digesta SS (reticulum, omasum, and abomasum) using three MS (single, double, and triple), and to develop an adjustment while using only the abomasum as the SS, ^{15}N as the MM, and the triple MS.
2) Investigate the influence of DG and FR on apparent total tract digestibility, performance, N balance, and fat deposition in Holstein × Gyr cows.
3) Evaluate the influence of DG and FR on maternal and fetal visceral organ mass and fetal development in Holstein × Gyr cows.

4) Evaluate the placental characteristics and expression of the genes VEGFA, VEGFB, ANGPT2, FGF2, NOS3, IGFR1, IGFR2, GUGY1B3, and HIF1A in placentome cells of dairy cows fed AL or ML at different DG.

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2- Effects of day of gestation and feeding regimen in Holstein × Gyr cows:I. Apparent total tract digestibility, nitrogen balance, and fat deposition. 2015. **Journal of Dairy Science**, accepted on January, 11, 2015. JDS 14-8280.

3- Effects of day of gestation and feeding regimen in Holstein × Gyr cows:II. Maternal and fetal visceral organ mass. **Journal of Dairy Science**, accepted on January, 11, 2015. JDS 14-8282.

4- Effects of day of gestation and feeding regimen in Holstein × Gyr cows:III. Placental adaptations and placentome gene expression. **Journal of Dairy Science**, accepted on January, 11, 2015. JDS 14-8283.

LITERATURE CITED

Belenguer, A., Yanez, D., Balcells, J., Ozdemir, N.H.B, Gonzalez, R.M., 2002. Urinary excretion of purine derivative and prediction of rumen microbial out flow in goats. Livest. Prod. Sci. 77, 127-135.

Blummel, M., Lebzien, P., 2001. Predicting ruminal microbial efficiencies of dairy rations by in vitro techniques. Livest. Produc. Sci. 68, 107-117.

Borowicz, P. P., D. R. Arnold, M. L. Johnson, A. T. Grazul-Bilska, D. A. Redmer, and L. P. Reynolds. 2007. Placental growth throughout the last two thirds of pregnancy in sheep: vascular development and angiogenic factor expression. Biol. Reprod. 76:259-267.

Carmeliet, P., V. Ferreira, G. Breier, S. Pollefeyt, L. Klenkens, M. Gertsenstein, M. Fahrig, A. Vandenhoeck, K. Harpal, C. Eberhardt, C. Declercq, J. Pawling, L. Moons, D. Collen, W. Risau, and A. Nagy. 1996. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature. 380:435–439.

Clark, J. H., K. C. Olson, T. B. Schmidt, M. L. Linville, D. O. Alkire, D. L. Meyer, G. K. Rentfrow, C. C. Carr, and E. P. Berg. 2007. Effects of dry matter intake restriction on diet digestion, energy partitioning, nutrient retention, and ruminal fermentation by beef steers. J. Anim. Sci. 85:3383-3390.

Dorshorst, M. E., and R. R. Grummer. 2002. Effects of day relative to parturition and dietary crude protein on rumen fermentation in prepartum transition cows. J. Dairy Sci. 85:2290-2298.

Drackley, J. K., R. L. Wallace, D. Graugnard, J. Vasquez, B. F. Richards and J. J. Loor. 2014. Visceral adipose tissue mass in nonlactating dairy cows fed diets differing in energy density. J. Dairy Sci. 97:3420-3430.

Duarte, M. S., M. P. Giombelli, P. V. R. Paulino, N. V. L. Serao, T. S. Martins, P. I. S. Totaro, C. A. Neves, S. C. Valadares Filho, M. V. Dodson, M. Zhu, and M. Du. 2013. Effects of maternal nutrition development of gastrointestinal tract of bovine fetus at different stages of gestation. Livest. Sci. 153:60-65.

Faichney, G. J. 1993. Digesta flow. In J. M. Forbes and J. France, editors, Quantitative aspects of ruminant digestion and metabolism. CAB International, Wallingford, UK. p. 53-85.

Ferrara, N., K. Carver-Moore, H. Chen, M. Dowd, L. Lu, K. S. O'Shea, L. Powell- Braxton, K. J. Hillan, and M. W. Moore. 1996. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature. 380:439–442.

Ferrell, C. L. 1988. Energy metabolism. Pages 250-268 in The Ruminant Animal: Digestive Physiology and Nutrition. D. C. Church, ed. Prentice Hall, Englewood Cliffs, NJ.

Ford, S. P. 2000. Cotyledonary placenta. In: Knobil E, Neil JD, editors. Encyclopedia of Reproduction, vol. 1. San Diego, CA: Academic Press p. 730e8.

Fowden, A. L. 2013. The insulin-like growth factors and feto-placental growth. Placenta. 24:803-812.

French, P. D. 2006. Dry matter intake and blood parameters of nonlactating Holstein and Jersey cows in late gestation. J. Dairy Sci. 89:1057-1061.

Galyean, M. L., D. G. Wagner, and F. N. Owens. 1979. Level of feed intake and site and extent of digestion of high concentrate diets by steers. J. Anim. Sci. 49:199-203.

Godfrey, K. M. 2002. The role of the placenta in fetal programming—a review. Placenta. Suppl A:S20–7.

Godfrey, K. M., and Barker, D. J. 2000. Fetal nutrition and adult disease. Am. J. Clin. Nutr. 7:1344–1352.

Gonzales, J., Arroyo, J.M., Ouarti, M., Guevara-Gonzales, J., Rodriguez, C.A., Alvir, M.R., Moya, V.J., Piquer, O., 2012. Composition of free and adherent ruminal bacteria inaccuracy of the microbial nutrient supply estimates obtained using free bacteria as reference samples and 15N as the marker. Anim. 6, 468-475.

Gonzalez-Ronquillo, M., Balcells, J., Belenguer, A., Castrillo, C., Mota, M., 2004. A comparison of purine derivated excretion with conventional methods as indices of microbial yield in dairy cows. J. Dairy Sci. 87, 2211-2221

Harmon, D. L., and C. J. Richards. 1997. Considerations for gastrointestinal cannulations in ruminants. J. Anim. Sci. 75:2248-2255.

Hayirli, A., and R. R. Grummer. 2004. Factors affecting dry matter intake prepartum in relationship to etiology of peripartum lipid-related metabolic disorders: A review. Can. J. Anim. Sci. 84:337-347.

He, Z. X., D. Q. Wu, Z. H. Sun, Z. L. Tan, J. Y. Qiao, T. Ran, S. X. Tang, C. S. Zhou, X. F. Han, M. Wang, J. H. Kang, and K. A. Beauchemin. 2013. Protein or energy restriction during late gestation alters fetal growth and visceral organ mass: An evidence of intrauterine programming in goats. Anim. Reprod. Sci. 137:177-182.

Herd, R. M., J. A. Archer, and M. E. van Amburgh. 2003. Reducing the cost of beef cattle production through genetic improvement in residual feed intake: Opportunity and challenges to application. J. Anim. Sci. 81:E9-E17.

Hristov, A. N. 2007. Comparative characterization of reticular and duodenal digesta and possibilities of estimating microbial outflow from the rumen based on reticular sampling in dairy cows. J. Anim. Sci. 85:2606–2613.

Ipharraguerre, I.R., Reynal, S.M., Lineiro, M., Broderick, G.A., 2007. A comparison of sampling sites, digesta and microbial markers, and microbial references for assessing the post ruminal supply of nutrients in dairy cows. J. Dairy Sci. 90, 1904-1919.

Krizsan, S. J., S. Ahvenjärvi, H. Volden, and G. A. Broderick. 2010. Estimation of rumen outflow in dairy cows fed grass silage-based diets by use of reticular sampling as an alternative to sampling from the omasal canal. J. Dairy Sci. 9:1138–1147.

Laviola, L., S. Perrini, G. Belsanti, A. Natalicchio, C. Montrone, A. Leonardini, A. Vimercati, M. Scioscia, L. Selvaggi, R. Giorgino, P. Greco, and F. Giorgino. 2005. Intrauterine growth restriction in humans is associated with abnormalities in placental insulin-like growth factor signaling. Endocrinology. 146:1498-1505.

Ma, T., Deng, K.D., Tu, Y., Zhang, N.F., Jiang, C.G., Liu, J., Zhao, Y.G., Diao, Q.Y., 2014. Effect of dietary forage-to-concentrate ratios on urinary excretion of purine derivatives and microbial nitrogen yields in the rumen of Dorper crossbred sheep. Livest. Sci. 160, 37-44.

Meyer, A. M., J. J. Reed. T. L. Neville, J. B. Taylor, C. J. Hammer, L. P. Reynolds, D. A. Redmer, K. A. Vonnahme, and J. S. Caton. 2010b. Effects of plane of nutrition and selenium supply during gestation on ewe and neonatal offspring performance, body composition, and serum selenium. J. Anim. Sci. 88:1786−1800.

Murphy, T. A., S. C. Loerch, and F. E. Smith. 1994. Effects of feeding high-concentrate diets at restricted intakes on digestibility and nitrogen metabolism in growing lambs. J. Anim. Sci. 72:1583-1590.

National Research Council. 2001. Nutrients Requirements of Dairy Cattle. 7th rev. ed. Natl. Acad. Press, Washington, DC.

Nennich, T. D., J. H. Harrison, L. M. van Wieringen, N. R. St-Pierre, R. L. Kincaid, M. A. Wattiaux, D. L. Davidson, and E. Block. 2006. Prediction and evaluation of urine and urinary nitrogen and mineral excretion from dairy cattle. J. Dairy Sci. 89:353-364.

Redmer, D. A., J. M. Wallace, and L. P. Reynolds. 2004. Effect of nutrient intake during pregnancy on fetal and placental growth and vascular development. Domestic Anim. Endocrin. 27:199-217.

Reed, J. J., M. A. Ward, K. A. Vonnahme, T. L. Neville, S. L. Julius, P. P. Borowicz, J. B. Taylor, D. A. Redmer, A. T. Grazul- Bilska, L. P. Reynolds, and J. S. Caton. 2007. Effects of selenium supply and dietary restriction on maternal and fetal body weight, visceral organ mass and cellularity estimates, and jejunal vascularity in pregnant ewe lambs. J. Anim. Sci. 85:2721–2733.

Reynal, S.M., Broderick, G.A., Bearzi, C., 2005. Comparison of four markers for quantifying microbial protein flow from the rumen of lactating dairy cows. J. Dairy Sci. 88, 4065-4082.

Reynal, S.M., Broderick, G.A., Bearzi, C., 2005. Comparison of four markers for quantifying microbial protein flow from the rumen of lactating dairy cows. J. Dairy Sci. 88, 4065-4082.

Reynolds, L. P., and D. A. Redmer. 1992. Growth and microvascular development of the uterus during early pregnancy in ewes. Biol. Reprod. 47:698–708.

Rincker, L. E. D., M. S. W. Nielsen, L. T. Chapin, J. S. Liesman, and M. J. CandeHaar. 2008. Effects of feeding prepubertal heifers a high-energy diet for three, six, or twelve weeks on feed intake, body growth, and fat deposition. J. Dairy Sci. 91:1913-1925.

Rotta, P.P., Valadares Filho, S.C., Detmann, E., Costa e Silva, L.F., Paulino, M.F., Marcondes, M.I., Lobo, A.A.G., Villadiego, F.A.C. 2014. Digesta sampling sites and marker methods for estimation of ruminal outflow in bulls fed different proportions of corn silage or sugarcane. J. Anim. Sci. 92, 1-11.

Santana, M. L., R. J. Pereira, A. B. Bignardi, L. El Faro, H. Tonhati, and L. G. Albequerque. 2014. History, structure, and genetic diversity of Brazilian Gyr cattle. Livest. Sci. 163:26-33.

Scheaffer, A. N., J. S. Caton, D. A. Redmer, D. R. Arnold, and L. P. Reynolds. 2004a. Effect of dietary restriction, pregnancy, and fetal type on intestinal cellularity and vascularity in Columbia and Romanov ewes. J. Anim. Sci. 82:3024–3033.

Sejrsen, K., J. T. Huber, and H. A. Tucker. 1983. Influence of amount fed on hormone concentrations and their relationship to mammary growth in heifers. J. Dairy Sci. 66:845-855.

Shalaby, F., J. Rossant, T. P. Yamaguchi, M. Gertsenstein, X. F. Wu, M. L. Breitman, and A. C. Schuh. 1995. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature. 376:62–66.

Silva, A. A., A. L. S. Azevedo, R. S. Verneque, K. Gasparini, M. G. C. D. Peixoto, M. V. G. B. Silva, P. S. Lopes, S. E. F. Guimaraes, and M. A. Machado. 2011. Quantitative trait loci affecting milk production traits on bovine chromosome 6 in Zebuine Gyr breed. J. Dairy Sci. 94:971-980.

Smith, R. L., and R. L. Baldwin. 1973. Effects of breed, pregnancy, and lactation on weight of organs and tissues in dairy cattle. J. Dairy Sci. 57:1055-1060.

Smith, R. L., and R. L. Baldwin. 1973. Effects of breed, pregnancy, and lactation on weight of organs and tissues in dairy cattle. J. Dairy Sci. 57:1055-1060.

Smith, R. L., and R. L. Baldwin. 1973. Effects of breed, pregnancy, and lactation on weight of organs and tissues in dairy cattle. J. Dairy Sci. 57:1055-1060.

Titgemeyer, E. C. 1997. Design and interpretation of nutrient digestion studies. J. Anim. Sci. 75:2235–2247.

Tucker, H. A. 1981. Physiological control of mammary growth, lactogenesis, and lactation. J. Dairy Sci. 64:1403-1421.

Vonnahme, K. A., B. W, Hess, T. R. Hansen, R. J. McCormick, D. C. Rule, G. E. Moss, W. J. Murdoch, M. J. N. Nijland, D. C. Skinner, P. W. Nathanielsz, and S. P. Ford. 2003. Maternal undernutrition from early- to mid-gestation leads to growth retardation, cardiac ventricular hypertrophy, and increased liver weight in the fetal sheep. Biol. Reprod. 69:133-140.

Vonnahme, K. A., C. O. Lemley, P. Shukla, and S. T. O'Rourke. 2013. 2011 and 2012 Early Careers Achievement Awards: Placental programming: How the maternal environment can impact placental function. J. Anim. Sci. 91:2467-2480.

Vonnahme, K. A., M. J. Zhu, P. P. Borowicz, T. W, Geary, B. W. Hess, L. P. Reynolds, J. S. Caton, W. J. Means, and S. P. Ford. 2007. Effect of early gestational undernutrition on angiogenic factor expression and vascularity in the bovine placentome. J. Anim. Sci. 85:2464-2472.

Zhu, M. J., M. Du., and S. P. Ford. 2013. Impacts of maternal obesity on placental and gut inflammation and health. J. Anim. Sci. 92:1840-1849.

CHAPTER 1

Comparison of purine bases and ^{15}N for quantifying microbial nitrogen yield using three marker systems and different sampling sites in Zebu cross breed bulls

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ABSTRACT

The present experiment was designed to evaluate the use of microbial markers (MM), sampling sites (SS), and marker systems (MS) to estimate microbial nitrogen (MN) synthesis in bulls and to develop equations to correct MN estimates when only one of the aforementioned techniques was utilized. The MM systems evaluated were 1) purine bases (PB) and 2) ^{15}N labeling. The SS evaluated were: 1) reticulum, 2) omasum, and 3) abomasum, and the single, double and triple MS were evaluated. Eight crossbred (Holstein × Zebu) bulls (353 ± 36.9 kg of BW; 24 ± 1 mo) with ruminal and abomasal cannulas were utilized in this experiment. The following experimental diets were used: 1) 60% corn silage + 40% concentrate, 2) 40% corn silage + 60% concentrate, 3) 60% fresh sugarcane + 40% concentrate, and 4) 40% fresh sugarcane + 60% concentrate. Four experimental periods lasting 16 d each were completed with 10 d for adaptation to the experimental diet and 6 d for sampling. Bulls were randomly distributed into two 4 × 4 Latin squares balanced for residual effects. Data were analyzed in a Latin square design using PROC MIXED. Interactions were observed ($P < 0.05$) in MN, microbial crude protein/total digestible nutrients (MCP/TDN), microbial nitrogen/rumen fermented organic matter (MN/RFOM), microbial nitrogen/rumen truly fermented organic matter (MN/RTFOM), and microbial dry matter/rumen fermented total carbohydrates (MDM/RFTCHO) between SS and MM. For PB, the greatest ($P < 0.01$) values of MN were observed for the digesta sampled in the reticulum and abomasum. In contrast, for ^{15}N, the greatest ($P < 0.01$) values were observed for digesta sampled in the omasum and abomasum. Microbial nitrogen yield was only different ($P < 0.05$) when using reticulum and ^{15}N from those estimated using abomasum and ^{15}N. Thus, the equation developed to correct MN value was: $MN\ (g/d) = 27.93 \pm 2.46 + 0.99 \pm 0.09 \times \text{reticulum}\ ^{15}N$. The triple MS exhibited the greatest ($P < 0.01$) value of MN compared to the single and double MS. No interactions ($P > 0.05$) were observed between MS and MM or SS; thus, the equation established to correct MN value used only the MS. In conclusion, we have demonstrated that there is no difference using ^{15}N to estimate MN yield if omasum or abomasum are used. Therefore, the omasum can be used as an accurate SS to predict MN. The triple MS presented higher values than the single and double MS. Thus, if single or double MS is used the value must be corrected by the equation obtained using the triple MS.

Keywords: abomasum; omasum; purine bases; reticulum; Zebu; ^{15}N

1. Introduction

Microbial nitrogen yield (MN) is of great importance to protein metabolism in ruminants (Bach et al., 2005; Broderick et al., 2010). The quantification of its flow to the small intestine is therefore important in calculating the amount of digestible MN available to the animal. Various microbial markers (MM) can be used to estimate MN flow (Carro and Miller, 2002; Ipharraguerre et al., 2007). Among the techniques used, ^{15}N, purine bases (PB), and urinary excretion of purine derivatives are the most common (Blummel and Lebzien, 2001; Belenguer et al., 2002; Ma et al., 2014).

However, according to Reynal et al. (2005) and Ipharraguerre et al. (2007), when omasal sampling of digesta and bacteria are performed to calculate MN flow, the use of ^{15}N is recommended. In contrast, these authors did not observe differences between ^{15}N and PB when the samples were taken from the duodenum. Using ^{15}N, Krizsan et al. (2010) suggested that digesta sampled from the reticulum could be used, as opposed to digesta sampled from the omasum. However, there is no study with other MM using the digesta sampled in the reticulum. In addition, studies whose objective was to evaluate the flow of MN used the triple marker system (MS) (Reynal et al., 2005; Krizsan et al., 2010) without reference to single and double MS.

Most of the studies that evaluate MN yield have used digesta sampled in the abomasum or duodenum (Gonzalez-Ronquillo et al., 2004; Ipharraguerre et al., 2007). However, fitting animals with abomasum and/or duodenal cannulas is costly, and the cannulas can be difficult to maintain (Harmon and Richards, 1997). If the digesta sampling could be performed in the omasum or reticulum through a ruminal cannula, it would be easier to obtain the samples and manage the animals. Additionally, due to the high cost and labor-intensive nature of performing triple MS experiments, it would be beneficial to be able to use a single or double MS and make adjustments with equations derived from triple markers.

Our hypothesis is that reticulum and omasum could be used as SS to estimate MN yield and that the double MS could be used instead of the triple MS. We also believe that different diets could influence the MN yield depending on the SS.

The objectives of this study were to estimate MN yield and its efficiency calculated using PB and ^{15}N, with data obtained from three digesta SS (reticulum, omasum, and abomasum) using three MS (single, double, and triple) in beef cattle fed diets characteristic of a tropical climate. The objectives were also to develop an adjustment for using only the abomasum as the SS, ^{15}N as the MM, and the triple MS.

2. Materials and methods

2.1. Animals, experimental design, and diets

This study was approved by the Institutional Animal Care and Use Committee at the Federal University of Viçosa.

The experiment was conducted at the Experimental Feedlot of Animal Science Department in Viçosa, Brazil. Laboratory analyses were conducted at the Ruminant Nutrition Laboratory at Animal Science Department at Federal University of Viçosa, Brazil.

Eight crossbred (Holstein × Zebu) bulls (353 ± 36.9 kg of BW; 24 ± 1 mo) with ruminal and abomasal cannulas were randomly distributed into two 4 × 4 Latin squares balanced for residual effects. The bulls were offered feed as TMR twice daily at 7:00 and at 15:00 h, in amounts that allowed ad libitum access to feed throughout the day. Bulls were housed in tie stalls with free access to water throughout the experiment. Four experimental diets, three digesta SS, three MS and two MM were assessed for the estimation of ruminal outflow of MN and efficiency calculated as: microbial crude protein (CP)/total digestible nutrients (MCP/TDN), microbial nitrogen/rumen fermented organic matter (MN/RFOM), microbial nitrogen/rumen truly fermented organic matter (MN/RTFOM), and microbial dry matter/rumen fermented total carbohydrates (MDM/RFTCHO). The following experimental diets were used: (dry matter (DM) basis): 1) 60% corn silage (CS) + 40% concentrate (CO), 2) 40% CS + 60% CO, 3) 60% fresh sugar cane (SC) + 40% CO, and 4) 40% SC + 60% CO.

The DM of the CS and SC diets were determined daily to adjust the amount of urea (U) and ammonium sulfate [(AS); 9:1, U:AS] supplied to the bulls, and the U:AS mixture was used to adjust the CP content of the diets to 120 g/kg DM (19.2 g of nitrogen (N)/kg DM). The DM content was analyzed daily in duplicate, using a conventional microwave oven, according to recommendations from the National Forage Testing Association (1993). Feeds and orts were weighed daily, sampled, and frozen for later analysis.

The chemical compositions of the feeds used in the experimental diets are shown in Table 1. The concentrate used in all diets consisted of 90.4% ground corn, 7.90% soybean meal, 0.85% mineral mixture, and 0.85% NaCl (on a DM basis; Table 2). The experimental isonitrogenous diets were formulated to provide an average daily gain of 1.10 kg/d according to the Brazilian BR-CORTE system described by Valadares Filho et al. (2010).

Besides the samplings procedures performed in the animals used in this study, the DM intake ranged between 7.32 to 8.64 kg/d on DM basis. Animals fed CS 60:40 and 40:60 had 8.08 and

8.63 kg/d DM intake, respectively. Animals fed SC 60:40 and 40:60 had 7.32 and 8.64 kg/d DM intake, respectively (Rotta et al., 2014).

2.2. Procedures for sampling and preparation of digesta samples

Four experimental periods lasting 16 d each were completed. Each period consisted of 10 d of adaptation to the next experimental diet and three days of fecal sampling. On days 14 to 16 of the experiment, reticular, omasal, and abomasal digesta were sampled, and feed intake was measured. Additionally, orts and forages were sampled.

Continuous infusion of the flow markers Yb acetate (2.50 g/d of Yb acetate or 1.05 g/d of Yb) and Co-EDTA (5.00 g/d of Co-EDTA or 0.70 g/d of Co) were performed using two peristaltic pumps (model BP-600.4 - Milan Scientific Equipment, Inc., Colombo, Paraná, Brazil) from day 11 of the experimental period until the last sampling of digesta on day 16. From the 12th day of the experimental period, 7.03 g ammonium sulfate enriched with 10 atom% ^{15}N [ammonium sulfate $(^{15}NH_4)_2SO_4$] [Sigma Aldrich (Isotec), Miamisburg, OH] was included in the marker solution, providing a daily supply of 150 mg of ^{15}N/total N ratio to each animal.

A total of eight digesta samples were collected from the reticulum, omasum, and abomasum at 9 hour intervals over a 3 days period. The sampling times were 0:00, 9:00, 18:00, 3:00, 12:00, 21:00, 6:00, and 15:00 h. The sampling sequence was abomasum, omasum, and reticulum with equal time between each SS.

Reticular, omasal, and abomasal digesta sampling and sample preparations were detailed in Rotta et al. (2014). Briefly, reticular digesta was sampled according to Krizsan et al. (2010) using a 250 mL screw cap container inserted through the rumen with the lid closed and then opened in the reticulum. The technique of Huhtanen et al. (1997) with adaptations described in Leão (2002), was used for omasal digesta sampling. The abomasal digesta were sampled through an abomasal cannula.

After every four samplings of 250 mL digesta (12:00, 9:00, 18:00, and 3:00 then 0:00, 21:00, 6:00, and 15:00 h), samples for bacterial isolation were taken according to Reynal et al. (2005) with modifications suggested by Krizsan et al. (2010).

All samples were stored at -80 °C and were lyophilized once frozen. After lyophilization, the samples were ground in a knife mill with 2-mm and 1-mm sieves (Wiley mill; A. H. Thomas, Philadelphia, PA).

2.3. Indigestible neutral detergent fiber and chemical analysis

The ground digesta samples were analyzed for DM, organic matter (OM) and N (AOAC, 2000; method number 934.01 for DM, 930.05 for OM, and 981.10 for N); ether extract (EE) was analyzed according to AOAC (2006); neutral detergent fiber (NDF) was analyzed according to the technique described by Mertens (2002), without the addition of sodium sulfite but with the addition of thermostable alpha-amylase to the detergent (Ankom Tech. Corp., Fairport, NY); NDF, neutral detergent insoluble nitrogen and acid detergent insoluble nitrogen were analyzed as described by Licitra et al. (1996); and acid detergent fiber was determined as described by Van Soest et al. (1991).

Indigestible neutral detergent fiber (iNDF) was quantified in triplicate on reticular, omasal, and abomasal digesta samples (single MS; double MS: particles and fluid; and triple MS: large particles and small particles), and for orts and feeds. The samples used for quantifying iNDF were ground in a knife mill with a 2-mm sieve (Valente et al., 2011). Subsamples weighing approximately 1.5 g were added to pre-weighed polyester bags with a pore size of 12 µm and a pore area equal to 6% of the total surface (Saatifil PES 12/6, Saatitech S.p.A., Veniano, Como, Italy). The bags were incubated for 288 hours in the rumen of two bulls fed a diet consisting of 50% CS and 50% CO (DM basis). After removal from the rumen, the bags were rinsed in a household washing machine, dried at 45 °C for 48 hours, and weighed. Residues were then analyzed for NDF in an Ankom 200/220 Fiber Analyzer (Ankom Technology Corp., Fairport, NY). Heat-stable α-amylase (Mertens et al., 2002) was used in the determination of NDF.

Non-fiber carbohydrates (NFC) were calculated according to Detmann and Valadares Filho (2010), where NFC (g/kg of DM) = 1000 – [CP – (CP derived from urea + urea) + NDF + EE + minerals]. Starch concentration was analyzed according to method 7611 of the American Association of Cereal Chemists (2003) on a Roch Cobas Mira S instrument (Roche Diagnostics, Basel, Switzerland). Water-soluble carbohydrate in the forages was determined as described by Thomas (1977). The OM content of forages and effluents was analyzed by combustion at 550 °C for 6 hours in a muffle furnace.

For non-protein nitrogen (NPN) determination, a sample suspension containing 5 g of ground forage and 45 mL of NaCl solution (9 g/L) was prepared and homogenized (5 minutes, 1,000 × g, and 20 °C) with an Ultra-Turrax T25 (Janke and Kundel, IKA-Labortechnik, Staufel, Germany). Twenty mL of the suspension was mixed with 20 mL of trichloroacetic acid and was homogenized (2 minutes, 1,000 × g, and 20 °C). The mixture was incubated (1 h and 25 °C) and filtered through Whatman 42 paper. The NPN filtrates were analyzed with the Kjeldahl method.

Cobalt was analyzed by an atomic absorption spectrophotometer (Spctr AA-800; Varian spectrometer, Harbour City, CA) according to the method described by Kimura and Miller (1957).

Ytterbium was analyzed by an atomic absorption spectrophotometer (Spctr AA-800; Varian spectrometer, Harbour City, CA) according to Siddons et al. (1985).

The PB analyses were performed according to Ushida et al. (1985) with modifications from Zinn and Owens (1986). For the analysis of ^{15}N, samples of liquid-associated bacteria (LAB), solid-associated bacteria (SAB), and the digesta were analyzed according to Machado et al. (2013). Briefly, ^{15}N atom excess was measured using an isotope ratio mass spectrometer (Delta S; Finnigan MAT, Bremen, Germany). Samples containing approximately 100 µg of N were weighed and placed in 5 by 8 mm capsules for future readings. The ratios of stable isotopes of the same chemical element (^{15}N:^{14}N) were evaluated in terms of Δ per thousand, according to international standards, and were converted to percentages of atoms in excess.

2.4. Calculations

Digesta flow was calculated based on single marker (iNDF) and the reconstitution technique pioneered by Faichney (1975) using a combination of two or three markers. Use of the double and triple markers systems is well established, and their use has been extensively documented in the literature (Faichney, 1975; France and Siddons, 1986). Measurements of digesta flow using double marker systems were based on Co as the fluid phase marker and on iNDF as the independent particle phase marker. Flows estimated according to triple marker systems were based on Co as the fluid, Yb as the small particle, and iNDF as large particle phase markers.

According to Ahvernjarvi et al. (2003), markers exhibited substantial variation in the distribution between digesta phases. This finding can be interpreted as an indication of the extent of unrepresentative sampling. None of the markers studied by Ahvenjarvi et al. (2003) was uniformly distributed across all digesta phases. However, Co was primarily associated with the liquid phase, Yb with small particles, and iNDF with large particulate matter.

In the double and the triple marker method calculations, the reconstitution factor (RF) was calculated based on the concentrations of markers in the different phases of digesta (France and Siddons, 1986). The RF of the small and large particle phases were used to mathematically reconstruct the composition of reticular, omasal, and abomasal true digesta.

2.5. Statistical analysis

^{15}N atom excess of digesta, chemical composition, and ^{15}N atom excess of LAB and SAB were analyzed in a Latin square design using PROC MIXED (SAS Inst. Inc., Cary, NC) according to the following the model:

$$Y_{ijklmn} = \mu + l_i + D_j + a_{(i)k} + p_l + S_m + DS_{jm} + e_{ijklm}$$

where μ = the overall mean; D_j = effect of the diet j; $a_{(i)k}$ = effect of the animal k nested in the Latin square i; p_l = effect of the experimental period l; S_m = effect of sampling site m; DS_{jm} = effect of the interaction between D_j and S_m; e_{ijklm} = is the random residual error. Reported values are least squares means. Animal and period were included as random effects.

Microbial CP/total digestible nutrients (g of microbial CP/kg of total digestible nutrients/day], MN/RFOM (g of MN/kg of rumen fermented /day), MN/RTFOM (g of MN/kg of truly rumen fermented OM/day) and MDM/RFTCHO (g of microbial DM/kg of ruminal fermented total carbohydrates/day) were analyzed with repeated measurements in space using the PROC MIXED (SAS Inst. Inc., Cary, NC) according to the following model:

$$Y_{ijklmn} = \mu + l_i + D_j + a_{(i)k} + p_l + S_m + DS_{jm} + M_n + DM_{jn} + R_o + DR_{jo} + SM_{mn} + SSR_{mo} + MR_{no} + SMR_{mno} + DSMR_{jmno} + e_{ijklmno}$$

where μ = the overall mean; D_j = effect of the diet j; $a_{(i)k}$ = effect of the animal k nested in the Latin square i; p_l = effect of the experimental period l; S_m = effect of sampling site m; M_n = effect of the marker system n; R_o = effect of the microbial marker o; $e_{ijklmno}$ = is the random residual error. Reported values are least squares means. Interactions between the factors are also indicated in the model. Sampling site represented repeated measures for each animal, and marker systems and microbial markers represented repeated measures for each sampling site. Animal and period were included as random effects. Statistical significance was declared at $P < 0.05$

If differences among SS, MS, and MM were observed, a regression using linear and nonlinear models on MIXED and NLMIXED procedures, respectively (SAS Inst. Inc., Cary, NC) was performed. For all comparisons, the critical level of probability for type I error was $P < 0.05$.

