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EFFECTS OF PREGNANCY ON QUANTITATIVE ASPECTS OF NUTRITION, PHYSIOLOGY AND METABOLISM OF BEEF HEIFERS

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Prof. Dr. Mateus Pies Gionbelli
Orientador

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GABRIEL MIRANDA MOREIRA

EFEITOS DA PRENHEZ SOBRE ASPECTOS QUANTITATIVOS DA NUTRIÇÃO, FISIOLOGIA E METABOLISMO DE NOVILHAS DE CORTE

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APROVADA em 01 de setembro de 2020
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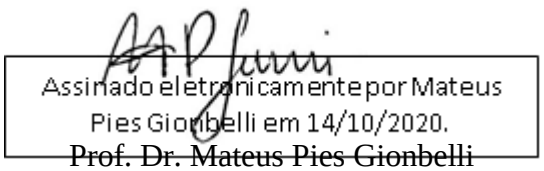

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À minha maior incentivadora e conselheira, minha mãe, Josiane.

Ao meu exemplo de caráter e humildade, meu pai, Carlos.

Dedico

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MUITO OBRIGADO POR NÃO DESISTIREM DE MIM!

RESUMO

Existem poucas informações sobre o efeito da gestação e do tempo de gestação sobre o consumo, digestão total e parcial e utilização de nutrientes, redução do volume ruminal e mudanças na taxa de passagem em vacas zebuínas. No Brasil existem 55 milhões de cabeças que podem ser beneficiadas com os avanços do conhecimento na área. Portanto, esta pesquisa foi realizada com o objetivo de quantificar os efeitos da prenhez sobre a nutrição, fisiologia e metabolismo de novilhas de corte. Doze novilhas Zebu, canuladas no rúmen, foram divididas aleatoriamente em dois grupos [gestantes (n = 7) e não gestantes (n = 5)]. Todas as novilhas receberam a mesma dieta durante o experimento. Novilhas gestantes acumularam reservas corporais (+ 35 kg) até 240 dias de gestação (DOP), quando então iniciaram a mobilização de tecidos (- 36 kg) até 286 DOP. Os consumos de matéria seca (MS), matéria orgânica (MO), proteína bruta (PB), fibra em detergente neutro (FDN) e nutrientes digestíveis totais (NDT) diminuíram (P <0,06) com o decorrer da gestação. A digestibilidade aparente do trato total de MS e FDN e a digestibilidade ruminal de MO e FDN foram menores (P ≤0,09) para novilhas gestantes em comparação com novilhas não gestantes. A digestibilidade aparente do trato total (P <0,01) de PB aos 267 e 286 DOP foi maior (P <0,01) em novilhas gestantes em comparação com não gestantes. O pool ruminal de matéria fresca (- 7,10 kg), MS (- 1,30 kg) e FDN (- 0,63 kg) foi menor (P <0,02) em novilhas gestantes do que em não gestantes aos 267 DOP. Em todos os períodos experimentais, a taxa de passagem da MS em novilhas gestantes foi maior (P <0,09) que em novilhas não gestantes. Nenhuma diferença foi encontrada no balanço de nitrogênio e nos parâmetros de fermentação ruminal. Novilhas gestantes foram mais eficientes ao longo do tempo para sintetizar proteína microbiana. A frequência cardíaca de novilhas em final de gestação em comparação com as não gestantes aumentou em oito batimentos/min quando avaliadas pouco antes da alimentação matinal, chegando a 11 batimentos/min quatro horas após a alimentação matinal. As concentrações de glicose antes da alimentação matinal foram semelhantes durante todos os períodos de coleta, com exceção daquela aos 286 dias (interação DOP × estado fisiológico; P = 0,05) quando a glicose foi mais baixa nas novilhas gestantes (83 mg/dL) em comparação com não gestantes (107 mg/dL). Os genes relacionados à remodelação, inflamação e transporte de ácidos graxos voláteis, H^+, HCO_3^- e glicose no epitélio ruminal foram regulados negativamente no final da gestação. Esses resultados sugerem que o epitélio ruminal economiza energia no final da gestação para beneficiar o desenvolvimento fetal. Além disso, o aumento da frequência cardíaca e a mobilização de tecidos podem ser considerados mecanismos homeorréticos que auxiliam no atendimento das necessidades nutricionais fetais. O estado fisiológico e o estágio de gestação devem ser incluídos nos modelos de predição de desempenho, uma vez que novilhas de corte no final da gestação são menos eficientes na extração de energia da dieta em comparação com animais não gestantes, alterando os nutrientes digestíveis totais preditos na alimentação.

Palavras-chave: Capacidade Ruminal, Consumo de Matéria Seca, Digestibilidade Intestinal, Digestibilidade Ruminal, Epitélio Ruminal, Expressão gênica, Frequência Cardíaca, Homeorrese, Tempo de Retenção Ruminal, Zebu

ABSTRACT

There is a lack of information about the effect of gestation and gestation time on intake, total and partial digestion and nutrient utilization, reduction of ruminal volume and changes in the feed passage rate in Zebu cows. Brazil has 55 million heads that could be benefited from the knowledge advances in this area. Therefore, this research was carried out to quantify the effects of pregnancy on the nutrition, physiology and metabolism of beef heifers. Twelve ruminally cannulated Zebu beef heifers were divided at random into two groups [pregnant (n = 7) and non-pregnant (n = 5)]. All heifers received the same diet throughout the experiment. Pregnant heifers accumulated body reserves (+ 35 kg) up to 240 days of pregnancy (DOP), then started mobilizing tissues (- 36 kg) until 286 DOP. The intake of dry matter (DM), organic matter (OM), crude protein (CP), neutral detergent fiber (NDF) and total digestible nutrients (TDN) reduces ($P < 0.06$) with the course of pregnancy. The apparent total-tract digestibility of DM and NDF and ruminal digestibility of OM and NDF was lower ($P \leq 0.09$) for pregnant compared with non-pregnant heifers. Crude protein apparent total-tract digestibility at 267 and 286 DOP was greater ($P < 0.01$) to pregnant compared with non-pregnant heifers. The ruminal pool of wet matter (- 7.10 kg), DM (- 1.30 kg) and NDF (- 0.63 kg) was lower ($P < 0.02$) to pregnant than non-pregnant heifers at 267 DOP. In all experimental periods, DM passage rate was greater ($P < 0.09$) to pregnant than non-pregnant heifers. No difference was found on nitrogen balance and ruminal fermentation parameters. Pregnant heifers were more efficient over time to synthetize microbial protein. The heart rate of late-pregnant heifers compared to controls increased by eight beats/min when evaluated just before morning feeding, and the difference reached 11 beats/min when evaluated four hours after morning feeding. Glucose levels before morning feeding were similar during all collection periods with an exception at 286 days (DOP × physiological status interaction; $P = 0.05$) when glucose was lower in pregnant (83 mg/dL) compared to non-pregnant (107 mg/dL) heifers. Both genes related to remodeling, inflammation, and volatile fatty acids, H^+, HCO_3^-, and glucose transport in the ruminal epithelium were downregulated at late gestation. These results suggest that the ruminal epithelium saves energy at late pregnancy to benefit fetal development. In addition, the increase in heart rate coupled with tissue mobilization can be considered homeorhetic mechanisms that help meet the fetal nutrient requirements. The physiological status, as well as the stage of gestation, should be included in performance prediction models since late-gestating beef heifers are less efficient at extracting energy from feed compared to non-pregnant animals, changing the feed predicted total digestible nutrients.

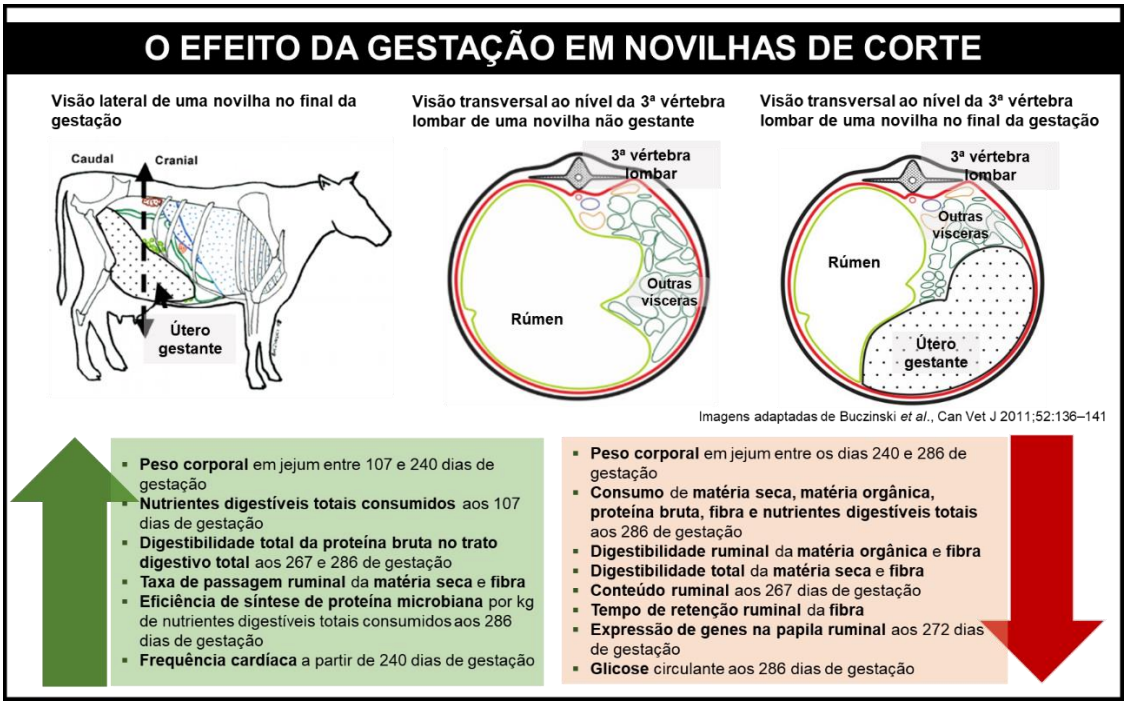
Keywords: Dry Matter Intake, Gene Expression, Heart Rate, Homeorhesis, Intestinal Digestibility, Ruminal Capacity, Ruminal Digestibility, Ruminal Epithelium, Ruminal Retention Time, Zebu Cattle

Efeito da gestação sobre aspectos quantitativos da nutrição, fisiologia e metabolismo de novilhas de corte

Elaborado por **Gabriel Miranda Moreira** e orientado por **Mateus Pies Gionbelli**

Para formular corretamente a dieta de um animal faz-se necessário estimar sua capacidade de consumo alimentar. No entanto, o cenário observado ao final da gestação é de alta exigência nutricional e baixa capacidade de consumo. Logo, essa pesquisa foi realizada com objetivo de quantificar os efeitos da gestação sobre a nutrição, fisiologia e metabolismo de novilhas de corte.

Foram observados maior consumo de nutrientes aos 107 dias de gestação e aumento do peso corporal (+ 35 kg) até 240 dias de gestação nas novilhas gestantes. No final da gestação (> 240 dias) houve redução da capacidade ruminal e do consumo de alimentos e, consequentemente, perdas de peso corporal (- 36 kg) nesse período. Também foi verificado aumento da velocidade em que o alimento passa pelo rúmen (taxa de passagem) em novilhas gestantes. Entretanto, devido ao menor tempo de retenção no rúmen, tanto a digestibilidade ruminal quanto a digestibilidade total da fibra foram diminuídas. Contudo, novilhas gestantes foram mais eficientes em utilizar a energia consumida para produzir proteína microbiana. A digestão da proteína bruta no trato digestório total aumentou no final da gestação. Houve menor expressão de genes relacionados à remodelação, inflamação e transporte de ácidos graxos voláteis, H^+, HCO_3^- e glicose no epitélio ruminal. A frequência cardíaca das novilhas gestantes foi maior no final da gestação. Portanto, concluímos que a gestação induz alterações sobre a nutrição, fisiologia e metabolismo de novilhas de corte. A quantificação dessas alterações fornece embasamento científico para a melhoria de modelos de ajuste do consumo de alimento e, consequentemente, melhor direcionamento das decisões a serem tomadas no sistema produtivo em relação a novilhas/vacas de corte gestantes criadas em condições tropicais.



Efeitos da gestação em novilhas zebuínas de corte. Ao final da gestação o útero ocupa grande parte da cavidade abdominal comprimindo o rúmen induzindo alterações nutricionais, fisiológicas e metabólicas em novilhas de corte.

Tese de Doutorado em Zootecnia na UFLA, defendida em 01/09/2020.

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FIRST SECTION

1 INTRODUCTION

The Brazilian bovine herd has 213.5 million heads (INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA, 2019), with an estimated 80% zebu component (*Bos taurus indicus*), composed mainly of Nellore cattle (NASCIMENTO, 2020). Of the total herd, around 55 million are breeding beef cows (FERRAZ et al., 2018). Breeding cows are permanent on the herd and have a high maintenance cost. Ritchie (1995) suggests that 50% of the total energy expended in beef production is used for maintenance. In beef cow-calf operation systems, 60 to 70% of the energy demand is for lactation and reproduction. Thus, it is essential to know adequately the nutritional requirements to balance the best diet for that cow category.

Pregnancy is the most physiologically complex stage in the production cycle of a cow. Notably, in pregnant Zebu cows, nutritional requirements and prediction of feed intake are much less known, and the first studies are only dated from 2010 onwards (GIONBELLI, 2013). Research dealing with pregnancy in cows is more difficult because of the inherently long experimental evaluation time.

In order to balance a diet correctly, it is necessary to accurately predict animal feed intake. However, it is difficult to predict the ingestion capacity of pregnant cows, since physical and physiological factors are not considered in traditional ruminant feed intake regulation models. Particularities, such as the influence of the gravid uterus on the reduction of rumen capacity, hormonal regulation of pregnancy, or the homeorhetic mechanism of nutrient usage, are difficult to model and are probably the main causes of the variations in voluntary intake observed in pregnant cattle.

Thus, this study aimed:

a. to increase information on the effect of gestation period on the intake, digestion, and use of nutrients in Zebu beef heifers;
b. to yield accurate information on rumen volume reduction and ingesta flow rate according to the gestation length in Zebu beef heifers;
c. to generate data on the effect of gestation time on the partial digestibility of dry matter and other dietary components in the rumen and intestine in Zebu beef heifers.
d. to investigate possible metabolic and physiological changes induced by pregnancy, as well as the behavior of important genes linked to the ruminal epithelium activity towards the advance of gestation in Zebu beef heifers.

The information obtained in this experiment may help to provide scientific basis to propose improved models for adjusting feed intake and, consequently, improving decision making in production systems relating to pregnant beef heifers/cows under tropical conditions.