3. Results

3.1. Enrichment of digesta with ^{15}N atoms

No interaction ($P > 0.05$) was detected between SS and diet for ^{15}N atom excess in the digesta. There was no difference ($P > 0.05$) in ^{15}N enrichment for the digesta evaluated for the single MS considering the SS evaluated (Table 3).

However, a difference ($P < 0.01$) was observed for the double MS. The greatest ($P < 0.001$) ^{15}N enrichment was observed for the digesta collected in the reticulum and abomasum compared to the omasum for the particle phase. For the fluid phase, the greatest ($P < 0.001$) enrichment was observed for the digesta collected from the reticulum.

For the triple MS, the greatest ($P < 0.001$) value for ^{15}N enrichment was observed for the digesta collected in the reticulum and in the abomasum of the large- and small-particles phases. However, for the fluid phase of digesta collected in the reticulum, the ^{15}N enrichment was greater ($P < 0.05$) than for the digesta collected in the omasum and abomasum.

3.2. Isolated bacteria

There was no interaction ($P > 0.05$) between SS and diet for OM (% in DM), N (% OM), and ^{15}N (atoms percent excess) in LAB and SAB sampled in the reticulum, omasum, and abomasum (Table 4). However, the percentages of OM (% in DM) of LAB collected in the omasum and abomasum were greater ($P < 0.001$) relative to the LAB collected in the reticulum. In contrast, the OM (% in DM) of SAB was the lowest ($P < 0.001$) for the digesta collected in the reticulum, intermediate ($P < 0.001$) for the digesta collected in the omasum, and greatest ($P < 0.001$) for the digesta collected in the abomasum.

Nitrogen (% OM) in LAB and SAB were greater ($P < 0.05$) for the digesta collected in the reticulum and omasum compared with the digesta collected in the abomasum.

However, the greatest ($P < 0.001$) values for ^{15}N enrichment in LAB and SAB, were observed for the digesta collected in the reticulum. Similar values ($P > 0.05$) were observed for LAB and SAB in the digesta collected in the omasum and abomasum.

A difference ($P < 0.05$) was observed in the OM (%DM) between LAB and SAB sampled in the reticulum and abomasum. The greatest ($P < 0.05$) values were observed in the SAB. No difference ($P > 0.05$) was observed between LAB and SAB sampled in the omasum.

Nitrogen (% OM) content was greater ($P < 0.05$) in the LAB than in the SAB independent of the SS used. The ^{15}N values (atoms percent excess) did not differ ($P > 0.05$) between LAB and SAB in the different SS studied.

3.3. Microbial nitrogen yield and efficiency

Interactions between diets and SS, MS, or MM were not significant ($P > 0.05$; Table 5). However, differences ($P < 0.05$) in MN, MCP/TDN, MN/RFOM, MN/TRFOM, and MDM/RFTCHO among the diets evaluated were observed (Tables 5 and 6). Microbial nitrogen yield was greater ($P < 0.01$) in animals fed with CS 40:60 and SC 40:60 diets, indicating that diets with higher CO proportion provide greater MN.

MCP/TDN, MN/RFOM, and MN/TRFOM were lower ($P < 0.05$) in animals fed SC 40:60. However, no differences ($P > 0.05$) were observed among SC 40:60, CS 60:40, and CS 40:60 fed animals for MN/RFOM and MN/TRFOM. The MDM/RFTCHO was lower ($P < 0.01$) in animals receiving the CS 60:40 when compared to animals fed other diets.

Interactions were observed ($P < 0.05$) between MN, MCP/TDN, MN/RFOM, MN/RTFOM, and MDM/RFTCHO for SS and MM. Interaction was also observed ($P < 0.05$) for MS and MM to MCP/TDN (Table 5).

Using PB, the greatest ($P < 0.01$) value of MN was observed for digesta sampled in the abomasum compared with the digesta sampled in the omasum (Table 7). In contrast, greater (P <

0.01) values were observed when digesta were sampled in the abomasum and omasum compared to digesta sampled in the reticulum using the ^{15}N as MM. Microbial nitrogen yield was similar ($P > 0.05$) for digesta sampled in the abomasum and in the omasum using PB or ^{15}N. However, for digesta sampled in the reticulum, the greatest ($P < 0.01$) value was observed for PB.

There was no difference ($P > 0.05$) between the values of MN using abomasum PB, omasum ^{15}N, omasum PB, and reticulum PB. However, the MN value was different ($P < 0.01$) between abomasum ^{15}N and reticulum ^{15}N. In this way, an equation was developed to estimate MN value when reticulum ^{15}N was used:

$$\text{MN (g/d)} = 27.93 \pm 2.46 + 0.99 \pm 0.09 \times \text{reticulum } ^{15}\text{N}$$

where MN is the microbial nitrogen yield in g/d, reticulum ^{15}N is the MN observed using the reticulum as the SS and ^{15}N as the MM.

With respect to MCP/TDN, MN/RFOM, and MN/TRFOM, the use of PB showed the greatest ($P < 0.01$) values for digesta sampled from the abomasum compared to digesta sampled from the omasum and reticulum. However, differences in ^{15}N values in the digesta sampled from the abomasum and the omasum exhibit the greatest ($P < 0.01$) values. MCP/TDN, MN/RFOM, MN/TRFOM, and MDM/RFTCHO were similar ($P > 0.05$) when the sampling was performed in the abomasum using PB or ^{15}N. However, when the SS was performed in the omasum, differences in MCP/TDN, MN/TRFOM, and MDM/RFTCHO ($P < 0.05$) was observed when ^{15}N was used compared to when PB was used. Estimation of MN/TRFOM and MDM/RFTCHO in the reticulum gave greater ($P < 0.05$) values as compared with ^{15}N (Table 7).

There was no interaction ($P < 0.05$) between MS and other variables used to estimate MN (Table 8). The greatest ($P < 0.001$) value of MN was observed for the triple MS. No difference ($P > 0.05$) in MN was observed between the single and the double MS. The hypothesis that the double

MS could be used instead of the triple MS was therefore not confirmed in this study. Use of the triple MS has been recommended by several authors (Hristov, 2007; Krizsan et al., 2010). Based on our results, we developed 2 equations to estimate MN when using the single and the double MS.

$$\text{MN (g/d)} = 49.71 \pm 5.86 + 0.66 \pm 0.05 \times \text{single MS}$$

$$\text{MN (g/d)} = 43.04 \pm 5.71 + 0.71 \pm 0.04 \times \text{double MS}$$

Microbial CP/TDN showed an interaction ($P < 0.05$) between MS and MM. When PB was used, there was no difference ($P > 0.05$) in MCP/TDN among the MS studied. However, ^{15}N incorporation showed the greatest ($P < 0.05$) value in the triple MS, and no difference ($P > 0.05$) was observed between single and double MS. Moreover, similar ($P > 0.05$) results were observed in single and double MS when PB or ^{15}N incorporation were used.

No interaction ($P > 0.05$) was observed in MN/RFOM, MN/TRFOM, and MDM/RFTCHO between MS and MM. The greatest ($P < 0.05$) values in MN/RFOM, MN/TRFOM, and MDM/RFTCHO were observed for the triple MS. The double MS showed intermediate ($P < 0.05$) values, and the single MS showed the lowest ($P < 0.05$) values of MN/RFOM and MN/TRFOM. No difference ($P > 0.05$) was observed for MDM/RFTCHO between the single and double MS.

4. Discussion

4.1. Enrichment of digesta with ^{15}N atoms

The enrichment of digesta with ^{15}N atoms seems to be influenced when the digesta is separated into different phases. When the whole digesta (single MS) was analyzed, no difference was observed in the ^{15}N enrichment, but when digesta was divided into two and three phases, ^{15}N values varied with the different SS. The variation observed in the different phases of the different SS may be an indicator that use of a single MS may not have be accurate in the estimation of MN yield. These results indicated that reticulum and omasum are totally different in ^{15}N enrichment because they gave different results for all phases evaluated in the double and triple MS.

4.2. Isolated bacteria

In agreement with some researchers (Ben-Ghedalia et al., 1978, Martin et al., 1994), bacteria composition was unaffected by dietary treatments in the present study. The influence of replacing 10% of the urea nitrogen in a purified diet with casein, maize gluten, or white fish meal on the efficiency of conversion of dietary-N into microbial N was examined by Ben-Ghedalia et al. (1978) in sheep, and no difference in bacterial composition was observed among these diets. Moreover, Martin et al. (1994) evaluated the cocksfoot hay diet and a diet consisting of 65% hay and 35% pelleted ground barley and also did not observe differences in bacterial composition.

Krizsan et al. (2010) evaluated three grass silages differing mainly in NDF concentrations (412, 530, or 639 g/kg of DM, each combined with one of two different levels of concentrate feed) and observed differences in OM (% DM) between omasal and reticular digesta for LAB. In our study, the LAB from the omasum had the greatest OM value. However, Krizsan et al. (2010) did not find any differences in the OM (% DM) of SAB assayed from the omasum and reticulum. However, the higher concentrations of OM in SAB as compared to OM in LAB observed in the present study agree with previous results (Merry and McAllan, 1983; Legay-Carmier and Bauchart, 1989; Rodriguez et al., 2000; Gonzales et al., 2012).

The lower value of N (% OM) observed for LAB and SAB in abomasal digesta may be due to the low pH in the abomasum (Faichney et al., 1993) where bacterial lysis may be occurring. This demonstrates the problem of using digesta from the abomasum. According to Harmon and Richards (1997) cannulation of the abomasum was quite common in early digestion experiments, but only 11% of the laboratories that they surveyed reported current use of cannulas in the abomasum for digestion studies. The decreased use of abomasal cannulas is a result of the special challenges that they present. The low pH of abomasal contents makes the choice of cannula material critical and may also be responsible for bacterial lysis.

Literature data reporting on the N content in SAB and LAB are inconsistent. Although some authors observed no differences (Bauchart et al., 1986; Craig et al., 1987; Rodriguez et al., 2000), others observed lower N content either for LAB (Benchaar et al., 1995) or SAB (Merry and McAllan, 1983; Komisarczuk et al., 1987).

Gonzalez et al. (2012) observed lower ^{15}N enrichment in SAB compared with LAB in ruminal samples. In agreement with these authors, the lower ^{15}N enrichment in SAB compared with LAB has been associated with a higher incorporation of AA by SAB (Komisarczuk et al., 1987; Carro and Miller, 2002) and with a lower ^{15}N/^{14}N ratio in ammonia molecules in the microenvironment of the SAB. This may result from diffusion impediments within this microenvironment (Rodrıguez et al., 2000).

4.3. Microbial nitrogen yield and efficiency

It is important to note that in this study, we only evaluated the MN from bacteria and did not consider the contribution of MN from protozoa. Belanche et al. (2011) evaluated the effect of presence or absence of protozoa on rumen fermentation and microbial protein in lambs. They observed that the presence of protozoa decreased the bacterial-N flow through the abomasum by 33%, whereas the protozoa-N contribution to the microbial N flow increased from 1.9 to 14.1% when barley grain was added to the alfalfa hay. Protozoa may represent up to 50% of the microbial biomass in the rumen and play a key role in ruminal N recycling though their intensive bacterial predation (Koenig et al., 2000).

Substances such as 2,6-aminoethylphosphonic acid (Abou Akkada et al., 1968) and ^{14}C (Faichney et al., 1997) have been investigated as potential protozoal markers. However the determination of the protozoal-N flow in the duodenum has been hindered by the lack of appropriate protozoal markers. In classical approaches, the contributions of protozoa and bacteria to microbial flow are not distinguished and it is assumed that both populations have a similar microbial marker/N ratio, in spite of a large body of experimental evidence showing the opposite (Vicente et al., 2004). It is therefore possible that the differences observed among SS may be influenced by the fact that the contribution of protozoa was not differentiated from that of bacterial populations.

This is the first study designed to evaluate the MS, SS, and MM together, and there is little information in literature about those factors. Reynal et al. (2005) compared four markers for quantifying microbial protein flow from the rumen of lactating dairy cows and concluded that microbial NAN flow from rumen estimated using ^{15}N appeared to be more accurate and precise than PB, amino acid profiles, and urinary excretion of purine derivatives. These authors also suggested caution when interpreting results obtained using PB as the MM.

The results of this study suggest that if ^{15}N is used to estimate MN, the sample can be taken from either the omasum or abomasum. Rotta et al. (2014) evaluated digesta SS and MS for estimation of ruminal outflow and concluded that digesta performed in the omasum and abomasum showed similar values, and thus, suggested the omasum should be used as the SS. Moreover, Rotta et al. (2014) did not recommend the use of digesta from the reticulum to estimate rumen outflow in beef cattle.

Some laboratories may not be able to use ^{15}N as the MM (due to the high cost of this method). Our results demonstrate that if the digesta is sampled from the abomasum, PB can be used as an alternative to estimate MN and its efficiencies.

Because of problems related to maintenance of abomasal and duodenal fistulas in beef cattle, alternatives to the sampling the abomasal or duodenal digesta are important. Sampling performed from the omasum when ^{15}N is used as the MM is recommended to estimate MN and MCP/TDN. This can replace digesta sampling performed in the abomasum; because the values observed between digesta sampled from the abomasum and omasum were similar (MN and MCP/TDN: 1.04% difference). However, for beef cattle fed diets typical of regions with tropical climates, the sampling of reticular digesta requires further studies to determine correction factors that allow for MN to be calculated accurately. In this study, we suggest an equation to correct the values of MN when the SS is the reticulum with ^{15}N as the MM.

The results using different MS to estimate MN yield indicated that neither the single or double MS may be used. The value observed for the triple MS was different from those observed for the single and double MS. The equations proposed in the present study may be used to correct those values when the laboratory cannot utilize the triple MS.

The efficiency evaluated as MCP/TDN was the only variable that showed an interaction between MS and MM. If the ^{15}N is used as MM, the triple MS seems to be the most reliable system, because it demonstrates values close to those suggested by the NRC (2000). The values estimated using the single and double MS are 30 and 21% lower than values observed with the triple MS, respectively.

5. Conclusion

Solid-associated bacteria showed greater OM content than LAB in the reticulum and abomasum. However, N content was greater in LAB regardless of the SS used. Different diets were responsible for changes in MN yield and its efficiency. Diets having a greater concentrate ratio had greater MN yield than those with a lower concentrate ratio. Digesta sampled in the abomasum showed similar results using ^{15}N and PB in MN yield and its efficiency. Using ^{15}N as MM provides similar results in MN yield and efficiency if the omasum is the SS. Digesta sampled in the reticulum using ^{15}N may be corrected using the equation: MN (g/d) = $27.93 \pm 9.46 + 0.99 \pm 0.09 \times$ reticulum ^{15}N. The use of the single and double MS should be corrected with the following equations to provide better estimations:

for single MS: MN (g/d) = $49.71 \pm 5.86 + 0.66 \pm 0.05 \times$ single MS and

for double MS: MN (g/d) = $43.04 \pm 5.71 + 0.71 \pm 0.04 \times$ double MS

Conflict of interest

We confirm that the manuscript has been read and approved by all named authors and that there are no other person who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

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References

Abou Akkada, A. R., D. A. Messmer, L. R. Fina, and E. E. Bartley. 1968. Distribution of 2-aminoethylphosphonic acid in some rumen microorganisms. J. Anim. Sci. 51:78‑81.

American Association of Cereal Chemists, 2003. Approved Methods of the American Association of Cereal Chemists. 10th ed. Am. Assoc. Cereal Chem., St. Paul, MN.

AOAC, 2000. Official Methods of Analysis. 17th ed. Association of Official Analytical Chemists, Arlington, VA.

AOAC, 2006. Official Methods of Analysis. 18th ed. Assoc. Off. Anal. Chem., Gaithersburg, M.D.

Ahvenjarvi, S., Vanhatalo, A., Shingfield, K.J., Huhtanen, P., 2000. Determination of digesta flow entering the omasal canal of dairy cows using different marker systems. Brit. J. Nutr. 90, 41-52.

Bach, A., Calsamiglia, S., Ster, M.D., 2004. Nitrogen metabolism in the rumen. J. Dairy Sci. 88, suppl.E9-E21.

Bauchart, D., Legay-Carmier, F., Doreau, M., Jouany, J.P., 1986. Effects of the addition of non-protected fat in rations for milk cows over the concentration and chemical composition of rumen bacteria and protozoa. Reprod. Nutr. Develop. 26, 309-310.

Belanche, A., Abecia, L., Holtrop, G., Guada, J.A., Castrillo, C., de la Fuente, G., Balcells, J., 2011. Study of the effect of presence or absence of protozoa on rumen fermentation and microbial protein contribution to the chymel. J. Anim. Sci. 89, 4163-4174.

Belenguer, A., Yanez, D., Balcells, J., Ozdemir, N.H.B, Gonzalez, R.M., 2002. Urinary excretion of purine derivative and prediction of rumen microbial out flow in goats. Livest. Prod. Sci. 77, 127-135.

Benchaar, C., Bayourthe, C., Vernay, M., Moncoulon, R., 1995. Composition chimique des bacteries libres ou adherents au contenu du rumen et du duodenum chez la vache (Chemical composition of free or attached bacteria from rumen or duodenum contents of cows). Annales Zootech. 44, 139.

Ben-Ghedalia, D.J., McMeniman, N.P., Armstrong, D.G. 1978. The effect of partially replacing urea nitrogen with protein N on N capture in the rumen of sheep fed a purified diet. Br. J. Nutr. 39, 37-44.

Blummel, M., Lebzien, P., 2001. Predicting ruminal microbial efficiencies of dairy rations by in vitro techniques. Livest. Produc. Sci. 68, 107-117.

Broderick, G.A., Huhtanen, P., Ahvenjarvi, S., Reynal, S.M., Shingfield, K.J., 2010. Quantifying ruminal nitrogen metabolism using the omasal sampling technique in cattle – a meta-analysis. J. Dairy Sci. 93, 3216-3230.

Carro, M.D., Miller, E.L., 2002. Comparison of microbial markers (^{15}N and purine bases) and bacterial isolates for the estimation of rumen microbial protein synthesis. J. Anim. Sci. 75, 315-321.

Cecava, M.J., Merchen, N.R., Gay, L.C., Berger, L.L., 1990. Composition of ruminal bacteria harvested from steers as influenced by dietary energy level, feeding frequency, and isolation techniques. J. Dairy Sci. 73, 2480-2488.

Craig, W.M., Browm, D.R., Broderick, G.A., Ricker, D.B., 1987. Post-prandial compositional changes of fluid- and particle-associated ruminal microorganisms. J. Anim. Sci. 65, 1042-1048.

Detmann, E., Valadares Filho, S.C., 2010. On the estimation of non-fibrous carbohydrates in feeds and diets. Arq. Bras. Med. Veter. Zoot. 62, 980-984.

Faichney, G.J., 1975. The use of markers to partition digestion within the gastrointestinal tract of ruminants. In Digestion and Metabolism in the Ruminant. In: Proc. IV Int. Symp. Rumin. Physiol., Sydney, Australia. p. 277–291.

Faichney, G.J., 1993. Digesta flow. In J. M. Forbes and J. France, editors, Quantitative aspects of ruminant digestion and metabolism. CAB International, Wallingford, UK. p. 53-85.

Faichney, G. J., C. Poncet, B. Lassalas, J. P. Jouany, L. Millet, J.Dore, and A. G. Brownlee. 1997. Effect of concentrates in a hay diet on the contribution of anaerobic fungi, protozoa and bacteria to nitrogen in rumen and duodenal digesta in sheep.Anim. Feed Sci. Technol. 64:193–213.

France, J., Siddons, R.C., 1986. Determination of digesta flow by continuous marker infusion. J. Theor. Biol. 121, 105–120.

Gonzalez-Ronquillo, M., Balcells, J., Belenguer, A., Castrillo, C., Mota, M., 2004. A comparison of purine derivated excretion with conventional methods as indices of microbial yield in dairy cows. J. Dairy Sci. 87, 2211-2221.

Gonzales, J., Arroyo, J.M., Ouarti, M., Guevara-Gonzales, J., Rodriguez, C.A., Alvir, M.R., Moya, V.J., Piquer, O., 2012. Composition of free and adherent ruminal bacteria inaccuracy of the microbial nutrient supple estimates obtained using free bacteria as reference samples and 15N as the marker. Anim. 6, 468-475.

Harmon, D.L., Richards, C.J., 1997. Considerations for gastrointestinal cannulations in ruminants. J. Anim. Sci. 75, 2248-2255.

Hristov, A.N., 2007. Comparative characterization of reticular and duodenal digesta and possibilities of estimating microbial outflow from the rumen based on reticular sampling in dairy cows. J. Anim. Sci. 85, 2606–2613.

Huhtanen, P., Brotz, P.G., Satter, L.D., 1997. Omasal sampling technique for assessing fermentative digestion in the fore stomach of dairy cows. J. Anim. Sci. 75, 1380–1392.

Ipharraguerre, I.R., Reynal, S.M., Lineiro, M., Broderick, G.A., 2007. A comparison of sampling sites, digesta and microbial markers, and microbial references for assessing the post ruminal supple of nutrients in dairy cows. J. Dairy Sci. 90, 1904-1919.

Kimura, F.T., Miller V.L., 1957. Improved determination of chromic oxide in calf feed and feces. J. Agric. Food Chem. 5, 216.

Koenig, K.M., Newbold, C.J., McIntosh, F.M., Rode L.M., 2000. Effects of protozoa on bacterial nitrogen recycling in the rumen. J. Anim. Sci. 78, 2431‑2445.

Komisarczuk, S., Durand, M., Beaumatin, P.H., Hannequart, G., 1987. Utilization de l’azote 15 pour la mesure de la proteosynthese microbianne dans les phases solide et liquid d’un fermenteur semi-continu (Rusitec) (Use of 15N to measure the microbial protein synthesis in the solid and liquid phases of a semi continuous fermenter (Rusitec)). Reprod. Nutr. Develop. 27, 261-262.

Krizsan, S.J., Ahvenjärvi, S., Volden, H., Broderick, G.A., 2010. Estimation of rumen outflow in dairy cows fed grass silage-based diets by use of reticular sampling as an alternative to sampling from the omasal canal. J. Dairy Sci. 93, 1138-1147.

Leão, M.I., 2002. Metodologias de coletas de digesta omasal e abomasal em novilhos submetidos a três níveis de ingestão: consumo, digestibilidade e produção microbiana. Master Diss. Federal University of Viçosa, Viçosa, Brazil.

Legay-Carmier, F., Bauchart, D., 1989. Distribution of bacteria in the rumen contents of dairy cows given a diet supplemented with soya-bean oil. Brit. J. Nutr. 61, 725-740.

Licitra, G., Hernandez, T.M., Van Soest, P.J., 1996. Standardization of procedures for nitrogen fractionation of ruminant feeds. Anim. Feed Sci. Techno. 57, 347-358.

Ma, T., Deng, K.D., Tu, Y., Zhang, N.F., Jiang, C.G., Liu, J., Zhao, Y.G., Diao, Q.Y., 2014. Effect of dietary forage-to-concentrate ratios on urinary excretion of purine derivatives and microbial nitrogen yields in the rumen of Doper crossbred sheep. Livest. Sci. 160, 37-44.

Machado, P.A.S., Valadares Filho, S.C., Detmann, E., Santos, S.A., Valadares, R.F.D., Ducatti, C., Rotta, P.P., Costa e Silva, L.F., 2013. Development of equations to estimate microbial contamination in ruminal incubation residues of forage produced under tropical conditions using 15N as a label. J. Anim. Sci. 91, 3836-3846.

Martin, C., Williams, A.G., Michalet-Doreau, B., 1994. Isolation and characteristics of the protozoa and bacterial fractions from bovine ruminal contents. J. Anim. Sci. 72, 2962-2968.

Merry, R.J., McAllan, A.B., 1983. A comparison of the chemical composition of mixed bacteria harvested from the liquid and solids fractions of rumen digesta. Brit. J. Nutr. 50, 701-709.

Mertens, D.R., Allen, M., Carmany J., Clegg, J., Davidowicz, A., Drouches, M., Frank, K., Gambin, D., Garkie, M., Gildemeister, B., Jeffress, D., Jeon, C.S., Jones, D., Kaplan, D., Kim, G.N., Kobata, S., Main, D., Moua, X., Paul, B., Robertson, J., Tayson, D., Thiex, N., Williams, J., Wolf, J.M.,2002. Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beakers or crucibles: Collaborative study. J. AOAC Intern. 85, 1217-1240.

National Forage Testing Association, 1993. Laboratory dry matter by microwave drying to constant weight**.** In: Forage analyses procedures. Available at http://www.foragetesting.org/lab_procedure/sectionB/2.1/part2.1.3.htm.

Reynal, S.M., Broderick, G.A., Bearzi, C., 2005. Comparison of four markers for quantifying microbial protein flow from the rumen of lactating dairy cows. J. Dairy Sci. 88, 4065-4082.

Rodriguez, C.A., Gonzalez, J., Alvir, M.R., Repetto, J.L., Centeno, C., Lamrani, F., 2000. Composition of bacteria harvested from the liquid and solid fractions of the rumen of sheep as influenced by intake level. Brit. J. Nutr. 84, 369-376.

Rotta, P.P., Valadares Filho, S.C., Detmann, E., Costa e Silva, L.F., Paulino, M.F., Marcondes, M.I., Lobo, A.A.G., Villadiego, F.A.C. 2014. Digesta sampling sites and marker methods for estimation of ruminal outflow in bulls fed different proportions of corn silage or sugarcane. J. Anim. Sci. 92, 1-11.

Siddons, B.Y.R.C., Paradine, J., Beever,D.E., Cornell, P.R., 1985. Ytterbium acetate as a particulate-phase digesta flow marker. Brit. J. Nutr. 54, 509-519.

Thomas, T.A., 1977. An automated procedure for the determination of soluble carboydrates in herbage. J. Sci. Food Agric. 28, 639-642.

Ushida, K., Lassalas, B., Jouany, J.P., 1985. Determination of assay parameters for RNA analysis in bacterial and duodenal samples by spectrophotometry: Influence of sample treatment and preservation. Reprod. Nutr. Develop. 25, 1037–1046.

Valadares Filho, S.C., Marcondes, M.I., Chizzotti, M.L., Paulino, P.V.R., 2010. Nutrient requirements of Zebu beef cattle – BR CORTE. 2nd. UFV.

Valente, T.N.P., Detmann, E., Valadares Filho, S.C., Cunha, M., Queiróz, A.C., Sampaio, C. B., 2011. In situ estimation of indigestible compounds contents in cattle feed and feces using bags made from different textiles. Braz. J. Anim. Sci. 40, 666-675.

Van Soest, P.J., Robertson, J.B. Lewis, B.A. 1991. Methods for dietary fiber neutral detergent fiber, and non-starch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74, 3583–3597.

Vicente, F., J. A. Guada, J. Surra, J. Balcells, and C. Castrillo. 2004. Microbial contribution to duodenal purine flow in fattening cattle given concentrate diets, estimated by purine N labelling (15N) of different microbial fractions. Anim. Sci. 78:159‑167.

Zinn, R.A., Owens, F.N., 1986. A rapid procedure for purine measurement and its use for estimating net ruminal protein synthesis. Can. J. Anim. Sci. 66, 157–166.

Table 1

Ingredients and chemical composition of feed used in experimental diets.

Feed	DM	OM	CP	EE	NDF	NFC	iNDF
	g/kg DM						
Corn silage	301	947	66	32	516	333	140
Sugar cane	282	974	28	16	459	471	222
Corn	902	989	95	36	144	715	1.7
Soybean meal	875	952	528	15	124	288	0.6
Mineral mix[a]	975	160	-	-	-	-	-
Urea + ammonium sulfate	951	985	2,63	-	-	-	-

DM = Dry matter, OM = Organic matter, CP = Crude protein, EE = Ether extract, NDF = Neutral detergent fiber, NFC = Non fiber carbohydrates, iNDF = Indigestible neutral detergent fiber.

[a] (266 g/kg calcium; 147 g/kg phosphorus; 7 g/kg magnesium; 3 g/kg potassium; 7 g/kg sulfur; 2 g/kg sodium; 118 mg/kg chromium; 1,191 mg/kg copper; 5,070 mg/kg iron; 1,728 mg/kg manganese; 4,198 mg/kg zinc; and 136 mg/kg cobalt).

Table 2

Feed and chemical composition of concentrate and diets used in the experiment.

Ingredients	Concentrate	Corn silage diet		Sugar cane diet	
	g/kg, of dry matter				
Forage	-	600	400	600	400
Corn	904	362	542	362	542
Soybean meal	79	32	47	32	47
Mineral mix	8.5	3.4	5.1	3.4	5.1
Salt	8.5	3.4	5.1	3.4	5.1
Urea + ammonium sulfate	-	21	21	35	35
Items	Chemical composition, g/kg dry matter				
Organic matter	973	958	963	974	963
Crude protein	126	125	129	118	129
Ether extract	34	33	33	23	26
Neutral detergent fiber	140	367	296	324	263
Non fiber carbohydrates	673	454	531	553	574
Indigestible neutral detergent fiber	3.3	98	75	145	107
Starch	641	392	477	287	405
Water-soluble carbohydrate	31	35	34	237	169
Neutral detergent insoluble	10.5	14	13	4.6	6.6
Acid detergent insoluble nitrogen	1	6.5	4.6	0.8	0.8
Non protein nitrogen	19	27	24	15	16

Table 3

Effect of different marker systems on ^{15}N atom excess in digesta collected from the reticulum, omasum, and abomasum in bulls fed corn silage or sugar cane.

MS	Phase	Sampling site			SEM	P	
		Reticulum	Omasum	Abomasum		SS	SS×D
Single		0.076	0.072	0.074	0.0102	0.11	0.28
Double	Particles	0.070^a	0.063^b	0.072^a	0.0101	***	0.72
	Fluid	0.095^a	0.080^b	0.077^b	0.0119	***	0.16
Triple	Large particles	0.069^a	0.063^b	0.069^a	0.0114	***	0.45
	Small particles	0.078^a	0.073^b	0.077^a	0.0102	***	0.74
	Fluid	0.120^a	0.100^b	0.098^b	0.0109	*	0.65

MS = Marker system, SEM = Standard error of mean, SS = Sampling site, D = Diet.

*$P < 0.05$

***$P < 0.001$

Table 4

Effect of sampling site (reticular, omasal and abomasal digesta) on organic matter, nitrogen, and ^{15}N atom excess of liquid-associated bacteria (LAB) and solid-associated bacteria (SAB) in bulls fed corn silage or sugar cane.

	Sampling site				P	
	Reticulum	Omasum	Abomasum	SEM	SS	SS×D
	Organic matter (% in DM)					
LAB	76.6^{b}	82.3^{a}	82.4^{a}	0.05	***	0.71
SAB	78.0^{c}	81.1^{b}	85.8^{a}	0.05	***	0.51
SEM	0.07	0.07	0.06			
P-value	*	0.12	***			
	Nitrogen (% OM)					
LAB	8.3^{a}	8.2^{a}	8.0^{b}	0.01	*	0.07
SAB	8.1^{a}	7.8^{a}	6.5^{b}	0.01	***	0.50
SEM	0.03	0.03	0.03			
P-value	*	**	***			
	^{15}N (atoms percent excess)					
LAB	0.104^{a}	0.092^{b}	0.091^{b}	0.0220	***	0.19
SAB	0.106^{a}	0.093^{b}	0.089^{b}	0.0315	***	0.51
SEM	0.0011	0.0012	0.001			
P-value	0.72	0.69	0.59			

SEM = Standard error of mean, SS = Sampling site, D = Diet, DM = Dry matter.

*$P < 0.05$

**$P < 0.01$

***$P < 0.001$

Table 5

P-values for MN, MCP/TDN, MN/RFOM, MN/RTFOM and MDM/RFTCHO using PB or ^{15}N in three different sampling sites and three different marker methods of bulls fed corn silage or sugar cane.

	P														
	D	SS	MS	MM	D×SS	D×MS	D×MM	D×SS×MS	D×SS×MM	D×MS×MM	D×SS×MS×MM	SS×MS	SS×MM	SS×MS×MM	MS×MM
MN	**	**	***	0.22	0.49	0.99	0.62	0.72	0.38	0.83	0.99	0.45	***	0.54	0.07
MCP/TDN	*	***	**	0.10	0.53	0.96	0.65	0.64	0.16	0.94	0.98	0.24	***	0.48	*
MN/RFOM	*	**	***	0.64	0.92	0.59	0.50	0.09	0.67	0.98	0.99	0.16	*	0.85	0.20
MN/RTFOM	*	***	***	0.65	0.90	0.56	0.52	0.07	0.08	0.69	0.99	0.14	*	0.87	0.20
MDM/RFTCHO	**	*	0.07	0.64	0.15	0.95	0.17	0.98	0.41	0.98	0.99	0.35	**	0.56	0.09

D = Diet, SS = Sampling site, MS = Marker system, MM = Microbial marker, MN = Microbial nitrogen synthesis, MCP/TDN = Microbial crude protein/total digestible nutrients, MN/RFOM = Microbial nitrogen/rumen fermented organic matter, MN/RTFOM = Microbial nitrogen/rumen truly fermented organic matter, MDM/RFTCHO = Microbial dry matter/rumen fermented total carbohydrates.

*P < 0.05

**P < 0.01

***P < 0.001

Table 6

Effect of diet on microbial nitrogen synthesis and its efficiency expressed as MCP/TDN, MN/RFOM, MN/TRFOM and MDM/RFTCHO in bulls fed corn silage or fresh sugar cane in different ratios.

	Corn silage		Sugar cane		SEM	P
	60:40	40:60	60:40	40:60		
MN	102.1^{b}	127.0^{a}	109.4^{b}	120.3^{a}	5.31	**
MCP/TDN	105.4^{ab}	117.1^{a}	109.0^{a}	104.2^{b}	5.10	*
MN/RFOM	29.4^{ab}	31.8^{ab}	34.3^{a}	28.3^{b}	2.05	*
MN/TRFOM	30.7^{ab}	32.8^{ab}	35.6^{a}	29.1^{b}	2.18	*
MDM/RFTCHO	293.2^{b}	359.3^{a}	381.1^{a}	358.0^{a}	19.25	**

SEM = Standard error of mean, MN = Microbial nitrogen synthesis, MCP/TDN = Microbial crude protein/total digestible nutrients, MN/RFOM = Microbial nitrogen/rumen fermented organic matter, MN/RTFOM = Microbial nitrogen/rumen truly fermented organic matter, MDM/RFTCHO = Microbial dry matter/rumen fermented total carbohydrates.

*P<0.05

**P<0.01

Table 7

Effects of different sampling sites and different microbial markers on microbial nitrogen synthesis (MN) and its efficiency expressed as MCP/TDN, MN/RFOM, MN/TRFOM and MDM/RFTCHO in bulls fed corn silage or fresh sugar cane in different ratios.

	Sampling site			SEM	P
	Reticulum	Omasum	Abomasum		SS×MM
MN	104.0	114.1	124.9	4.59	***
Purine bases	114.2^{abA}	106.3^{bA}	130.0^{aA}	4.78	***
^{15}N	94.1^{bB}	123.2^{aA}	120.1^{aA}	4.79	***
MCP/TDN	101.2	108.0	117.8	4.39	***
Purine bases	107.0^{bA}	93.3^{bB}	117.2^{aA}	4.44	***
^{15}N	95.0^{bA}	122.3^{aA}	118.2^{aA}	4.42	***
MN/RFOM	24.8	31.8	36.2	2.05	*
Purine bases	26.8^{bA}	29.2^{bA}	37.7^{aA}	2.09	*
^{15}N	22.7^{bA}	34.4^{aA}	34.6^{aA}	2.08	*
MN/TRFOM	25.5	33.0	37.7	1.89	*
Purine bases	27.6^{bA}	30.3^{bB}	39.4^{aA}	1.96	*
^{15}N	23.4^{bB}	35.7^{aA}	36.0^{aA}	1.97	*
MDM/RFTCHO	321.0	354.3	367.7	16.64	**
Purine bases	352.2^{aA}	323.3^{aB}	378.0^{aA}	18.22	**
^{15}N	290.1^{bB}	384.1^{aA}	359.0^{aA}	18.41	**

SEM = Standard error of mean, SS = Sampling site, MM = Microbial marker, MCP/TDN = Microbial crude protein/total digestible nutrients, MN/RFOM = Microbial nitrogen/rumen fermented organic matter, MN/RTFOM = Microbial nitrogen/rumen truly fermented organic matter, MDM/RFTCHO = Microbial dry matter/rumen fermented total carbohydrates.

Capital letters in the same column are different at $P < 0.05$ and small letters in the same line are different at $P < 0.05$.

*$P < 0.05$

**$P < 0.01$

***$P < 0.001$

Table 8

Main effect means of microbial nitrogen synthesis (MN) and its efficiency expressed as MCP/TDN, MN/RFOM, MN/TRFOM and MDM/RFTCHO using different marker systems and different microbial markers.

	Marker system			SEM	Microbial marker		SEM	P
	Single	Double	Triple		PB	^{15}N		MS×MM
MN	109.1^{b}	111.0^{b}	123.2^{a}	4.59	117.2	112.3	3.75	0.07
MCP/TDN	100.9	107.2	119.0	4.39	106.0	112.1	3.58	*
Purine bases	103.4^{aA}	106.4^{aA}	108.3^{aB}	4.78				*
^{15}N	99.1^{bA}	107^{bA}	129^{aA}	4.84				*
MN/RFOM	25.9^{c}	31.0^{b}	35.9^{a}	1.78	31.3	30.6	1.45	0.20
MN/TRFOM	26.6^{c}	32.2^{b}	37.4^{a}	1.89	32.4	31.7	1.54	0.20
MDM/RFTCHO	338.2^{b}	335.3^{b}	370.1^{a}	16.62	351.3	344.2	13.61	0.14

SEM = Standard error of mean, MS = Marker system, MM = Microbial marker, MCP/TDN = Microbial crude protein/total digestible nutrients, MN/RFOM = Microbial nitrogen/rumen fermented organic matter, MN/RTFOM = Microbial nitrogen/rumen truly fermented organic matter, MDM/RFTCHO = Microbial dry matter/rumen fermented total carbohydrates.

Capital letters in the same column are different at $P < 0.05$ and small letters in the same line are different at $P < 0.05$.

*$P < 0.05$

CHAPTER 2

Interpretive summary

Investigating the effect of feeding regimen and day of gestation in dairy cows

Rotta

This study evaluated 2 feeding regimens for dry cows during the entire gestation, and the effects on digestion, nutrient excretion, and tissue composition in dairy production. We concluded that feeding dairy cows at 1.15% of body weight with a diet containing 93% of roughage is adequate during the gestation period and results in less fat deposition and less N excretion in pregnant dairy cows.

DAY OF GESTATION AND DIET LEVELS IN COWS

Effects of day of gestation and feeding regimen in Holstein × Gyr cows:I. Apparent total tract digestibility, nitrogen balance, and fat deposition

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ABSTRACT

This study investigates how the feeding regimen (FR) alters apparent total tract digestibility, performance, N balance, excretion of purine derivatives, and fat deposition in Holstein × Gyr cows at different day of gestation (DG). Forty-four pregnant multiparous Holstein × Gyr cows with an average initial body weight of 480 ± 10.1 kg and an initial age of 5 ± 0.5 years old were allocated to 1 of 2 FR: ad libitum (AL; n = 20) and maintenance level (ML; n = 24). Maintenance level was considered to be 1.15% of body weight in dry matter (DM) basis and met 100% of the energy requirements, while AL provided 190% of the total net energy requirements. Hot and cold carcass dressing, fat deposition, average daily gain, empty body gain, and average daily gain without the gravid uterus data were analyzed as a 4 × 2 factorial design. Intake, apparent total tract digestibility, N balance, urinary concentration of urea, and purine derivatives data were analyzed as repeated measurements taken over the 28 d time period (122, 150, 178, 206, 234, and 262 d of gestation). Cows were individually fed a corn silage-concentrate based diet composed of 93% roughage and 7% concentrate (DM basis) as a total mixed ration. Pregnant cows were slaughtered at 4 DG: 139 (n = 11), 199 (n = 11), 241 (n = 11), and 268 d (n = 11). Overall, DM intake decreased as DG increased. The decrease that was observed in DM intake may be associated with the reduction in ruminal volume caused by the rapid increase in fetal size during late gestation. We observed an interaction for DM and organic matter apparent total tract digestibility between FR and DG; at 150, 178, and 206 d of gestation, ML-fed cows had greater DM and organic matter apparent total tract digestibility values than AL-fed cows. Rib fat thickness, mesentery, and kidney, pelvic, and heart fat were greater in AL- than in ML-fed cows at all DG, with the exception of rib fat thickness on d-139. Ad libitum-fed cows excreted more N in their feces and urine compared to ML-fed cows. Pregnant cows that were fed at maintenance had greater digestibility during some DG, excreted less N in feces and less N and urea in urine, and deposited less fat in the body. We therefore recommend ML (1.15% of body weight with 93% of roughage) as a FR for pregnant dry cows; however, during the last month of gestation, AL seems to be the most appropriate FR to avoid loss of body weight.

Key words: ad libitum, maintenance, N balance, performance

INTRODUCTION

According to the National Research Council (2001), DMI is an important factor in nutrition because it establishes the amount of nutrients that are available for maintenance and production. Underfeeding nutrients can restrict production and may affect the pregnancy (Vonnahme et al., 2007). Moreover, feeding in excess of requirements increases feed costs (Herd et al., 2003), and may result in increased excretion of nutrients into the environment (Nennich et al., 2006) and fat deposition (Rincker et al., 2008; Duarte et al., 2013).

Some studies have demonstrated that DMI decreases during late gestation (Dorshorst and Grummer, 2002; Hayirli and Grummer, 2004; French, 2006); however, most of these experiments have used Holstein, and thus, information for Holstein × Gyr cows are lacking. The Gyr cattle breed (Bos indicus) is important because of its tolerance to heat and parasites, its rusticity, and its adaptation to the tropics (Silva et al., 2011; Santana et al., 2014). Also, a considerable amount of data describing changes in voluntary DMI of Holstein cows during the prepartum period is available (Hayirli et al., 2002, 2003). However, information regarding DMI of Holstein × Gyr cows during gestation is lacking.

Limit-feeding has been shown to increase diet digestibility when compared to animals with ad libitum (**AL**) access to feed (Galyean et al., 1979; Murphy et al., 1994; Clark et al., 2007). However, no study has been conducted using pregnant Holstein × Gyr cows to evaluate diet digestibility during early, mid, and late-gestation of cows that are fed at maintenance level (**ML**) or with AL access to feed.

Our hypothesis was that cows can be fed at ML throughout the gestation period without changing the apparent total tract digestibility and carcass characteristics, while resulting in a N balance close to zero (Eriksson et al., 2004). Our objectives in this study, therefore, were to investigate the influence of day of gestation (**DG**) and feeding regimen (**FR**) on apparent total tract digestibility, performance, N balance, and fat deposition in Holstein × Gyr cows.

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, prior to initiation of the experiment.

Animals and Management

A vaginal progesterone insert (CIDR, Zoetis, Madson, NJ) was used for 7 d in unsuckled multiparous Holstein × Gyr cows (n = 120) to synchronize estrus, after which a PGF2α injection (25 mg, Lutalyse, Zoetis, Madson, NJ) was administered at CIDR removal. Cows were monitored for estrus every 12 h and were artificially inseminated by using semen from a single Holstein bull 12 h after cow estrus. On d 40 of gestation, cows were housed in 30 m^2 individual pens, of which 8 m^2 were covered with concrete floors, equipped with individual feed bunks, and an automatic water system. On d 60 of gestation, the pregnancy was verified by palpating, fetal sexing was performed by using transrectal ultrasound (Aloka 500 with a 5-MHz linear probe, Aloka, Wallingford, CT), and 44 cows were randomly selected for this experiment. The diets were provided from d 60 of gestation until cows reached the previously designated DG.

Forty-four multiparous dry Holstein × Gyr cows with an average initial BW of 480 ± 10.1 kg and an age of 5 ± 0.5 years old were allocated to 1 of 2 FR: AL (n = 20) or ML (n = 24). The cows used in this experiment was chosen in an attempt to represent the average age of cows raised in Brazil (IBGE, 2009). Maintenance was considered to be 1.15% of BW according to a previous study conducted by our research group (Duarte et al., 2013). The average milk production of the last lactation was 12.1 ± 1.25 L/d for AL-fed cows and 12.8 ± 1.11 L/d for ML-fed cows. The AL-fed cows had an average number of lactations of 2.7 ± 0.20 and for the ML-fed cows, the number of lactations was 2.8 ± 0.16. Every 28 d, cows were weighed in the morning before feeding and after a 16 h fast to obtain the shrunk BW. The shrunk BW was estimated after collecting all of the feed in the feeders, 16 h before weighing, in order to standardize the BW. Feed intake was adjusted based on values of shrunk BW in order to maintain ML throughout the entire gestation period.

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 6 cows from ML slaughtered at 139, 199, 241, and 268 d of gestation. However, 1 abortion was verified in a cow from the ML treatment at 139 d. Thus, data from 43 cows were used for analyses, and 5 ML cows were evaluated at 139 d of gestation.

Cows were fed corn silage and concentrate based diet at a ratio of 93:7 on a DM basis as a TMR twice daily, with 60% of the amount offered in the morning and 40% in the afternoon feeding (DM basis). The composition of the experimental diet is presented in Table 1. The amounts of corn silage and concentrate that were supplied were recorded daily. In order to allow AL cows ad libitum access to feed, feed delivery was adjusted to allow approximately 5% orts daily on an as-fed basis. All cows had ad libitum access to water. Corn silage was sampled daily and stored at -20ºC until analyzed. Corn silage samples were dried weekly in a forced air drying oven at 55ºC for 72 h and ground through a 1 mm screen (Wiley mill; A. H. Thomas, Philadelphia, PA) for further analysis.

Urine and Fecal Measurements

In the present study, we had 6 periods of spot fecal and urine collections and each period lasted for 28 d. For the apparent total tract digestibility evaluation, feces from all cows were collected during the last 5 d of each 28 d period. Fecal collections were performed at 0600, 0900, 1200, 1500, and 1800 h on days 1, 2, 3, 4, and 5, respectively. Samples of approximately 200 g were collected by rectal stimulation or as the animal defecated. Feces were dried in a forced air drying oven at 55ºC for 72 h and then ground through a 1 mm screen (Wiley mill; A. H. Thomas, Philadelphia, PA). A composite sample was obtained per collection period for each cow by utilizing 15 g of the dried and ground sample per collection time. Indigestible neutral detergent fiber (**iNDF**) was used as an internal marker to estimate total fecal excretion (Lippke et al., 1986).

Spot urine samples were collected at 0600 and 1500 h on days 1 and 4 of each 28-d period, respectively, with day 1 considered to be the first day of the period composed of 28 d. Urine samples were taken midstream after manual stimulation of the vulva, acidified to a pH below 4.0 with concentrated sulfuric acid to prevent NH_3 volatilization, and then frozen at −20°C for further analyses of N, urea, allantoin, creatinine, and uric acid.

Slaughter and Laboratory Analyses

Pre-harvest handling of animals was in accordance with good animal welfare practices, and slaughtering procedures followed the Sanitary and Industrial Inspection Regulation for Animal Origin Products (Brasil, 1997). Feed was withheld overnight, but cows had ad libitum access to water. Cows were slaughtered at Universidade Federal de Viçosa by stunning them with a captive bolt and subsequent exsanguination. The gravid uterus was immediately collected and weighed. Maternal mesentery was removed from the reticulum-rumen, omasum, abomasum, small, and large intestines; kidney, pelvic, and heart fat (**KPH**) were also removed before splitting and weighing the carcass. The left half of the carcass was weighed and cooled in a cold chamber at 4ºC for 24 h. After cooling, carcasses were weighed to evaluate the cold carcass weight and cold carcass dressing percentage. The 12th rib fat thickness was taken at 3/4 of the length ventrally over the Longissimus muscle (Greiner et al., 2003).

Corn silage, ingredients in concentrate, and feces were analyzed for DM, OM, and N concentrations (AOAC, 2000; method number 934.01 for DM, 930.05 for OM, and 981.10 for N). Ether extract (**EE**) was analyzed according to the described by AOAC (2006). The NDF was determined according to the technique described by Mertens et al. (2002) without the addition of sodium sulfite, but with the addition of thermostable alpha-amylase to the detergent (Ankom Tech. Corp., Fairport, NY).

The concentration of iNDF was quantified in triplicate for corn silage, feed, and fecal samples. The samples that were used to quantify iNDF were ground in a knife mill with a 2-mm sieve (Valente et al., 2011). Samples of 1.5 g were added to pre-weighed polyester bags with a pore size of 12 μm and a pore area equal to 6% of the total surface (Saatifil PES 12/6, Saatitech S.p.A., Veniano, Como, Italy). The bags were incubated for 288 h in the rumens of 2 cannulated bulls that were fed a diet consisting of 50% corn silage and 50% concentrate on a DM basis at the maintenance level. After removal from the rumen, the bags were rinsed in a household washing machine, dried at 45°C for 48 h, and weighed. Residues were then analyzed for NDF in an Ankom 200/220 Fiber Analyzer (Ankom Technology Corp., Fairport, NY). Heat-stable α-amylase (Mertens et al., 2002) was used in the determination of NDF. Non-fiber carbohydrates were calculated according to Detmann and Valadares Filho (2010), where NFC (%) = 100 – [%CP – (%CP derived from urea + %urea) + %NDF + %EE + %ash]. The content of TDN was estimated based on the following equation: digestible CP + 2.2 digestible EE + digestible NDF + digestible NFC (NRC, 2001). Allantoin, creatinine, and uric acid were analyzed by using an HPLC (Agilent 1100 series, Agilent Technologies, Waldbronn, Germany) as previously described by Czauderna and Kowalczyk (2000), with modifications by George et al. (2006).

Estimation of Purine Derivatives

The equations outlined below were used to estimate the excretion of purine derivatives (**PD**) have been described previously (Chen, 1989; Verbic et al., 1990; Chen et al., 1995). To estimate microbial N supply based on urinary excretion of PD, the PD index was calculated based on total PD [allantoin (mmol/L) + uric acid (mmol/L)] where PD index = {[total PD (mmol/L)]/ creatinine (mmol/L)} × $BW^{0.75}$. The excretion of creatinine (mmol/kg of $BW^{0.75}$) was extrapolated by using the estimated daily urinary volume (L), as calculated from the equation by Pacheco et al. (2009). The estimated urinary creatinine excretion (0.9 mmol/kg of $BW^{0.75}$) was included in the following equation to estimate the daily excretion of PD (mmol/kg of $BW^{0.75}$): daily excretion of PD (**dPD**; mmol/kg of $BW^{0.75}$) = PD index × 0.90. From this, the amount of purines that were absorbed daily was estimated: daily absorbed purines (**daP**) = [dPD (mmol/kg of $BW^{0.75}$) − 0.385 × $BW^{0.75}$] +

0.85; microbial N (g of N/d) supply was determined by using the following equation: microbial N (g of N/d) = (daP × 70)/(0.116 × 0.83 × 1,000).

Statistical Analysis

Hot and cold carcass dressing, rib fat thickness, fat deposition, ADG, empty body gain, and ADG without the gravid uterus data were analyzed as a 4 × 2 factorial design by using the PROC MIXED (SAS Inst. Inc., Cary, NC) and the following model:

$$Y_{ijk} = \mu + F_i + D_j + (F \times D)_{ij} + e_{ijk}$$

where μ = the overall mean, F_i = the effect of FR i, D_j = the effect of DG j, F × D = the interaction between FR and DG, and e_{ijk} = the random error associated with Y_{ijk}.

Intake, apparent total tract digestibility, N balance, urinary concentration of urea, and PD data were analyzed as repeated measurements taken over the 28 d time period by using the PROC MIXED (SAS Inst. Inc., Cary, NC) and the following model:

$$Y_{ijklm} = \mu + F_i + D_j + (F \times D)_{ij} + e_{ijk} + M_l + (F \times M)_{il} + (D \times M)_{jl} + (F \times D \times M)_{ijl} + e_{ijklm}$$

where μ = the overall mean, F_i = the effect of FR i, D_j = the effect of DG j, F × D = the interaction between FR and DG, F × M = the interaction between FR and time of measurement, D × M = the interaction between DG and time of measurement, F × D × M = the interaction among FR, DG, and time of measurement, and e_{ijkl} = the random error associated with the replicate of F within D, M_l = the effect of time of measurement l, and e_{ijklm} = random error associated with Y_{ijklm}.

The DG and FR were considered to be the fixed effects for the repeated measurements taken over the 28 d time period, and cows were considered to be the random effect. Distinct residual variances were modeled by using the command REPEATED. An ARH [1] was used. The PDIFF option, adjusted by the Tukey method, was included in the LSMEANS statement to account for multiple comparisons. Least squares means were estimated for all effects and were compared by using Tukey's method at $\alpha = 0.10$. A quadratic regression was performed for DMI expressed as gram per kilogram of BW during DG by using data from AL cows to provide an equation to predict DMI.

RESULTS

Feed Intake

Holstein × Gyr cows with AL access to feed had greater ($P < 0.01$) DMI expressed as kilogram per day at 150 and 178 d compared to DMI at 234 and 262 d of gestation (Table 2). Given that the average gestation period for these cows is approximately 284 d (Mellado et al., 2011), we observed that from 50 d before parturition, DMI decreased. Dry matter intake at 262 d in Holstein × Gyr cows was approximately 22% ($P < 0.01$) less than at 234 d. No difference ($P = 0.27$) was observed in DMI in ML-fed cows during gestation, and this may be due to the fact that they were weighed every 28 d and DMI was adjusted according to 1.15% BW. Moreover, in all DG that were studied, DMI was greater ($P = 0.03$) in AL supplemented cows when compared to ML-fed cows. However, at 262 d of gestation, treatment did not influence ($P = 0.43$) DMI when it was analyzed as a percent of BW (Figure 1). When using the percentage of BW to evaluate DMI, we observed no difference ($P = 0.74$) at 122, 150, 178, and 206 d of gestation in AL-fed cows (Figure 1). However, these DG were different ($P < 0.01$) from those observed at 234 and 262 d, and the lowest ($P < 0.01$) value was observed in cows at 262 d of gestation (Figure 1). Organic matter, CP, and NDF intakes were also less ($P < 0.01$) in AL-fed cows at 234 and 262 d than those at 150 and 178 d of gestation (Table 2). Figure 2 illustrates the DMI expressed as gram per kilogram of BW in AL-fed cows from 118 d until 262 d of gestation. In addition, an estimated equation to predict the DMI in pregnant dairy cows is presented: DMI (g/kg BW) = $-0.0007d^2 + 0.189d + 9.3942$, where d = days of gestation (122 to 262 d).

Apparent Total Tract Digestibility

Dry matter and OM apparent total tract digestibility had a significant interaction effect ($P = 0.03$) between FR and DG. Maintenance fed cows showed greater ($P < 0.01$) DM and OM apparent total tract digestibility than AL-fed cows at 150, 178, and 206 d of gestation. However, at 122, 234, and 262 d of gestation, DM and OM apparent total tract digestibility were similar ($P = 0.42$) between ML- and AL-fed cows. We observed the lowest ($P < 0.01$) value of DM apparent total tract digestibility in ML- and AL-fed cows at 234 and 262 d of gestation.

An interaction ($P = 0.03$) was observed for CP apparent total tract digestibility between FR and DG. Both AL- and ML-fed cows showed the greatest ($P = 0.03$) value in CP apparent total tract digestibility at 122 d (Table 3). These results are similar to those reported for DM apparent total tract digestibility, where the decrease in digestibility begins at 150 d of gestation. However, no difference ($P = 0.24$) for CP apparent total tract digestibility was observed among 150, 178, 206, 234, and 262 d of gestation.

No difference ($P = 0.19$) was observed for NDF apparent total tract digestibility between FR and DG (Table 3). However, we observed differences ($P < 0.01$) between FR. The greatest ($P < 0.01$) value of NDF apparent total tract digestibility was detected in ML-fed cows. Also, the greatest ($P < 0.01$) NDF apparent total tract digestibility value was observed in cows at 122 d of gestation, and the lowest ($P < 0.01$) value was observed at 262 d. Intermediate ($P < 0.01$) values were observed in cows at 150, 178, 206, and 234 d of gestation.

Nitrogen Balance

No difference ($P = 0.54$) was observed in mass of N excretion in the urine of AL-fed cows for the different DG that were evaluated (Figure 3). Nevertheless, N intake was less ($P < 0.01$) at 234 and 262 d than 122, 150, 178, and 206 of gestation. Nitrogen excreted in feces was less ($P < 0.01$) at 234 and 262 d than at 122, 150, 178, and 206 d of gestation in AL-fed cows, and it was similar ($P = 0.36$) to ML-fed cows (Figure 3). Nitrogen excreted in feces was greater ($P < 0.01$) in AL- than ML-fed cows at 122, 150, 178, 206, and 234 d of gestation (Figure 3). However, at 262 d of gestation, we observed similar ($P = 0.21$) estimate in AL- and ML-fed cows. This can be explained by the similar DMI expressed as percentage of BW at 262 d of gestation for the 2 FR (Figure 1). For AL-fed cows, the lowest ($P < 0.01$) value of N balance was observed at 262 d of gestation (Figure 3).

Excretion of Purine Derivatives

The values for urea, allantoin, uric acid, absorbed purines, microbial N, and microbial efficiency are presented in Table 4. Allantoin, uric acid, and absorbed purines were greater ($P < 0.01$) in AL- than ML-fed cows. However, no difference ($P = 0.18$) was observed for microbial efficiency expressed as gram of CP per kilogram of TDN between AL- and ML-fed cows. The DG influenced (P = 0.02) uric acid.

Carcass Characteristics, Fat Deposition, and Performance

Final BW and empty BW were heavier (P < 0.01) in AL- than in ML-fed cows (Table 5). Although no difference (P = 0.21) was observed in final BW for the different DG, we observed a lighter (P = 0.02) empty BW in cows at 139 and 199 d of gestation. No difference (P = 0.27) was observed for hot carcass and cold carcass dressings between the FR that were evaluated. However, differences (P < 0.01) were observed when comparing DG. Heavier (P < 0.01) values for hot carcass and cold carcass dressings were observed at 139, 199, and 241 d than at 268 d of gestation.

Rib fat thickness was greater (P = 0.04) in AL- than ML-fed cows during gestation, with an exception at 139 d (Figure 4). Differences in rib fat thickness (P = 0.08) were also observed for DG; the greatest value was observed in cows at 268 d followed by 199 and 241 d, while the lowest value was observed in cows at 139 d of gestation in AL-fed cows.

Mesentery tissue mass, expressed as kilogram and gram per kilogram of empty BW, were heavier (P < 0.01) in AL- than ML-fed cows (Figure 4). Day of gestation influenced (P = 0.01) mesentery tissue mass expressed as kilogram and gram per kilogram of empty BW, and the lightest mass was observed in AL-fed cows at 139 d of gestation. As expected, KPH expressed as kilogram and gram per kilogram of empty BW were heavier (P < 0.01) in AL- than ML-fed cows.

Average daily gain was different (P < 0.01) between AL- and ML-fed cows in the 4 DG that were evaluated (Table 5). No difference (P = 0.85) was observed for DG in AL- and ML-fed cows. The ADG during gestation of AL-fed cows was 1.13 ± 0.051 kg/d, while that of ML-fed cows was

0.30 ± 0.063 kg/d. Average daily gain in AL-fed cows was about 3.8 times greater than in ML-fed cows. When evaluating ADG without the gravid uterus, differences ($P < 0.01$) were observed between AL- and ML-fed cows depending on DG. At 139, 199, and 241 d of gestation, similar ($P = 0.54$) values were observed. The lowest ($P < 0.01$) observed value was at 268 d, but it was not different ($P = 0.78$) than the values at 241 d of gestation. Maintenance-fed cows had similar ($P = 0.44$) ADG without the gravid uterus at 139 and 199 d of gestation. An intermediate ($P = 0.02$) value was observed at 241 d, and the lowest ($P = 0.04$) value was observed at 268 d of gestation. Empty BW gain was different ($P < 0.01$) between AL- and ML-fed cows, but no difference ($P = 0.85$) was observed within FR among DG. The empty BW gain during gestation was 1.23 ± 0.050 kg/d in AL-fed cows and 0.38 ± 0.051 kg/d in ML-fed cows.

DISCUSSION

Feed Intake

When comparing the DMI observed at 150 d and 262 d of gestation, the decrease in DMI was approximately 40%. Thus, it appears that a diet containing more energy and protein during late gestation should be provided, as this period is the critical time during which energy and protein supplementation support fetal development (Bell, 1995). According to Forbes (1986), the decrease in DMI is associated with a reduction in ruminal volume caused by the rapid increase in fetal size during late gestation; this creates a physical impingement on ruminal volume, because approximately 60% of fetal growth occurs during the last 2 months of gestation (Bauman and Currie, 1980). In this study, we observed an approximately 50 ± 2.6% increase in the fetal BW from d-239 to d-268 (Rotta et al., 2014). During the last 70 d of gestation, we observed that approximately 70 ± 3.5% of fetal growth occurred during this period (Rotta et al., 2014). Moreover, concentrations of many hormones in the blood increase or decrease dramatically at parturition and may be potent modifiers of DMI (NRC, 2001). For example, plasma estrogen of placental origins increases in the blood as parturition approaches. Exogenous estrogen administration inhibits DMI (Grummer et al., 1995; Eckel, 2004). Reduced DMI during estrus and late pregnancy may reflect greater endogenous estrogen production.

Inert fill in the rumen has also been shown to cause a decrease in feed intake (Dado and Allen, 1995). Moreover, Dado and Allen (1995) observed that there was an increase in the frequency of small meals when high-forage and rumen-inert fill were added to the diet. This may therefore explain the decrease in DMI that is often associated with animals on diets with high NDF content. In this study, we used a diet that contained approximately 50% NDF, which may also be responsible for the decrease in DMI during late gestation. According to Arelovich et al. (2008), diets presenting with 45% or more of NDF are considered to be high NDF diets. Forbes (1970) observed that the DMI of ewes changed dramatically during late gestation. This adjustment during pregnancy in ewes has been attributed to changes in ruminal capacity due to the growing fetus and the increased concentration of estrogen (Forbes, 1986). In the present study, we observed that the decreased DMI, expressed as a percentage of BW, started at week 25 of gestation, which corresponds to 178 d. In agreement with this finding, Ingvartsen and Anderson (2000) reported that Holstein cows decreased DMI from week 26 of gestation until calving.

Apparent Total Tract Digestibility

According to Clark et al. (2007), the level of intake influences apparent total tract DM digestibility in beef steers, and limit-feeding has been shown to increase digestibility of the diet when compared to AL-fed animals (Galyean et al., 1979; Murphy et al., 1994; Clark et al., 2007). However, in the present study, we observed that differences between the 2 FR occurred only from 150 to 206 d of gestation (Table 3).

Independently of the FR that were studied, we observed that apparent total tract DM digestibility decreased with advancing gestation. Beharka et al. (1988), Faichney and White (1988), and Scheaffer et al. (2001) also observed a decrease in apparent total tract DM digestibility in ewes, beef cows, and beef heifers, respectively. Beharka et al. (1988) observed that apparent total tract DM digestibility decreased, whereas the rate of passage increased. According to these authors, because the rate of passage and digestibility are competing forces, the decreased apparent total tract DM digestibility may in part be explained by the increased passage rate. Okine and Mathison (1991) suggested that the passage rate of NDF increased with increasing DMI. Cows fed ad libitum are therefore supposed to have a greater passage rate of NDF than ML-fed cows, which likely explains the reduction in total tract apparent digestibility of NDF.

Nitrogen Balance

There has been great interest in investigating the potential of specific on-farm alternatives to reduce N losses (Spek et al., 2013). Previous studies have shown that the excretion of N in urine is linearly related to N intake in beef (Hoekstra et al., 2007) and lactating dairy cows (Tas et al., 2006; Higgs et al., 2012). Nevertheless, in pregnant AL-fed cows, it seems that N excretion in urine does not exhibit the same response (Figure 3). Despite the lower values of N intake at 234 and 262 d of gestation, the excretion of N in urine did not change. This may be due to a lower energy balance during the last phase of gestation (Bauman and Currie, 1980). The urea excretion in urine (Table 4) was also similar among the different DG in AL-fed cows, again showing that although N intake was less, N excretion in urine did not change.