2 BACKGROUND

During cow pregnancy, several challenges occur to the normal physiological status to support and allocate resources for the conception and development of a new individual (GIONBELLI *et al.*, 2015). Beef cows have a dramatic increase in nutrient demand during late gestation. To illustrate this, the maintenance requirements for metabolizable energy (ME) increase 28% in the last 90 days of pregnancy (DOP), considering a 500 kg zebu beef cow with no variation in body condition score (VALADARES FILHO *et al.*, 2016). Concurrently with nutritional demand increases, feed intake seems to decrease, leading to the establishment of additional mechanisms to maintain pregnancy and development of a healthy calf.

Fundamentally, pregnancy in cows impacts metabolism through changes in feed intake, ruminal fill, digesta passage rate, feed digestibility, ruminal fermentation parameters, maternal body weight (BW), circulating serum metabolites, and steroid hormones.

2.1 Effects of pregnancy on feed intake and ruminal capacity

The advancement of gestation may reduce feed intake in ruminant females and, during the days closer to parturition, the reduction in feed intake is even more drastic (FORBES, 2007). Initially, the reduction in feed intake observed in the late gestation of ruminants was related to ruminal compression generated by the growth of the gravid uterus (MAKELA, 1956). This association became known as Makela's compression theory. In the 1960s, several studies were carried out to assess intake and possible factors that can change it during pregnancy (BROSTER *et al.*, 1964; CAMPLING, 1966; FORBES, 1968; GRAHAM; WILLIAMS, 1962; JOHNSON *et al.*, 1966; LAMBERTH, 1969; REID; HINKS, 1962). At the end of the decade, the conclusion was that pregnancy had a physical impact on ruminal capacity. However, intake was not always changed or the variation was insignificant, where changes in the passage rate of digesta, as well as metabolic and hormonal mechanisms, were suggested as possible regulators of feed intake.

Until the end of the 20th century, more studies showed the effect of pregnancy on the intake and nutrients use in ruminants (COFFEY *et al.*, 1989; FORBES, 1970; FORBES, 1986;

GUNTER *et al.*, 1990; HANKS *et al.*, 1993; VANZANT *et al.*, 1991). Feed intake is directly related to rumen capacity (MERTENS, 1987), although during pregnancy this effect is not entirely clear. Hanks *et al.* (1993) verified lower gastrointestinal fill in pregnant than in non-pregnant limit-fed beef cows during the last trimester of pregnancy. Similarly, Stanley *et al.* (1993) using pregnant beef cows given *ad libitum* access to feed, reported a decrease in ruminal fill between 65 and 24 days prepartum, whereas dry matter (DM) intake increased. This information provides sufficient evidence to indicate that there is a physical effect of gestation on the reduction of ruminal capacity of ruminants at late gestation. Forbes (1986) remember that the effects of physical compression coincide with the changes in endocrine factors, such as estrogen levels, and body reserves, mediated in response to the advancement of gestation and preparation for the future lactation.

In the last 20 years, researchers remain evaluating the effect of pregnancy on feed intake (HARE *et al.*, 2019; LINDEN *et al.*, 2014; ROTTA *et al.*, 2015b; SCHEAFFER *et al.*, 2001; WOOD *et al.*, 2013). Scheaffer *et al.* (2001) showed that pregnant crossbred beef heifers had lower total ruminal fill (g/kg BW) than non-pregnant heifers at 200 and 270 DOP. However, DM intake and ME intake did not change from early-to-late gestation. Similarly, Wood *et al.* (2013) did not found difference in DM intake between pregnant and non-pregnant mature beef cows (Angus and Simmental cross-breeding). Conversely, Holstein × Gyr dairy cows decreased DM intake from 50 days before parturition (ROTTA *et al.*, 2015b). Also, Hare *et al.* (2019) verified that pregnant Hereford cross heifers increased DM intake between eight to two weeks prepartum by 18%, then decreased it by 8.0% in the week before parturition.

The effects of pregnancy on DM intake of forage-fed beef heifers were compared to mature beef cows (LINDEN *et al.*, 2014), where over the seven weeks prepartum, DM intake as a percentage of BW was similar between cows and heifers. Moreover, a tendency for an age × pregnancy × time interaction was also observed for DM intake once pregnant heifers demonstrated increases in intake from seven through two weeks prepartum followed by a small decline in the final week. However, pregnant cows demonstrated relatively stable intakes for most of the prepartum period with a minor increase in the final week.

Therefore, the effect of pregnancy on voluntary feed intake of ruminant depends on an interaction between physical and physiological factors, rendering intake estimation a complex task. The third edition of Nutrient Requirements of Zebu and Crossbred cattle (BR-CORTE 3.0) proposes a reduction of 0.0204 grams of DM per kg shrunk body weight (SBW) for each day over 135 DOP by pregnant zebu cows (VALADARES FILHO *et al.*, 2016). Another key point is that most of the studies investigating the effect of pregnancy on feed intake were carried

out in temperate regions and with taurine animals, and information for zebu cattle raised in tropical regions is still lacking.

2.2 Effects of pregnancy on passage rate of digesta and feed digestibility

The main justification for absence of a reduction in feed intake during pregnancy is the increase in the digesta passage rate. Passage rate is a measure of the time by which a portion of digesta is exposed to the processes of mixing, digestion, and absorption in the gastrointestinal tract or a defined segment; it is measured as the mean retention time, which is the ratio of the amount of any component of digesta in a segment to the flow of that digesta component from that segment (DIJKSTRA *et al.*, 2005). Passage rates are affected by a wide variety of factors that have different effects, including animal and feed factors (MOYO; NSAHLAI, 2018).

Most of the published studies propose that movement of digesta through the gastrointestinal tract of pregnant ruminants is increased such that it compensates for the loss of ruminal capacity caused by fetal growth in late gestation (ewes: (COFFEY *et al.*, 1989; FAICHNEY; WHITE, 1988; KASKE; GROTH, 1997; WESTON, 1988); beef cows: (HANKS *et al.*, 1993; LINDEN *et al.*, 2014; STANLEY *et al.*, 1993; VANZANT *et al.*, 1991). Okine and Mathison (1991) indicated that the ruminal passage rate of neutral detergent fiber (NDF) increased concomitantly with intake. But, during late gestation feed intake seems to be not the only factor that changes passage rate. Pregnant cows and heifers fed *ad libitum* or on the pasture had faster digesta passage rates than their non-pregnant counterparts (LINDEN *et al.*, 2014; STANLEY *et al.*, 1993; VANZANT *et al.*, 1991). Likewise, pregnant ewes fed *ad libitum* decreased mean retention time of digesta compared to non-pregnant ewes (COFFEY *et al.*, 1989; KASKE; GROTH, 1997). On the other hand, Hanks *et al.* (1993) found that limit-fed pregnant cows had increased particulate passage rate and decreased ruminal and total-tract mean retention time when compared to non-pregnant cows. Likewise, an increased passage rate of digesta from the reticulorumen has also been demonstrated for late pregnancy ewes fed at a constant level throughout pregnancy (FAICHNEY; WHITE, 1988; GUNTER *et al.*, 1990; WESTON, 1988). With this in mind, it can be concluded that the impact of gestation on the passage rate of digesta occurs through factors other than feed intake. Neural or hormonal factors might contribute to an increase in prepartum passage rates (STANLEY *et al.*, 1993). According to Forbes (1986), the circulating estrogen levels may increase passage rate.

Feed digestibility and passage rate of digesta are inversely proportional, in other words, decreases in apparent total tract digestibility of diet components may be explained in part by

the increase in passage rate (COLUCCI *et al.*, 1982; RIBEIRO *et al.*, 2015). Although the passage rate increases during gestation, studies evaluating the effects of pregnancy on digestibility in cattle are not consistent. Scheaffer *et al.* (2001) reported lower *in vitro* DM digestibility in pregnant heifers when compared with non-pregnant heifers. Vanzant *et al.* (1991) and Hanks *et al.* (1993) found no difference in organic matter (OM) and DM digestibility, respectively, between pregnant and non-pregnant animals. On the other hand, Linden *et al.* (2014) noted that DM digestibility was greater for pregnant animals than for non-pregnant animals and decreased more overtime for non-pregnant than for pregnant animals. However, these results represent apparent total-tract digestibility which is the sum of ruminal, and post-ruminal digestibilities. Therefore, it is necessary to establish more clearly the relationship between feed digestibility and passage rate in pregnant cows, mainly zebu cattle, as well as to assess changes in partial digestions (ruminal and intestinal) as a function of pregnancy time.

2.3 Effects of pregnancy on ruminal parameters and microbial protein synthesis

Since pregnancy can cause changes in feed intake, passage rate, and digestibility, it is expected that the ruminal parameters should also change. For instance, ruminal pH depends on saliva production, volatile fatty acids (VFA) balance, type and level of feed intake, and on the exchange of bicarbonates and phosphates through the ruminal epithelium (ASCHENBACH *et al.*, 2011). Rumen VFA concentration reflects the balance between production and clearance by passage with the fluid phase into the omasum or by absorption through the ruminal wall (LOPEZ *et al.*, 2003). Additionally, rumen ammoniacal nitrogen (N-NH_3) is a very potent buffer once NH_3 can immediately bind H^+ to form NH_4^+ in the ruminal content. Ruminal N-NH_3 removal occurs through the use of ruminal bacteria, efflux to the omasum, or absorption across the ruminal wall (ASCHENBACH et al. 2011).

Hanks et al. (1993) found that ruminal N-NH_3 did not differ between pregnant and non-pregnant cows until 10 days before parturition when pregnant cows (6.9 mg/dL) had lower concentrations than non-pregnant cows (8.0 mg/dL). The same authors also observed that total VFA concentration was lower in pregnant than non-pregnant cows at 203 DOP; however, this relationship was reversed at 230 DOP. On the other hand, ruminal pH and VFA concentration did not differ between pregnant and non-pregnant heifers, while ruminal N-NH_3 was reduced due to pregnancy (SCHEAFFER et al., 2001). Besides, Hare et al. (2019) verified that ruminal N-NH_3 had a treatment × day interaction, where pregnant animals fed excess (+ 33%)

metabolizable protein (MP) decreased as parturition approached (10.1 to 8.6 mg/ dL); whereas, N-NH_3 was not affected for pregnant animals fed to meet MP requirements (1.0 to 1.3 mg/dL).

Nitrogen (N) availability and its synchronization with energy sources are the greatest determinant of the amount of microbial crude protein (MCP) synthesized in the rumen (CLARK *et al.*, 1992). Conceptually, microbial efficiency is the amount of MCP obtained from a determined energy unit, or else, it is the amount of protein produced by ruminal microorganisms from energy substrates available in the rumen, under the interference of a series of factors (SANTOS *et al.*, 2016). However, there is a lack of studies evaluating the direct effects of pregnancy on MCP synthesis in the literature. In conditions where the passage rate is high, there is an expected reduction in microbial maintenance costs due to a reduction in the ruminal retention time (GIONBELLI, 2013).

2.4 Effects of pregnancy on maternal body weight

According to Bauman and Currie (1980), metabolism control during pregnancy besides homeostasis also involves homeorhesis regulations. These authors define homeorrhesis as orchestrated control in the metabolism of body tissues necessary to support a physiological state. For example, nutrient partitioning during pregnancy prioritizes fetal growth (fetus and fetal membranes) and gravid uterus as well as the development of the mammary gland (BAUMAN; CURRIE, 1980).

During pregnancy, and especially in its last trimester, nutrient requirements increase significantly, while rumen capacity is reduced due to fetal growth. Lower ruminal volume leads to lower feed intake capacity. To support the high nutritional demand, pregnant cows increase digesta passage rate, and consequently intake increases. However, this mechanism does not always meet the requirements, and tissue mobilization must take place in order to fulfill pregnancy demands. Nevertheless, Vanzant *et al.* (1991) observed that pregnant heifers were 50 kg heavier than non-pregnant at 55 and 12 days before calving. However, BW assessment may not accurately represent maternal tissues weights due to the growth of pregnancy-related components, such as the gravid uterus and mammary gland (GIONBELLI *et al.*, 2015). Scheaffer *et al.* (2001) investigated the impact of pregnancy and advancing gestation on BW in crossbred beef heifers. The carcass weight and eviscerated BW of pregnant heifers were greater at 200 DOP, but the carcass weight of pregnant compared with that of non-pregnant heifers tended to be lower at 270 DOP. In another study, cows and heifers were fed tallgrass prairie hay (low nutritional quality) for *ad libitum* intake and 450 g/day of soybean meal. It was

observed that over the prepartum phase (7 to 1 week before calving) BW decreased more in pregnant than in non-pregnant cows, indicating that the low-quality forage did not meet the energy requirements of pregnancy even with protein supplementation from soybean meal (LINDEN *et al.*, 2014). Hare *et al.* (2019) observed that increasing MP supply by 33% over predicted requirements improved N balance and decrease indicators of skeletal muscle catabolism, hence increasing BW gain during late gestation. Some authors suggested that to compensate restricted diets pregnant ruminants may alter the maternal visceral organ mass to benefit the offspring (MEYER *et al.*, 2010; REED *et al.*, 2007; ROTTA *et al.*, 2015a; SCHEAFFER *et al.*, 2001; WOOD *et al.*, 2013).

2.5 Effects of pregnancy on circulating serum metabolites and steroid hormones

Circulating serum metabolites are used as indicators of the nutritional and metabolic status of the dam. Beef cows may alter metabolism in response to the increase in nutrient demands especially during mid- to late gestation (WOOD et al., 2013). Glucose is a major energy source used for both maternal tissues as well as fetal growth and development during pregnancy (DOORNENBAL; TONG; MURRAY, 1984). During late gestation, there is an increase in hepatic gluconeogenesis along with decreased glucose utilization by tissues (BELL, 1995). In beef cows and heifers, plasma glucose concentrations were lower in pregnant than in non-pregnant animals in the last 50 DOP, likely due to glucose use by the fetus according to Linden et al. (2014). In contrast, glucose concentrations increase during the last week of pregnancy in dairy cows (INGVARTSEN; ANDERSEN, 2000).

Circulating non-esterified fatty acid (NEFA) and beta-hydroxybutyrate (BHB) concentrations are indicators of fat mobilization and ketogenic metabolic status. Bell (1995) suggests that increased BHB as a result of incomplete NEFA oxidation. In mid-to-late gestation, pregnant cows had increased circulating NEFA, and BHB concentrations and reduced circulating total cholesterol concentrations when compared to non-pregnant cows, indicative of greater fat catabolism and a more ketogenic metabolic status during pregnancy (WOOD et al., 2013). Similarly, pregnant cows and heifers had higher plasma BHB than their non-pregnant counterparts (LINDEN et al., 2014). This observation is justified by insufficient dietary energy, as a result of low feed quality, during pregnancy leading to increased lipolysis.