Nitrogen excretion in urine was greater at 234 and 262 d of gestation in ML-fed cows, although N intake was similar among the different DG, and this may be related to AA catabolism (Bauman and Currie, 1980). Moreover, Kwon et al. (2004) demonstrated that maternal malnutrition reduces concentrations of AA in ovine maternal and fetal plasma as well as in fetal fluids. Since d 234 is considered to be the final third of gestation, the net requirements are greater (Bell, 1995). Maintenance-fed diets should provide sufficient energy and AA content for fetal development. Thus, greater AA catabolism may be responsible for increasing N excretion in urine. The main indicator of this increased catabolism is that the greatest urea excretion concentration occurred at 234 and 262 d of gestation in ML-fed cows. Under prolonged fasting conditions in dairy cows, the AA catabolism by the conceptus may increase to provide approximately 70% of the fetal glucose (Bell, 1995). Thus, despite the fact that ML-fed cows may not have enough N to meet their requirements, the greater N excretion in urine cannot be avoided since part of the N comes from AA catabolism.

The negative N balance value at 262 d of gestation can be explained by the lowest N intake at that time, as well as due to the fact that the conceptus (fetus, placenta, associated fetal membranes, and supporting uterine tissues) makes extensive, direct demands upon maternal supplies of glucose and AA during late pregnancy (Ferrell, 1991). During late gestation, 35 to 40% of fetal energy is supplied by glucose and the fetal-placental metabolite, lactate, while an additional 55% is supplied by AA (Comline and Silver, 1976; Reynolds et al., 1986; Ferrell, 1991). Thus, AA catabolism may result in increased urea excretion and explain the increased N excretion in urine, and thus, contribute to a lower value for N balance.

Maintenance-fed cows had lower values for N balance in all DG that were studied when compared with AL-fed cows; moreover, the N balance was slightly negative at 150 until 206 d of gestation in ML-fed cows, as they were approximately -1.30 g/d of N. However, at d-234 and d-262 of gestation, an increase in the negative values of N balance was observed, which were approximately -17.0 g/d of N. Thus, the last 2 months that were evaluated in this study demonstrated a greater negative magnitude of N balance in ML-fed cows. In the last DG that was evaluated in this study, a negative value for ADG without the gravid uterus was observed. The FR that decreases the amount of excreted N may be considered a good alternative to try to limit the environmental impact of N that is produced by the dairy industry. According to Spears et al. (2003), the dairy industry has been identified as a potential contributor to point and nonpoint source environmental pollution. Van Egmond et al. (2002) reported that the main sources of reactive N in the environment are fertilizers and manure. The N in urine is rapidly converted into ammonia by urease in the environment (Tamminga, 1992).

Excretion of Purine Derivatives

According to Barbosa et al. (2011), allantoin and uric acid in urine are linearly related to DMI, which is supported by the findings of the present study. Johnson et al. (2003) suggested that the diet is one of the main factors that contribute to greater values of uric acid excretion in urine. Ad libitum-fed cows had about a 90% greater microbial N than ML-fed cows. According to Clark and Davis (1983), the deficiency of digestible OM and protein may decrease microbial N synthesis in the rumen. The amount of consumed feed is the nutritional factor that limits performance when balanced diets are fed to dairy cows, and microbial N content increases when cows consume increasing amounts of the same diet (Clark et al., 2007). These authors demonstrated that microbial N is linearly related to OM intake. Thus, the greater OM intake that was observed in AL-fed cows may explain the greater microbial N synthesis for AL- than ML-fed cows. The microbial efficiency values that we reported in this study are close (137 ± 2.1 g CP/kg of TDN) to those suggested by the National Research Council (2001; 130 g CP/kg of TDN).

Carcass Characteristics, Fat Deposition, and Performance

The greater values for hot carcass and cold carcass dressings that were observed at 139, 199, and 241 d versus 268 d of gestation may be related to gravid uterine growth (fetus, uterus, placenta, and liquids). At 268 d of gestation, the gravid uterus was approximately 500, 170, and 60% heavier than at 139, 199, and 241 d of gestation, respectively (Rotta et al., 2014). Thus, we can infer that at 139 and 199 d of gestation, the rumen capacity was greater than at 241 and 268 d of gestation. The greater rumen capacity is likely due to the presence of increased digesta and growth of the fetus during these DG.

Cows that were fed AL gained body fat during gestation and had an increased rib fat thickness, whereas ML-fed cows had a constant amount of body fat and rib fat thickness. Differences in the mass of viscera and internal fat have been reported in heifers that were fed with different levels of energy (Lage et al., 2012). This also occurred in our study because AL-fed cows typically had heavier mass of viscera and internal fat (Rotta et al., 2014) when compared to those of ML-fed cows. These differences may contribute to differences in empty BW, and concomitantly, the similarity of cold carcass dressings between FR. Nutritional management is one of the main factors that can affect fat deposition. Cattle that are fed ML must prioritize energy utilization in order to maintain vital functions and internal organs of the body such as the liver, kidneys, heart, and gastrointestinal tract. These animals utilize about 40% of the energy requirement for maintenance (Koong et al., 1985). Maintenance-fed cows have less available energy for rib fat thickness deposition than AL-fed cows.

As expected, the increased mass of mesentery observed in AL-fed cows was likely the result of increased caloric intake. Thus, part of the excess energy in the diet of pregnant AL-fed cows was converted to mesentery, which is related to increased energy requirements. A persistent positive energy balance leads to deposition of fat in the animal (Rincker et al., 2008). Fat deposition can be chemically characterized by a continued accumulation of lipids, primarily in the form of triacylglycerides, and morphologically characterized by hyperplasia and hypertrophy (Nürnberg et al., 1998). In the present study, cows were approximately 5 years old, and consequently, the fat deposition may have occurred for the most part due to hypertrophy (Okumura et al., 2007).

CONCLUSIONS

We conclude that DMI expressed as a percentage of BW decreased as gestation progressed in Holstein × Gyr cows. Cows fed at 1.15% of BW with a diet containing 93% of roughage had greater DM and OM apparent total tract digestibility than AL-fed cows between 150 to 206 d of gestation; however, DM apparent total tract digestibility is reduced in late-gestation. Urinary N excretion in pregnant cows that are fed AL was not related to N intake. Cows that are fed at 1.15% of BW may impact the environment less because they have less N excretion in feces and urine.

Part of the excess energy from the AL diet was stored as mesenteric or KPH fat. During the last month of gestation, AL seems to be the most appropriate FR to avoid loss of BW.

REFERENCES

AOAC International. 2000. Official Methods of Analysis. 17th ed. AOAC International Arlington, VA.

AOAC International. 2006. Official methods of analysis. 18th ed. AOAC International Gaithersburg, MD.

Arelovich, H. M., C. S. Abney, J. A. Vizcarra, and M. L. Galyean. 2008. Effects of dietary neutral detergent fiber on intakes of dry matter and net energy by dairy and beef cattle: analysis of published data. Prof. Anim. Sci. 24:375-383.

Barbosa, A. M., R. F. D. Valadares, S. C. Valadares Filho, D. S. Pina, E. Detmann and M. I. Leao. 2011. Endogenous fraction and urinary recovery of purine derivatives obtained by different methods in Nellore cattle. J. Anim. Sci. 89:510-519. http://dx.doi.org/jas.2009-2366.

Bauman, D. E., and W. B. Currie. 1980. Partitioning of nutrients during pregnancy and lactation: a review of mechanisms involving homeostasis and homeorhesis. J. Dairy Sci. 63:1514–1529. http://dx.doi.org/10.3168/jds.S0022-0302(80)83111-0.

Beharka, A., B. Cochran, D. Harmon, and T. Avery. 1988. The influence of stage of pregnancy on digestion characteristics of beef cows. Kansas State Univ. Prog. Rep. 539:13-15.

Bell, A. W. 1995. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. J. Anim. Sci. 73:2804–2819.

Brasil. 1997. Ministério da Agricultura, Pecuária e Abastecimento, Regulamento da Inspeção Industrial e Sanitária de Produtos de Origem Animal [Food of animal origin sanitary and industry inspection].

Chen, X. B. 1989. Excretion of purine derivatives by sheep and cattle and its use for the estimation of absorbed microbial protein. PhD Thesis. Univ. of Aberdeen, Aberdeen, Scotland.

Chen, X. B., P. Susmel, B. Stefanon, and E. R. Orskov. 1995. Protein metabolism and nutrition. Pages 325–329 in Proc. 7th Int. Symp. Protein Metab. Nutr. Vale de Santarem, Portugal.

Clark, J. H., and C. L. Davis. 1983. Future improvement of milk production: potential for nutritional improvement. J. Anim. Sci. 57:750-764.

Clark, J. H., K. C. Olson, T. B. Schmidt, M. L. Linville, D. O. Alkire, D. L. Meyer, G. K. Rentfrow, C. C. Carr, and E. P. Berg. 2007. Effects of dry matter intake restriction on diet digestion, energy partitioning, nutrient retention, and ruminal fermentation by beef steers. J. Anim. Sci. 85:3383-3390. http://dx.doi.org/10.2527/jas.2006-741.

Comline, R. S., and M. Silver. 1976. Some aspects of foetal and utero-placental metabolism in cows with indwelling umbilical and uterine vascular catheters. J. Physiol. 260:571–586.

Czauderna, M., and J. Kowalczyk. 2000. Quantification of allantoin, uric acid, xanthine and hypoxanthine in ovine urine by high-performance liquid chromatography and photodiode array detection. J. Chromatogr. B. Biomed. Sci. Appl. 744:129–138.

Dado, R. G., and M. S. Allen. 1995. Intake limitations, feeding behavior, and rumen function of cows challenged with rumen fill from dietary fiber or inert bulk. J. Dairy Sci. 78:118-133. http://dx.doi.org/10.3168/jds.S0022-0302(95)76622-X.

Detmann, E., and S. C. Valadares Filho. 2010. On the estimation of non-fibrous carbohydrates in feeds and diets. Braz. J. Vet. Anim. Sci. 62:980-984. http://dx.doi.org/10.1590/S0102-09352010000400030.

Dorshorst, M. E., and R. R. Grummer. 2002. Effects of day relative to parturition and dietary crude protein on rumen fermentation in prepartum transition cows. J. Dairy Sci. 85:2290-2298. http://dx.doi.org/10.3168/jds.S0022-0302(02)74309-9.

Duarte, M. S., M. P. Giombelli, P. V. R. Paulino, N. V. L. Serao, T. S. Martins, P. I. S. Totaro, C. A. Neves, S. C. Valadares Filho, M. V. Dodson, M. Zhu, and M. Du. 2013. Effects of maternal nutrition development of gastrointestinal tract of bovine fetus at different stages of gestation. Livest. Sci. 153:60-65. http://dx.doi.org/10.1016/j.livsci.2013.01.006.

Eckel, L. A. 2004. Estradiol: a rhythmic, inhibitory, indirect control of meal size. Physiol. Behav. 82:35-41. http://dx.doi.org/10.1016/j.physbeh.2004.04.023.

Eriksson, T., M. Murphy, P. Ciszuk, and E. Burstedt. 2004. Nitrogen balance, microbial protein production, and milk production in dairy cows fed fodder beets and potatoes, or barley. J. Dairy Sci. 87:1057-1070. http://dx.doi.org/10.3168/jds.S0022-0302(04)73252-X.

Faichney, G. A., and G. A. White. 1988. Partition of organic matter, fibre and protein digestion in ewes fed at a constant rate throughout gestation. Aust. J. Agric. Res. 39:493-504.

Ferrell, C. L. 1991. Maternal and fetal influences on uterine and conceptus development in the cow: II. Blood flow and nutrient flux. J. Anim. Sci. 69:1954–1965.

Forbes, J. M. 1970. Voluntary food intake of pregnant ewes. J. Anim. Sci. 31:1222–1227.

Forbes, J. M. 1986. The effects of sex hormones, pregnancy, and lactation on digestion, metabolism, and voluntary food intake. Pages 420-435 in: Control of Digestion and

Metabolism in Ruminants. L. P. Milligan, W. L. Grovum and A. Dobson, ed. Prentice- Hall, Englewood Cliffs, NJ.

French, P. D. 2006. Dry matter intake and blood parameters of nonlactating Holstein and Jersey cows in late gestation. J. Dairy Sci. 89:1057-1061. http://dx.doi.org/10.3168/jds.S0022-0302(06)72173-7.

Galyean, M. L., D. G. Wagner, and F. N. Owens. 1979. Level of feed intake and site and extent of digestion of high concentrate diets by steers. J. Anim. Sci. 49:199-203.

George, S. K., M. T. Dipu, U. R. Mehra, P. Singh, A. K. Verma, and J. S. Rangaokar. 2006. Improved HPLC method for the simultaneous determination of allantoin, uric acid and creatinine in cattle urine. J. Chromatogr. B. 832:134–137. http://dx.doi.org/10.1016/j.jchromb.2005.10.051.

Greiner, S. P., G. H. Rouse, D. E. Wilson, L. V. Cundiff, and T. L. Wheeler. 2003. The relationship between ultrasound measurements and carcass fat thickness and longissumus muscle area in beef cattle. J. Anim. Sci. 81:676-682. http://dx.doi.org/10.1016/j.meatsci.2013.01.008.

Grummer, R. R., P. C. Hoffman, M. L. Luck, and J. Bertics. 1995. Effect of prepartum and postpartum dietary energy on growth and lactation of primiparous cows. J. Dairy Sci. 78:172-180. http://dx.doi.org/10.3168/jds.S0022-0302(95)76627-9.

Hayirli, A., R. R. Grummer, E. V. Nordheim, and P. M. Crump. 2002. Animal and dietary factors affecting feed intake during the prefresh transition period in Holsteins. J. Dairy Sci. 85:3430–3443. http://dx.doi.org/10.3168/jds.S0022-0302(02)74431-7.

Hayirli, A., R. R. Grummer, E. V. Nordheim, and P. M. Crump. 2003. Models for predicting dry matter intake of Holsteins during the prefresh transition period J. Dairy Sci. 86:1771–1779. http://dx.doi.org/10.3168/jds.S0022-0302(03)73762-X.

Hayirli, A., and R. R. Grummer. 2004. Factors affecting dry matter intake prepartum in relationship to etiology of peripartum lipid-related metabolic disorders: A review. Can. J. Anim. Sci. 84:337-347.

Herd, R. M., J. A. Archer, and M. E. van Amburgh. 2003. Reducing the cost of beef cattle production through genetic improvement in residual feed intake: Opportunity and challenges to application. J. Anim. Sci. 81:E9-E17.

Higgs, R. J., L. E. Chase, and M. E. van Amburgh. 2012. Development and evaluation of equations in the Cornell Net Carbohydrate and Protein System to predict nitrogen excretion in lactating dairy cows. J. Dairy Sci. 95:2004–2014. http://dx.doi.org/10.3168/jds.2011-4810.

Hoekstra, N. J., R. P. O. Schulte, P. C. Struik, and E. A. Lantinga. 2007. Pathways to improving the N efficiency of grazing bovines. Eur. J. Agron. 26:363–374. http://dx.doi.org/10.1016/j.eja.2006.12.002.

IBGE. 2009. Censo Agropecuario. Accessed Dec. 8, 2014. http://www.ibge.gov.br/home/estatistica/economia/agropecuaria/censoagro/.

Ingvartsen, K. L., and J. B. Andersen. 2000. Integration of metabolism and intake regulation: A 511 review focusing on periparturient animals. J. Dairy Sci. 83:1573-1597. http://dx.doi.org/10.3168/jds.S0022-0302(00)75029-6.

Johnson, R. J., D. Kang, D. Feig, S. Kivlighn, J. Kanellis, S. Watanabe, K. R. Tuttle, B. Rodriguez-Iturbe, J. Herrera-Acosta, and M. Mazzali. 2003. Is there a pathogenetic role for uric acid in hypertension and cardiovascular and renal disease? Hypertension. 41:1183-1190. http://dx.doi.org/10.1161/01.HYP.0000069700.62727.C5.

Koong, L. J., C. L. Ferrell, and J. A. Nienaber. 1985. Assessment of interrelationships among levels of intake and production, organ size and fasting heat production in growing animals. J. Nutr. 115:1383-1388.

Kwon, H., S. P. Ford, F. W. Bazer, T. E. Spencer, P. W. Nathanielsz, M. J. Nijland, B. W. Hess, and G. Wu. 2004. Maternal nutrient restriction reduces concentrations of amino acids and polyamines in ovine maternal and fetal fluids. Biol. Reprod. 71:901-908. http://dx.doi.org/10.1095/biolreprod.104.029645.

Lage, J. F., P. V. R. Paulino, S. C. Valadares Filho, E. J. O. Souza, M. S. Duarte, P. B. Benedeti, N. K. P. Souza, and R. B. Cox. 2012. Influence of genetic type and level of concentrate in the finishing diet on carcass and meat quality traits in beef heifers. Meat Sci. 90:770–774. http://dx.doi.org/10.1016/j.meatsci.2011.11.012.

Lippke, H., W. C. Ellis, and B. F. Jacobs. 1986. Recovery of indigestible fiber from feces of sheep and cattle on forage diets. J. Anim. Sci. 69:403-412.

Mellado, M., F. Coronel, A. Estrada, and F. G. Rios. 2011. Lactation performance of Holstein and Holtein x Gyr cattle under intensive condition in a subtropical environment. Trop. Subtrop. Agroecosyt. 14: 927-931.

Mertens, D. R., M. Allen, J. Carmany, J. Clegg, A. Davidowicz, M. Drouches, K. Frank, D. Gambin, M. Garkie, B. Gildemeister, D. Jeffress, C. S. Jeon, D. Jones, D. Kaplan, G. N. Kim, S. Kobata, D. Main, X. Moua, B. Paul, J. Robertson, D. Tayson, N. Thiex, J. Williams, and M. Wolf. 2002. Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beakers or crucibles: Collaborative study. J. AOAC Intern. 85:1217-1240.

Murphy, T. A., S. C. Loerch, and F. E. Smith. 1994. Effects of feeding high-concentrate diets at restricted intakes on digestibility and nitrogen metabolism in growing lambs. J. Anim. Sci. 72:1583-1590.

Nennich, T. D., J. H. Harrison, L. M. van Wieringen, N. R. St-Pierre, R. L. Kincaid, M. A. Wattiaux, D. L. Davidson, and E. Block. 2006. Prediction and evaluation of urine and urinary nitrogen and mineral excretion from dairy cattle. J. Dairy Sci. 89:353-364. http://dx.doi.org/10.3168/jds.S0022-0302(06)72101-4.

National Research Council. 2001. Nutrients Requirements of Dairy Cattle. 7th rev. ed. Natl. Acad. Press, Washington, DC.

Nürnberg, K., J. Wegner and K. Ender. 1998. Factors influencing fat composition in muscle and adipose tissue of farm animals. Livest. Prod. Sci. 56:145-156. http://dx.doi.org/10.1016/S0301-6226(98)00188-2.

Okine, E. K., and G. W. Mathison. 1991. Effects of feed intake on particle distribution, passage of digesta, and extent of digestion in the gastrointestinal tract of cattle. J. Anim. Sci. 69:3435-3445.

Okumura, T., K. Saito, H. Sakuma, T. Nade, S. Nakayama, K. Fujita, and T. Kawamura. 2007. Intramuscular fat deposition in principal muscles from twenty-four to thirty months of age using identical twins of Japanese Black steers. J. Anim. Sci. 85:1902-1907. http://dx.doi.org/10.2527/jas.2006-752.

Pacheco, D., K. Lowe, J. L. Burke, and G. P. Cosgrove. 2009. Urinary nitrogen excretion from cows at different stage of lactation grazing different ryegrass cultivars during spring or autumn. Proc. New Zeal Soc. Anim. Prod. 69:196–200.

Reynolds, L. P., C. L. Ferrell, D. A. Robertson, and S. P. Ford. 1986. Metabolism of the gravid uterus, foetus and utero-placenta at several stages of gestation in cows. J. Agric. Sci. 106:437–444. http://dx.doi.org/10.1017/S0021859600063309.

Rincker, L. E. D., M. S. W. Nielsen, L. T. Chapin, J. S. Liesman, and M. J. CandeHaar. 2008. Effects of feeding prepubertal heifers a high-energy diet for three, six, or twelve weeks on feed intake, body growth, and fat deposition. J. Dairy Sci. 91:1913-1925. http://dx.doi.org/10.3168/jds.2006-210.

Rotta, P. P., S. C. Valadares Filho, T. R. Santos, L. F. Costa e Silva, T. E. Engle, M. I. Marcondes, M. M. Campos, A. C. B. Menezes, and A. A. Lobo. 2014. Effects of stage of gestation and feeding regimen in Holstein × Gyr cows: II. Maternal and fetal visceral organ mass. J. Dairy Sci. in press.

Santana, M. L., R. J. Pereira, A. B. Bignardi, L. El Faro, H. Tonhati, and L. G. Albequerque. 2014. History, structure, and genetic diversity of Brazilian Gir cattle. Livest. Sci. 163:26-33. http://dx.doi.org/10.1016/j.livsci.2014.02.007.

Scheaffer, A. N., J. S. Caton, M. L. Bauer, and L. P. Reynolds. 2001. Influence of pregnancy on body weight, ruminal characteristics, and visceral organ mass in beef heifers. J. Anim. Sci. 79:2481-2490.

Silva, A. A., A. L. S. Azevedo, R. S. Verneque, K. Gasparini, M. G. C. D. Peixoto, M. V. G. B. Silva, P. S. Lopes, S. E. F. Guimaraes, and M. A. Machado. 2011. Quantitative trait loci affecting milk production traits on bovine chromosome 6 in zebuine Gyr breed. J. Dairy Sci. 94:971-980. http://dx.doi.org/10.3168/jds.2009-2970.

Spears, R. A., R. A., Kohn, and A. J. Young. 2003. Whole-farm nitrogen balance on western dairy farms. J. Dairy Sci. 86:4178-4186. http://dx.doi.org/10.3168/jds.S0022-0302(03)74033-8.

Spek, J. W., J. Dijkstra, G. van Duinkerken, W. H. Hendriks, and A. Bannink. 2013. Prediction of urinary nitrogen and urinary nitrogen excretion by lactating dairy cattle in northwestern Europe and North America: A meta-analysis. J. Dairy Sci. 96:4310-4322. http://dx.doi.org/10.3168/jds.2012-6265.

Tamminga, S. 1992. Nutrition management of dairy cows as a contribution to pollution control. J. Dairy Sci. 75:345–357. http://dx.doi.org/10.3168/jds.S0022-0302(92)77770-4.

Tas, B. M., H. Z. Taweel, H. J. Smit, A. Elgersma, J. Dijkstra, and S. Tamminga. 2006. Effects of perennial ryegrass cultivars on milk yield and nitrogen utilization in grazing dairy cows. J. Dairy Sci. 89:3494–3500. http://dx.doi.org/10.3168/jds.S0022-0302(06)72388-8.

Valente, T. N. P., E. Detmann, S. C. Valadares Filho, M. Cunha, A. C. Queiróz, and C. B. Sampaio. 2011. In situ estimation of indigestible compounds contents in cattle feed and feces using bags made from different textiles. Braz. J. Anim. Sci. 40:666-675. http://dx.doi.org/10.1590/S1516-35982011000300027.

Van Egmond, K., T. Bresser, and L. Bouwman. 2002. The European nitrogen case. Ambio. 31:72–78. http://dx.doi.org/10.1579/0044-7447-31.2.72.

Verbic, J., X. B. Chen, N. A. Macleod, and E. R. Orskov. 1990. Excretion of purine derivatives by ruminants. Effect of microbial nucleic acid infusion on purine derivative excretion by steers. J. Agric. Sci. 114:243–248. http://dx.doi.org/10.1017/S0021859600072610.

Vonnahme, K. A., M. J. Zhu, P. P. Borowicz, T. W, Geary, B. W. Hess, L. P. Reynolds, J. S. Caton, W. J. Means, and S. P. Ford. 2007. Effect of early gestational undernutrition on angiogenic factor expression and vascularity in the bovine placentome. J. Anim. Sci. 85:2464-2472. http://dx.doi.org/10.2527/jas.2006-805.

Table 1. Ingredients and chemical composition of feed (means ± SD)

Item	DM, %
Ingredients	
Corn silage	93.0
Cotton meal	5.0
Limestone	0.5
Salt	0.5
Urea	0.9
Ammonium sulfate	0.1
Mineral mix[1]	0.02
Chemical composition	
DM	37.6 ± 0.36
OM	92.9 ± 0.60
CP	11.1 ± 0.12
NDF	49.7 ± 0.29
Ether extract	3.7 ± 0.06
NFC	28.4 ± 0.21
Indigestible neutral detergent fiber	15.7 ± 0.16

[1] Mineral mix composition = 29.2 g/kg of calcium, 0.70 g/kg of phosphorus, 2.11 g/kg of magnesium, 0.89 g/kg of potassium, 0.31 g/kg of sodium, 63.5 g/kg of sulfur, 348 mg/kg of cobalt, 2.56 mg/kg of chromium, 3,296 mg/kg of copper, 2,088 mg/kg of iron, 4,673 mg/kg of manganese, 7,817 mg/kg of zinc, and 318 mg/kg of selenium.

Table 2. Effects of different day of gestation and feeding regimens on intake (kg/d) in Holstein × Gyr cows (means ± SEM)

Feeding regimen	Day of gestation						P-value[1]
	122	150	178	206	234	262	
DM							
n (ad libitum)	20	15	15	10	10	5	
n (maintenance)	23	18	18	12	12	6	
Ad libitum	12.3^{b} ± 0.31	12.7^{ab} ± 0.31	13.5^{a} ± 0.31	12.0^{b} ± 0.34	9.22^{c} ± 0.402	7.73^{d} ± 0.521	< 0.01
Maintenance	6.63 ± 0.271	6.49 ± 0.271	6.46 ± 0.271	6.25 ± 0.304	6.12 ± 0.370	6.34 ± 0.434	0.27
P-value	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.03	
OM							
Ad libitum	11.4^{b} ± 0.29	11.8^{ab} ± 0.29	12.5^{a} ± 0.29	11.1^{b} ± 0.32	8.50^{c} ± 0.374	7.12^{d} ± 0.480	< 0.01
Maintenance	6.13 ± 0.252	6.00 ± 0.252	5.98 ± 0.252	5.88 ± 0.281	5.78 ± 0.344	5.86 ± 0.404	0.27
P-value	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.03	
CP							
Ad libitum	1.36^{b} ± 0.031	1.40^{ab} ±0.031	1.47^{a} ± 0.031	1.32^{b} ± 0.044	1.08^{c} ± 0.048	0.85^{d} ± 0.062	< 0.01
Maintenance	0.73 ± 0.030	0.71 ± 0.030	0.70 ± 0.030	0.69 ± 0.031	0.67 ± 0.042	0.68 ± 0.055	0.26
P-value	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	
NDF							
Ad libitum	6.34^{b} ± 0.160	6.56^{ab} ± 0.160	6.95^{a} ± 0.160	6.17^{b} ± 0.181	4.72^{c} ± 0.203	3.96^{d} ± 0.140	< 0.01
Maintenance	3.39 ± 0.145	3.34 ± 0.145	3.33 ± 0.145	3.22 ± 0.152	3.19 ± 0.191	3.24 ± 0.220	0.32
P-value	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.04	

[1] Main effects.

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

Table 3. Effects of different day of gestation and feeding regimens on apparent total tract digestibility (%) in Holstein × Gyr cows (means ± SEM)

Items	Feeding regimen		Day of gestation						P-value		
	Ad libitum	Maintenance	122	150	178	206	234	262	FR^{1}	DG^{2}	FR × DG^{3}
DM	68.7 ± 0.22	69.9 ± 0.20	72.3 ± 0.30	68.8 ± 0.30	69.4 ± 0.30	70.2 ± 0.33	67.7 ± 0.38	66.0 ± 0.48	< 0.01	0.01	0.03
Ad libitum	-	-	72.3^{aA} ± 0.45	68.4^{bB} ± 0.45	68.5^{bB} ± 0.45	68.9^{bB} ± 0.49	67.7^{cA} ± 0.56	65.6^{cA} ± 0.73	-	-	-
Maintenance	-	-	72.4^{aA} ± 0.40	70.2^{bA} ± 0.40	70.3^{bA} ± 0.40	70.4^{bA} ± 0.44	67.8^{cA} ± 0.52	66.4^{cA} ± 0.61	-	-	-
P-value	-	-	0.65	0.05	0.03	0.74	0.72	0.54	-	-	-
OM	70.1 ± 0.21	72.4 ± 0.19	74.7 ± 0.32	70.9 ± 0.32	71.4 ± 0.32	71.5 ± 0.35	72.2 ± 0.35	69.6 ± 0.52	< 0.01	< 0.01	0.02
Ad libitum	-	-	74.3^{aA} ± 0.48	70.3^{bB} ± 0.48	70.3^{bB} ± 0.48	70.6^{bB} ± 0.52	71.8^{bA} ± 0.60	68.9^{cA} ± 0.79	-	-	-
Maintenance	-	-	75.2^{aA} ± 0.43	72.5^{bA} ± 0.43	72.4^{bA} ± 0.43	72.8^{bA} ± 0.47	72.5^{bA} ± 0.56	70.3^{cA} ± 0.66	-	-	-
P-value	-	-	0.85	0.04	0.02	0.68	0.64	0.59	-	-	-
CP	70.0 ± 0.30	69.7 ± 0.26	73.6 ± 0.44	66.6 ± 0.44	67.8 ± 0.44	70.7 ± 0.49	72.2 ± 0.57	68.3 ± 0.71	0.23	< 0.01	0.03
Ad libitum	-	-	74.1^{aA} ± 0.66	67.9^{bA} ± 0.66	67.8^{bA} ± 0.66	70.6^{bA} ± 0.72	70.4^{bA} ± 0.84	69.1^{bA} ± 1.10	-	-	-
Maintenance	-	-	73.2^{aA} ± 0.59	67.8^{bA} ± 0.59	68.2^{bA} ± 0.59	70.5^{bA} ± 0.65	70.5^{bA} ± 0.77	67.9^{bA} ± 0.92	-	-	-
P-value	-	-	0.64	0.39	0.45	0.50	0.46	0.48	-	-	-
NDF	61.1 ± 0.32	65.7 ± 0.28	66.4^{a} ± 0.44	63.7^{b} ± 0.44	64.1^{b} ± 0.44	64.2^{b} ± 0.48	64.2^{b} ± 0.56	61.4^{c} ± 0.70	< 0.01	< 0.01	0.19

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

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

[1] Feeding regimen main effects.

[2] Day of gestation main effects.

[3] Interaction between feeding regimen and day of gestation.

Table 4. Effects of different day of gestation and feeding regimens on urinary concentrations of urea and purine derivatives in Holstein × Gyr cows (means ± SEM)

	Feeding regimen		Day of gestation						P-value		
Items	Ad libitum	Maintenance	122	150	178	206	234	262	FR[1]	DG[2]	FR×DG[3]
Urea, g/d	205 ± 4.4	143 ± 4.2	152 ± 4.3	161 ± 4.3	161 ± 4.3	174 ± 7.4	186 ± 8.0	210 ± 10.7	< 0.01	< 0.01	< 0.01
Ad libitum	-	-	187^{aA} ± 6.2	203^{aA} ± 6.2	202^{aA} ± 6.2	215^{aA} ± 10.7	197^{aA} ± 11.5	224^{aA} ± 15.6	-	-	-
Maintenance	-	-	117^{bB} ± 5.9	118^{bB} ± 5.9	120^{bB} ± 5.9	132^{bB} ± 10.3	175^{aA} ± 11.1	196^{aA} ± 14.6	-	-	-
P-value	-	-	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	-	-	-
Allantoin, mmol/d	309 ± 8.8	185 ± 8.3	259 ± 8.1	253 ± 8.1	263 ± 8.1	249 ± 13.4	233 ± 16.0	228 ± 23.2	< 0.01	0.54	0.81
Uric acid, mmol/d	36.3 ± 1.60	13.1 ± 1.49	22.2^{b} ± 1.46	18.4^{b} ± 1.46	25.0^{ab} ± 1.46	24.9^{ab} ± 2.43	29.3^{a} ± 2.89	30.5^{a} ± 4.20	< 0.01	0.02	0.54
AP[4], mmol/d	302 ± 9.0	158 ± 8.4	241 ± 8.2	228 ± 8.2	247 ± 8.2	229 ± 13.7	218 ± 16.2	219 ± 19.7	< 0.01	0.61	0.79
Microbial[5]	182 ± 5.6	103 ± 5.3	158 ± 5.2	153 ± 5.2	161 ± 5.2	144 ± 8.6	122 ± 10.2	138 ± 14.8	-	-	-
Microbial efficiency[6]	134 ± 4.2	141 ± 3.9	132 ± 3.9	134 ± 3.9	139 ± 3.9	136 ± 6.4	142 ± 7.6	143 ± 11.0	0.18	0.79	0.84

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

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

[1] Feeding regimen main effects.

[2] Day of gestation main effects.

[3] Interaction between feeding regimen and day of gestation.

[4] Absorbed purines.

[5] Determined by assuming daily purine derivative excretion (dPD; mmol/kg of $BW^{0.75}$) = PD index × 0.9; daily absorbed purines (daP) = [dPD (mmol/kg of $BW^{0.75}$) − $0.385 \times BW^{0.75}$] + 0.85; and microbial N (g of N/d) = (daP × 70)/(0.116 × 0.83 × 1,000).

[6] Expressed as g of CP/kg TDN.

Table 5. Effects of different day of gestation and feeding regimens on carcass characteristics of Holstein × Gyr cows (means ± SEM)

Item	Feeding regimen		Day of gestation				P-value		
	Ad libitum	Maintenance	139	199	241	268	FR[1]	DG[2]	FR × DG[3]
n	20	23	10	11	11	11			
Initial BW, kg	482 ± 10.6	477 ± 9.6	488 ± 14.2	480 ± 13.9	480 ± 13.9	468 ± 13.9	-	-	-
Final BW, kg	644 ± 12.9	521 ± 11.7	553 ±17.2	577 ± 16.9	596 ± 16.9	603 ± 16.9	< 0.01	0.21	0.64
Empty BW, kg	580 ± 11.0	456 ± 10.0	484^{b} ± 14.7	505^{b} ± 14.4	537^{a}± 14.4	545^{a} ± 14.4	< 0.01	0.02	0.51
ADG, kg	1.11 ± 0.06	0.30 ± 0.05	0.73 ± 0.09	0.73 ± 0.08	0.70 ± 0.08	0.68 ± 0.08	< 0.01	0.85	0.65
ADG without gravid uterus, kg	0.94 ± 0.05	0.11 ± 0.04	0.61 ± 0.07	0.61 ± 0.06	0.48 ± 0.06	0.41 ± 0.06	< 0.01	0.04	0.02
Ad libitum	-	-	1.05^{aA} ± 0.11	1.00^{aA} ± 0.10	0.90^{abA} ± 0.10	0.82^{bA} ± 0.10	-	-	-
Maintenance	-	-	0.17^{aB}± 0.06	0.22^{aB} ± 0.05	0.05^{bB} ± 0.05	-0.01^{cB} ± 0.05	-	-	-
P-value	-	-	< 0.01	< 0.01	< 0.01	< 0.01	-	-	-
Empty body gain, kg	1.27 ± 0.07	0.41 ± 0.06	0.82 ± 0.09	0.88 ± 0.10	0.84 ± 0.10	0.82 ± 0.10	< 0.01	0.85	0.87
Gastrointestinal content, kg	64.1 ± 0.59	65.3 ± 0.52	69.0^{a} ± 0.84	72.2^{a} ± 0.78	58.9^{b} ± 0.78	57.8^{b} ± 0.78	0.21	0.01	0.63
Hot carcass dressing, %	52.2 ± 0.48	51.5 ± 0.44	54.2^{a} ± 0.67	52.5^{a} ± 0.63	52.0^{a} ± 0.63	48.8^{b} ± 0.63	0.27	< 0.01	0.42
Cold carcass dressing, %	51.6 ± 0.46	50.4 ± 0.41	52.9^{a} ± 0.64	51.8^{a} ± 0.60	51.0^{a} ± 0.60	48.1^{b} ± 0.60	0.16	< 0.01	0.42

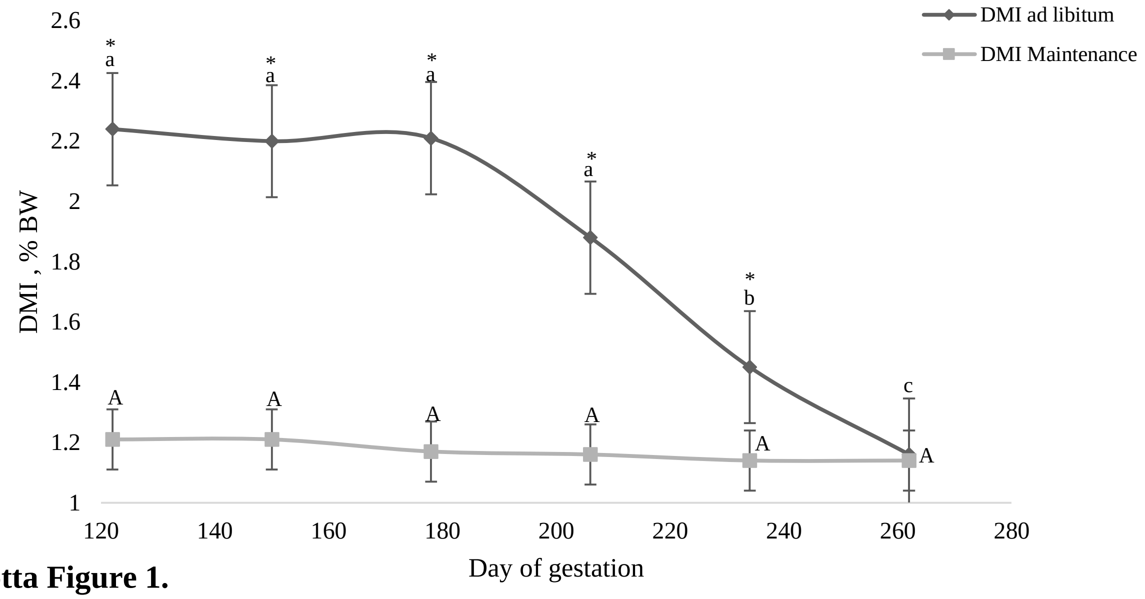
$^{a\text{-}b}$Means within a row with different superscripts differ ($P \leq 0.10$).

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

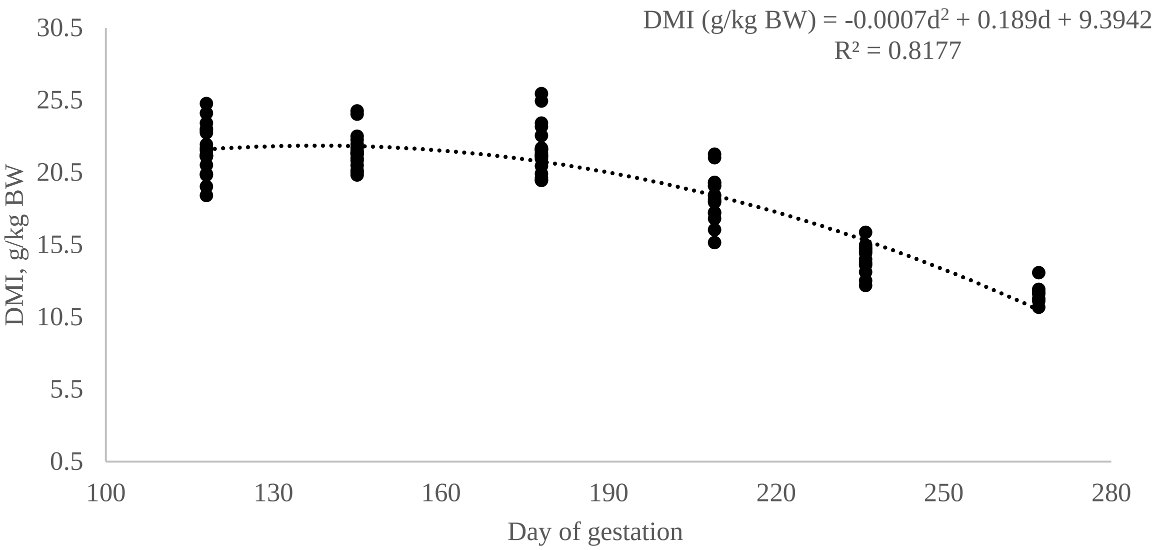
[1] Feeding regimen.

[2] Day of gestation.

[3] Interaction between feeding regimen and day of gestation.



Rotta Figure 1.



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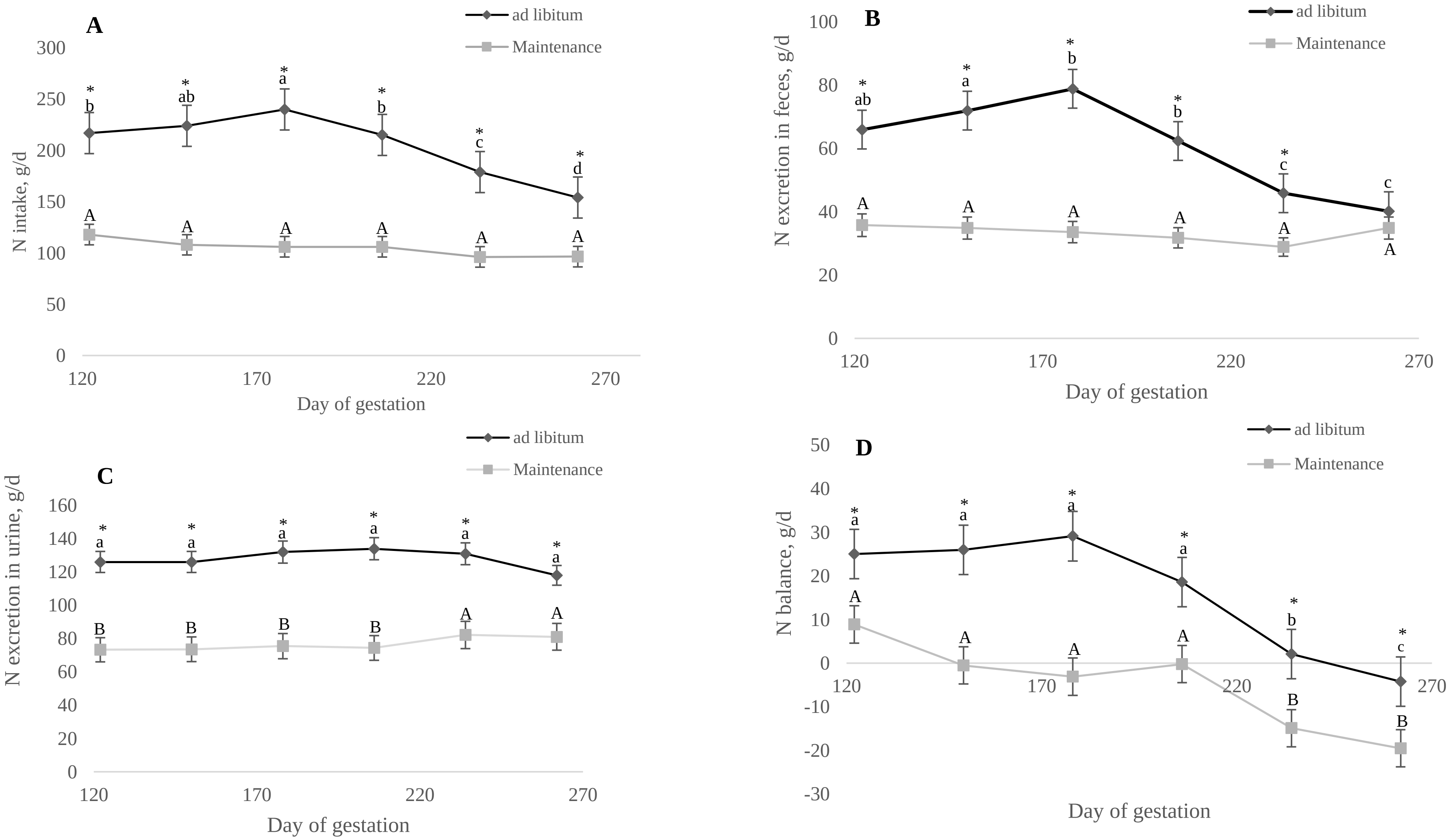
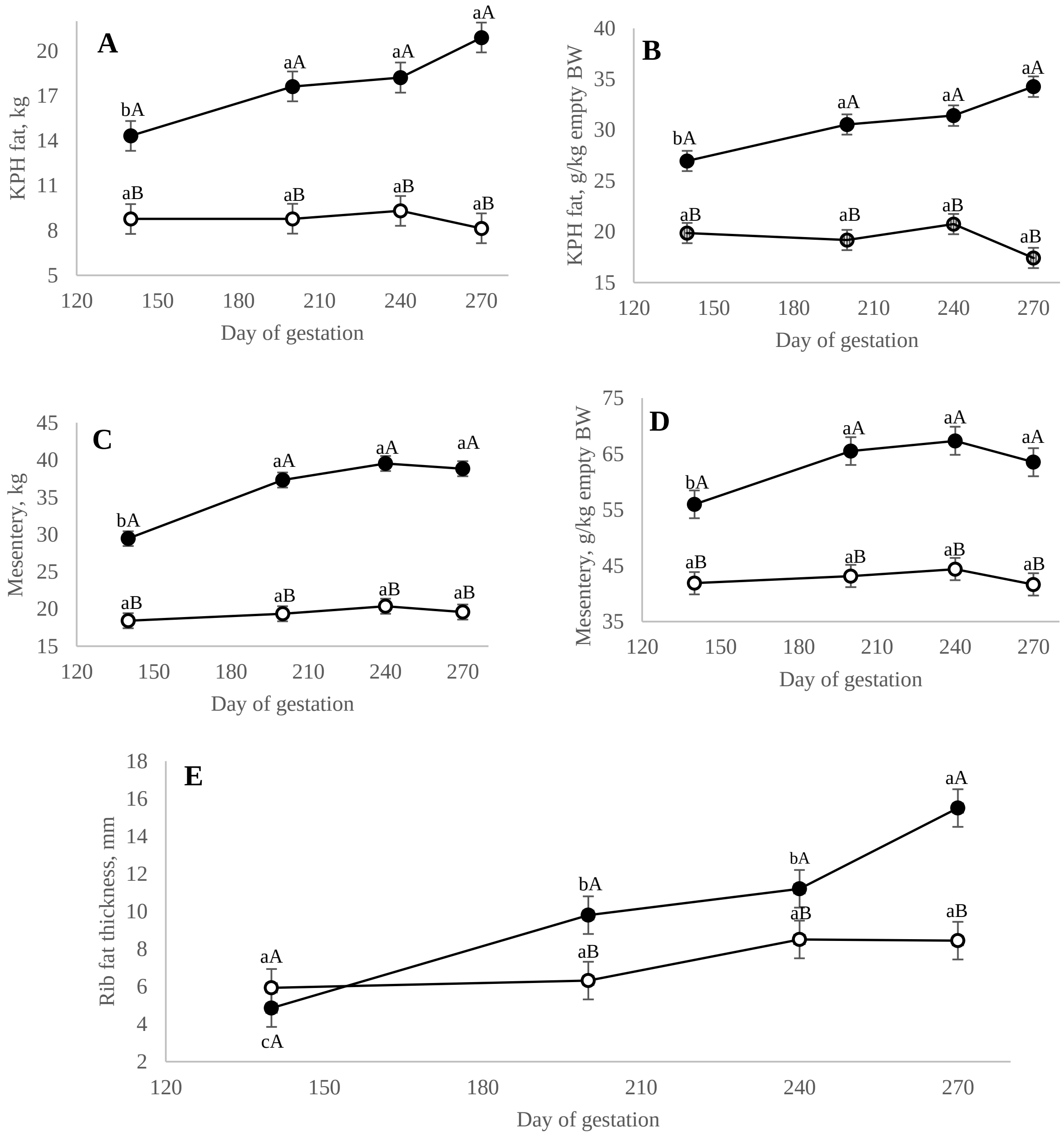


Rotta Figure 3.



Rotta Figure 4.

Figure 1. Dry matter intake calculated as percentage of BW in cows fed ad libitum and maintenance levels during gestation. Results are means ± SEM. *Different at $P \leq 0.10$ between feeding regimens. Different lowercase letters are different at $P \leq 0.10$ for ad libitum diets. Different uppercase letters are different at $P \leq 0.10$ for maintenance diets.

Figure 2. Dry matter intake expressed as gram per kilogram of BW in cows fed ad libitum during gestation and an estimated equation to predict DMI in Holstein × Gyr pregnant cows (118 to 268 d of gestation).

Figure 3. Nitrogen intake (**A**), N excretion in feces (**B**) and urine (**C**), and N balance (**D**) in cows fed ad libitum or maintenance levels during gestation. Results are means ± SEM. *Different at $P = 0.04$ between feeding regimens. Different lowercase letters are different at $P \leq 0.10$ for ad libitum diets. Different uppercase letters are different at $P \leq 0.10$ for maintenance diets.

Figure 4. Kidney, pelvic, and heart fat (KPH) (kg; **A**), KPH (g/kg empty BW; **B**), mesentery mass (kg; **C**), mesentery mass (g/kg empty BW; **D**), and rib fat thickness (mm; **E**) in cows fed ad libitum (•) or maintenance (○) levels during gestation. Results are means ± SEM. Different lowercase letters differed among day of gestation and different capital letters differed between feeding regimens at $P \leq 0.10$.

CHAPTER 3

Interpretive summary

Fetal development of dairy cows

Rotta

In the present study, we evaluated the effects of day of gestation and feeding regimen in dairy cows on maternal and fetal visceral organ mass. We found that maternal feeding regimen affects mass of heart, liver, mammary gland, and gastrointestinal tract in cows. However, fetal development was not observed to be affected by maternal feeding regimen.

DAY OF GESTATION AND DIET LEVELS IN COWS

Effects of day of gestation and feeding regimen in Holstein × Gyr cows:II. Maternal and fetal visceral organ mass

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ABSTRACT

This study investigates the influence of day of gestation (DG) and feeding regimens (FR) on maternal and fetal visceral organ mass in Holstein × Gyr cows. Forty-four pregnant multiparous Holstein × Gyr cows with an average initial body weight of 480 ± 10.1 kg and an average initial age of 5 ± 0.5 years old were allocated to 1 of 2 FR: ad libitum (AL; n = 20) or maintenance level (ML; n = 24). Maintenance level was considered to be 1.15% of body weight (dry matter basis) and met 100% of the energy requirements and AL provided 190% of the total net energy requirements. Cows were individually fed a corn silage-concentrate based diet composed of 93% roughage and 7% concentrate (dry matter basis) as a total mixed ration twice daily. Pregnant cows were slaughtered at 4 DG: 139 (n = 11), 199 (n = 11), 241 (n = 11), and 268 (n = 11) d, which was followed by necropsy. Mass of heart, liver, and gastrointestinal were heavier in AL- than in ML-fed cows. Mammary gland mass was heavier in AL- than in ML-fed cows, and the heaviest mass was observed at 268 d of gestation. Feeding regimen did not influence fetal body weight in this study. The majority of the visceral organ masses were similar in fetuses from cows fed AL or ML. This data indicates that maternal feed-restriction does not affect the development of most fetal organs and fetal development; however, some maternal organs are affected according to the FR that is provided. Moreover, the negative impact on mammary gland mass caused by the ML probably will not affect the subsequent lactation because the crude protein concentration in the mammary gland increased with this FR. However, we suggest that the AL diet in pregnant dry cows should be provided with caution since the amount of fat in the mammary gland increased at 268 d of gestation.

Key words: fetal development, gastrointestinal tract, mammary gland, small intestine

INTRODUCTION

Caloric needs for gestation in beef cattle increase approximately 5.5 times from early to late gestation, but approximately 50% of this increase can be accounted for the needs of the gravid uterus (Scheaffer et al., 2001). Moreover, according to Ferrell (1988), visceral tissues are responsible for making up about 41% of the total energy expenditure even though they only represent 6% of the empty body weight (**EBW**) in beef cows. In nonlactating dairy cows, Smith and Baldwin (1973) found that the liver accounts for 22.5% of the total animal energy expenditure. Thus, a feeding regimen (**FR**) that supplies nutritional requirements without compromising fetal development may provide an alternative that increases savings in nutritional costs for dairy cow production.

There are few studies (He et al., 2013; Drackley et al., 2014) that have been conducted to evaluate the effects of FR on maternal and fetal organ mass in dairy cows. Some studies have demonstrated that underfeeding during gestation can cause intrauterine growth restriction, which results in impaired development and potential long-term consequences (He et al., 2013). Growth of the mammary gland is significantly retarded by overfeeding in the later stages of the pre pubertal period, thus resulting in reduced milk yield (Tucker, 1981; Sejrsen et al., 1983). However, overfeeding during post puberty appears to have no ill effect on mammary growth (Tucker, 1981). However, no study has been conducted with pregnant Holstein × Gyr cows fed at different levels in order to evaluate the mammary gland composition and growth.

Some authors (Scheaffer et al., 2004a; Reed et al., 2007; Meyer et al., 2010b) have suggested that the dam may compensate for restricted diets by altering the size and morphology of the maternal gastrointestinal tract in response to gestation status and nutritional level to spare problems to her offspring. However, in dairy cows, there are few studies (Drackley et al., 2014) investigating the influence of FR on the gastrointestinal tract. We hypothesized that cows fed a maintenance level (**ML**) diet that does not result in body weight loss during gestation would not negatively affect fetal development when compared to an ad libitum (**AL**) diet; moreover AL diet increases the costs of production. Our objectives were therefore to evaluate the influence of DG and FR on maternal and fetal visceral organ mass and fetal development in Holstein × Gyr cows.

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, prior to initiation of the experiment.

Animals and Management

The management practices that were adopted for the animals in this present experiment were previously described in Rotta et al. (2014a). However, a brief description is provided as follows: 44 multiparous dry Holstein × Gyr cows with an average initial BW of 480 ± 10.1 kg and age of 5 ± 0.5 years were allocated to 1 of 2 FR: AL (n = 20) or ML (n = 24). The average age of the cows that were used in this experiment was chosen with the aim of representing the average age of cows raised in Brazil (IBGE, 2009). Maintenance level was assumed to be 1.15% of BW (in DM basis). To evaluate the effects of different day of gestation (**DG**), pregnant cows were slaughtered at 4 DG. Each FR group (ML and AL) was randomly divided into 4 groups, with 5 cows from AL and 6 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 (DM basis) as a TMR twice daily, with 60% in the morning and 40% in the afternoon (DM basis).

Tissue Collection

Pre-harvest handling of animals 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. Feed was withheld overnight, but cows had ad libitum access to water. Cows were slaughtered at Universidade Federal de Viçosa by stunning them with a captive bolt followed by exsanguination.

After exsanguination, the gravid uterus was immediately collected, and the fetus was removed. Maternal viscera were removed, dissected, and weighed. The digestive tract was gently stripped of fat and digesta, and the stomach complex and intestines were dissected. The esophagus was removed from its entry at the dorsal sac of the rumen and abomasum as well as from the intestine at the pyloric valve. The stomach complex was then divided into the reticulum-rumen, omasum, and abomasum, and each component was weighed. The dissection of the fetus and isolation of the gastrointestinal tract was performed similarly to that described by Meyer et al. (2010a); briefly, fetuses were dissected and the entire gastrointestinal tract was collected and gently stripped of fat and digesta. The stomach complex was isolated from the esophagus and the intestine at the pyloric valve and divided into reticulum-rumen, omasum, and abomasum. Each component was gently emptied and weighed. Small and large intestines were isolated, and their weight and length were recorded separately. The small intestine was then divided into duodenum, jejunum, and ileum similarly to that described by Soto-Navarro et al. (2004) as follows. The duodenum was identified as the segment between the pylorus to a point directly adjacent to the entry of the gastrosplenic vein into the mesenteric vein. The jejunum was the segment from the caudal end of the duodenum to the junction of jejunum and ileum. This junction was determined by measuring 15 cm up the mesenteric vein from the convergence of the mesenteric and ileocecal veins, and then up the mesenteric arcade to the point of intestinal intersection. The ileum measurement was terminated at the ileocecal junction. The mammary gland was removed and cut in slices of approximately 4 cm^2, which were then ground in a commercial meat mill. A sample of mammary gland tissue was collected after grinding, which was then lyophilized for later analyses. Mammary tissue samples were analyzed for DM (AOAC, 2000) and ether extract (AOAC, 2006).

Statistical Analysis

Before performing the statistical analysis, we realized the test of 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 (139, 199, 241, and 268 d), maternal FR (AL vs. ML), 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 an interaction between maternal FR × DG, main effects are reported; otherwise, interaction means are discussed.

RESULTS

Maternal Viscera and Organ Mass

We did not observe any interactions (P = 0.14) between FR and DG for the mass of claws (phalanxes and external tissues), head, hide, blood, or mammary gland (Table 1). However, heavier (P = 0.07) masses were observed for the claws and head in AL- than in ML-fed cows. In contrast, mass of both the claws and head were heavier (P < 0.01) in ML-fed cows expressed as gram per kilogram of EBW due to the ML-fed cows' lower BW.

Mass of hide and blood were heavier (P < 0.01) in AL- than in ML-fed cows (Table 1). Nevertheless, no difference (P = 0.27) was observed when those characteristics are expressed as gram per kilogram of EBW. Mammary gland mass was affected (P < 0.01) by FR. Ad libitum-fed cows had heavier (P < 0.01) mammary glands than ML-fed cows (Table 1). However, concentration of CP in the mammary gland of ML-fed cows was greater (P = 0.09) than AL-fed cows at 268 d of gestation (Figure 1). Moreover, the concentration of fat in the mammary gland was greater (P = 0.04) in AL-fed cows at 268 d of gestation than ML-fed cows (Figure 2). Also, DG influenced (P < 0.01) mammary gland mass (Table 1). Cows at 268 d of gestation were observed to have the heaviest (P < 0.01) mammary gland. The mammary gland mass was heavier during late gestation, which coincides with the period of the most rapid fetal growth; in the last month of gestation, mammary gland mass increased by about 80.1 ± 3.58% when compared with the previous month (241 d of gestation).

No interaction (P = 0.11) was observed between DG and FR for mass of heart, liver, lungs, spleen, diaphragm, trachea plus esophagus and when those were expressed as gram per kilogram of EBW, and kidney (Table 2). However, an interaction (P = 0.06) between DG and FR was observed for the kidneys expressed as gram per kilogram of EBW. Maintenance-fed cows had heavier (P < 0.01) kidneys expressed as gram per kilogram of EBW at 241 and 268 d of gestation than did the AL-fed cows. We did not observe an effect ($P = 0.41$) on kidneys expressed as gram per kilogram of EBW in ML-fed cows among DG. However, AL-fed cows had lighter ($P = 0.36$) mass of kidney at 241 and 268 d than those observed at 139 d of gestation.

Mass of heart, liver, kidneys, lungs, spleen, diaphragm, trachea plus esophagus, and the liver and spleen expressed as gram per kilogram of EBW were heavier ($P = 0.02$) in AL- than in ML-fed cows (Table 2). However, mass of the heart and lungs expressed as gram per kilogram of EBW were heavier ($P < 0.01$) in ML-fed cows.

Day of gestation affected ($P < 0.01$) mass of the heart, liver, and spleen expressed as gram per kilogram of EBW as well as the mass of the diaphragm and trachea plus esophagus (Table 2). Heavier ($P < 0.01$) mass of heart, liver, and spleen expressed as gram per kilogram of EBW were observed for cows at 139 and 199 d than at 241 and 268 d of gestation. In contrast, heavier ($P = 0.08$) mass of diaphragm and trachea plus esophagus were observed at 268 d than 139 d of gestation, and no difference ($P = 0.65$) was found across 199, 241, and 268 d of gestation for the mass of trachea plus esophagus.

There was no interaction ($P = 0.19$) between DG and FR for the mass of the reticulum-rumen, omasum, abomasum, small, and large intestines expressed as kilogram and gram per kilogram of EBW (Table 3). Effects ($P = 0.09$) of FR were observed on the mass of reticulum-rumen, omasum, abomasum, small, and large intestines and for the abomasum, small, and large intestines expressed as gram per kilogram of EBW. Abomasum, small, and large intestines in AL-fed cows were heavier ($P = 0.09$) than in ML-fed cows; however, these organs expressed as gram per kilogram of EBW were heavier in ML-fed cows ($P = 0.07$).

Day of gestation influenced ($P = 0.07$) reticulum-rumen expressed as kilogram and gram per kilogram of EBW, and omasum and large intestine expressed as gram per kilogram of EBW. The reticulum-rumen was heavier (P = 0.07) at 139 than 268 d of gestation, but reticulum-rumen expressed as gram per kilogram of EBW was heavier (P < 0.01) at 139 d than 199, 241, and 268 d of gestation. Omasum and large intestine expressed as gram per kilogram of EBW were heavier (P < 0.01) at 139 d than 241 and 268 d of gestation.

Fetal Viscera and Organ Mass

Fetal BW and measurements are presented in Table 4. No interaction (P = 0.11) between FR and DG was observed, although differences (P = 0.09) were observed between FR for the mass of claws, carcass weight expressed as gram per kilogram of fetal BW, fetal length, fetal chest length, fetal cranial circumference, and fetal hip height. Ad libitum-fed cows had the greatest values (P = 0.09) for these characteristics.

Day of gestation influenced (P < 0.01) all characteristics that were evaluated to investigate fetal development (fetal BW, fetal eviscerated BW, head, claws, and carcass weight, fetal length, chest length, cranial and neck circumferences, hip and withers height). Fetal BW, eviscerated BW, head, claws, and carcass weights were different (P < 0.01) among the studied DG, with the heaviest values observed at 268 d and the lowest (P < 0.01) values noticed at 139 d of gestation. Fetal BW increased approximately 50% (241 d = 21.8 ± 1.00 kg and 268 d = 32.3 ± 1.00 kg) in the last month of gestation. During the last 4 months of gestation, fetal BW increased approximately 20 times.

Fetal length, fetal chest length, cranial and neck circumference, hip and withers height were longer (P < 0.01) at 268 than 139, 199, and 241 d of gestation. One exception to this was that cranial circumference was similar at 241 and 268 d of gestation.

Table 5 lists the mass of fetal visceral organs. We only observed an interaction ($P = 0.02$) between FR and DG for the reticulum-rumen expressed as gram per kilogram of fetal BW. We observed that the mass of fetal reticulum-rumen expressed as gram per kilogram of fetal BW differed ($P < 0.01$) between AL- and ML-fed cows at 139 d of gestation, and the fetal reticulum-rumen expressed as gram per kilogram of fetal BW was heavier in AL- than ML-fed cows. Ad libitum and ML-fed cows had lighter ($P < 0.01$) fetal reticulum-rumen expressed as gram per kilogram of fetal BW at 241 and 268 d than at 139 and 199 d of gestation. However, for ML-fed cows, the mass was similar ($P = 0.03$) at 139 and 199 d of gestation.

The only effects of FR observed on fetal visceral organ mass were on the bladder and diaphragm expressed as gram per kilogram of fetal BW. Maintenance-fed cows had heavier ($P < 0.01$) bladders expressed as gram per kilogram of fetal BW and lighter ($P = 0.01$) diaphragms expressed as gram per kilogram of fetal BW than AL-fed cows.