Prepartum changes in feed intake and digesta passage rate are more consistent with changes in blood concentrations of steroid hormones. Grummer et al. (1990) speculated that the surge in blood estrogen might be responsible for the depression in feed intake before

parturition. Plasma estrogen concentrations increase to around tenfold to twentyfold at about one month before calving (FORBES, 2007). Studies in rats also indicate that the effect of estrogen on feed intake is due, at least in part, to direct actions in the brain, where the paraventricular nucleus of the hypothalamus is the main site of action for estrogen on feed intake (BUTERA; BEIKIRCH, 1989). Estrogen has been proposed to interacts with orexigenic (neuropeptide Y, ghrelin, and melanin-concentrating hormone) and anorexigenic (insulin, leptin, serotonin, and cholecystokinin) neuropeptides influencing feed intake (BROWN; CLEGG, 2010). For example, neuropeptide Y (NPY) is a potent orexigenic, which increases feeding behavior in fed and fasted animals. Estrogen acts via the estrogen receptors in the hypothalamus to reduce feed intake and may mediate its anorectic effects by decreasing NPY expression or release. Conversely, injection of estradiol 17β at day 276 of gestation to achieve blood concentrations similar to those at parturition did not depress feed intake (BREMMER et al., 1999). This indicated that other hormones besides estrogen must be involved in regulating prepartum intake because the decrease in feed intake is initiated before the rise in estrogen before parturition. Progesterone does not seem to have a direct effect on feed intake in cattle, but, since it blocks estrogen effects it may reduce its effects on feed intake.

The faster passage rate also is linked to circulating estrogen. Forbes (1986) reviewed the literature regarding the effects of sex hormones on voluntary intake and concluded that decreased ruminal retention time of particles during the last trimester of pregnancy probably resulted from high circulating estrogen concentrations. Furthermore, both estrogen and progesterone increase gut motility in non-pregnant cattle and sheep (FORBES, 1986). Indeed, these hormones exhibited greater concentrations in pregnant than in non-pregnant cows, coinciding with greater particulate passage rate and lower gastrointestinal mean retention time, ruminal retention time, and intestinal transit time to pregnant animals (HANKS et al., 1993).

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SECOND SECTION – ARTICLE

ARTICLE 1 - Pregnancy affects maternal performance, ruminal digestibility, digesta passage rate, and efficiency of microbial protein synthesis in zebu beef heifers

Article formatted according to Livestock Science guidelines

Pregnancy affects maternal performance, ruminal digestibility, digesta passage rate, and efficiency of microbial protein synthesis in zebu beef heifers

Abstract

The aim was to quantify the effects of physiological status (PS; pregnant and non-pregnant) and pregnancy time on maternal performance, feed intake, digestibility and digestion kinetics in zebu beef heifers. Twelve rumen-cannulated Zebu beef heifers (7 pregnant and 5 non-pregnant) receiving the same diet during all pregnancy until calving were assigned to an experimental design. Samples were obtained at six collection periods throughout gestation [107, 170, 208, 240, 267, and 286 days of pregnancy (DOP)]. Pregnant heifers accumulated body reserves (+ 35 kg) from 107 until 240 DOP, but diminished body weight (- 36 kg) from this point until 286 DOP. Dry matter (DM), organic matter (OM), crude protein (CP), neutral detergent fiber (NDF) and total digestible nutrients (TDN) intake decreased ($P < 0.06$) as pregnancy progressed. Apparent total-tract digestibilities of DM and NDF and ruminal digestibilities of OM and NDF were lower ($P \leq 0.09$) in pregnant compared with non-pregnant heifers. There was an interaction effect ($P = 0.04$) between physiological

state (PS) and DOP on CP apparent total-tract digestibility. Crude protein digestibility was greater ($P < 0.01$) at 267 and 286 DOP in pregnant compared to non-pregnant heifers. Intestinal digestibility of diet components was similar independently of PS. Ruminal pool of wet matter (- 7.10 kg), DM (- 1.30 kg) and NDF (- 0.63 kg) were lower ($P < 0.02$) in pregnant than in non-pregnant heifers at 267 DOP. In all collection periods, DM passage rate was greater ($P < 0.09$) in pregnant than in non-pregnant heifers, and the difference was more evident at late gestation. Nitrogen balance and ruminal fermentation parameters were similar between PS. Pregnant heifers were more efficient ($P \leq 0.09$) over time to synthetize microbial protein. Late-gestating beef heifers have faster digesta passage rate and are less efficient at extracting energy from feed compared to non-pregnant animals, changing the predicted feed total digestible nutrients.

Keywords: dry matter intake, gestation, intestinal digestibility, ruminal capacity, ruminal digestibility, ruminal retention time

1. Introduction

Properly estimating feed intake is essential to accurately meet the requirements for the maintenance and production of beef cattle (Allen, 1996). However, traditional models of feed intake regulation in ruminants normally do not include physical and physiological factors that affect feed intake of pregnant cows (NASEM, 2016). Particularities, such as the influence of fetal growth in reducing ruminal capacity, are difficult to model and are the main causes of the variations in voluntary intake observed at this physiological status (PS) of cattle (Ingvartsen, 1994). It is known that beef cows have a dramatic increase in energy and protein demand during late gestation (Gionbelli

et al., 2016), however their ruminal capacity is limited (Stanley et al., 1993). Although ruminal volume is reduced close to 40% at the end of gestation (Gionbelli, 2013), the reduction in feed intake is not proportional (Ingvartsen et al., 1992), suggesting the development of compensation mechanisms to increase feed intake. These compensatory mechanisms may be related to the adjustment of feed digestibility and passage rate.

Digestibility and passage rate are inversely proportional; in other words, decreases in apparent total-tract digestibility of diet components may be explained in part by the increase in passage rate (Colucci et al., 1982; Ribeiro et al., 2015). The movement of digesta through the gastrointestinal tract of pregnant beef cows is increased in late gestation (Vanzant et al., 1991; Hanks et al., 1993; Stanley et al., 1993; Linden et al., 2014). Conversely, studies evaluating the effects of pregnancy on digestibility in cattle are not consistent, noticeably in zebu cattle. The *in vitro* dry matter (DM) digestibility in pregnant heifers was lower compared with non-pregnant heifers (Scheaffer et al., 2001). Vanzant et al. (1991) and Hanks et al. (1993) found no difference in organic matter (OM) and DM digestibility, respectively, in pregnant and non-pregnant animals. On the other hand, DM digestibility was greater for pregnant animals than for non-pregnant animals and decreased more overtime for non-pregnant animals than for pregnant animals (Linden et al., 2014). However, these results represent the total-tract apparent digestibility, which is the sum of ruminal and post-ruminal digestibility. Therefore, to our understanding, this is the first study evaluating, in pregnant and rumen cannulated zebu beef heifers, the changes in partial digestions (ruminal and intestinal) as a function of pregnancy time. Once digestion is altered, changes in ruminal fermentation parameters can be verified (Allen, 1997).

We hypothesized that as a way to compensate for the reduction in ruminal volume caused by the compression generated due to fetal growth, beef heifers increase the passage rate and reduce the ruminal digestibility of diet components without altering intestinal digestibility. The aim was to quantify the effects of physiological status (pregnant and non-pregnant) and pregnancy time on maternal performance, feed intake, digestibility and digestion kinetics in zebu beef heifers.

2. Material and Methods

The experiment was carried out in the Department of Animal Sciences Beef Unit of the Federal University of Lavras (UFLA) in Lavras, MG, Brazil. All procedures involving animal care and management were approved by the UFLA Ethics Committee on Animal Use (protocol number: 048/16).

2.1. Animals, housing, and feeding

Twelve rumen cannulated zebu beef heifers (body weight, BW = 417 ± 95.6 kg) were used. Heifers were artificially inseminated following an ovarian synchronization protocol. Pregnancy was detected sixty days later via transrectal ultrasonography. Seven heifers were grouped as pregnant, and five non-pregnant heifers were used as controls. The control group was used to compare with pregnant heifers at different stages of gestation since the nutritional composition of the diet ingredients and climate conditions could vary throughout time. The heifers were group-housed in pastures with water and mineral supplement available up to 85 days of pregnancy (DOP). Then, heifers were allocated to individual pens (80 m^2 with 16 m^2 of covered area) until calving. A period of ten days was allowed for housing and diet adaptation, during which the DM offered was gradually increased until voluntary intake was reached.

All heifers received the same diet throughout the experiment. The experimental diet was based on medium-quality corn silage and concentrate supplement containing ground corn, soybean meal, urea plus ammonium sulfate, and mineral mixture (Table 1). Experimental diets were formulated according to the Nutrient Requirements of Zebu and Crossbred Cattle - BR-CORTE 3.0 (Valadares Filho et al., 2016), to allow *ad libitum* intake without large accumulation of body reserves and an adequate maintenance of gestation. The use of 95.95% medium-quality corn silage sought to approach the nutritional quality of the diet to that of to grazing conditions plus a protein-based supplement. Heifers were fed twice a day at 0800 h and 1400 h. Daily orts were removed and weighed before the morning feeding. Free access to good-quality water was available.

2.2. Collection periods and sampling

Samples were obtained at six collection periods throughout gestation (107, 170, 208, 240, 267, and 286 DOP). Collection periods 107, 208, and 267 DOP were performed for 11 straight days, while periods 170, 240, and 286 DOP for five consecutive days. The feed intake control started at 95 DOP until parturition. Corn silage samples were collected daily, and concentrate-ingredient samples were obtained immediately before mixing. Orts per animal were sampled daily during each collection period. Heifer BW was measured at the beginning and the end of each collection period, before the morning feeding. Body condition score (BCS) was assessed on day five of each collection period and was obtained by the average score of three trained observers using a 9-point scale (1 = emaciated; 9 = obese).

Feces spot collection was performed on days one to five of each collection period to determine the coefficient of the apparent total-tract digestibility of dietary

components. Fecal samples were collected at 10-h intervals within a day and at 16-h intervals between days (day one, 0600 and 1600 h; day two, 0800 and 1800 h; day three, 1000 and 2000 h; day four, 1200 and 2200 h; day five, 1400 h), totaling nine samples per collection period. Fecal samples were immediately frozen at −20°C until further analyses. On the same days of fecal sampling, spot urine samples were collected to assess microbial crude protein (MCP) synthesis and the excretion of urinary nitrogenous compounds. Five-spot urine samples were obtained by stimulating the area below the vulva (day one, 0600 h; day two, 1800 h; day three, 1000 h; day four, 2200 h; day five, 1400 h). Urine samples were filtered, and a 12 mL aliquot was immediately acidified by diluting one volume of urine with four volumes of sulfuric acid (H_2SO_4) at 0.036 mol/L to avoid N loss. Then, samples were frozen at -20 °C for further analysis.

During the 107, 208, and 267 DOP collection periods, samplings of omasal and ruminal digesta were performed. Omasal digesta sampling was performed according to Huhtanen et al. (1997), with modifications described by Leão (2002). Omasal digesta was collected twice a day, at 12-h intervals within a day, and at 16-h intervals between days to avoid possible variation in digesta flux related to collection time. Samples are collected at 0600 and 1800 h on day six, at 1000 and 2200 h on day seven, and 1400 and 0200 h of the next day on day eight, totaling six samples per collection period. A sample of approximately 600 mL of digesta per animal was collected and frozen at −20°C for further analysis. For determination of ruminal DM outflow, two indicators were utilized: Co-EDTA as the fluid phase and small particles indicator (Udén et al., 1980) and indigestible neutral detergent fiber (iNDF) as the solid-phase indicator. The Co-EDTA was wrapped in 1.5 g paper cartridges, and a total of six g was provided daily, administered four times in 6-h intervals (0600, 1200, 1800,

and 0000 h), directly through the ruminal cannula. The administration of Co-EDTA started three days before the first omasal digesta collection until the last collection.

To determine the ruminal pools, rates of intake (ki), passage (kp), and digestion (kd) of diet components, on day nine, complete evacuation of the rumen was performed approximately four hours after feeding, according to procedures described by Allen and Linton (Allen and Linton, 2007). After evacuation, the total digesta was weighed and then filtered through four layers of cheesecloth to separate the solid and fluid phases, which were weighed and sampled for further analysis. Then, the digesta was put back into the respective rumen. On day 11, ruminal evacuation was also performed one hour before feeding, theoretically the time the rumen is at its least volume.

To evaluate the pH, volatile fatty acids (VFA), and concentration of ruminal ammoniacal N ($N\text{-}NH_3$), ruminal fluid was sampled on collection periods 107, 208, and 267 DOP. Samples were collected manually from the ventral sections of the rumen before (time 0) and four hours after (time 4) morning feeding. Ruminal fluid samples were filtered through a triple layer of gauze, and 50 mL of the ruminal liquid were used immediately to determine pH, in a pH-meter (model HI 2221, Hanna Instruments, Woonsocket, RI, USA). At the same time, 20 mL aliquots of filtered ruminal fluid were shocked frozen in liquid N to inhibit microbial growth. Then, samples were frozen at -20 °C for VFA analysis. Additionally, 1 mL of H_2SO_4 diluted in distilled water (1:1) was added to 50 mL aliquots of filtered ruminal fluid and frozen at -20 °C for $N\text{-}NH_3$ analysis.

2.3. Laboratory procedures, analyses, and calculations

Heifer BW was determined by the average between the BWs at the beginning and end of each collection period. Pregnancy was considered as an extra component of the heifer (referred mathematically as pregnancy component, PREG). The PREG

allows the estimating portion of the BW of a pregnant heifer that is a function of pregnancy. The PREG includes all tissues that increase due to the pregnancy and is equal to the gravid uterus plus udder accretion during pregnancy. The PREG was estimated using DOP, heifer BW and BCS, and calf BW at calving (Gionbelli et al., 2015). The BW of a pregnant heifer minus PREG or the BW of a non-pregnant heifer was called BWnp. To reduce the fill effect and improve the accuracy of measurements, BW was used to estimate the shrunk body weight (SBW), as follows: $SBWnp = 0.8084 \times BWnp^{1.0303}$ (Gionbelli et al., 2015). The SBW of a pregnant heifer minus PREG or the SBW of a non-pregnant heifer was called SBWnp.

At the end of each collection period, omasal digesta were thawed at room temperature, and an animal composite sample was obtained. Two-thirds of composite samples were filtered through a 100-µm nylon mesh filter with a 44% open area (Sefar Nitex 100/44; Sefar, Thal, Switzerland), resulting in two phases. The portion retained in the mesh was designated as particle phase, and the filtered portion was named fluid + small particle phase. Thawed samples of corn silage, orts, feces, ruminal and omasal digesta were pre-dried in a forced-air oven at 65°C for 72 h. Then, samples were ground in a knife-mill with a 2-mm sieve for iNDF, and with a 1-mm sieve for DM, OM, crude protein (CP), ash- and protein-free neutral detergent fiber (apNDF), and ether extract (EE).

Corn silage samples were composed weekly or by collection period. Furthermore, composite samples of orts, feces, ruminal digesta for each heifer, and collection period were obtained. A composite sample of ruminal digesta was obtained from the dry samples of the solid and fluid phases of the two ruminal evacuations (one hour before and four hours after feeding), based on the dry weight of each sample.