Alternatively, DG affected fetal visceral organ mass. Mass of heart, liver, kidneys, lungs, reticulum-rumen, abomasum, spleen, bladder, gallbladder, and diaphragm were heavier ($P < 0.01$) at 268 d than 139, 199, and 241 d of gestation. At 241 d, those masses were heavier ($P < 0.01$) than at 139 and 199 d of gestation; at 199 d, heavier ($P < 0.01$) masses were observed than at 139 d of gestation. However, mass of omasum was similar ($P = 0.36$) between 241 and 268 d of gestation, but heavier ($P < 0.01$) than 139 and 199 d of gestation. If we compare fetal visceral organs expressed as gram per kilogram of fetal BW, heart was heavier ($P < 0.01$) at 139 and 199 d than at 241 and 268 d of gestation. Liver and kidneys were heavier ($P < 0.01$) at 139 d than 199, 241, and 268 d of gestation, and at 199 d, a heavier ($P < 0.01$) mass was observed than at 241 and 268 d of gestation. The lungs had the heaviest ($P < 0.01$) mass at 139 d, and the lightest ($P < 0.01$) mass was observed at 268 d of gestation. Omasum expressed as gram per kilogram of fetal BW had different ($P < 0.01$) results for all evaluated DG, with the heaviest mass observed at 139 d of gestation. The mass of the abomasum, spleen, and diaphragm expressed as gram per kilogram of fetal BW did not differ ($P = 0.15$) between the different DG.

The effects of FR and DG on the small and large intestines are presented in Table 6. We observed an interaction ($P = 0.06$) of small intestine expressed as gram per kilogram of fetal BW. At 139 d of gestation, fetuses from AL-fed cows had heavier ($P = 0.04$) small intestine than fetuses from ML-fed cows. No effect ($P = 0.47$) was observed at 199, 241, and 268 d of gestation in fetuses from AL- and ML-fed cows. Fetuses from AL-fed cows had heavier ($P < 0.01$) small intestine at 139 and 199 d than at 241 and 268 d of gestation. Fetuses from ML-fed cows had heavier ($P < 0.01$) small intestine at 199, 241, and 268 d than at 139 d of gestation.

Feeding regimen influenced ($P = 0.04$) the length of the large intestine. Fetuses from AL-fed cows had longer large intestines than those of ML-fed cows. However, DG had a diverse effect on the mass and length of small and large intestines. Mass of small and large intestinal were different in all studied DG. Heavier ($P < 0.01$) masses were found at 268 d than at 139, 199, and 241 d of gestation. The lowest ($P < 0.01$) masses of the small and large intestine were observed at 139 d of gestation.

The lengths of the small and large intestine were longer ($P < 0.01$) at 241 and 268 d, with the shortest ($P < 0.01$) lengths observed at 139 d of gestation. The length of the duodenum did not differ ($P = 0.30$) at 139, 199, and 241 d. The lengths of the jejunum were highly variable ($P < 0.01$) among the studied DG. The longest ($P < 0.01$) value was observed at 268 d and the shortest at 139 d of gestation. The lengths of the ileum were similar ($P = 0.85$) at 199, 241, and 268 d of gestation, with the shortest ($P < 0.01$) value at 139 d of gestation.

DISCUSSION

Maternal Viscera and Organ Mass

Blood plays many important roles in the body. Its major roles during gestation include transport of nutrients, oxygen, and wastes, as well as temperature regulation, immunity, communication, and defense (Ford et al., 1995). We therefore expected a greater amount of blood in AL-fed cows, since those animals have more nutrients, oxygen, and wastes to be transported. The amount of blood in the body is related to BW, and AL-fed cows had heavier BW than ML-fed cows. However, we can infer that the amount of blood in the body is related mainly to BW because no difference was observed when considering the measurement expressed as gram per kilogram of EBW.

Sejrsen et al. (1982) evaluated the effect of nutrition on mammary development of pre pubertal and post pubertal heifers that were fed 60:40 concentrate:roughage, either ad libitum or restricted to 60% of ad libitum. Feed restricted heifers' ADG was 0.61 vs. 1.22 kg/d for ad libitum heifers, and they observed that ad libitum feeding in the pre pubertal group decreases mammary parenchymal tissue mass by 23% and decreases mammary DNA by 32% when compared with the feed restricted group. However, feeding level had no effect on post pubertal heifers and the composition of mammary parenchyma was not affected by plane of nutrition.

Maintenance-fed cows demonstrated growth restriction in their mammary glands when compared with AL-fed cows. However, this negative impact on the mammary gland caused by the ML might not have a negative influence on the subsequent lactation, because the CP concentration in the mammary gland increased with this FR. On the other hand, cows fed AL presented with a greater concentration of fat in the mammary gland, which suggests a negative influence on the subsequent lactation since the excess fat pad negatively influences the milk production (Capuco et al., 1997). Roche et al. (2007) evaluated the relationships among BCS, BW, and milk production variables in pasture-based dairy cows and concluded that the optimum calving BCS for milk production was approximately 3.5 in the 5-point scale. Considering that in the present study the BCS of AL-fed cows was approximately 4.5 in the 5-point scale, we may infer that these cows were obese and that the milk production may be decreased in the next lactation. Silva et al. (2002) studied the relationship between body growth and mammary development in dairy heifers and concluded that increased body fat was a better predictor of impaired mammary development than rapid BW gain; thus, if we consider that the same may occur in cows, we may infer that AL-fed cows may have impaired mammary glands because of the greatest amount of fat deposition in the body, as observed by Rotta et al. (2014a).

Carlson et al. (2009) observed that when considering the absolute mass, the heart was heavier in ewes that were fed 100% of requirements than in those that were underfed. Ekpe et al. (2000) also described a heavier heart mass in ewes fed an AL-diet in comparison with those fed restricted diets. They also observed a positive relationship between heart mass and the density of β1 adrenoceptors. According to Guyton (1976), a large heart may be associated with a larger force of contraction, and a greater number of β1 adrenoceptors may favor development of a large force of contraction. Ekpe et al. (2000) reported that animals fed a restricted diet had fewer β1 adrenoceptors in their heart membranes than those fed an AL-diet.

A study by Carlson et al. (2009) supports the results that were observed in the present study regarding the liver, as they evaluated the effects of feed restriction during defined periods of gestation on maternal adaptations in ewes, and observed that animals fed approximately 0.9 kg/d had heavier liver mass than those fed 0.7 and 0.5 kg/d. According to Reed et al. (2007) the liver is easily affected by nutrient restriction. Meyer et al. (2010a) studied the effect of nutrient restriction and day of gestation (125 and 245 d of gestation) in beef cows and observed an interaction between feeding regimen and day of gestation when evaluating the liver mass. On d 125, mass of the liver from nutrient-restricted cows were lighter than those of control cows with gain of 0.72 kg/d according to the NRC (2000), but no difference was observed at 245 d of gestation (Meyer et al., 2010a). In contrast, in the present study, we did not observe interactions between FR and DG or any effects of DG on liver mass. Similar results were shown by Scheaffer et al. (2001) who evaluated the mass of liver at 40, 120, 200, and 270 d of gestation. These authors indicated that there was no effect of pregnancy and day of gestation on liver mass.

The increased kidney mass in AL- vs. ML-fed cows that was observed in the current study corroborates with results from Carlson et al. (2009), in which feeding nutrient restricted diets to pregnant ewes reduced kidney mass. These studies evaluated different nutrient restricted diets in pregnant sheep, and showed lower mass of kidney in ewes that were fed nutrient restricted diets. The present results also agree with those of Ekpe et al. (2000), who studied different nutritional levels in ewes, and observed that animals fed an AL-diet had heavier mass of kidney. Kasiske et al. (1988) demonstrated in obesity-prone animal models that excess body-fat mass results in renal-specific consequences. Rotta et al. (2014a) demonstrated that fat deposition was greater in AL- than ML-fed cows, and if we consider that the cows that were fed AL-diet in this experiment received 190% of their energy for maintenance, we can infer that those cows were obese. Also, Williams et al. (2007) observed an increased incidence of renal disease with obesity in rats, and that prenatal malnutrition eliminated any renal histopathology associated with juvenile obesity.

According to Scarpace and Yu (1986), diet restriction in rats retarded the age-related loss of beta-adrenergic receptors and adenylate cyclase activity in the lungs. They observed that dietary restriction prolonged the life span and retarded a variety of physiological processes that change with age. In dairy cattle, there are several studies evaluating the effect of different physiological stages (prepartum, early lactation, and late lactation), different diets for heifers, and days relative to calving on organ mass (Andrew et al., 1994; Moallem et al., 2004; Reynolds et al., 2004). In beef cows, Wood et al. (2013) did not observe a difference in lung mass between cows that were fed 85 and 140% of their nutritional requirements. According to Cesta (2006), the 3 main factors that affect spleen development are species, age, and genetic composition. However, we demonstrated in this study that FR also influences spleen mass.

Wood et al. (2013) demonstrated a heavier rumen mass in cows that were fed 140% of their nutrient energy requirements when compared to those fed 85%. According to these authors, this may be due to a greater rumen fill, which could influence rumen mass. However, when the rumen was measured relative to BW or hot carcass weight, no difference was observed in FR by those authors. We observed a relationship between reticulum-rumen mass and DMI (Table 3 and Rotta et al., 2014a). From 199 d of gestation, DMI expressed as percentage of BW began decreasing in AL-fed cows, which coincides with the DG during which the reticulum-rumen mass also started decreasing (Rotta et al., 2014a). The lower rumen fill beginning at 199 d of gestation may be related to the decrease in reticulum-rumen mass expressed as kilogram and gram per kilogram of EBW.

Similar to our findings in the present study, Meyer et al. (2010a) also observed a heavier omasum mass in beef cows that were fed a control diet, which exceeded the energy requirements with a gain of 0.72 kg/d according to the NRC (2000) of cows that were fed a nutrient restricted diet. Carlson et al. (2009) presented similar results for omasum mass; these authors studied the effect of a diet with 100% of NRC (2007) requirements and another with 60% of NRC (2007) requirements on pregnant ewes. Thus, both the mass of reticulum-rumen and omasum are affected by FR in pregnant dairy cows.

The greatest mass that were observed in the small and large intestines expressed as gram per kilogram of EBW in ML-fed cows in this study may be due to the fact that the gastrointestinal tract serves as the main site for nutrient absorption. Maintenance-fed cows show changes in maternal visceral organs during pregnancy, which positively affect the fetuses, since no difference was observed in fetal weight between ML- and AL-fed cows (Table 4). Scheaffer et al. (2004 a, b) and Reed et al. (2007) suggested that a dam may compensate for nutritional deficiency, thus sparing her offspring. In this way, gastrointestinal tissues in dairy cattle seem to be responsive to nutrient restriction. In addition, the large intestine expressed as gram per kilogram of EBW may be responsive to DG, since it decreased at 241 and 268 d when compared to 139 d of gestation.

Fetal Viscera and Organ Mass

Maternal FR during gestation had no effect upon fetal weight and fetal eviscerated BW in the current study. These results are in agreement with previous studies conducted with dairy cows (Graugnard et al., 2012, 2013) and beef cows (Freetly et al., 2000, 2008; Meyer et al., 2010a). Some authors (Peel et al., 2012; Meyer et al., 2010b) observed similar birth weights in lambs born to ewes that were fed maintenance and high levels of nutrition. Differences between species seem to exist, even among ruminants. These differences may result from differences in timing of placental development and vascularization between species (Vonnahme et al., 2007). Osorio et al. (2013) observed that calf birth weight was affected by maternal diet, where cows that were fed a control diet had calved that were 5 kg heavier than calves from cows that were overfed. Moreover, Gao et al. (2012) found that the weights of the calves from cows that were fed a low energy diet was reduced when compared with those of the high energy group, thus demonstrating that weight and measurements in neonatal calves were influenced by maternal energy density in the last 21 d of pregnancy. Although no effect of maternal FR was observed on fetal weight, FR affected fetal carcass weight expressed as gram per kilogram of fetal BW when the dams were fed ML. Similarly, fetal length, chest length, cranial circumference, and hip height were affected in fetuses from ML-fed cows. However, these differences were only about 5% and may not have a significant impact on dairy production, since those characteristics are more related to beef cattle performance (Fernandes et al., 2010; De Paula et al., 2013). Meyer et al. (2010a) also observed the same results regarding fetal visceral organ growth for fetuses from beef cows that were fed control and nutrient restricted diets. According to these authors, accelerated or compensatory fetal organ growth rate may have occurred due to increased efficiency of nutrient usage by the dam, the fetus, or both during maternal restriction. Reduced mass of heart, liver, fat deposition (Rotta et al., 2014a), reticulum-rumen, omasum, abomasum, small, and large intestine for ML-fed cows that were observed in this study, combined with decreased heat production during nutrient restriction (Freetly et al., 2006), may have resulted in lower maintenance requirements for ML-fed cows.

Studies with ruminant and non-ruminant animals observed that fetuses from dams that were subjected to nutrient restriction during early to mid-gestation demonstrated decreased growth of the gastrointestinal tract (Trahair et al., 1997; Wang et al., 2008). Even with adequate postnatal nutrition, the suboptimal growth caused permanent changes in gastrointestinal functions such as epithelial permeability (Trahair et al., 1997). However, these studies applied an intensive nutrient restriction that did not meet the energy requirements, and in the present experiment, we applied a feed restriction but we met the energy requirements for maintenance. Moreover, when studying ad libitum or maintenance-fed beef cows, Duarte et al. (2013) observed that maternal feed-restriction did not affect the development of most of the fetal gastro-intestinal parts, with the exception that the small intestine had an increased surface area as a response to maternal feed nutrition. Our observation that no effect of FR on the reticulum-rumen mass may be due to the non-functionality of these compartments at the fetal stage; theoretically, there is no need to expand the surface area of the stomach to increase nutrient absorption, as this occurs in the small intestine (Duarte et al., 2013).

As expected, fetal BW and all fetal visceral organs that were measured increased in weight during gestation; greater masses were observed at 268 d than 139, 199, and 241 d of gestation, with the exception of the omasum, which had similar masses at 241 and 268 d of gestation. Only the abomasum, spleen, and diaphragm showed growth proportional to that of the fetus, as no differences were observed in mass per unit of EBW in these tissues. Heart, liver, kidneys, lungs, omasum, bladder, and gallbladder had greater proportional masses expressed as gram per kilogram of fetal BW at 139 d of gestation, suggesting that these organs increased in weight more in early and mid-gestation than in late-gestation. Meyer et al. (2010a) observed that the abomasum and large intestine grew faster than the fetal body in later gestation in beef cows. However, none of the organs and tissues that were evaluated in this study demonstrated this behavior. Similar results were observed by Duarte et al. (2013) in fetuses from beef cows that were fed ad libitum or maintenance diets. According to Weaver et al. (1991), the phase of rapid growth of the gastrointestinal tract occurs at the third trimester of gestation in species that have a long gestational period. However, in this study, we observed that approximately 70% of fetal growth occurred during the last 2 DG evaluated. For the organs and tissues, this value was around 60% of total growth in the last 2 DG. It has also been suggested that even though the dam is undernourished, the placental system can compensate to provide the fetus an adequate amount of nutrients, mainly by increasing the number of caruncles (Clarke et al., 1998). This may have also contributed to the lack of effects of maternal feed restriction on fetal gastrointestinal tract mass. In this study, we observed a greater placental mass and number of placentomes in cows that were fed the ML-diet (Rotta et al., 2014b), which may have compensated for maternal feed restriction.

CONCLUSIONS

These data indicate that maternal feed-restriction does not affect the development of most fetal organs and fetal development; however, some maternal organs are affected according to the FR provided. The negative impact on mammary gland mass caused by the ML probably will not affect the subsequent lactation because the CP concentration in the mammary gland increased with this FR. However, we suggest that an AL diet in pregnant dry cows should be provided with caution since the amount of fat in the mammary gland increased at 268 d of gestation.

REFERENCES

Andrew, S. M., D. R. Waldo and R. A. Erdman. 1994. Direct analysis of body composition of dairy cows at three physiological stages. J. Dairy Sci. 77:3022-3033. http://dx.doi.org/10.3168/jds.1994-77244-1.

AOAC International. 2000. Official Methods of Analysis. 17th ed. AOAC International Arlington, VA.

AOAC International. 2006. Official methods of analysis. 18th ed. AOAC International Gaithersburg, MD.

Capuco, A. V., R. M. Akers, and J. J. Smith. 1997. Mammary growth in Holstein cows during the dry period: quantification of nucleic acids and histology. J. Dairy Sci. 80:477-487.

Carlson, D. B., J. J. Reed, P. P. Borowicz, J. B. Taylor, L. P. Reynolds, T. L. Neville, D. A. Redmer, K. A. Vonnahme, and J. S. Caton. 2009. Effects of dietary selenium supply and timing of nutrient restriction during gestation on maternal growth and body composition of pregnant adolescent ewes. J. Anim. Sci. 87:669-680. http://dx.doi.org/10.2527/jas.2007-0837.

Cesta, M. F. 2006. Normal structure, function, and histology of the spleen. Toxicol. Pathol. 34:455-465. http://dx.doi.org/10.1080/01926230600867743.

Clarke, L., L. Heasman, D. T. Juniper, and M. E. Symonds. 1998. Maternal nutrition in early-mid gestation and placental size in sheep. Br. J. Nutr. 79:359–364.

De Paula, N. E., L. O. Tedeschi, M. F. Paulino, H. J. Fernandes, and M. A. Fonseca. 2013. Predicting carcass and body fat composition using biometric measurements of grazing beef cattle. J. Anim. Sci. 91:3341-3351. http://dx.doi.org/10.2527/jas.2012-5233.

Drackley, J. K., R. L. Wallace, D. Graugnard, J. Vasquez, B. F. Richards and J. J. Loor. 2014. Visceral adipose tissue mass in nonlactating dairy cows fed diets differing in energy density. J. Dairy Sci. 97:3420-3430. http://dx.doi.org/10.3168/jds.2014-8014.

Duarte, M. S., M. P. Giombelli, P. V. R. Paulino, N. V. L. Serao, T. S. Martins, P. I. S. Totaro, C. A. Neves, S. C. Valadares Filho, M. V. Dodson, M. Zhu, and M. Du. 2013. Effects of maternal nutrition development of gastrointestinal tract of bovine fetus at different stages of gestation. Livest. Sci. 153:60-65. http://dx.doi.org/10.1016/j.livsci.2013.01.006.

Ekpe, E. D., J. A. Moibi, and R. J. Christopherson. 2000. Effects of temperature and plane of nutrition on beta-adrenergic receptors in heart, kidney, and liver of lambs. J. Anim. Sci. 78:1907-1916.

Fernandes, H. J., L. O. Tedeschi, M. F. Paulino, and L. M. Paiva. 2010. Determination of carcass and body fat compositions of grazing crossbred bulls using measurements. J. Anim. Sci. 88:1442-1453. http://dx.doi.org/10.2527/jas.2009-1919.

Ferrell, C. L. 1988. Energy metabolism. Pages 250-268 in The Ruminant Animal: Digestive Physiology and Nutrition. D. C. Church, ed. Prentice Hall, Englewood Cliffs, NJ.

Ford, S. P. 1995. Control of blood flow to the gravid uterus of domestic livestock species. J. Anim. Sci. 73:1852–1860.

Freetly, H. C., C. L. Ferrell, and T. G. Jenkins. 2000. Timing of realimentation of mature cows that were feed-restricted during pregnancy influences calf birth weights and growth rates. J. Anim. Sci. 78:2790–2796.

Freetly, H. C., J. A. Nienaber, and T. Brown-Brandl. 2006. Changes in heat production by mature cows after change in feeding level. J. Anim. Sci. 84:1429-1438.

Freetly, H. C., J. A. Nienaber, and T. Brown-Brandl. 2008. Partitioning of energy in pregnant beef cows during nutritionally induced weight fluctuation. J. Anim. Sci. 86:370–377. http://dx.doi.org/10.2527/jas.2007-0250.

Gao, F. Y. C. Liu, Z. H. Zhang, C. Z. Zhang, H. W. Su and S. L. Li. 2012. Effect of prepartum maternal energy density on the growth performance, immunity, and antioxidation capability of neonatal calves. J. Dairy Sci. 95:4510-4518. http://dx.doi.org/ 10.3168/jds.2011-5087.

Graugnard, D. E., M. Bionaz, E. Trevisi, K. M. Moyes, J. L. Salak-Johnson, R. L. Wallace, J. K. Drackley, G. Bertoni, and J. J. Loor. 2012. Blood immunometabolic indices and polymorphonuclear neutrophil function in peripartum dairy cows are altered by level of dietary energy. J. Dairy Sci. 95:1749-1758. http://dx.doi.org/10.3168/jds.2011-4579.

Graugnard, D. E., K. M. Moyes, E. Trevisi, M. J. Khan, D. Keisler, J. K. Drackley, G. Bertoni, and J. J. Loor. 2013. Liver lipid content and inflammometabolic indices in peripartal dairy cows are altered in response to prepartal energy intake and postpartal intramammary inflammatory challenge. J. Dairy Sci. 96:918-935. http://dx.doi.org/10.3168/jds.2012-5676.

Guyton, A. C. 1976. Textbook of Medical Physiology. W. B. Saunders Company Philadelphia, PA.

He, Z. X., D. Q. Wu, Z. H. Sun, Z. L. Tan, J. Y. Qiao, T. Ran, S. X. Tang, C. S. Zhou, X. F. Han, M. Wang, J. H. Kang, and K. A. Beauchemin. 2013. Protein or energy restriction during late gestation alters fetal growth and visceral organ mass: An evidence of intrauterine programming in goats. Anim. Reprod. Sci. 137:177-182. http://dx.doi.org/10.1016/j.anireprosci.2013.01.005.

IBGE. 2009. Censo Agropecuario. Accessed Dec. 8, 2014. http://www.ibge.gov.br/home/estatistica/economia/agropecuaria/censoagro/.

Kasiske, B. L., M. P. O’Donnell, M. P. Cleary, and W. F. Keane. 1988. Treatment of hyperlipidemia reduces glomerular injury in obese Zucker rats. Kidney Int. 33:667-672.

Osorio, J. S., E. Trevisi, M. A. Ballou, G. Bertoni, J. K. Drackley, and J. J. Loor. 2013. Effect of the level of maternal energy intake prepartum on immunometabolic markers, polymorphonuclear leukocyte function, and neutrophil gene network expression in neonatal Holetin heifer calves. J. Dairy Sci. 96:3573-3587. http://dx.doi.org/10.3168/jds.2012-5759.

Meyer, A. M., J. J. Reed, K. A. Vonnahme, S. A. Soto-Navarro, L. P. Reynolds, S. P. Ford, B. W. Hess, and J. S. Caton. 2010a. Effects of stage of gestation and nutrient restriction during early to mid-gestation on maternal and fetal visceral organ mass and indices of jejunal growth and vascularity in beef cows. J. Anim. Sci. 88:2410-2424. http://dx.doi.org/10.2527/jas.2009-2220.

Meyer, A. M., J. J. Reed. T. L. Neville, J. B. Taylor, C. J. Hammer, L. P. Reynolds, D. A. Redmer, K. A. Vonnahme, and J. S. Caton. 2010b. Effects of plane of nutrition and selenium supply during gestation on ewe and neonatal offspring performance, body composition, and serum selenium. J. Anim. Sci. 88:1786−1800. http://dx.doi.org/10.2527/jas.2009-2435.

Moallem, U., G. E. Dahl, E. K. Duffey, A. V. Capuco, D. L. Wood, K. R. McLeod and R. L. Baldwin. 2004. Bovine somatotropin and rumen-undegradable protein effects in prepubertal dairy heifers: effects on body composition and organ and tissue weight. J. Dairy Sci. 87:3869-3880. http://dx.doi.org/ 10.3168/jds.2004-73526-2.

NRC. 2007. Nutrient Requirements of Small Ruminants. 1st. ed. Natl. Acad. Press, Washington, DC.

NRC. 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed. Natl. Acad. Press, Washington, DC.

Peel, R. K., G. J. Eckerke, and R. V. Anthony. 2012. Effects of overfeeding naturally-mated adolescent ewes on maternal, fetal, and postnatal lamb growth. J. Anim. Sci. 90:3698-3708. http://dx.doi.org/10.2527/jas.2012-5140.

Reed, J. J., M. A. Ward, K. A. Vonnahme, T. L. Neville, S. L. Julius, P. P. Borowicz, J. B. Taylor, D. A. Redmer, A. T. Grazul- Bilska, L. P. Reynolds, and J. S. Caton. 2007. Effects of selenium supply and dietary restriction on maternal and fetal body weight, visceral organ mass and cellularity estimates, and jejunal vascularity in pregnant ewe lambs. J. Anim. Sci. 85:2721–2733. http://dx.doi.org/ 10.2527/jas.2006-785.

Reynolds, C. K., B. Durst, B. Lupoli, D. J. Humphries and D. E. Beever. 2004. Visceral tissue mass and rumen volume in dairy cows during the transition from late gestation to early lactation. J. Dairy Sci.87:961-971. http://dx.doi.org/ 10.3168/jds.2004-73240-3.

Roche, J. R., J. M. Lee, K. A. Macdonald, and D. P. Berry. 2007. Relationships among body condition score, body weight, and milk production variables in pasture-based dairy cows. J. Dairy Sci. 90:3802-3815. http://dx.doi.org/10.3168/jds.2006-740.

Rotta, P. P., S. C. Valadares Filho, T. R. Santos, L. F. Costa e Silva, T. E. Engle, M. I. Marcondes, F. S. Machado, F. A. C. Villadiego and L. H. R. Silva. 2014a. Effects of days of gestation and feeding regimen in Holstein × Gyr cows:I. Apparent total tract digestibility, nitrogen balance, microbial protein supply, and fat deposition. J. Dairy Science. in press.

Rotta, P. P., S. C. Valadares Filho, T. R. Santos, L. F. Costa e Silva, T. E. Engle, M. I. Marcondes, S. E. F. Guimaraes, C. S. Nascimento, B. C. Carvalho, F. A. S. Silva, and J. R. S. Oliveira. 2014b. Effects of days of gestation and feeding regimen in Holstein × Gyr cows:III. Placental adaptations and placentome gene expression. J. Dairy Sci. in press.

Scarpace, P. J., and B. P. Yu. 1986. Diet restriction retards the age-related loss of beta-adrenergic receptors and adenylate cyclase activity in rat lung. J. Gerentol. 42:442-446.

Scheaffer, A. N., J. S. Caton, M. L. Bauer, and L. P. Reynolds. 2001. Influence of pregnancy on body weight, ruminal characteristics, and visceral organ mass in beef heifers. J. Anim. Sci. 79:2481-2490.

Scheaffer, A. N., J. S. Caton, D. A. Redmer, D. R. Arnold, and L. P. Reynolds. 2004a. Effect of dietary restriction, pregnancy, and fetal type on intestinal cellularity and vascularity in Columbia and Romanov ewes. J. Anim. Sci. 82:3024–3033.

Scheaffer, A. N., J. S. Caton, D. A. Redmer, and L. P. Reynolds. 2004b. The effect of dietary restriction, pregnancy, and fetal type in different ewe types on fetal weight, maternal body weight, and visceral organ mass in ewes. J. Anim. Sci. 82:1826–1838.

Sejrsen, K., J. T., Huber, H. A. Tucker and R. M. Akers. 1982. Influence of nutrition on mammary development in pre- and postpubertal heifers. J. Dairy Sci. 65:793-800. http://dx.doi.org/10.3168/jds.1982-82268-6.

Sejrsen, K., J. T. Huber, and H. A. Tucker. 1983. Influence of amount fed on hormone concentrations and their relationship to mammary growth in heifers. J. Dairy Sci. 66:845-855.

Silva, L. F. P., M. J. VandeHaar, B. K. Whitlock, R. P. Radcliff, and H. A. Tucker. 2002. Short communication: relationsgip between body growth and mammary development in dairy heifers. J. Dairy Sci. 85:2600-2602. http://dx.doi.org/10.3168/jds.S0022-0302(02)74344-0.

Smith, R. L., and R. L. Baldwin. 1973. Effects of breed, pregnancy, and lactation on weight of organs and tissues in dairy cattle. J. Dairy Sci. 57:1055-1060. http://dx.doi.org/10.3168/jds.1973-85008-3.

Soto-Navarro, S. A., T. L. Lawler, J. B. Taylor, L. P. Reynolds, J. J. Reed, J. W. Finley, and J. S. Caton. 2004. Effect of high-selenium wheat on visceral organ growth selenium wheat on visceral organ mass, and intestinal cellularity and vascularity in finishing beef steers. J. Anim. Sci. 82:1788–1793.

Teixeira, A. M., D. G. Jayme, G. A. Sene, L. O. Fernandes, A. C. Barreto, D. J. Rodrigues Junior, A. C. Coutinho, and J. R. Gloria. 2013. Performance of crossbred Holstein × Zebu cows rotationally grazing in Tifton 85 pasture irrigates or rainfed. Arq. Bras. Med. Vet. Zootec. 65:1447-1453.

Trahair, J. F., T. M. DeBarro, J. S. Robinson, and J. A. Owens. 1997. Restriction of nutrition in utero selectively inhibits gastrointestinal growth in fetal sheep. J. Nutr. 127:637–641.

Tucker, H. A. 1981. Physiological control of mammary growth, lactogenesis, and lactation. J. Dairy Sci. 64:1403-1421.

Vonnahme, K. A., M. J. Zhu, P. P. Borowicz, T. W. Geary, B. W. Hess, L. P. Reynolds, J. S. Caton, W. J. Means, and S. P. Ford. 2007. Effect of early gestational undernutrition on angiogenic factor expression and vascularity in the bovine placentome. J. Anim. Sci. 85:2464–2472. http://dx.doi.org/10.2527/jas.2006-805.

Wang, J., L. Chen, D. Li, Y. L. Yin, X. Wang, P. Li, L. J. Dangott, W. Hu, and G. Wu. 2008. Intrauterine growth restriction affects the proteomes of the small intestine, liver, and skeletal muscle in newborn pigs. J. Nutr. 138:60–66.

Weaver, L. T., S. Austin, and T. J. Cole. 1991. Small intestinal length: a factor essential for gut adaptation. Gut. 32:1321–1323

Williams, P. J., L. O. Kurlak, A. C. Perkins, H. Budge, T. Stephenson, D. Keisler, M. E. Symonds, and D. S. Gardner. 2007. Hypertension and impaired renal function accompany juvenile obesity: The effect of prenatal diet. Kidney Int. 72:279-289.

Wood, K. M., B. J. Awda, C. Fitzsimmons, S. P. Miller, B. W. Mc Bride, and K. C. Swanson. 2013. Effect of moderate dietary restriction on visceral organ weight, hepatic oxygen consumption, and metabolic proteins associated with energy balance in mature pregnant beef cows. J. Anim. Sci. 91:4245:4255. http://dx.doi.org/10.2527/jas.2013-6421.

Table 1. Effects of different day of gestation and feeding regimens on BW and non-carcass components of Holstein × Gyr cows (means ± SEM)

Item	Feeding regimen		Day of gestation				P-value		
	Ad libitum	Maintenance	139	199	241	268	FR[1]	DG[2]	FR × DG[3]
Initial BW, kg	482 ± 10.6	477 ± 9.6	488 ± 15.4	480 ± 13.9	480 ± 13.9	468 ± 13.9	0.74	0.80	0.68
Final BW, kg	644 ± 12.9	521 ± 11.7	553 ± 18.7	577 ± 16.9	596 ± 16.9	603 ± 16.9	< 0.01	0.21	0.64
Empty BW, kg	580 ± 11.0	456 ± 10.0	482^{b} ± 16.0	509^{ab} ± 14.4	537^{a} ± 14.4	545^{a} ± 14.4	< 0.01	0.02	0.51
Claws, kg	9.57 ± 0.471	8.37 ± 0.433	8.61 ± 0.684	9.16 ± 0.624	9.81 ± 0.624	9.30 ± 0.624	0.07	0.34	0.21
g/kg empty BW	16.5 ± 0.70	18.4 ± 0.64	17.9 ± 1.02	18.0 ± 0.92	18.3 ± 0.92	17.1 ± 0.92	0.08	0.77	0.11
Head, kg	15.5 ± 0.28	14.4 ± 0.26	14.5 ± 0.41	15.2 ± 0.37	15.2 ± 0.37	14.9 ± 0.37	< 0.01	0.56	0.90
g/kg empty BW	26.7 ± 0.47	31.6 ± 0.43	30.1^{a} ± 0.69	29.9^{a} ± 0.62	28.3^{ab} ± 0.62	27.3^{b} ± 0.62	< 0.01	0.03	0.24
Hide, kg	40.4 ± 1.55	33.8 ± 1.40	38.2 ± 2.25	38.5 ± 2.03	35.5 ± 2.03	36.3 ± 2.03	< 0.01	0.69	0.18
g/kg empty BW	69.7 ± 2.68	74.1 ± 2.43	79.3^{a} ± 3.90	75.6^{a} ± 3.52	66.1^{b} ± 3.52	66.6^{b} ± 3.52	0.27	0.04	0.14
Blood, kg	24.1 ± 1.08	19.7 ± 0.98	21.4 ± 1.58	21.5 ± 1.42	23.1 ± 1.42	21.6 ± 1.42	< 0.01	0.83	0.36
g/kg empty BW	41.6 ± 1.59	43.2 ± 1.44	44.4 ± 2.31	42.2 ± 2.08	43.0 ± 2.08	39.6 ± 2.08	0.46	0.48	0.19
Mammary gland, kg	12.6 ± 0.98	8.56 ± 0.890	7.14^{b} ± 1.431	7.86^{b} ± 1.290	9.77^{b} ± 1.290	17.6^{a} ± 1.290	< 0.01	< 0.01	0.26

[a-b] Means within a row with different superscripts differ ($P \leq 0.10$).