Ground samples were analyzed for DM (method INCT-CA G-003/1), OM determined by ash (method INCT-CA M-001/1), and CP obtained by total N, using the micro-Kjeldahl technique (method INCT-CA N-001/1). Additionally, the EE was determined after petroleum ether extraction (method INCT-CA G-005/1) and iNDF (INCT-CA F-009/1), according to Detmann et al. (2012). Ash- and protein-free NDF were analyzed using filtration in porous crucibles with heat-stable α-amylase and sodium sulfite (Van Soest et al., 1991). Non-fiber carbohydrates (NFC) were calculated according to Detmann and Valadares Filho (Detmann and Valadares Filho, 2010), where NFC (% of DM) = 100 – [CP – (CP derived from urea + urea) + NDF + EE + ash]. Cobalt content in omasal digesta was analyzed in a mineral solution prepared according to method INCT-CA M-004/1 (Detmann et al., 2012) using an atomic absorption spectrophotometer (model AA-7000; Shimadzu Corp., Kyoto, Japan).

The apparent total-tract digestibilities of DM, OM, CP, apFDN, and NFC were determined by the difference between intake and the fecal content divided by the intake. The content of total digestible nutrients (TDN) was obtained according to recommendations from the NRC (2001).

Ruminal DM and apNDF outflows were estimated by the double-indicator (Co-EDTA and iNDF) system (France and Siddons, 1986). For the double-indicator system calculation, concentrations of indicators in different digesta phases were used to calculate the reconstitution factor. The reconstitution factor indicates the units of the digesta phase that must be removed (when negative) or added (when positive) to non-representative digesta to reconstitute the true digesta (France and Siddons, 1986).

The composite samples of ruminal digesta were used to calculate the ruminal pool of DM and apNDF. Rates of intake, passage, and digestion were estimated using the pool-and-flux method (Robinson et al., 1987). The ki was calculated by dividing the

655 diet component intake by its respective ruminal pool size. The kp was obtained by
656 dividing the ruminal outflow of the diet component by its respective ruminal pool size.
657 The kd was calculated as ki minus kp.

658 Urine samples were thawed, and a composite for each heifer and collection
659 period was performed. Urine total N was determined using the micro-Kjeldahl
660 technique [method INCT-CA N-001/1; (Detmann et al., 2012)]. Urine creatinine
661 concentration was analyzed using a commercial kit (Creatinine K016, Bioclin, Belo
662 Horizonte, Brazil). Allantoin concentration was quantified according to the colorimetric
663 method (Chen and Gomes, 1992). Uric acid was estimated using allantoin
664 concentration (Santos et al., 2016), as follows: uric acid (mmol/d) = 0.1104 × allantoin
665 (mmol/d). Microbial CP synthesis was estimated by using the technique of the purine
666 derivatives in urine (Chen and Gomes, 1992). Urine volume was estimated using
667 creatinine concentration as a marker and assuming a daily creatinine excretion (mg/d)
668 of $37.88 \times SBW^{0.9316}$, where SBW is the shrunk body weight in kg (Santos et al., 2016).
669 Excretion of purine derivatives in urine was calculated by the sum of the allantoin and
670 uric acid excretions, which were obtained by the product between their concentrations
671 in urine by the daily urinary volume. Absorbed purines were calculated from the
672 excretion of purine derivatives (Prates et al., 2012), as follows: absorbed purines
673 (mmol/d) = excretion of purine derivates (mmol/d) − $(0.389 \times BW^{0.75})/0.99$; where 0.99
674 = recovered absorbed purines. The $0.389 \times BW^{0.75}$ value = endogenous excretion of
675 purine derivates (mmol/d). Ruminal MCP synthesis was calculated as a function of the
676 absorbed purines (Prates et al., 2012), as follows: MCP = 70 × absorbed purines
677 (mmol/d)/(0.93 × 0.11 × 1000); where 70 = purine N content (mg N/mol), 0.93 = purine
678 digestibility and 0.11 = relation of purine N:total N of microorganisms. The efficiency of
679 MCP synthesis was calculated dividing the amount of MCP by intake of CP, digestible

680 OM, and TDN. The total-tract N balance was calculated subtracting from the N intake
681 the amount excreted via urine and feces. Ruminal N balance was considered the
682 difference between N intake and ruminal outflow of N.

683 Ruminal fluids for VFA analyses were thawed, filtered through sterile syringe
684 filter with 0.45 µm pore sizes, centrifuged at 1,000 × g for 30 min at 4°C, and acidified
685 with 0.2 mL of H_2SO_4 at 0.1 mol/L. Volatile fatty acid concentrations were determined
686 using a gas chromatograph (Varian Model CP3800; Varian, Inc, Walnut Creek, CA)
687 equipped with a Nukol capillary GC column (length: 15 m; inner diameter: 0,53 mm;
688 stationary phase film thickness: 0,50 µm) bonded phase - acid modified polyethylene
689 glycol [Supelco, Bellefonte, PA]), where N_2 was used as a carrier gas at a flow rate of
690 2.5 mL/min. The following temperature programming was used in the oven: 110°C for
691 1 min followed by a ramp of 6°C/min to 160°C and ramp of 30°C/min up to 195°C a
692 with a final plateau of 5 min. The injection port and detector port temperatures were
693 220 and 250°C, respectively. Ruminal N-NH_3 concentrations were determined
694 according to method INCT-CA N-006/1 described by Detmann et al., 2012.
695
696 *2.4. Statistical analyses*

697 Data were analyzed through the mixed model methodology (procedure MIXED
698 of SAS 9.2, SAS Inst. Inc., Cary, NC), considering the PS effect (pregnant or non-
699 pregnant) and the DOP as fixed effects and animal as the random effect. When
700 appropriate, initial BW was included as a covariate in the model. Once repeated
701 measurements were taken from the same animal (for DOP), the subject animal nested
702 within treatment was included in the repeated measurement statement. For every
703 DOP, the PS effect on the measured variable was estimated using the “estimate
704 statement” of SAS. The *P*-value < 0.10 was adopted as a critical level of probability for

the occurrence of Type I error. Tendency was determined as 0.15 > *P* ≥ 0.10. Except for body weight variables, the isolated effect of DOP is not discussed, as it is related to environmental and dietary and environmental variations over time and not exactly to the study factor.

3. Results

3.1. Body weight

Mean pregnancy length and calf BW at birth were 292 ± 4 days and 31.3 ± 6.7 kg, respectively. Initial BW and BCS did not differ (*P* ≥ 0.80) between pregnant and non-pregnant heifers (Table 2). Moreover, BW, SBW, and BCS also did not vary (*P* ≥ 0.29) between PS. However, BW and SBW increased (*P* < 0.01) over days in both groups. On the other hand, BWnp and SBWnp decreased (*P* = 0.01) in pregnant compared with non-pregnant heifers over days (Fig. 1A and 1B). In pregnant heifers BWnp and SBWnp were lower (*P* < 0.01) than in non-pregnant heifers at late gestation (267 and 286 DOP). Whereas, at 107, 170, 208, and 240 DOP, BWnp and SBWnp were similar (*P* = 0.27) between pregnant and non-pregnant heifers. The estimated PREG increased over DOP (Fig. 1C).

3.2. Feed intake

There was an interaction (*P* ≤ 0.08) between the effects of DOP and PS on DM, OM, CP, apNDF, iNDF, and TDN intake (kg/d) such that these parameters decreased in pregnant heifers over gestation time, but not in non-pregnant heifers. (Table 3). At 107 DOP, TDN intake was greater (*P* = 0.05), while at 286 DOP, TDN intake tended to be lower (*P* = 0.10) in pregnant compared to non-pregnant heifers. Furthermore, at

286 DOP, OM and apNDF intake were lower (P = 0.09), and DM, CP, and iNDF intake tended to be lower (P = 0.11) in pregnant compared to non-pregnant heifers. Likewise, when expressed as grams per kilogram of BW, DM (Fig. 2), apNDF, iNDF, and TDN (Fig. 3) intakes also were affected by an interaction effect ($P \leq 0.05$). At 107 DOP, TDN intake was greater (P = 0.04) and DM, apNDF, and iNDF intake tended to be greater ($P \leq 0.13$) in pregnant compared to non-pregnant heifers, whereas, at 286 DOP, these same intakes were lower ($P \leq 0.09$) in pregnant heifers.

3.3. Apparent total-tract, ruminal, and intestinal digestibility

The apparent total-tract digestibility (g/kg of DM) of DM and apNDF were lower ($P \leq 0.09$) and OM digestibility tended to be lower (P = 0.11) in pregnant compared to non-pregnant heifers (Table 4). There was an interaction effect (P = 0.04) between PS and DOP on CP apparent total-tract digestibility. Pregnant heifers had similar ($P \geq 0.22$) CP apparent total-tract digestibility until 240 DOP, but greater ($P < 0.01$) digestibility at 267 and 286 DOP compared with non-pregnant heifers (Fig. 4). In contrast, the ruminal digestibility (g/kg of DM) of OM and apNDF was lower ($P \leq 0.09$) in pregnant compared to non-pregnant heifers, but the PS did not affect ($P \geq 0.22$) the DM, CP or NFC ruminal digestibility. Regarding intestinal digestibility (g/kg of the amount reaching the omasum), no difference was observed in the diet components depending on PS.

3.4. Ruminal pool and passage rate

There was an interaction between PS and DOP (P = 0.01) on the ruminal WM pool (kg; Table 5). In pregnant heifers the ruminal WM pool at 107 and 208 DOP were similar ($P \geq 0.30$), but was lower ($P < 0.01$) at 267 DOP than in non-pregnant heifers (Fig. 5). Similarly, there was a PS × DOP interaction effect ($P \leq 0.02$) on the ruminal

pool of DM and apNDF. In pregnant heifers the ruminal pool of DM (- 1.30 kg) and apNDF (- 0.63 kg) were lower ($P < 0.01$) than in non-pregnant heifers at 267 DOP. Conversely, the ruminal outflow of DM and apNDF were greater ($P \leq 0.04$) at 107 and 208 DOP (average of 0.74 and 0.40 kg, respectively) in pregnant heifers. Additionally, an interaction effect ($P \leq 0.09$) between PS and DOP was found on DM kp and ki and apNDF ki and kd (Table 5). Pregnant heifers had greater DM kp (Fig. 6) and ki in all collection periods; however, the difference was numerically greater at 267 DOP. Concerning apNDF ki and kd, at 267 DOP, pregnant compared to non-pregnant heifers increased by 0.85 %/h and 0.30 %/h, respectively. Also, pregnant heifers had greater ($P \leq 0.05$) DM kd and kp apNDP, and lower ($P < 0.01$) retention time for DM and apNDF than non-pregnant heifers.

3.5. Nitrogen balance and efficiency of microbial protein synthesis

No difference was found between PS on N balance, with the exception of N ruminal outflow (Table 6). An interaction between the effect of PS and DOP was detected ($P = 0.03$) on N ruminal outflow (g/day), but this effect was likely a consequence of variation over time than PS. There was an interaction effect ($P \leq 0.09$) between PS anf DOP on MCP expressed per unit of CP, digestible OM (DOM), and TDN intakes (Fig. 7). Pregnant heifers were more efficient over pregnancy time, achieving greater ($P \leq 0.06$) difference at 286 DOP.

3.6. Ruminal fermentation parameters

Before morning feeding, there was no difference between PS in ruminal fermentation parameters (Table 7). On the other hand, four hours after the morning feeding, total volatile fatty acids concentration (mmol/L) tended to be greater ($P = 0.12$)

780 in pregnant heifers. Additionally, there was a tendency (*P* = 0.14) for an interaction
781 effect between PS and DOP on the propionic acid concentration (mmol/L) at four hours
782 after the morning feeding. At late gestation (267 DOP), the concentration of propionic
783 acid in the ruminal fluid were 15.1 mmol/L and 19.1 mmol/L in pregnant and non-
784 pregnant heifers, respectively.
785
786 **4. Discussion**
787 *4.1. Body weight*
788 During pregnancy, BW increase was probably due to the deposition of body
789 tissue reserves or due to the growth of pregnancy-related components, such as the
790 gravid uterus and mammary gland (Gionbelli et al., 2015). Rendering the use of BW to
791 assess beef cow pregnancy performance unsuitable. In this trial, when estimating
792 pregnancy components, a loss of heifer maternal body reserve was detected at late
793 gestation. This observation indicates the mobilization of maternal stores to support the
794 growing fetus and developing mammary gland requirements (Scheaffer et al., 2001;
795 Linden et al., 2014). Insufficient DM intake and low-quality diet may have led to such
796 mobilization.
797 Another pattern, commonly observed during pregnancy, is the transition from
798 an anabolic to a catabolic state as pregnancy progresses (Robinson, 1986). Results
799 indicate that this transition in maternal metabolism occurs after 240 days of gestation,
800 corroborating results of previous studies (Scheaffer et al., 2001; Meyer et al., 2010).
801
802 *4.2. Feed intake*
803 The current study showed that pregnant heifers had a greater TDN intake at the
804 end of the second third of gestation (170 DOP), suggesting animal adaptation to body

reserve accumulation to sustain late pregnancy high metabolic demand. Similarly, voluntary DM intake in pregnant dairy heifers reached a peak between 15 and 17 weeks before calving (Ingvartsen and Andersen, 2000), i.e., at the end of the second third of gestation. Indeed, in this trial, the accumulation of maternal tissues occurred at the same stage of pregnancy. Additionally, the greater intake at this period may be the result of a faster DM kp observed in pregnant heifers.

On the other hand, DM and TDN intakes decreased throughout pregnancy, coincidently with a lower ruminal pool of wet matter (WM), DM, and apNDF, which are indicative of low ruminal capacity. These results agree with studies that showed a reduction in feed intake and ruminal fill at late pregnancy (Hanks et al., 1993; Stanley et al., 1993; Scheaffer et al., 2001). These observations provide sufficient evidence to support a physical effect of pregnancy in reducing ruminant DM intake at late gestation. However, it is unlikely that the decrease in ruminal volume is the only cause of the late gestation intake decline. It is important to note that the effects of physical compression coincide with the changes in endocrine factors and body reserves, mediated in response to the advancement of gestation and preparation for future lactation (Forbes, 2013).

Nevertheless, the reduction observed in intake was not proportional to the reduction in the ruminal pool of DM at late gestation. While the ruminal pool was reduced to around 23%, the intake reduction was only 11%. Consequently, this disproportionality caused an increase in DM ki for pregnant heifers in the present study, considering that DM ki is a ratio between DM intake and ruminal pool (Robinson et al., 1987).

4.3. Passage rate

The movement of digesta through the rumen of pregnant beef heifers was increased probably to compensate for the loss of ruminal capacity caused by fetal growth in late gestation. Passage rates are affected by a wide variety of factors exerting different effects, including animal and feed factors (Moyo and Nsahlai, 2018). Okine and Mathison (Okine and Mathison, 1991) indicated that ruminal kp increased concomitantly with intake. But, during late gestation, feed intake seems to be not the causative factor for changes in kp. Pregnant cows and heifers fed for *ad libitum* intake had faster digesta kp than their non-pregnant counterparts (Linden et al., 2014). Likewise, ruminal indigestible ADF kp increased from 61 days to six days prepartum in mature beef cows fed *ad libitum* (Stanley et al., 1993).