[1] Feeding regimen main effects.

[2] Day of gestation main effects.

[3] Interaction between feeding regimen and day of gestation.

Table 2. Effects of different day of gestation and feeding regimens on maternal organs in Holstein × Gyr cows (means ± SEM)

Item	Feeding regimen		Day of gestation				P-value		
	Ad libitum	Maintenance	139	199	241	268	FR[1]	DG[2]	FR × DG[3]
Heart, kg	2.07 ± 0.061	1.83 ± 0.054	1.89 ± 0.082	2.11 ± 0.072	1.97 ± 0.072	1.95 ± 0.072	< 0.01	0.11	0.56
g/kg of empty BW	3.57 ± 0.081	4.01 ± 0.076	3.92^{a} ± 0.110	4.15^{a} ± 0.101	3.67^{b} ± 0.101	3.58^{b} ± 0.101	< 0.01	< 0.01	0.19
Liver, kg	6.75 ± 0.173	4.94 ± 0.150	6.01 ± 0.252	6.22 ± 0.220	5.41 ± 0.220	5.64 ± 0.220	< 0.01	0.18	0.11
g/kg empty BW	11.6 ± 0.240	10.8 ± 0.224	12.5^{a} ± 0.35	12.2^{a} ± 0.31	10.1^{b} ± 0.31	10.3^{b} ± 0.31	0.02	< 0.01	0.12
Kidneys, kg	1.17 ± 0.035	0.97 ± 0.031	1.10 ± 0.054	1.09 ± 0.045	0.99 ± 0.045	1.10 ± 0.045	< 0.01	0.24	0.32
g/kg empty BW	2.02 ± 0.044	2.13 ± 0.047	2.28 ± 0.061	2.14 ± 0.053	1.84 ± 0.053	2.01 ± 0.053	0.13	< 0.01	0.06
Ad libitum	-	-	2.34^{aA} ± 0.104	2.16^{abA} ± 0.104	1.73^{bB} ± 0.104	1.87^{bB} ± 0.104	-	-	-
Maintenance	-	-	2.23^{aA} ± 0.102	2.09^{aA} ± 0.094	2.00^{aA} ± 0.094	2.22^{aA} ± 0.094	-	-	-
P-value	-	-	0.66	0.26	0.04	< 0.01	-	-	-
Lungs, kg	3.37 ± 0.121	2.98 ± 0.102	3.06 ± 0.173	3.27 ± 0.157	3.36 ± 0.157	3.42 ± 0.157	0.02	0.16	0.96
g/kg empty BW	5.81 ± 0.170	6.53 ± 0.155	6.35 ± 0.254	6.42 ± 0.228	6.26 ± 0.228	6.28 ± 0.228	< 0.01	0.13	0.51
Spleen, kg	1.52 ± 0.055	1.08 ± 0.054	1.30 ± 0.088	1.44 ± 0.075	1.26 ± 0.075	1.18 ± 0.075	< 0.01	0.20	0.88
g/kg empty BW	2.62 ± 0.072	2.37 ± 0.075	2.70^{a} ± 0.115	2.83^{a} ± 0.104	2.35^{b} ± 0.104	2.17^{b} ± 0.104	0.01	< 0.01	0.51
Diaphragm, kg	3.10 ± 0.102	2.33 ± 0.090	2.58^{b} ± 0.156	2.78^{ab} ± 0.140	2.79^{ab} ± 0.140	2.99^{a} ± 0.140	< 0.01	0.08	0.90
g/kg empty BW	5.34 ± 0.154	5.11 ± 0.140	5.35 ± 0.227	5.46 ± 0.208	5.20 ± 0.208	5.52 ± 0.208	0.19	0.12	0.34
Trachea plus esophagus, kg	5.35 ± 0.259	4.18 ± 0.236	4.54^{b} ± 0.365	5.28^{a} ± 0.339	5.19^{a} ± 0.339	5.04^{a} ± 0.339	< 0.01	0.04	0.93
g/kg empty BW	9.22 ± 0.388	9.17 ± 0.345	9.42 ± 0.550	10.4 ± 0.508	9.66 ± 0.508	9.25 ± 0.508	0.91	0.13	0.90

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

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

[1] Feeding regimen main effects.

[2] Day of gestation main effects.

[3] Interaction between feeding regimen and day of gestation.

Table 3. Effects of different day of gestation and feeding regimens on the mass of gastrointestinal tract in Holstein × Gyr cows (means ± SEM)

Item	Feeding regimen		Day of gestation				P-value		
	Ad libitum	Maintenance	139	199	241	268	FR[1]	DG[2]	FR × DG[3]
Reticulum-rumen, kg	10.1 ± 0.25	8.10 ± 0.222	9.90^{a} ± 0.361	9.14^{ab} ± 0.324	8.80^{ab} ± 0.324	8.64^{b} ± 0.324	< 0.01	0.07	0.70
g/kg empty BW	17.4 ± 0.45	17.8 ± 0.41	20.5^{a} ± 0.662	18.0^{b} ± 0.590	16.4^{b} ± 0.590	15.9^{b} ± 0.590	0.71	< 0.01	0.37
Omasum, kg	3.90 ± 0.132	2.95 ± 0.122	3.54 ± 0.191	3.43 ± 0.178	3.13 ± 0.178	3.41 ± 0.178	< 0.01	0.15	0.61
g/kg empty BW	6.72 ± 0.264	6.47 ± 0.233	7.34^{a} ± 0.381	6.74^{ab} ± 0.349	5.83^{b} ± 0.349	6.26^{b} ± 0.349	0.38	< 0.01	0.36
Abomasum, kg	1.52 ± 0.050	1.36 ± 0.048	1.44 ± 0.082	1.43 ± 0.074	1.45 ± 0.074	1.43 ± 0.074	0.04	0.99	0.34
g/kg empty BW	2.62 ± 0.091	2.98 ± 0.082	2.99 ± 0.132	2.81 ± 0.125	2.70 ± 0.125	2.62 ± 0.125	< 0.01	0.26	0.19
Small intestine, kg	5.46 ± 0.185	4.78 ± 0.165	5.05 ± 0.264	5.40 ± 0.239	5.26 ± 0.239	5.26 ± 0.239	< 0.01	0.25	0.42
g/kg empty BW	9.41 ± 0.344	10.5 ± 0.314	10.5 ± 0.49	10.8 ± 0.45	9.86 ± 0.451	9.65 ± 0.451	0.03	0.15	0.32
Large intestine, kg	3.35 ± 0.155	2.99 ± 0.145	3.46 ± 0.225	3.08 ± 0.204	3.11 ± 0.204	3.02 ± 0.204	0.09	0.47	0.74
g/kg empty BW	5.78 ± 0.298	6.56 ± 0.267	7.18^{a} ± 0.420	6.05^{ab} ± 0.388	5.79^{b} ± 0.388	5.54^{b} ± 0.388	0.07	0.03	0.75

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

[1] Feeding regimen main effects.

[2] Day of gestation main effects.

[3] Interaction between feeding regimen and day of gestation.

Table 4. Effects of different day of gestation and feeding regimens on fetal characteristics in Holstein × Gyr cows (means ± SEM)

Item	Feeding regimen		Day of gestation				P-value		
	Ad libitum	Maintenance	139	199	241	268	FR^{1}	DG^{2}	FR × DG^{3}
Fetal BW, kg	17.2 ± 0.75	15.8 ± 0.69	1.77^{d} ± 0.11	10.2^{c} ± 1.00	21.8^{b} ± 1.00	32.3^{a} ± 1.00	0.20	< 0.01	0.56
Eviscerated BW, kg	13.2 ± 0.55	12.0 ± 0.51	1.30^{d} ± 0.822	7.80^{c} ± 0.741	16.6^{b} ± 0.74	24.7^{a} ± 0.74	0.10	< 0.01	0.52
Head, g	1,616 ± 56.5	1,509 ± 52.3	286^{d} ± 83.9	1,186^{c} ± 75.7	2,033^{b} ± 75.7	2,745^{a} ± 75.7	0.17	< 0.01	0.89
g/kg fetal BW	115 ± 2.10	116 ± 1.99	162^{a} ± 3.3	116^{b} ± 3.0	93.3^{c} ± 3.01	85.0^{d} ± 3.01	0.72	< 0.01	0.11
Claws, g	1,076 ± 42.7	973 ± 39.5	65.3^{d} ± 6.33	594^{c} ± 57.2	1,450^{b} ± 57.2	1,998^{a} ± 57.2	0.09	< 0.01	0.51
g/kg fetal BW	62.6 ± 1.13	61.6 ± 1.05	36.9^{c} ± 1.68	58.2^{b} ± 1.51	66.5^{ab} ± 1.51	61.9^{ba} ± 1.51	0.55	< 0.01	0.88
Carcass, g	10,535 ± 475	9,483 ± 441	945^{d} ± 70.6	6,017^{c} ± 638	13,100^{b} ± 638	19,979^{a} ± 638	0.11	< 0.01	0.49
g/kg fetal BW	595 ± 7.3	578 ± 6.8	534^{b} ± 10.9	590^{a} ± 9.8	601^{a} ± 9.8	619^{a} ± 9.8	< 0.10	< 0.01	0.78
Fetus length, cm	69.2 ± 1.24	65.4 ± 1.15	34.4^{d} ± 1.84	63.0^{c} ± 1.66	79.7^{b} ± 1.66	92.0^{a} ± 1.66	0.03	< 0.01	0.47
Fetus chest length, cm	50.6 ± 0.91	48.3 ± 0.84	25.7^{d} ± 1.35	46.2^{c} ± 1.22	58.9^{b} ± 1.22	66.9^{a} ± 1.22	0.07	< 0.01	0.41
Cranial circumference, cm	42.3 ± 0.75	39.3 ± 0.69	25.3^{c} ± 1.11	36.9^{b} ± 1.00	48.7^{a} ± 1.00	49.7^{a} ± 1.00	< 0.01	< 0.01	0.34
Neck circumference, cm	32.6 ± 0.82	30.9 ± 0.76	16.5^{d} ± 1.21	30.8^{c} ± 1.10	38.1^{b} ± 1.10	41.7^{a} ± 1.10	0.13	< 0.01	0.79
Hip height, cm	47.9 ± 0.64	45.6 ± 0.60	20.2^{d} ± 0.96	43.0^{c} ± 0.86	57.6^{b} ± 0.86	66.4^{a} ± 0.86	0.01	< 0.01	0.29
Withers height, cm	52.2 ± 0.80	50.4 ± 0.74	22.4^{d} ± 1.19	46.4^{c} ± 1.07	62.6^{b} ± 1.07	73.7^{a} ± 1.07	0.13	< 0.01	0.24

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

[1] Feeding regimen main effects.

[2] Day of gestation main effects.

[3] Interaction between feeding regimen and day of gestation.

Table 5. Effects of different day of gestation and feeding regimens on the mass of fetal organs in Holstein × Gyr cows (means ± SEM)

Item	Feeding regimen		Day of gestation				P-value		
	Ad libitum	Maintenance	139	199	241	268	FR[1]	DG[2]	FR × DG[3]
Heart, g	117 ± 1.4	113 ± 1.2	14.9^{d} ± 1.92	84.4^{c} ± 1.85	143^{b} ± 1.85	216^{a} ± 1.85	0.62	< 0.01	0.42
g/kg fetal BW	6.80 ± 0.209	7.16 ± 0.192	8.42^{a} ± 0.302	8.27^{a} ± 0.272	6.56^{b} ± 0.272	6.69b ± 0.272	0.85	< 0.01	0.23
Liver, g	383 ± 2.2	365 ± 1.8	57.1^{d} ± 2.3	281^{c} ± 2.0	447^{b} ± 2.0	710^{a} ± 2.0	0.52	< 0.01	0.85
g/kg fetal BW	22.3 ± 0.66	23.1 ± 0.61	32.3^{a} ± 0.98	27.5^{b} ± 0.89	20.5^{c} ± 0.89	22.0^{c} ± 0.89	0.45	< 0.01	0.86
Kidneys, g	100 ± 2.7	90.6 ± 2.61	18.8^{d} ± 2.99	88.7^{c} ± 2.87	124^{b} ± 2.9	151^{a} ± 2.9	0.16	< 0.01	0.79
g/kg fetal BW	5.81 ± 0.272	5.73 ± 0.250	10.6^{a} ± 0.41	8.69^{b} ± 0.37	5.69^{c} ± 0.37	4.67^{c} ± 0.37	0.94	< 0.01	0.96
Lungs, g	401 ± 2.3	381 ± 1.8	55.2^{d} ± 2.20	260^{c} ± 2.12	526^{b} ± 2.12	723^{a} ± 2.12	0.46	< 0.01	0.73
g/kg fetal BW	23.3 ± 0.70	24.1 ± 0.65	31.2^{a} ± 1.04	25.5^{b} ± 0.94	24.1^{b} ± 0.94	22.4^{c} ± 0.90	0.48	< 0.01	0.12
Reticulum-rumen, g	82.7 ± 2.91	79.6 ± 2.70	15.1^{d} ± 3.32	74.1^{c} ± 3.19	104^{b} ± 3.19	131^{a} ± 3.19	0.44	< 0.01	0.85
g/kg fetal BW	4.81 ± 0.175	5.04 ± 0.164	8.53 ± 0.261	7.26 ± 0.232	4.77 ± 0.232	4.06 ± 0.232	0.89	< 0.01	0.02
Ad libitum	-	-	9.34^{aA} ± 0.352	7.15^{bA} ± 0.352	4.54^{cA} ± 0.352	4.04^{cA} ± 0.352	-	-	-
Maintenance	-	-	7.89^{aB} ± 0.356	7.59^{aA} ± 0.322	5.32^{bA} ± 0.322	4.13^{cA} ± 0.322	-	-	-
P-value	-	-	< 0.01	0.19	0.36	0.85	-	-	-
Omasum, g	33.4 ± 0.73	32.0 ± 0.61	7.93^{c} ± 0.745	34.0^{b} ± 0.73	44.4^{a} ± 0.73	44.4^{a} ± 0.73	0.57	< 0.01	0.19
g/kg fetal BW	1.94 ± 0.181	2.03 ± 0.174	4.48^{a} ± 0.275	3.33^{b} ± 0.252	2.04^{c} ± 0.252	1.37^{d} ± 0.252	0.58	< 0.01	0.25
Abomasum, g	70.1 ± 0.69	66.4 ± 0.52	7.35^{d} ± 0.844	45.7^{c} ± 0.801	87.5^{b} ± 0.801	133^{a} ± 0.801	0.46	< 0.01	0.14
g/kg fetal BW	4.08 ± 0.192	4.20 ± 0.181	4.15 ± 0.290	4.48 ± 0.266	4.01 ± 0.266	4.11 ± 0.266	0.47	0.62	0.54
Spleen, g	42.8 ± 0.88	37.6 ± 0.76	4.19^{d} ± 0.98	32.1^{c} ± 0.94	50.7^{b} ± 0.94	74.0^{a} ± 0.94	0.19	< 0.01	0.80
g/kg fetal BW	2.49 ± 0.130	2.38 ± 0.125	2.37 ± 0.204	2.55 ± 0.185	2.33 ± 0.185	2.29 ± 0.185	0.39	0.15	0.36
Bladder, g	16.6 ± 0.11	18.2 ± 0.08	2.75^{d} ± 0.16	10.4^{c} ± 0.14	22.3^{b} ± 0.14	34.3^{a} ± 0.14	0.29	< 0.01	0.15
g/kg fetal BW	0.97 ± 0.075	1.15 ± 0.064	1.55^{a} ± 0.102	1.02^{b} ± 0.092	1.02^{b} ± 0.092	1.06^{b} ± 0.092	< 0.01	< 0.01	0.28
Gallbladder, g	3.60 ± 0.040	3.03 ± 0.031	0.64^{d} ± 0.052	2.72^{c} ± 0.049	4.21^{b} ± 0.049	5.70^{a} ± 0.049	0.23	< 0.01	0.68
g/kg fetal BW	0.21 ± 0.020	0.19 ± 0.017	0.36^{a} ± 0.034	0.27^{ab} ± 0.030	0.19^{b} ± 0.030	0.18^{b} ± 0.030	0.66	< 0.01	0.34
Diaphragm, g	59.7 ± 0.74	49.1 ± 0.69	6.29^{d} ± 0.86	36.3^{c} ± 0.83	72.0^{b} ± 0.83	103_{a} ± 0.8	0.11	< 0.01	0.80
g/kg fetal BW	3.47 ± 0.126	3.11 ± 0.121	3.55 ± 0.194	3.56 ± 0.172	3.30 ± 0.172	3.18 ± 0.172	0.01	0.19	0.21

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

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

[1] Feeding regimen main effects.

[2] Day of gestation main effects.

[3] Interaction between feeding regimen and day of gestation.

Table 6. Effects of different day of gestation and feeding regimens on fetal large and small intestines characteristics in Holstein × Gyr cows (means ± SEM)

Item	Feeding regimen		Day of gestation				P-value		
	Ad libitum	Maintenance	139	199	241	268	FR[1]	DG[2]	FR × DG[3]
Small intestine, g	258 ± 9.9	240 ± 9.2	25.1^{d} ± 10.62	165^{c} ± 10.5	321^{b} ± 10.5	485^{a} ± 10.5	0.42	< 0.01	0.73
g/kg fetal BW	15.0 ± 0.49	15.2 ± 0.45	14.2 ± 0.73	16.2 ± 0.66	14.7 ± 0.66	15.0 ± 0.66	0.62	0.28	0.06
Ad libitum	-	-	16.2^{aA} ± 0.97	16.9^{aA} ± 0.97	14.5^{bA} ± 0.97	14.7^{bA} ± 0.97	-	-	-
Maintenance	-	-	12.2^{bB} ± 0.97	15.3^{aA} ± 0.88	15.4^{aA} ± 0.88	15.1^{aA} ± 0.88	-	-	-
P-value	-	-	< 0.01	0.54	0.65	0.68	-	-	-
Large intestine, g	78.9 ± 4.78	71.9 ± 4.42	6.97^{d} ± 5.09	50.8^{c} ± 4.96	101^{b} ± 5.0	143^{a} ± 5.0	0.29	< 0.01	0.42
g/kg fetal BW	4.59 ± 0.281	4.55 ± 0.267	3.93 ± 0.412	4.98 ± 0.370	4.63 ± 0.370	4.43 ± 0.370	0.99	0.24	0.59
Small intestine, cm	891 ± 31.8	875 ± 29.4	363^{c} ± 37.2	855^{b} ± 35.6	1,117^{a} ± 35.6	1,197^{a} ± 35.6	0.72	< 0.01	0.46
Large intestine, cm	136 ± 3.9	125 ± 3.6	64.6^{c} ± 5.81	126^{b} ± 5.3	161^{a} ± 5.3	170^{a} ± 5.3	0.04	< 0.01	0.29
Duodenum, cm	31.3 ± 2.23	29.8 ± 2.07	21.6^{b} ± 3.31	27.0^{b} ± 2.99	30.8^{b} ± 2.99	42.4^{a} ± 2.99	0.69	< 0.01	0.60
Jejunum, cm	418 ± 26.4	373 ± 24.5	113^{d} ± 39.2	239^{c} ± 35.4	507^{b} ± 35.4	722^{a} ± 33.8	0.21	< 0.01	0.40
Ileum, cm	435 ± 34.7	477 ± 32.2	228^{b} ± 51.6	589^{a} ± 46.6	569^{a} ± 46.6	538^{a} ± 44.4	0.38	< 0.01	0.30

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

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

[1] Feeding regimen main effects.

[2] Day of gestation main effects.

[3] Interaction between feeding regimen and day of gestation.

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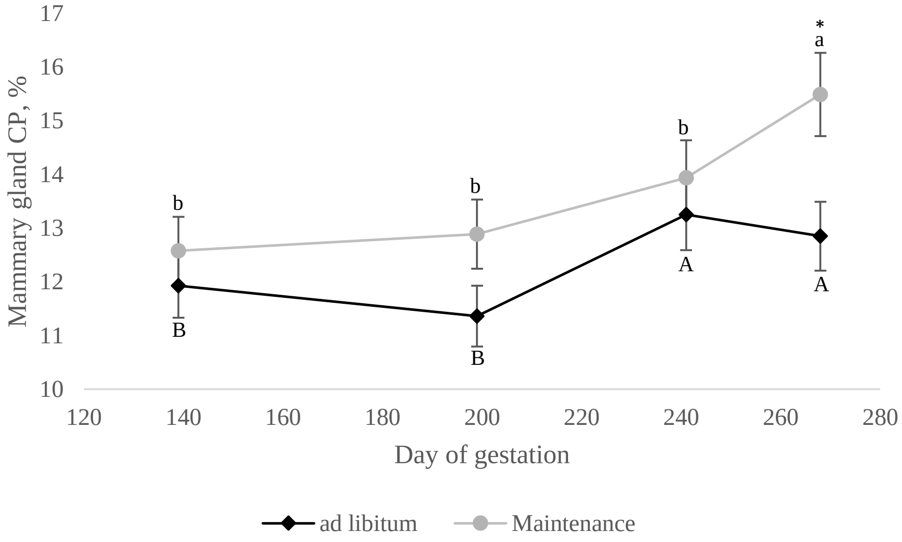
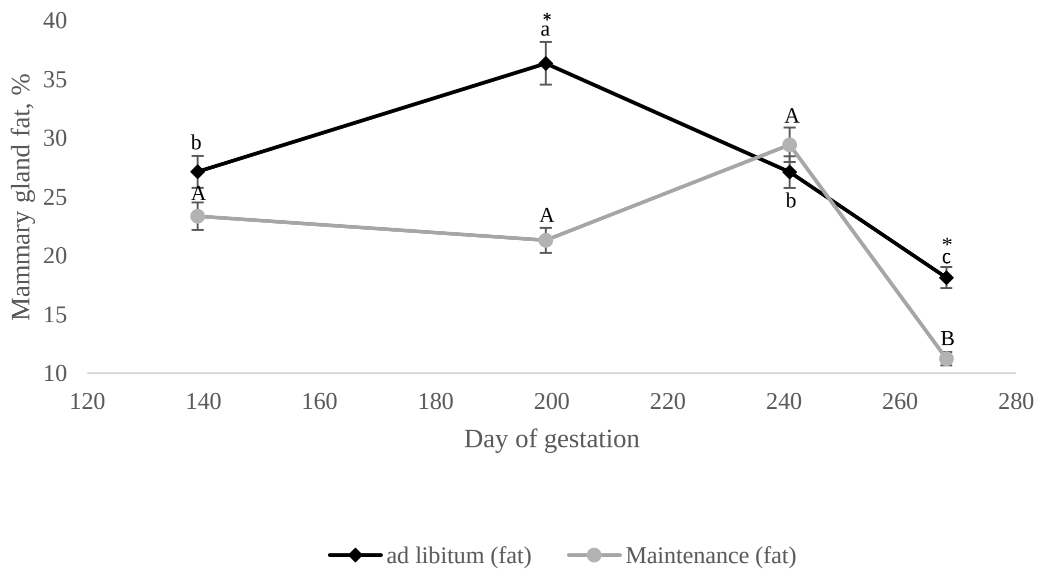


Rotta Figure 1



Rotta Figure 2

Figure 1. Crude protein concentrations in mammary gland of pregnant dry cows that were fed ad libitum or maintenance levels at different day of gestation. Results are means ± SEM. *Differed at $P \leq 0.10$. Different lowercase letters are different at $P \leq 0.10$ for ad libitum diets. Different uppercase letters are different at $P \leq 0.10$ for maintenance diets.

Figure 2. Fat concentrations in mammary gland of pregnant dry cows that were fed ad libitum and maintenance levels during gestation. Results are means ± SEM. *Differed at $P \leq 0.10$. Different lowercase letters are different at $P \leq 0.10$ for ad libitum diets. Different uppercase letters are different at $P \leq 0.10$ for maintenance diets.

CHAPTER 4

Interpretive summary

Placental changes to maintain normal fetal growth in feed restricted dairy cows

Rotta

This study investigated placental modifications in dairy cows that were fed 2 levels of diet: ad libitum and maintenance. We observed that placenta from maintenance fed cows developed 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.

THE ROLE OF THE PLACENTA IN FETAL DEVELOPMENT

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 years 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 kilogram and gram per kilogram of empty body weight (EBW) was heavier in ML- than in AL-fed cows at 268 d of gestation. Placenta expressed as kilogram and gram per kilogram of EBW was the lightest at 139 d of gestation, and the heaviest mass was observed at 268 d in ML-fed cows. However, in AL-fed cows, the heaviest placenta expressed as gram per kilogram of EBW was observed from 199 d of gestation. Placentomes expressed as gram 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 genes were involved in placenta adaptations when ML was provided, as their expression in placentome cells were greater in ML-fed cows at 268 d of gestation. The genes responsible for angiogenese were also greater in ML-fed cows. VEGFA, GUCYB3, HIFA, FGF2, and NOS3 were altered by FR and DG interaction and they were greater in ML-fed cows at 268 d of gestation. Also, VEGFB and ANGPT2 did not present interaction between FR and DG, but they were greater in ML-fed cows. Thus, we suggest that placenta from ML-fed cows develop 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, 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 impact fetal growth (Vonnahme et al., 2013). The relationship between maternal nutrient intake during pregnancy and the 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). Since profitability in the livestock industry is dependent upon efficiency of production characteristics such as growth and development after birth, the precursor of efficiency, namely fetal growth, must be optimal (Redmer et al., 2004; Zhu et al., 2013).

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 which develop adjacent to uterine caruncle on the uterine wall, and interdigitate with corresponding 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 × 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, 2013). 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 placenta of cows fed maintenance level (**ML**) diet is more efficient in nutrient transfer to the fetus than cows fed ad libitum level (**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, GUGY1B3, 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, prior to initiation of the experiment.

Animals and Management

The animals used in this present experiment such as the management adopted was previously described in Rotta et al. (2014a). However a brief description is provided as follow: 40 multiparous dry Holstein × Gyr cows with an average initial BW of 482 ± 10.8 kg and age of 5 ± 0.8 years 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 choose aiming to represent 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 were 12.5 ± 1.36 L/d for AL-fed cows and 13.4 ± 1.20 L/d for ML-fed cows. The AL-fed cows had an average number of lactation of 2.9 ± 0.25 and for the ML-fed cows the number of lactation was 3.0 ± 0.25. 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 (in DM basis) as a TMR twice daily, with 60% in the morning and 40% in the afternoon.

Slaughter and Laboratory Analyses

Pre-harvest 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.

Prior to slaughter, feed was withheld overnight, but cows had ad libitum access to water. Cows were slaughtered at Universidade Federal de Viçosa by stunning them with a captive bolt 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 -20ºC 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 -80ºC until further analysis. Placenta and uterus were analyzed for DM and N concentrations (AOAC, 2000; method number 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 re-suspended in RNase-free ultrapure water and stored at -70ºC until further use. RNA concentrations were checked by NanoVue Plus Spectrophotometer (GE. Healthcare, Piscataway, NJ) with an optimal 260/280 ratio 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, Carlsbad, CA) to remove any contaminating DNA. Reverse transcription was performed using GoScript Reverse Transcription System (Promega, Madison, WI) and oligo (dT) primers (Invitrogen, Carlsbad, CA). 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, Madison, WI) 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 (IDT, Coralville, IA) and are summarized in Table 1.

Before performing real time PCR, part of the reverse transcription product was diluted, pooled, then serial diluted to construct the 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 Schmittigen, 2001). The PCR amplification efficiencies were calculated for each reference gene assay using the formula E = (10 – 1 / slope - 1) × 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. This ensures that a specific gene amplification was obtained.

After the analysis of efficiency, the most adequate 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 Microsoft Excel files 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), glyceraldehyde 3-phosphate dehydrogenase, (GAPDH) and splicing factor 3a, subunit 1 (SF3A1) for each sample.

Data Analysis

Before performing the statistical analysis, we realized the test of 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, fetus 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 × 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, considering gram 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 gram per kilogram of EBW than AL-fed cows. Considering the fact 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 the 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 uterine 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 heavier in ML- than AL-fed cows, as it was 1.63 times heavier in ML-fed cows. Thus, we can assume that since fetal mass was not altered by FR, ML-fed cows may have had placenta adaptations to be more efficient in transferring nutrients to the fetus.

Another important change caused by FR was in regards to the fetal liquids expressed as gram per kilogram of EBW. Heavier (P = 0.04) fetal liquid mass was found in ML- than AL-fed cows. Fetal liquid mass were similar (P = 0.77) at 139, 199, and 241 d of gestation. However, at d-268 we observed the heaviest mass, being 1.88 times heavier 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 gram per kilogram of EBW, both caruncles and cotyledons were heavier (P = 0.07) in ML-fed cows. Since placentomes are the sum of caruncles and cotyledons, the same result was observed for placentomes. Maintenance-fed cows had greater (P = 0.02) placentomes expressed as gram per kilogram of EBW than AL-fed cows.

The mass of caruncles, cotyledons, and placentomes were different (P = 0.07) according to DG (Table 4). For caruncles and placentomes, the heaviest masses occurred at 241 and 268 d of gestation, and the lighest 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, while no difference (P = 0.66) occurred at 199, 241, and 268 d of gestation (Figure 3). 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 d-241. This indicates that 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

The gene expression expressed as relative abundance of insulin-like growth factor receptors (IGFR1 and IGFR2) are shown in Figures 5 and 6, respectively. We observed that IGFR1 and IGFR2 were constant in AL-fed cows throughout gestation, while 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 throughout gestation. However, in placentomes of ML-fed cows, a difference occurred in its expression; from d-241, greater ($P < 0.01$) values were observed than those at 139 and 199 d of gestation. 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.

On the contrary, 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, the DG affected their expression, and the lowest value was found at 139 d 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. 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 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.

DISCUSSION

Utero and Placental Characteristics

Wallace et al. (2001) evaluated adolescent ewes that were fed moderate or high amount of feed and observed that the placenta mass was heavier in those that were fed moderate. While 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 completed 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 × Gyr dairy cows (Rotta et al., 2014b). 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 d-268 was similar to that of 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 placenta growth pattern was different from AL-fed cows because it continued to grow until d-268. Thus, feeding cows at 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, while 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, there appears to be no significant effect of day of gestation on placentome number (Redmet et al., 2009; Liu et al., 2010). This is the first study evaluating the effect of DG and FR in Holstein × Gyr cows and our results suggest that Gyr breed may increase the number of placentomes. Previous studies with Holstein cattle observed that placentome number is about 100 units. However, our study found values about 2 to 3-fold greater for placentome units. Even though we did not test the influence of breed on placentome number, our results give us reason to suggest that the Gyr breed in the crossbreeding with Holstein animals may be responsible to increase the placentome number.