On the other hand, Hanks et al. (Hanks et al., 1993) found that limit-fed pregnant cows had increased particulate kp and decreased ruminal and total-tract mean retention time when compared to non-pregnant cows. Thus, an increase in kp occurs even when intake does not change. It is concluded that the impact of pregnancy on the digesta kp occurs through factors other than feed intake. Nevertheless, the reasons for the increase in the kp are still unclear. Neural or hormonal factors might contribute to an increase in kp (Stanley et al., 1993).

Changes in digesta passage from the ruminoreticulum are associated with the strength (Al-Shboul et al., 2019) and duration (Okine and Mathison, 1991) of reticular contraction. In this context, the frequency of primary ruminoreticular movements increased as the pregnancy progressed in pregnant sheep (Stafford, 1991); however, no studies were found evaluating ruminoreticular motility during pregnancy of cattle. In general, the ruminoreticular motility is controlled by smooth muscle contractility, which in turn is regulated by extrinsic (sympathetic and parasympathetic neurons) and intrinsic (sensory and motor neurons) factors of the enteric system and specific

hormones (Costa et al., 2000). Several hormones are linked to this motility function, such as secretin, peptide YY, neurotensin, gastrin, gastrin-releasing peptide, cholecystokinin, somatostatin, ghrelin, and motilin (Kitazawa and Kaiya, 2019). Recently, it was verified that total ghrelin concentration increases during the last months of pregnancy in adult cows but not in heifers (Chouzouris et al., 2018). According to Forbes (1986), circulating estradiol concentrations may increase kp. However, recent studies, in humans, have shown that estradiol is responsible for relaxing the smooth muscles of the stomach and other organs (Al-Shboul et al., 2018; Al-Shboul et al., 2019). Thus, although estradiol concentrations are higher in pregnant cows (Hanks et al., 1993), they are unlikely to be the cause of the increase in kp.

The motility of ruminant digestive tracts can also be controlled by tension receptors (located in the muscular wall) and epithelial receptors in the reticulorumen (Forbes and Barrio, 1992). Therefore, the lower ruminal volume of pregnant heifers can increase the mechanical stimulation caused by digesta on these receptors and can also increase ruminoreticular motility and, consequently, digesta passage rate.

4.4. Digestibility

The ruminal digestibility of apNDF and OM was impaired in pregnant heifers corroborating our hypothesis. To our knowledge, this is the first study that reports changes in the ruminal digestibility of beef cattle during pregnancy. The faster ruminal kp leads to lower digestibility due to the shorter time feed is exposed to digestive processes (Ribeiro et al., 2015). Indeed, our results showed that pregnant heifers had a faster passage of ruminal DM and apNDF and shorter retention time of these diet components in the rumen, mainly at late gestation. As a consequence of lower ruminal digestibility, the apparent total-tract digestibility of apNDF and DM was lower, and OM

tended to be lower in pregnant heifers since these components had no alteration in intestinal digestibility.

In contrast, it was observed that the CP apparent total-tract digestibility increased in pregnant heifers with the course of gestation. At the same time, pregnant heifers had greater efficiency of MCP synthesis. Therefore, assuming that MCP has high intestinal digestibility, around 80% (Mariz et al., 2018), the greater CP digestibility can be determined by the better quality of CP reaching the intestines.

4.5. Nitrogen balance

In grazing bovines, the excretion of N in urine is linearly related to N intake (Hoekstra et al., 2007). Despite the lower values of N intake in pregnant heifers at late gestation, the N excretion did not change. Nitrogen balance is correlated to energy balance in pregnant ruminants (Bauman and Currie, 1980). The conceptus has a high demand for glucose and amino acids at the end of pregnancy (Bell et al., 2005). Thus, although the lower N intake at late gestation, the catabolism of amino acids to support the demands of fetal energy possibly increased the excretion of nitrogen compounds (Rotta et al., 2015).

4.6. Efficiency of microbial crude protein synthesis

The efficiency of MCP synthesis is strongly correlated to DM intake (Broderick et al., 2010). Furthermore, MCP synthesis is increased by a longer digesta retention time in the rumen, or a reduced digesta kp (Huhtanen et al., 2016). For these reasons, it was expected that during late gestation, pregnant heifers would have a low efficiency in synthesizing MCP. This hypothesis, however, was not confirmed. Actually, the efficiency of MCP synthesis was greater in pregnant than in non-pregnant heifers

because even though the intake of pregnant heifers was lower, MCP synthesis was similar between PS. The MCP synthesis was estimated from the purine derivatives content in the urine (Chen and Gomes, 1992). In turn, urinary volume was estimated by the urinary creatinine concentration. Creatinine is formed from muscle metabolism and is excreted at a constant rate relative to muscle mass and, consequently, body weight (Costa e Silva et al., 2012). There is evidence that creatinine excretion changes depending on pregnancy status or pregnancy time (Hare et al., 2019; Whittet et al., 2019). However, more studies need to be carried out to corroborate such a hypothesis, especially in zebu cows raised in tropical conditions. Pregnant heifers underwent intense catabolism of skeletal muscle tissue at late pregnancy due to low protein intake and high requirements of gestation. Thus, this estimated microbial yield may not be representative of the real MCP synthesis since total collection of urine was not performed.

4.7. Ruminal fermentation parameters

Ruminal pH depends on saliva production, the balance of volatile fatty acids (VFA), the type and level of feed intake, and the exchange of bicarbonates and phosphates through the ruminal epithelium (Aschenbach et al., 2011). Whereas, rumen VFA concentration reflects the balance between production and clearance by passage with the fluid phase into the omasum or by absorption through the ruminal wall (Lopez et al., 2003). Although pregnant heifers have lower intake and digestibility and a higher DM passage rate, ruminal pH did not change and the VFA concentration in the rumen had only a neglectable change. Additionally, N-NH_3 is a very potent buffer once NH_3 can immediately bind H^+ to form NH_4^+ in the ruminal content. Ruminal N-NH_3 removal occurs through the use of ruminal bacteria, efflux to the omasum, or

absorption across the ruminal wall (Aschenbach et al., 2011). Similarly, to pH and VFA, $N\text{-}NH_3$ concentration was not different between PS.

5. Conclusions

As pregnancy progresses, ruminal capacity is reduced leading to lower feed intake and an increase of the digesta passage rate, impairing the ruminal digestibility of dietary dry matter and fiber. Thus, late-gestating beef heifers are less efficient at extracting energy from feed compared to non-pregnant animals, changing the feed predicted total digestible nutrients.

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Table 1

Ingredients and chemical composition of the corn silage, concentrate, and experimental diet.

Ingredient, g/kg of dry matter	Corn silage	Concentrate	Diet
Corn silage	100.0	-	959.5
Ground corn	-	239.5	9.7
Soybean meal	-	194.3	7.9
Mineral mixture[1]	-	377.5	15.3
Urea + ammonium sulfate[2]	-	188.7	7.6
Chemical composition, g/kg of dry matter			
Dry matter	303.7	938.5	329.5
Organic matter	953.3	605.6	939.2
Crude protein	63.5	647.3	87.2
apNDF	582.9	55.7	561.6
iNDF	209.7	8.5	201.5
Non-fibrous carbohydrates	277.6	233.1	275.8
Ether extract	29.2	13.3	28.6

apNDF = ash- and protein-free neutral detergent fiber, iNDF = indigestible neutral detergent fiber.

[1] Composition: calcium = 100 g/kg; phosphorus = 40 g/kg; sodium = 165 g/kg; sulfur = 6,000 mg/kg; magnesium = 5,000 mg/kg; copper = 680 mg/kg; zinc = 2,580 mg/kg; fluorine = 400 mg/kg; manganese = 750 mg/kg; iron = 350 mg/kg; selenium = 7 mg/kg; iodine = 45 mg/kg; cobalt = 35 mg/kg.

[2] Mixture of urea and ammonium sulfate (9:1 ratio).

Table 2

Effect of physiological status (PS) and days of pregnancy (DOP) on body weight (BW) and body condition score (BCS) of beef heifers.

Item	Physiological status		SEM	*P*-value		
	Non-pregnant	Pregnant		PS	DOP	PS×DOP
Initial BW, kg	466	460	54	0.93	-	-
Initial BCS	6.04	5.93	0.33	0.80	-	-
BW, kg	497	510	10	0.30	<0.01	0.22
BWnp, kg	497	477	10	0.16	<0.01	<0.01
SBW, kg	485	499	10	0.29	<0.01	0.23
SBWnp, kg	485	466	10	0.16	<0.01	<0.01
PREG [1], kg	-	33.0	3.7	-	<0.01	-
BCS	6.10	5.87	0.30	0.47	0.90	0.65

PS×DOP = interaction between physiological status and days of pregnancy; BWnp = maternal BW (subtracting gravid uterus weight and udder accretion); SBW = shrunk body weight; SBWnp = maternal SBW (subtracting gravid uterus weight and udder accretion); PREG = pregnancy component.

[1] Gravid uterus minus the non-pregnant uterus plus the accretion in udder related to pregnancy.

Table 3

Effect of physiological status (PS) and days of pregnancy (DOP) on intake and intake in relation to body weight (BW) of beef heifers.

Item	Physiological status		SEM	*P*-value		
	Non-pregnant	Pregnant		PS	DOP	PS×DOP
Intake, kg/d						
Dry matter	6.64	6.76	0.45	0.84	<0.01	<0.01
Organic matter	6.33	6.39	0.43	0.92	<0.01	<0.01
Crude protein	0.60	0.61	0.04	0.89	<0.01	0.01
apNDF	3.64	3.68	0.23	0.88	<0.01	0.04
iNDF	1.33	1.35	0.09	0.86	<0.01	0.08
NFC	2.01	2.03	0.14	0.91	<0.01	0.25
Ether extract	0.21	0.21	0.02	0.93	<0.01	0.14
TDN	3.93	3.94	0.22	0.99	0.01	0.06
Intake, g/kg of BW						
Dry matter	13.4	13.5	0.83	0.99	<0.01	<0.01
apNDF	7.29	7.30	0.47	0.99	<0.01	<0.01
iNDF	2.66	2.68	0.18	0.94	<0.01	0.02
TDN	7.94	7.80	0.43	0.80	<0.01	0.05

PS×DOP = interaction between physiological status and days of pregnancy; apNDF = ash- and protein-free neutral detergent fiber; iNDF = indigestible neutral detergent fiber; NFC = non-fibrous carbohydrates; TDN = total digestible nutrient.

Table 4

Effect of physiological status (PS) and days of pregnancy (DOP) on ruminal, intestinal, and apparent total-tract digestibility of beef heifers.

Item	Physiological status		SEM	*P*-value		
	Non-pregnant	Pregnant		PS	DOP	PS×DOP
Ruminal digestibility, g/kg of DM						
Dry matter	405	378	19	0.22	<0.01	0.19
Organic matter	483	464	19	0.09	<0.01	0.19
Crude protein	94.6	71.1	32.0	0.58	<0.01	0.51
apNDF	366	336	19	0.02	<0.01	0.26
NFC	789	802	12	0.37	0.83	0.78
Intestinal digestibility, g/kg of DM reaching the omasum						
Dry matter	271	272	36	0.98	0.77	0.98
Organic matter	244	239	33	0.90	0.58	0.95
Crude protein	519	541	25	0.52	0.18	0.38
apNDF	164	146	17	0.39	0.85	0.37
NFC	558	600	38	0.41	0.33	0.56
Apparent total-tract digestibility, g/kg of dry matter						
Dry matter	554	537	9	0.09	<0.01	0.56
Organic matter	598	576	13	0.11	<0.01	0.67
Crude protein	551	577	7	0.01	<0.01	0.04
apNDF	452	401	8	<0.01	<0.01	0.42
NFC	898	913	12	0.19	0.89	0.35
TDN	600	581	15	0.24	<0.01	0.72

PS×DOP = interaction between physiological status and days of pregnancy; apNDF = ash- and protein-free neutral detergent fiber; NFC = non-fibrous carbohydrates; TDN = total digestible nutrient.

Table 5

Effect of physiological status (PS) and days of pregnancy (DOP) on ruminal pool, outflow, rates of ingestion, passage, and digestion, and retention time of beef heifers

Item	Physiological status		SEM	*P*-value		
	Non-pregnant	Pregnant		PS	DOP	PS×DOP
Ruminal pool, kg of wet matter	34.4	31.8	1.5	0.20	<0.01	0.01
Ruminal dry matter						
Pool, kg	5.25	5.09	0.27	0.66	0.30	<0.01
Outflow, kg/day	3.95	4.48	0.19	0.06	<0.01	0.10
ki, %/h	5.43	6.33	0.17	<0.01	0.21	0.05
kp, %/h	3.20	3.74	0.15	<0.01	<0.01	0.01
kd, %/h	2.25	2.60	0.11	0.05	<0.01	0.34
Retention time, h	31.8	27.4	1.2	<0.01	<0.01	0.23
Ruminal apNDF						
Pool, kg	3.79	3.81	0.21	0.92	<0.01	0.02
Outflow, kg/day	2.32	2.52	0.11	0.21	0.03	<0.01
ki, %/h	3.95	4.45	0.14	0.01	<0.01	0.09
kp, %/h	2.57	2.94	0.15	<0.01	<0.01	0.22
kd, %/h	1.41	1.54	0.07	0.25	0.07	0.04
Retention time, h	40.0	35.6	2.1	<0.01	<0.01	0.74

PS×DOP = interaction between physiological status and days of pregnancy; apNDF = ash- and protein-free neutral detergent fiber; ki = ingestion rate; kp = passage rate; kd = digestion rate.

Table 6

Effect of physiological status (PS) and days of pregnancy (DOP) on nitrogen (N) balance and microbial crude protein synthesis of beef heifers.

Item	Physiological status		SEM	*P*-value		
	Non-pregnant	Pregnant		PS	DOP	PS×DOP
Nitrogen intake, g/day	95.5	96.6	5.4	0.88	<0.01	0.10
Fecal nitrogen						
g/day	45.8	44.9	2.6	0.80	0.27	0.21
% of N intake	47.8	46.6	0.8	0.25	0.18	0.57
Urinary nitrogen						
g/day	42.5	41.6	2.1	0.73	0.01	0.38
% of nitrogen intake	46.4	45.6	3.1	0.83	0.35	0.17
Total nitrogen excretion						
g/day	88.5	86.4	4.1	0.70	0.01	0.60
% of nitrogen intake	95.3	92.2	2.8	0.43	0.68	0.21
Nitrogen balance						
g/day	7.47	8.82	2.36	0.66	0.60	0.46
% of N intake	6.82	7.36	2.95	0.89	0.61	0.34
Ruminal outflow nitrogen						
g/day	88.3	88.9	7.5	0.90	<0.01	0.03
% of nitrogen intake	93.5	91.5	3.0	0.61	<0.01	0.55
Ruminal nitrogen balance						
g/day	6.55	9.09	2.70	0.49	<0.01	0.55
% of nitrogen intake	6.49	8.50	2.95	0.61	<0.01	0.55
Efficiency of microbial crude protein synthesis						
g/day	459	461	27	0.95	0.04	0.96
g/kg crude protein intake	797	801	23	0.90	<0.01	0.02
g/kg DOM intake	128	133	5	0.44	<0.01	0.09
g/kg TDN intake	122	125	4	0.65	<0.01	0.09

PS×DOP = interaction between physiological status and days of pregnancy; DOM = digestible organic matter; TDN = total digestible nutrient.

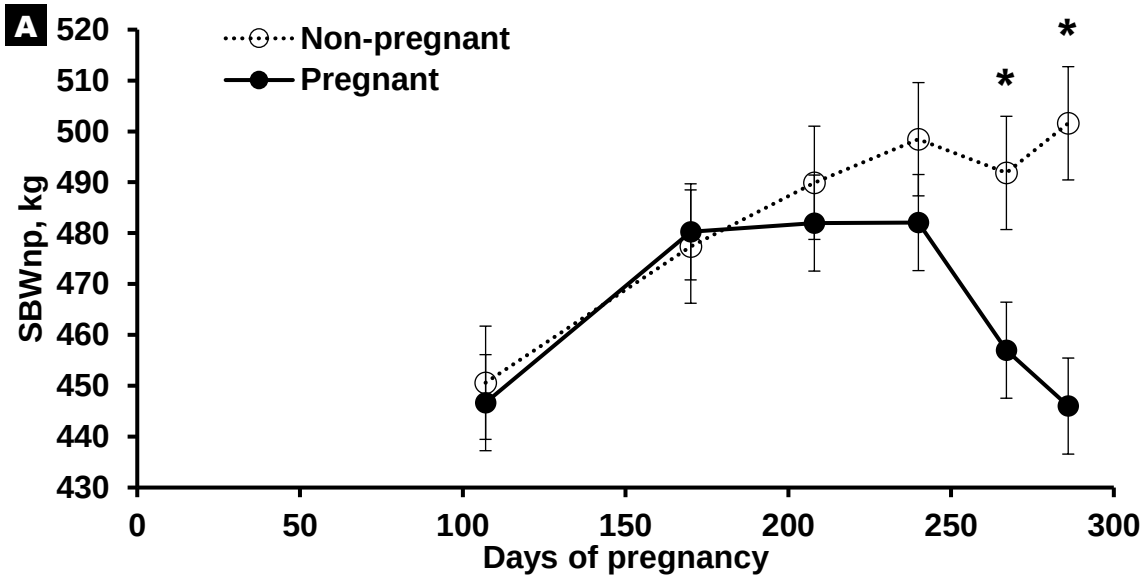
Table 7

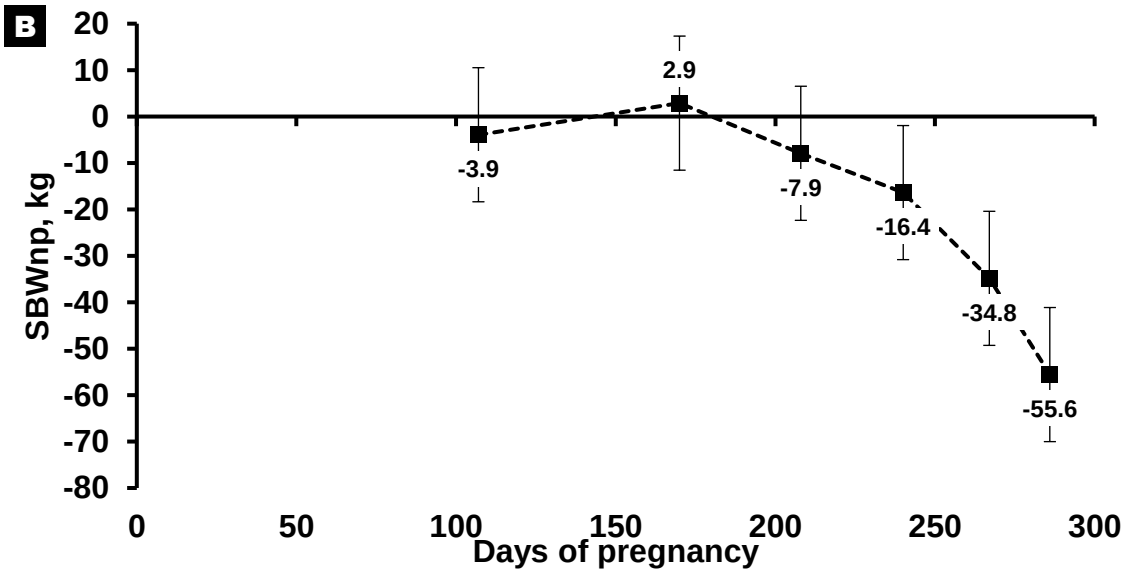
Effect of physiological status (PS) and days of pregnancy (DOP) on ruminal fermentation parameters of beef heifers.

Item	Physiological status		SEM	*P*-value		
	Non-pregnant	Pregnant		PS	DOP	PS×DOP
Before morning feeding						
pH	7.01	6.99	0.10	0.79	0.08	0.25
N-NH_3, mg/dL	14.9	15.4	1.0	0.71	0.69	0.34
VFA concentration, mmol/L						
Total VFA	61.4	56.6	11.8	0.66	0.45	0.75
Acetic acid	44.0	41.1	8.5	0.71	0.34	0.72
Propionic acid	14.7	13.2	2.6	0.56	0.97	0.83
Butyric acid	2.21	2.50	0.54	0.55	<0.01	0.97
VFA concentration, % of total of VFA						
Acetic acid	71.5	71.5	1.0	0.96	<0.01	0.19
Propionic acid	24.6	24.0	0.8	0.60	<0.01	0.33
Butyric acid	3.67	4.33	0.34	0.17	<0.01	0.22
A:P ratio	3.03	3.09	0.13	0.77	<0.01	0.16
Four hours after morning feeding						
pH	6.77	6.74	0.13	0.81	0.02	0.78
N-NH_3, mg/dL	22.9	23.3	1.3	0.76	0.19	0.16
VFA concentration, mmol/L						
Total VFA	60.3	67.7	3.3	0.12	<0.01	0.53
Acetic acid	42.1	46.4	2.4	0.20	<0.01	0.51
Propionic acid	15.3	17.2	0.7	0.06	<0.01	0.14
Butyric acid	2.88	3.06	0.19	0.46	0.14	0.70
VFA concentration, % of total VFA						
Acetic acid	70.0	68.9	1.3	0.52	0.32	0.60
Propionic acid	25.2	25.5	0.4	0.51	0.24	0.16
Butyric acid	4.79	4.51	0.23	0.38	0.80	0.97
A:P ratio	2.76	2.72	0.07	0.60	0.27	0.35

PS×DOP = interaction between physiological status and days of pregnancy; N-NH_3 = ammoniacal nitrogen; VFA = volatile fatty acids; A:P = acetic and propionic acids ratio

Figures


A
Non-pregnant
Pregnant
SBWnp, kg
520
510
500
490
480
470
460
450
440
430
*
*
0
50
100
150
200
250
300
Days of pregnancy


B
SBWnp, kg
20
10
0
-10
-20
-30
-40
-50
-60
-70
-80
2.9
-3.9
-7.9
-16.4
-34.8
-55.6
0
50
100
150
200
250
300
Days of pregnancy

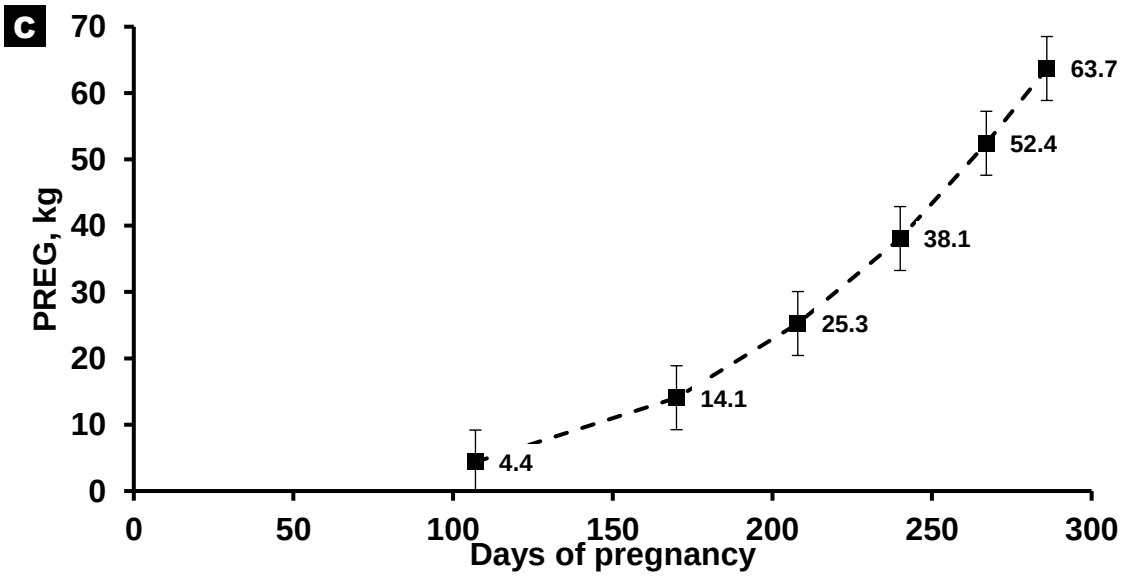

C
PREG, kg
70
60
50
40
30
20
10
0
63.7
52.4
38.1
25.3
14.1
4.4
0
50
100
150
200
250
300
Days of pregnancy

Fig. 1 Effect of physiological status and days of pregnancy on maternal shrunk body weight of beef heifers. (**A**) Maternal shrunk body weight (SBWnp; kg) of pregnant and non-pregnant beef heifers over days. (**B**) Estimated differences in SBWnp between pregnant and non-pregnant (kg) over days. (**C**) Estimated pregnancy component (kg) of pregnant heifers over days, according to Gionbelli et al. (Gionbelli et al., 2015). * $P \leq 0.10$.

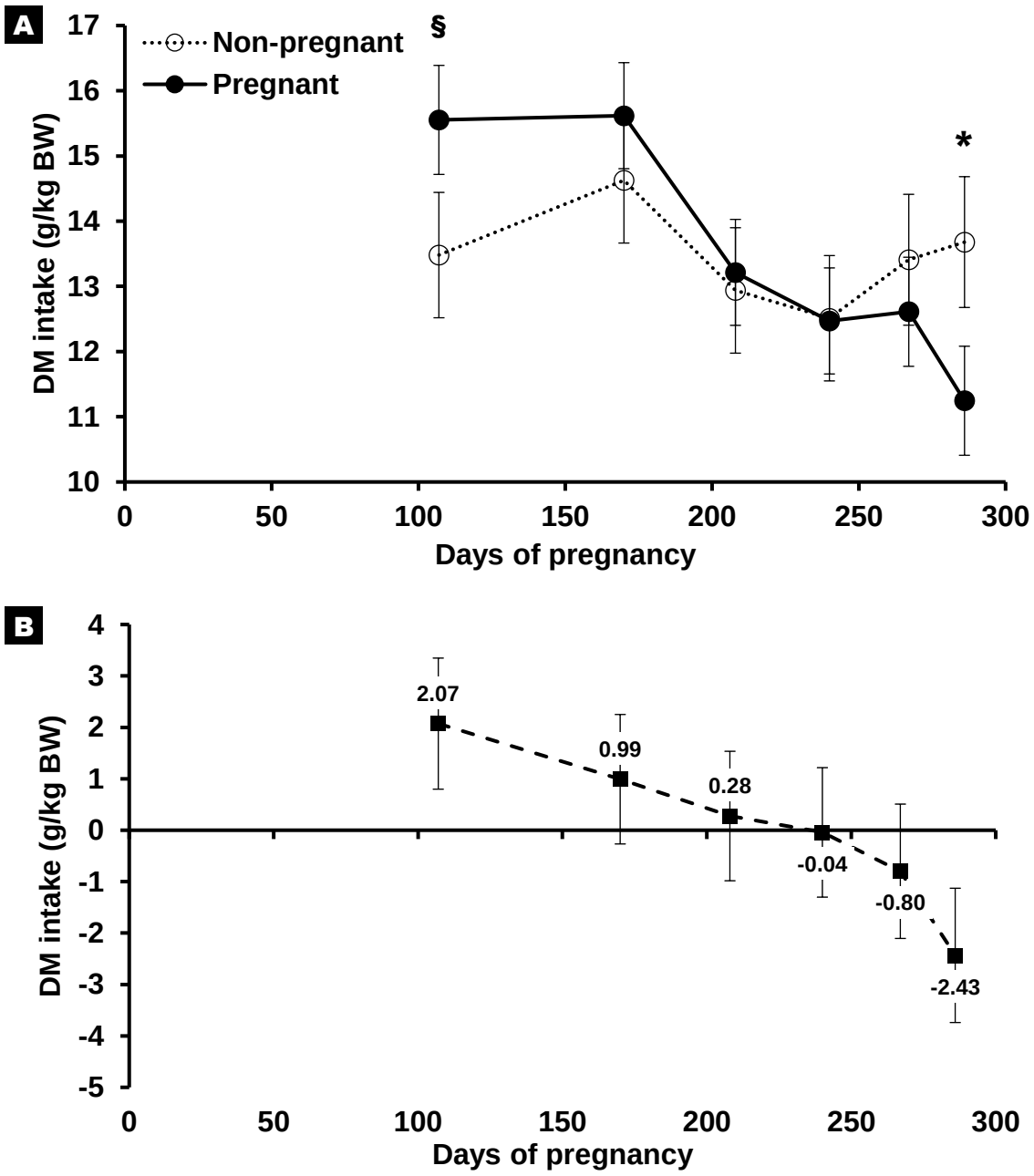


Fig. 2 Effect of physiological status and days of pregnancy on dry matter intake of beef heifers. (**A**) Dry matter (DM) intake of pregnant and non-pregnant beef heifers (g/kg of BW) over days. (**B**) Estimated differences in DM intake (g/kg of BW) between pregnant and non-pregnant beef heifers over days. § $P < 0.15$; * $P \leq 0.10$.