The heavier placentome expressed as gram 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 fairly constant (Ford et al., 1984; Dowell and Kauer, 1997) during pregnancy. Wallace et al. (2002; 2003a, b) 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. These authors also relayed 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; 2003a, b), because we can associate results based on a moderate amount of feed with data from ML-fed cows (approximatelly 0.30 kg/d ADG), and results from cows fed high amounts would associate with those from AL-fed cows (approximattely 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 this is an evidence that IGFR1 and IGFR2 are sensitive in placentoma alterations in the maternal metabolic environment, and hypothesize that an alternative endocrine factor may regulate the growth of fetuses from feed restricted cows. In order to provide the fetus and the newborn with sufficient energy and nutrients, the pregnant dam undergoes a number of physiological, metabolic, and endocrine adaptations (Prentice et al., 1995; Olausson and Sohlstrom, 2003). Changes in the IGFR system are one type of adaptation. Several studies have demonstrated the importance of IGFR1 and IGFR2 for gestation (DeChiara, et al., 1990; Constancia et al., 2002; Fowden, 2013), 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, and the control of angiogenesis and neovascularization in organs and tissues (Pfarrer et al., 2006). Thus, the greater its concentration in animal organism, the greater the potential for vascularization and 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 in part be explained by the greater nutrient requirements during the final phase of gestation, and because ML-fed cows had a lower DMI (Rotta et al., 2014a) 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 up-regulation of growth signaling pathways in cotyledons under maternal nutrient restriction (Reynolds et al., 2005). Other growth factors may also be responsible for the up-regulation 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, 2003). Thus, the greater expression of HIF1 observed in ML-fed animals during the final gestation period may suggest a decrease in oxygen concentration being necessary to increase the expression of HIF1.

CONCLUSIONS

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

REFERENCES

AOAC International. 2000. Official Methods of Analysis. 17th ed. AOAC International Arlington, VA.

Babaei, S., K. Teichert-Kuliszewska, J. C. Monge, F. Mohamed, M. P. Bendeck, and D. J. Stewart. 1998. Role of nitric oxide in the angiogenic response in vitro to basic fibroblast growth factor. Circ. Res. 82:1007-1015.

Bjellin, L., P. O. Sjoquist, and A. M. Carter. 1975. Uterine, maternal placental and ovarian blood flow throughout pregnancy in the guinea pig. Zeitschrift fur Geburtshilfe und Perinatologie. 179:179-187.

Borowicz, P. P., D. R. Arnold, M. L. Johnson, A. T. Grazul-Bilska, D. A. Redmer, and L. P. Reynolds. 2007. Placental growth throughout the last two thirds of pregnancy in sheep: vascular development and angiogenic factor expression. Biol. Reprod. 76:259-267. http://dx.doi.org/10.1095/biolreprod.106.054684.

Brasil. 1997. Ministério da Agricultura, Pecuária e Abastecimento, Regulamento da Inspeção Industrial e Sanitária de Produtos de Origem Animal [Food of animal origin sanitary and industry inspection].

Carmeliet, P., V. Ferreira, G. Breier, S. Pollefeyt, L. Klenkens, M. Gertsenstein, M. Fahrig, A. Vandenhoeck, K. Harpal, C. Eberhardt, C. Declercq, J. Pawling, L. Moons, D. Collen, W. Risau, and A. Nagy. 1996. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature. 380:435–439. http://dx.doi.org/10.1038/380435a0.

Claesson-Welsh, L. 2008. VEGF-B taken to our hearts: specific effect of VEGF-B in myocardial ischemia. Arterioscler. Tromb. Vasc. Biol. 28:1575-1576. http://dx.doi.org/10.1161/ATVBAHA.108.170878.

Constancia, M., M. Hemberger, J. Hughes, W. Dean, A. Ferguson-Smith, R. Fundele, F. Steward, G. Kelsey, A. L. Fowden, C. Sibley, and W. Reik. 2002. Placental-specific IGF-II is a major modulator of placental and fetal growth. Nature. 417:945–948. http://dx.doi.org/10.1038/nature00819.

DeChiara, T. M., E. J. Robertson, and A. Efstratiadis. 1990. A growth-deficient phenotype in heterozygous mice carry an insulin-like growth factor II gene disrupted by targeting. Nature. 344:78–80. http://dx.doi.org/10.1038/344078a0.

Dowell, R. T., and C. D. Kauer. 1997. Maternal hemodynamics uteroplacental blood flow throughout gestation in conscious rat. Meth Find Exp. Ciin Pharmacol. 19:613-625.

Ferrara, N., K. Carver-Moore, H. Chen, M. Dowd, L. Lu, K. S. O’Shea, L. Powell- Braxton, K. J. Hillan, and M. W. Moore. 1996. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature. 380:439–442. http://dx.doi.org/10.1038/380439a0.

Ferrel, C. L., W. N. Garret, and N. Hinman. 1976. Estimation of body composition in pregnant and non pregnant heifers. J. Anim. Sci. 42:1158-1166.

Ford, S. P., L. P. Reynolds, and C. L. Ferrell. 1984. Blood flow, steroid secretion and nutrient uptake of the gravid uterus during the periparturient period in sows. J. Anim. Sci. 59:1085-1091.

Ford, S. P. 2000. Cotyledonary placenta. In: Knobil E, Neil JD, editors. Encyclopedia of Reproduction, vol. 1. San Diego, CA: Academic Press p. 730e8.

Fowden, A. L. 2013. The insulin-like growth factors and feto-placental growth. Placenta. 24:803-812. http://dx.doi.org/10.1016/S0143-4004(03)00080-8.

George, L. A., A. B. Uthlaut, N. M. Long, L. Zhang, Y. Ma, D. T. Smith, P. W. Nathanielsz, and S. P. Ford. 2010. Different levels of overnutrition and weight gain during pregnancy have differential effects on fetal growth and organ development. Reprod. Biol. Endocrinol. 24:8-75. http://dx.doi.org/10.1186/1477-7827-8-75.

Godfrey, K. M., and Barker, D. J. 2000. Fetal nutrition and adult disease. Am. J. Clin. Nutr. 7:1344–1352.

Godfrey, K. M. 2002. The role of the placenta in fetal programming—a review. Placenta. Suppl A:S20–7. http://dx.doi.org/10.1053/plac.2002.0773.

Huusko, J., M. Merentie, M. H. Dijkstra, M. M. Ryhanen, H. Karvinen, T. T., Rissanen, M. Vanwildemeersch, M. Hedman, J. Lipponen, S. E. Heinonen, U. Eriksson, M. Shibuya, and S. Yla-Herttuala. 2010. The effects of VEGF-R1 and VEGF-R2 ligands on angiogenic responses and left ventricular function in mice. Cardiovac. Res. 86:122-130. http://dx.doi.org/10.1093/cvr/cvp382.PMID19955220.

IBGE. 2009. Censo Agropecuario. Accessed Dec. 8, 2014. http://www.ibge.gov.br/home/estatistica/economia/agropecuaria/censoagro/.

Laviola, L., S. Perrini, G. Belsanti, A. Natalicchio, C. Montrone, A. Leonardini, A. Vimercati, M. Scioscia, L. Selvaggi, R. Giorgino, P. Greco, and F. Giorgino. 2005. Intrauterine growth restriction in humans is associated with abnormalities in placental insulin-like growth factor signaling. Endocrinology. 146:1498-1505.

Li, C., C. J., Sun, J. C. Fan, N. Geng, C. H. Li, J. Liai, K. Mi, G. Q., Zhu, H. Ma, Y. F. Song, Y. L. Tang, and Y. Chen. 2013. Angiopoietin-2 expression is correlated with angiogenesis and overall survival in oral squamous cell carcinoma. Med. Oncol. 30:571. http://dx.doi.org/10.1007/s12032-013-0571-2.

Livak, K. J., and T. D. Schmittigen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2DDCt method. Methods. 25:402-408. http://dx.doi.org/10.1006/meth.2001.1262.

National Research Council. 2001. Nutrients Requirements of Dairy Cattle. 7th rev. ed. Natl. Acad. Press, Washington, DC.

Olausson, H., and A. Sohlstrom. 2003. Effects of food restriction and pregnancy on the expression of insulin-like growth factors-I and –II in tissues from guinea pigs. J. Endoc. 179:427-445. http://dx.doi.org/10.1677/joe.0.1790427.

Olofsson, B., K. Pajusola, A. Kaipainen, G. von Euler, V. Joukov, O. Sakselas, A. Orpanat, R. F. Petterson, K. Alitalo, and U. Eriksson. 1996. Vascular endothelial growth factor B, novel growth factor for endothelial cells. Cell Biol. 93:2576-2581.

Parenti, A., L. Morbidelli, X. L. Cui, J. G. Douglas, J. D. Hood, H. J. Granger, F. Ledda, and M. Ziche. 1998. Nitric oxide is an upstream signal of vascular endothelial growth factor-induced extracellular signal-regulated kinase ½ activation in postcapillary endothelium. J. Biol. Chem. 273:4220-4226.

Pfaffl, M. A. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29:9-45. http://dx.doi.org/10.1093/nar/29.9.e45.

Pfarrer, C. D., S. D. Ruziwa, H. Winther, H. Callesen, R. Leiser, D. Schams, and V. Dantzer. 2006. Localization of vascular endothelial growth factor (VEGF) and its receptors VEGFR-1 and VEGFR-2 in bovine placentomes from implantation until term. Placenta. 27:889-898. http://dx.doi.org/10.1016/j.placenta.2005.09.004.

Prentice, A. M., S. D. Poppitt, G. R. Goldberg, and A. Prentice. 1995. Adaptive strategies regulating energy balance in human pregnancy. Human Reprod. Update. 1:149–161. http://dx.doi.org/10.1093/humupd/1.2.149.

Ratcliffe, P. J. 2003. From erythropoietin to oxygen: hypoxia-inducible factor hydroxylases and the hypoxia signal pathway. Blood Purif. 20:445-450. http://dx.doi.org/10.1159/000065201.

Redmer, D. A., J. M. Wallace, and L. P. Reynolds. 2004. Effect of nutrient intake during pregnancy on fetal and placental growth and vascular development. Domestic Anim. Endocrin. 27:199-217. http://dx.doi.org/10.1016/j.domaniend.2004.06.006.

Reynolds, L. P., and D. A. Redmer. 1992. Growth and microvascular development of the uterus during early pregnancy in ewes. Biol. Reprod. 47:698–708. http://dx.doi.org/10.1095/biolreprod47.5.698.

Reynolds, L. P., P. P. Borowicz, K. A. Vonnahme, M. L. Johnson, A. T. Grazul-Bilska, D. A. Redmer, and J. S. Caton. 2005. Placental angiogenesis in sheep models of compromised pregnancy. J. Physiol. 565:46-58.

Reynolds, L. P., and J. S. Caton. 2012. Role of the pre- and post-natal environment in developmental programming of health and productivity. Mol. Cell. Endocrinol. 354:54-59. http://dx.doi.org/10.1016/j.mce.2011.11.013.

Rosenfeld, C. R., F. H. Morriss, E. L. Jr Makowski, G. Meschia, and F. C. Battaglia. 1974. Circulatory changes in the reproductive tissues of the ewes during pregnancy. Gynel. Invest. 5:252-268.

Rotta, P. P., S. C. Valadares Filho, T. R. Santos, L. F. Costa e Silva, T. E. Engle, M. I. Marcondes, F. S. Machado, F. A. C. Villadiego, and L. H. R. Silva. 2014a. Effects of stage of gestation and feeding regimen in Holstein × Gyr cows:I. Apparent total tract digestibility, nitrogen balance, microbial protein supply, and fat deposition. J. Dairy Sci. in press.

Rotta, P. P., S. C. Valadares Filho, T. R. Santos, L. F. Costa e Silva, T. E. Engle, M. I. Marcondes, M. M. Campos, A. C. B. Menezes, and A. A. G. Lobo. 2014b. Effects of stage of gestation and feeding regimen in Holstein × Gyr cows: II. Maternal and feral visceral organ mass. J. Dairy Sci. in press.

Shalaby, F., J. Rossant, T. P. Yamaguchi, M. Gertsenstein, X. F. Wu, M. L. Breitman, and A. C. Schuh. 1995. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature. 376:62–66. http://dx.doi.org/10.1038/376062a0.

Sousa, L. M. M. C., D. B. Campos, V. U. Fonseca, P. Viau, J. R. Kfoury Jr., C. A. Oliveira, M. Binelli, J. Burantini, and P. C. Papa. 2012. Vascular endothelial growth factor A (VEGFA) modulates bovine placenta steroidogenesis in vitro. Placenta. 33:788-794. http://dx.doi.org/10.1016/j.placenta.2012.07.009.

Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, and F. Speleman. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 18:3RESEARCH0034. http://dx.doi.org/10.1186/gb-2002-3-7-research0034.

Vonnahme, K. A., B. W, Hess, T. R. Hansen, R. J. McCormick, D. C. Rule, G. E. Moss, W. J. Murdoch, M. J. N. Nijland, D. C. Skinner, P. W. Nathanielsz, and S. P. Ford. 2003. Maternal undernutrition from early- to mid-gestation leads to growth retardation, cardiac ventricular hypertrophy, and increased liver weight in the fetal sheep. Biol. Reprod. 69:133-140. http://dx.doi.org/10.1095/biolreprod.102.012120.

Vonnahme, K. A., C. O. Lemley, P. Shukla, and S. T. O'Rourke. 2013. 2011 and 2012 Early Careers Achievement Awards: Placental programming: How the maternal environment can impact placental function. J. Anim. Sci. 91:2467-2480. http://dx.doi.org/10.2527/jas.2012-5929.

Wallace, J. M., D. A. Bourke, P. Da Silva, and R. P. Aitken. 2001. Nutrient partitioning during adolescent pregnancy. Reproduction. 122:347–57. http://dx.doi.org/10.1530/rep.0.1220347.

Wallace, J. M., D. A. Bourke, R. P. Aitken, N. Leitch, and W. W Hay Jr. 2002. Blood flows and nutrient uptakes in growth restricted pregnancies induced by overnourishing adolescent sheep. Am. J. Physiol. Regul. Integr. Comp. Physiol. 282:R1027–36. http://dx.doi.org/10.1152/ajpregu.00465.2001.

Wallace, J. M., J. S. Milne, N. Leitch, and R. P. Aitken. 2003a. Overnourishing singleton bearing adult ewes stimulates adiposity but does not influence nutrient partitioning to the gravid uterus. Pediatr. Res. 6:38-42. http://dx.doi.org/10.1203/00006450-197201000-00006.

Wallace, J. M., R. P. Aitken, V. Buchan, and W. W. Hay Jr. 2003b. Amino acid fluxes in growth-restricted pregnancies induced by overnourishing adolescent sheep. Placenta. 24:A9.

Zhang, F., Z. Tang, X. Hou, J. Lennartsson, Y. Li, A. W. Koch, P. Scotney, C. Lee, P. Arjunan, L. Dong, A. Kumar, T. T. Rissanen, B. Wang, N. Nagai, P. Fons, R. Fariss, Y. Zhang, E. Wawrousek, G. Tansey, J. Raber, G. H. Fong, H. Ding, D. A. Greenberg, K. G. Becker, J. M. Herbert, A. Nash, S. Yla-Herttuala, Y. Cao, R. J. Watts, and X. Li. 2009. VEGF-B is dispensable for blood vessel growth but critical for their survival, and VEGF-B targeting inhibits pathological angiogenesis. Proc. Natl. Acad. Sci. 106:6152-6157. http://dx.doi.org/10.1073/pnas.0813061106.

Zhu, M. J., M. Du, B. W. Hess, W. J. Means, P. W. Nathaniels, and S. P. Ford. 2007. Maternal nutrient restriction upregulates growth signaling pathways in the cotyledonary artery of cows placentomes. Placenta. 28:361-368. http://dx.doi.org/10.1016/j.placenta.2006.04.005.

Zhu, M. J., M. Du., and S. P. Ford. 2013. Impacts of maternal obesity on placental and gut inflammation and health. J. Anim. Sci. 15. http://dx.doi.org/10.2527/jas.2013-7106.

Ziche, M., L. Morbidelli, R. Choudhuri, H. T. Zhang, S. Donnini, H. J. Granger, and R. Bicknell. 1997. Nitric oxide synthase lies downstream from vascular endothelial growth factor-induced but not basic fibroblast growth factor-induced angiogenesis. J. Clin. Invest. 99:2625-2634.

Table 1. Details of target and reference genes

Gene name	Gene ID	Forward sequence	Reverse sequence	Amplicon size, bp
VEGFA	NM_174216.1	ATGACGAAAGTCTGGAGTGTG	TCTCCTATGTGCTGGCTTTG	94
VEGFB	NM_174487.2	AGAGTTGGATGAGGAGACCA	AGAGGAGCCAGCTGTTAGA	151
ANGPT2	NM_001098855.1	GCTGTACGACCACTTCTATCTC	GCTGGCTTATGCTGCTTATTT	101
FGF2	NM_174056.3	CCTACTCCTAGGCAATATGGTAAAT	CAACCCACCTAGTCAGAGATTG	96
NOS3	NM_181037.3	CTGTCATTCCACTATGGCTCTAC	GTACAGGGAATCCAACAGTCTC	109
IGFR1	NM_001077828.1	TCCCATCTCCCTGGATTTCT	GGGTTGGAAGACTGCTGATT	105
IGFR2	NM_174087.3	GGAAGTGGTCCAGCAAGATT	CGTCAATTTGGGCTCTGATTTC	98
GUCY1B3	NM_174641.1	GGAAGGGTTGTTGGATGTAGAG	GCTTCGGGCAAGTAGATCAT	105
HIF1A	NM_174339.3	GAGGCTCACCATCAGCTATTT	GCAATTCATCTGTGCCTTCATT	91
SF3A1	NM_001081510.1	ATGCCAACTCGCTGGCTTAC	AGAGCAGGCTTCTCCTACTT	100
BACTIN	NM_001033618.1	ACTCCTGCTTGCTGATCCACATCT	AAGATCAAGATCATCGCGCCTCCA	109
GAPDH	NM_001034034.1	GGCGTGAACCACGAGAAGTATAA	CCCTCCACGATGCCAAAGT	99

Table 2. PCR amplification efficiencies for each reference gene

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

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

Item	Feeding regimen		SEM	Day of gestation				SEM	P-value			
	Ad libitum	Maintenance		139	199	241	268		FR[1]	DG[2]	FR × DG[3]	Sex[4]
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[5]	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^{bA}	3.42^{aA}	4.06^{aA}	4.19^{aB}	0.456	-	-	-	-
Maintenance	-	-		1.51^{cA}	4.08^{bA}	4.62^{bA}	8.99^{aA}	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 ($P \leq 0.10$).

[1] Feeding regimen main effects.

[2] Day of gestation main effects.

[3] Interacion between feeding regimen and day of gestation.

[4] Sex of fetus main effects.

[5] Empty body weight.

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 ($P \geq 0.10$).

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

Items	Feeding regimen		SEM	Day of gestation				SEM	P-value			
	Ad libitum	Maintenance		139	199	241	268		FR[1]	DG[2]	FR × DG[3]	Sex[4]
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[5]	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
Placentomes, 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 ($P \leq 0.10$).

[1] Feeding regimen main effects.

[2] Day of gestation main effects.

[3] Interacion between feeding regimen and day of gestation.

[4] Sex of fetus main effects.

[5] Empty body weight.

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 ($P \geq 0.10$).

Table 5. Gene expression (relative abundance) of Holstein × Gyr cows fed ad libitum or maintenance at different day of gestation

Gene	Feeding regimen		SEM	Day of gestation				SEM	P-value			
	Ad libitum	Maintenance		139	199	241	268		FR[1]	DG[2]	FR × DG[3]	Sex[4]
ANGPT2	0.004	0.006	0.0001	0.003	0.005	0.006	0.004	0.0010	0.06	0.12	0.64	0.87
FGF2	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^{aA}	0.011^{aB}	0.012^{aB}	0.010^{aB}	0.0034	-	-	-	-
Maintenance	-	-	-	0.006^{bA}	0.023^{aA}	0.034^{aA}	0.031^{aA}	0.0034	-	-	-	-
P-value	-	-	-	0.44	0.02	< 0.01	< 0.01	-	-	-	-	-
NOS3	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^{aA}	0.004^{aB}	0.005^{aB}	0.005^{aB}	0.0012	-	-	-	-
Maintenance	-	-	-	0.004^{bA}	0.010^{aA}	0.011^{aA}	0.011^{aA}	0.0012	-	-	-	-
P-value	-	-	-	0.89	0.06	0.04	0.05	-	-	-	-	--
VEGFA	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^{aA}	0.126^{aA}	0.088^{aB}	0.093^{aB}	0.0120	-	-	-	-
Maintenance	-	-	-	0.046^{bA}	0.069^{bA}	0.320^{aA}	0.283^{aA}	0.0162	-	-	-	-
P-value	-	-	-	0.80	0.12	< 0.01	< 0.01	-	-	-	-	-
VEGFB	0.263	0.404	0.0266	0.292	0.351	0.368	0.321	0.0395	< 0.01	0.49	0.33	0.45
GUCYB3	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^{aA}	0.005^{aA}	0.002^{aB}	0.003^{aB}	0.0007	-	-	-	-
Maintenance	-	-	-	0.003^{cA}	0.004^{bcA}	0.008^{aA}	0.006^{abA}	0.0007	-	-	-	-
P-value	-	-	-	0.99	0.98	0.07	0.07	-	-	-	-	-
HIFA1	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^{aA}	0.004^{aA}	0.003^{aB}	0.003^{aB}	0.0019	-	-	-	-
Maintenance	-	-	-	0.003^{bA}	0.004^{bA}	0.015^{aA}	0.012^{aA}	0.0019	-	-	-	-
P-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 ($P \leq 0.10$).

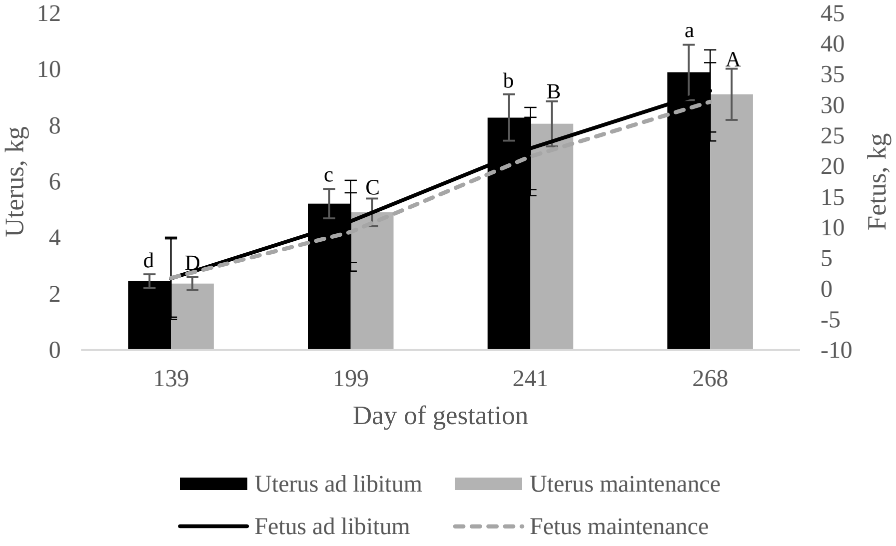
[1] Feeding regimen main effects.

[2] Day of gestation main effects.

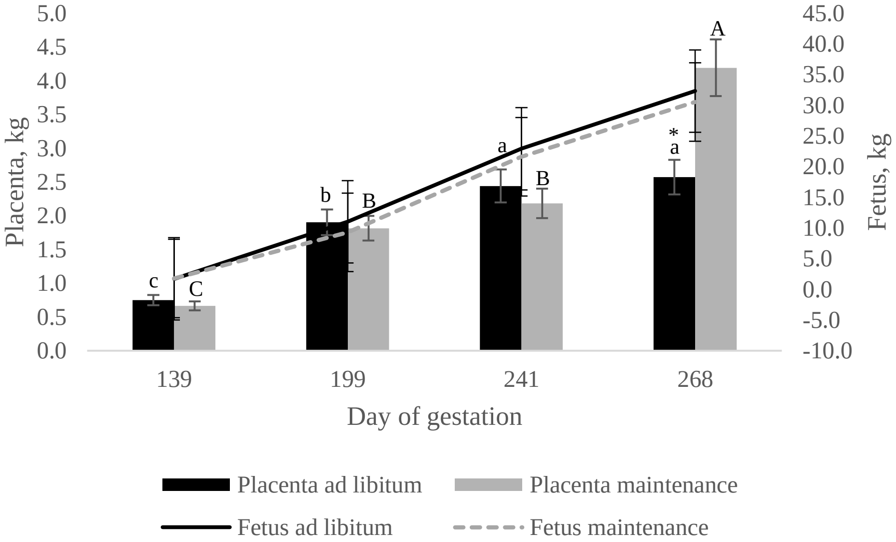
[3] Interacion between feeding regimen day of gestation.

[4] Sex of fetus main effects.

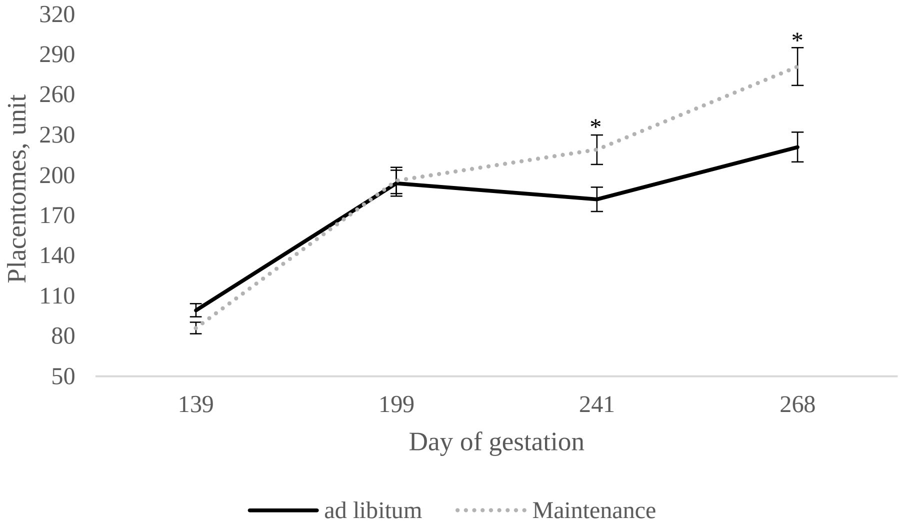
No interaction was observed between feeding regimen and sex ($P \geq 0.10$), day of gestation and sex ($P \geq 0.10$), and feeding regimen, day of gestation, and sex ($P \geq 0.10$).



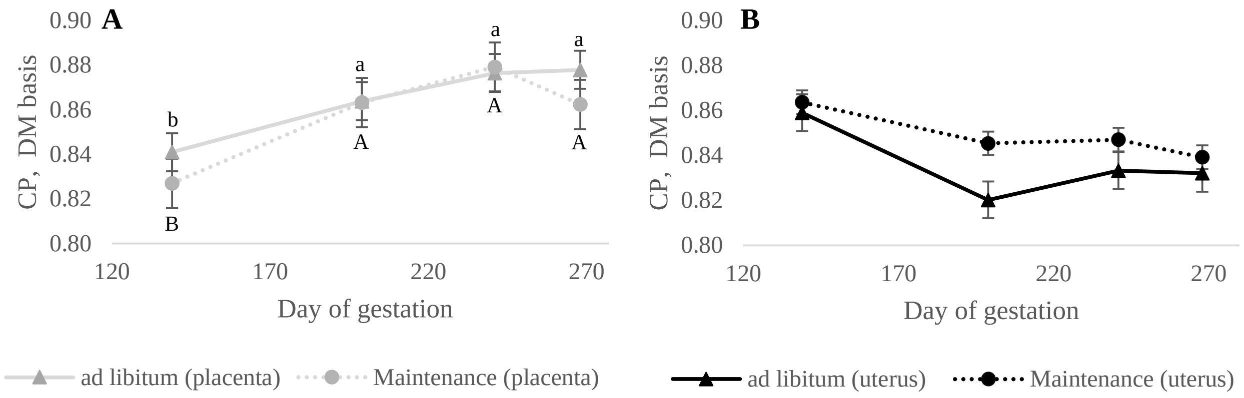
Rotta Figure 1



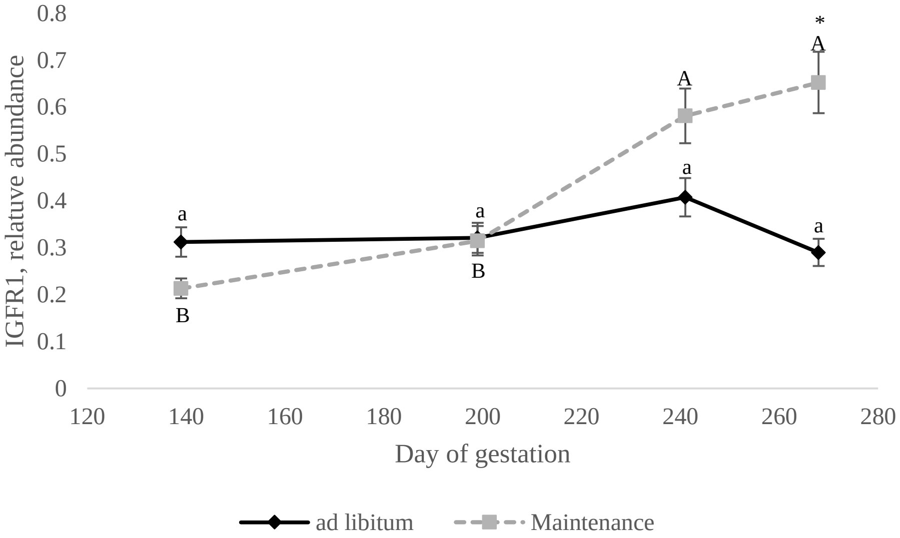
Rotta Figure 2



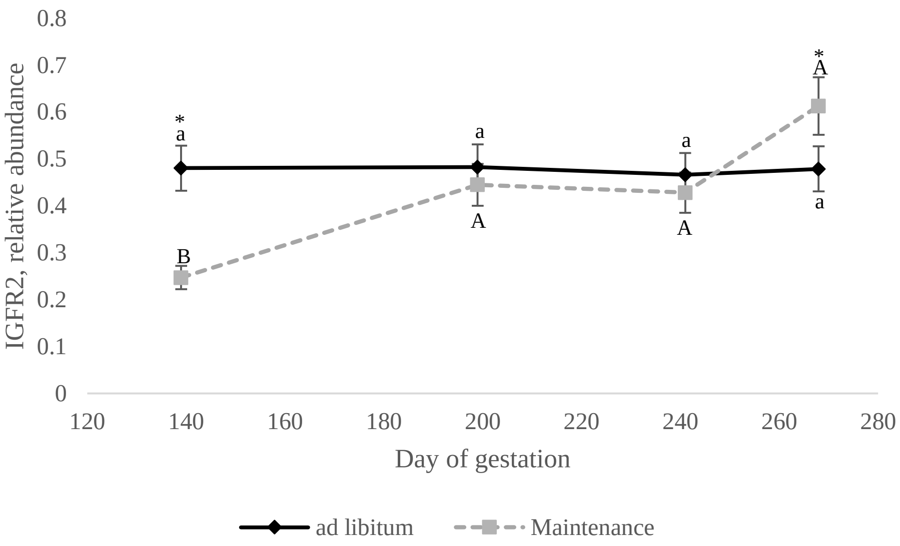
Rotta Figure 3



Rotta Figure 4



Rotta Figure 5



Rotta Figure 6

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.

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.

Figure 3. Placentomes units (means ± SEM) during gestation in Holstein × Gyr cows. *Different at $P \leq 0.10$.

Figure 4. Crude protein concentration (means ± SEM) in placenta (**A**) and uterus (**B**) in Holstein × Gyr cows at different day 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.

Figure 5. IGFR1 (means ± SEM) during gestation in Holstein × 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. IGFR2 (means ± SEM) during gestation in Holstein × 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.