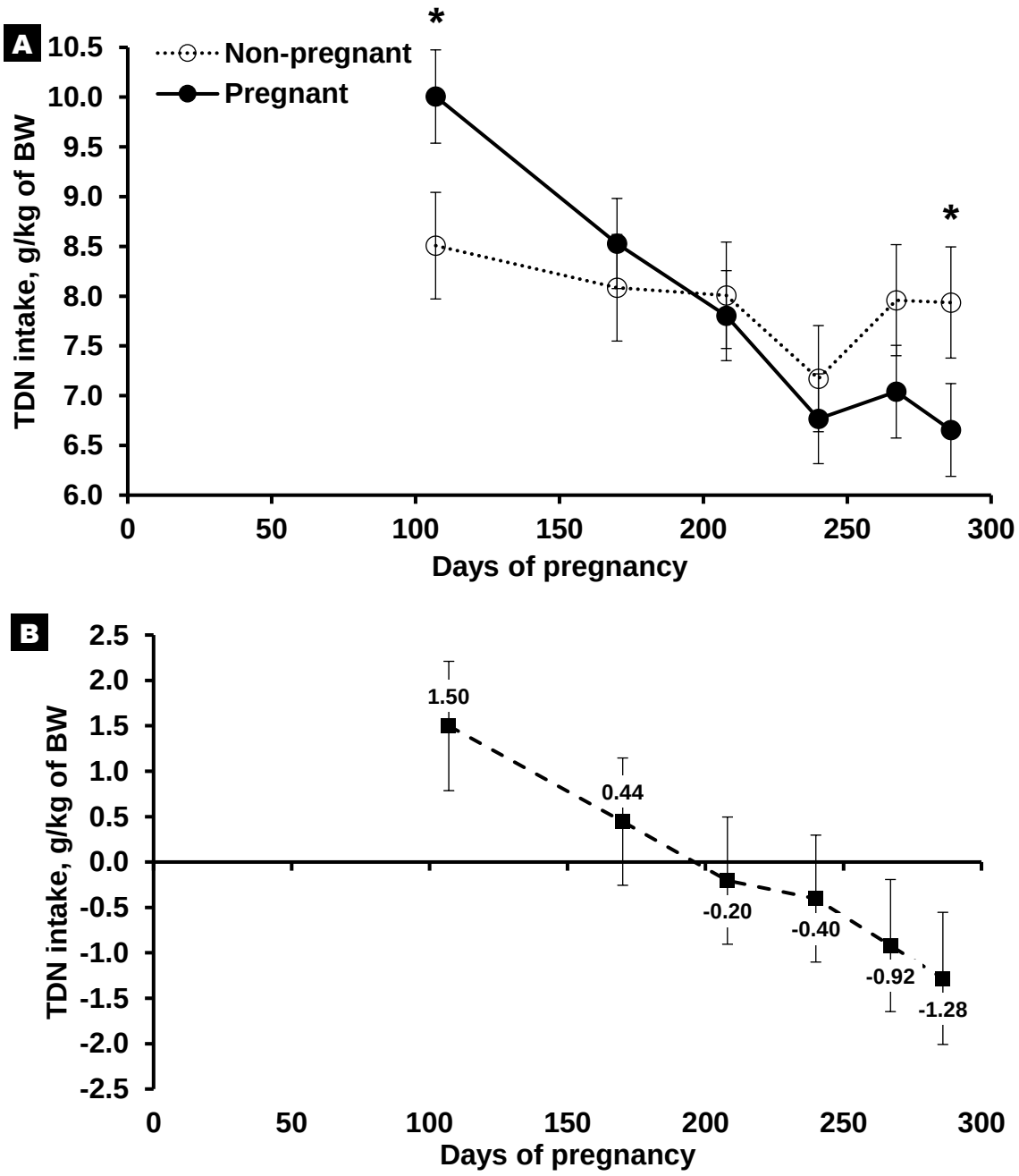


Fig. 3 Effect of physiological status and days of pregnancy on total digestible nutrients intake of beef heifers. (**A**) Total digestible nutrients (TDN) intake of pregnant and non-pregnant beef heifers (g/kg of BW) over days. (**B**) Estimated differences in TDN intake (g/kg of BW) between pregnant and non-pregnant beef heifers over days. * $P \leq 0.10$.

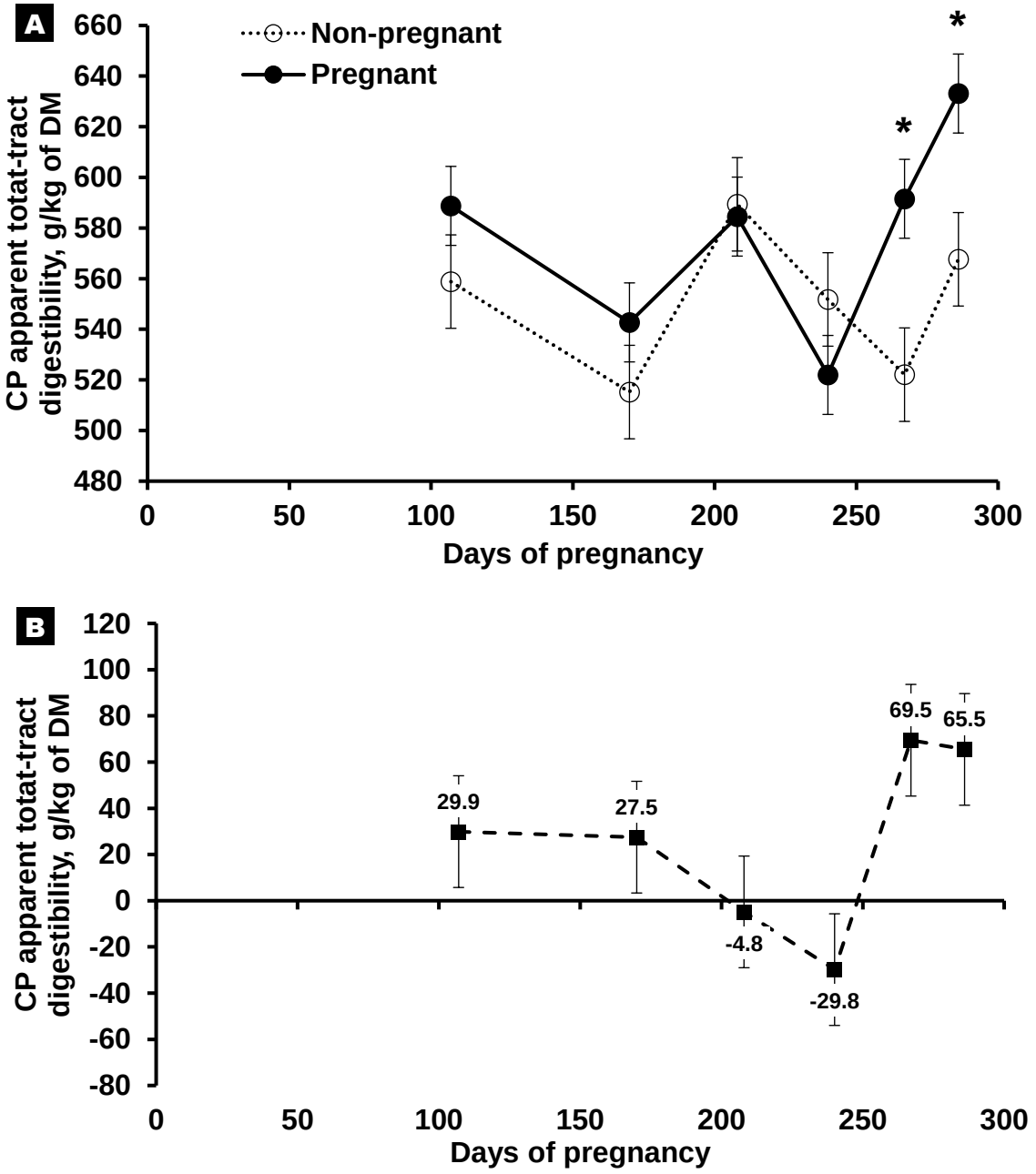


Fig. 4 Effect of physiological status and days of pregnancy on crude protein apparent total-tract digestibility of beef heifers. (**A**) Crude protein apparent total-tract digestibility of pregnant and non-pregnant beef heifers (g/kg of DM) over days. (**B**) Estimated differences in crude protein apparent total-tract digestibility (g/kg of DM) between pregnant and non-pregnant beef heifers over days. * $P \leq 0.10$.

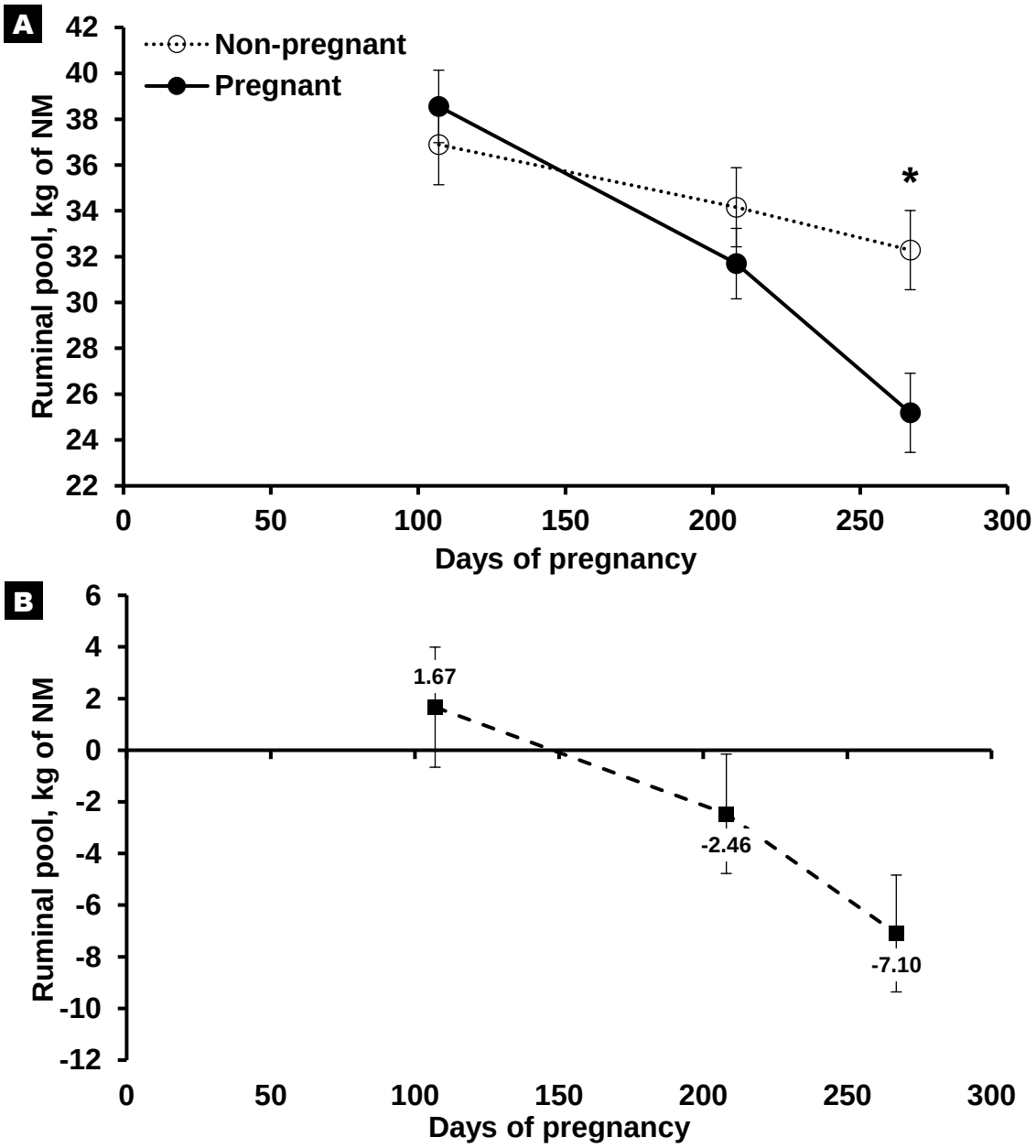


Fig. 5 Effect of physiological status and days of pregnancy on the ruminal pool of wet matter of beef heifers. (**A**) Ruminal pool of wet matter (NM) of pregnant and non-pregnant beef heifers (kg of NM) over days. (**B**) Estimated differences in ruminal pool of wet matter (kg) between pregnant and non-pregnant beef heifers over days. * $P \leq 0.10$.

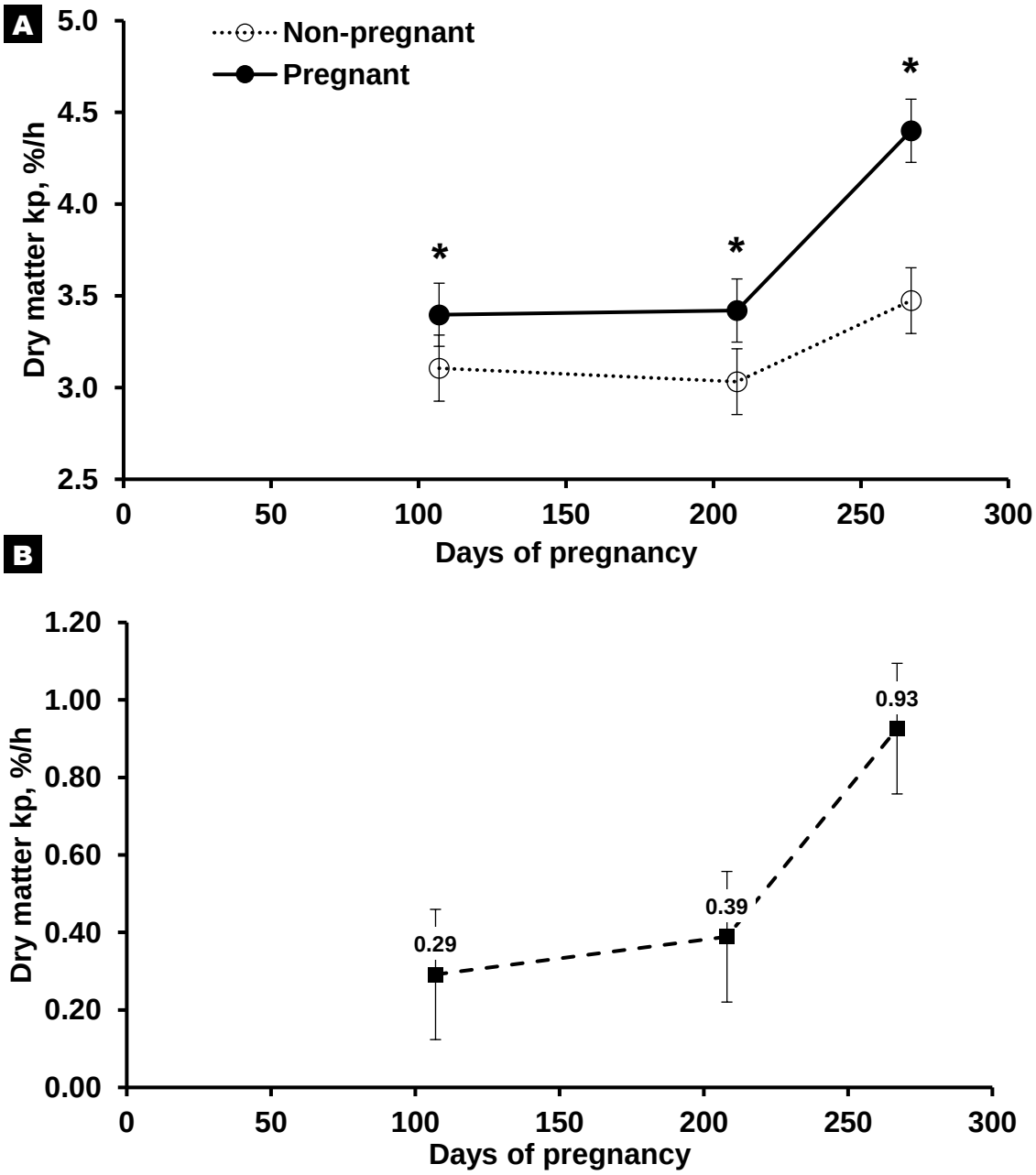


Fig. 6 Effect of physiological status and days of pregnancy on dry matter passage rate of beef heifers. (**A**) Dry matter (DM) passage rate (kp) of pregnant and non-pregnant beef heifers (%/h) over days. (**B**) Estimated differences in DM kp (%/h) between pregnant and non-pregnant beef heifers over days. * $P \leq 0.10$.

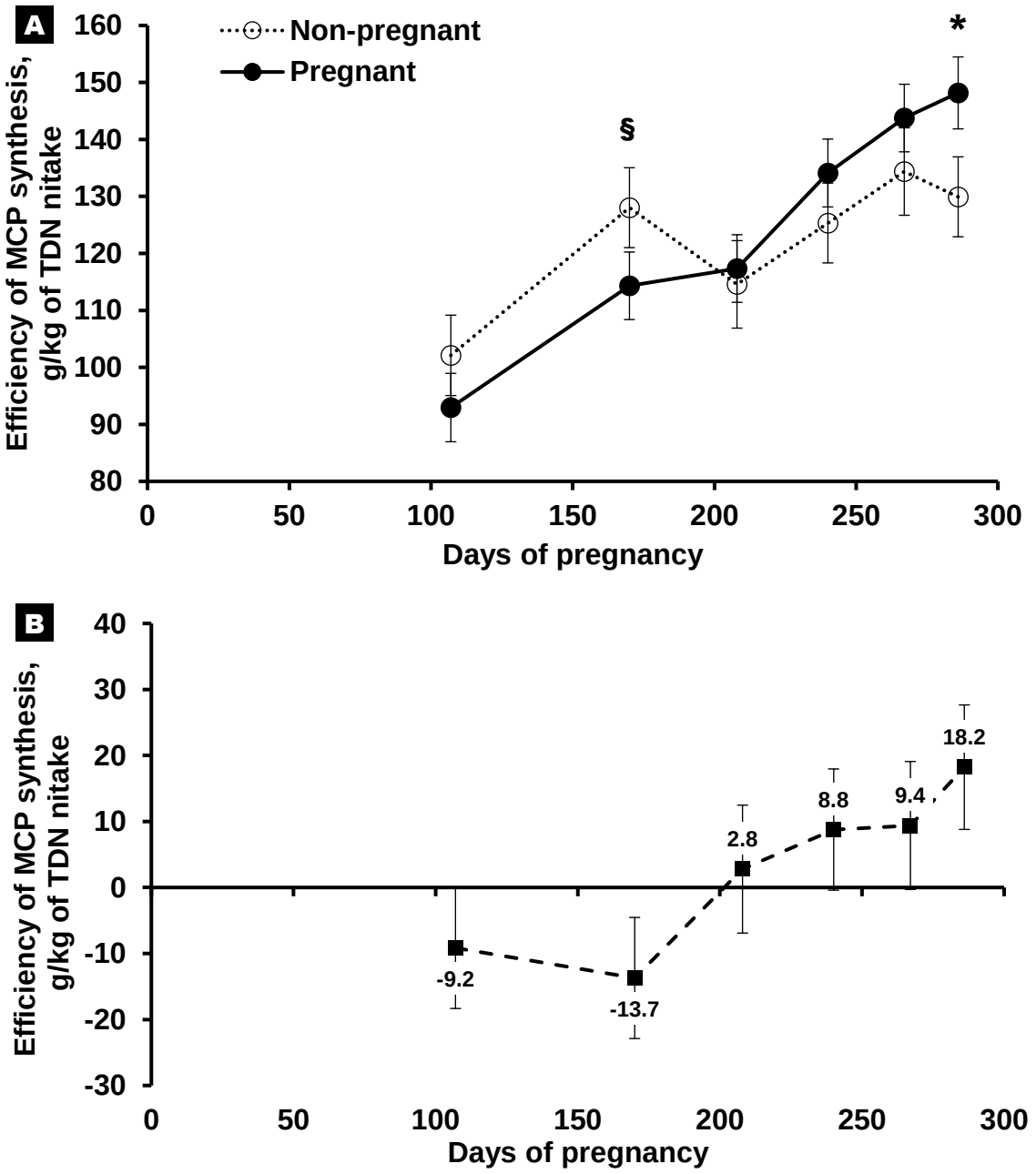


Fig. 7 Effect of physiological status and days of pregnancy on the efficiency of microbial crude protein synthesis of beef heifers. (**A**) Efficiency of microbial crude protein (MCP) synthesis of pregnant and non-pregnant beef heifers (g/kg of TDN intake) over days. (**B**) Estimated differences in efficiency of MCP synthesis (g/kg of TDN intake) between pregnant and non-pregnant beef heifers over days. § $P < 0.15$; * $P \leq 0.10$.

ARTICLE 2 - The course of pregnancy changes general metabolism and affects ruminal epithelium activity pattern in zebu beef heifers

Article formatted according to Livestock Science guidelines

The course of pregnancy changes general metabolism and affects ruminal epithelium activity pattern in zebu beef heifers

Abstract

The present study aimed to investigate metabolic and physiological changes induced by pregnancy, as well as the expression of key genes linked to the absorptive and proliferative activity of the ruminal epithelium towards the advance of the gestational period in zebu beef heifers. Twelve ruminally cannulated Zebu beef heifers were randomly assigned into two experimental treatments: a pregnant group (n = 7) and a control group (non-pregnant; n = 5). All heifers received the same diet throughout the experiment. Respiratory and heart rates and plasma glucose levels were assessed just before and four hours after morning feeding at 110, 171, 206, 242, 266, and 286 days of pregnancy (DOP). Blood samples were collected for serum non-esterified fatty acids (NEFA), beta-hydroxybutyrate (BHB), and urea analysis prior to the morning feedings at 110, 206, 266, and 286 DOP. At 215 and 272 DOP the ruminal epithelium was biopsied to evaluate the mRNA expression of key genes involved in remodeling, inflammation, and transport. The respiratory rate was similar ($P \geq 0.79$) between groups over days. However, the heart rate increased in late-pregnant heifers compared to controls. Blood concentrations of NEFA ($P = 0.11$) and BHB ($P = 0.67$) did not vary over the gestation period in pregnant heifers. Glucose levels before morning feeding

were similar during all collection periods with an exception at 286 days (DOP × physiological status interaction; P = 0.05) when glucose was lower in pregnant (83 mg/dL) compared to non-pregnant (107 mg/dL) heifers. The mRNA expression of genes related to cellular remodeling (*PCNA* and *CASP3*), inflammation (*KLK9* and *KLK10*), and transport of volatile fatty acids (*SLC16A1* and *SLC16A3*), H^+ (*SLC9A1*), HCO_3^- (*SLC26A3* and *SLC26A6*), and glucose (*SLC5A1*) in the ruminal epithelium were downregulated at late gestation. These results suggest that the ruminal epithelium saves energy at late pregnancy to benefit fetal development. In addition, the increase in heart rate coupled with tissue mobilization can be considered homeorhetic mechanisms that help meet fetal nutrient requirements.

Keywords: gene expression, heart rate, homeorhesis, rumen epithelium, Zebu cattle

1. Introduction

Regulation of nutrient partitioning during pregnancy involves homeorhetic controls arising from the conceptus (Bauman and Currie, 1980). It is suggested that late-gestation pregnant cows may be able to reduce maintenance energy costs to support the energetic demands of the conceptus (Freetly et al., 2008). Even though gestation is a physiologic state (PS) distinguished by increased nutritional requirements, a decrease in dry matter (DM) intake is usually observed as gestation advances (Gionbelli et al., 2016).

Furthermore, as pregnancy advances and the concept grow, the enlarged uterus compresses maternal organs such as the rumen (Forbes, 1968). The reduction in ruminal capacity can induce a decrease in feed intake (Hanks et al., 1993; Stanley et al., 1993; Scheaffer et al., 2001). This physical limitation can often be compensated

by an increase in the feed passage rate, hence allowing maintenance of the intake level. Still, the faster feed passes through the digestive tract; the smaller tends to be its digestibility (Ribeiro et al., 2015). As passage rate and digestibility change, other parameters of ruminal fermentation may also be modified (Hare et al., 2019). Production and absorption of volatile fatty acids (VFA), nitrogen compounds usage, microbial protein synthesis, and balance of ruminal pH are all subject to change as gestation progresses.

The ruminal epithelium has an essential role in the absorption VFA as well as the regulation of luminal pH (Aschenbach et al., 2011). In dairy cattle, ruminal epithelium transcriptome in the transition period has been a target in several studies (Minuti et al., 2015; Steele et al., 2015; Bach et al., 2018). However, both the type and amount of feed consumed by lactating and dry cows differ drastically in dairy cows, while in beef cattle, the changes are slighter. Thus, information about the pattern of activity of the ruminal epithelium during the gestation of beef cattle may lead to an increased understanding of cellular mechanisms involved in energetic partitioning.

Therefore, the present study aimed to investigate possible metabolic and physiological changes induced by pregnancy, as well as the mRNA expression of key genes involved in the absorptive and proliferative activity of the ruminal epithelium towards the advance of the gestational period in zebu beef heifers. It was hypothesized that gestation advancement promotes homeorhetic changes to reduce maternal maintenance energy expenditure.

2. Material and Methods

This study was conducted at the Department of Animal Sciences facilities of the Federal University of Lavras (UFLA), Lavras, Minas Gerais, Brazil. All the procedures

involving the use of animals in this study followed the guidelines established by the UFLA Ethics Committee on Animal Use (protocol number: 048/16).

2.1. Animals, housing, and feeding

Animal handling procedures have been previously reported (Moreira et al., 2020). Briefly, 12 ruminally cannulated Zebu heifers (BW = 417 ± 95.6 kg) were randomly assigned into two groups, a pregnant group (n = 7) and a control group (non-pregnant; n = 5). Heifers assigned to the pregnant group were submitted to an ovulation synchronization protocol and the day zero of gestation was considered the day of artificial insemination. On day 85 of gestation of the pregnant group, all heifers were housed in individual pens (80 m^2). After penning, heifers started the experimental adaptation phase. The feed intake control started on the 95^{th} day of gestation and ended at calving.

The experimental diet [DM = 32.95%; crude protein (CP) = 8.72%, and neutral detergent fiber (NDF) = 56.16%] was formulated according to the Nutrient Requirements of Zebu and Crossbred Cattle - BR-CORTE (Valadares Filho et al., 2016), to allow *ad libitum* intake without large accumulation of body reserves and adequate maintenance of gestation. The experimental diet (DM basis) was based on 95.95% of medium quality corn silage (DM = 30.37%; CP = 6.35%, and NDF = 58.29%), and 4.05% of concentrate supplement (DM = 93.85%; CP = 64.73%, and NDF = 5.57%) prepared from ground corn (0.97%), soybean meal (0.79%), urea plus ammonium sulfate (0.76%), and macro and micro mineral mixture (1.53%). All heifers received the same diet throughout the experiment. Animals were fed twice a day at 0800 and 1400 h.

2.2. Data collection and analysis

A timeline of sampling days was followed during the experiment (Fig. 1).

2.2.1. Respiratory and heart rates evaluation

Respiratory and heart rates were assessed just before and four hours after morning feedings at 110, 171, 206, 242, 266, and 286 days of pregnancy (DOP). Respiratory rates (RR) were monitored for 1 min by counting flank movements (Milan et al., 2016). Heart rates (HR) were determined by counting heartbeats with a stethoscope placed on the left chest wall over the cardiac area for 1 min (Ghizzi et al., 2018).

2.2.2. Blood metabolite levels

Blood samples were collected via coccygeal venipuncture in 4 mL sodium fluoride tubes (code: 50205, Labor Import, Osasco, Brazil) for plasma glucose analysis. For non-esterified fatty acid (NEFA), beta-hydroxybutyrate (BHB), and urea analysis a 10 mL spray-coated silica tube (code: 50208, Labor Import, Osasco, Brazil) was used. For NEFA, BHB, and urea analysis, blood samples were obtained just before morning feeding at 110, 206, 266, and 286 DOP. While for glucose analysis, samples were collected at 110, 171, 206, 242, 266, and 286 DOP just before and four hours after morning feedings. Blood was immediately centrifuged at 2700 × g for 20 min at 4°C. Serum/plasma was removed by pipette and frozen at −20°C until further analyses.

Plasma glucose concentrations were measured according to an enzymatic system by a commercial kit (GLICOSE Liquiform, Ref.: 133, Labtest Diagnóstica S.A., Lagoa Santa, Brazil). The NEFA concentrations were measured by Enzyme-Linked Immunosorbent Assay (ELISA) according to the recommendations of a commercial kit

(Bovine Non-esterified Fatty Acid ELISA Kit, Bioassay Technology Laboratory, Shanghai, China). Serum BHB levels were assayed using a kinetic enzymatic method (Ranbut kit no. RB 1007, Randox Laboratory, Antrim, UK). Serum urea was determined using an enzymatic system by a commercial kit (Ureia 500, Doles Reagentes e Equipamentos para Laboratórios Ltda, Goiânia, Brazil).

2.2.3. Gene expression in ruminal papillae

At 215 and 272 DOP, total rumen evacuation was performed one hour before morning feeding for ruminal papillae collection. The rumen papillae were collected at the most ventral site of the ventral rumen sac using curved surgical scissors. Papillae were immediately washed with a 0.9% NaCl solution, stored in 2 mL cryotubes, and snap-frozen in liquid nitrogen. Papillae samples were stored at −80°C until RNA extraction and mRNA expression analysis.

The design of primers for target and housekeeping genes was performed using sequences that are registered and published in the GenBank public data bank, a National Center for Biotechnology Information (NCBI) platform (Table 1). Primers were designed using OligoPerfect Designer software (Invitrogen, Karlsruhe, Germany) and synthesized (Invitrogen, Carlsbad, CA, USA). Total RNA was extracted from papillae samples using QIAzol (QIAGEN, Valencia, CA, USA) and treated with DNA-free DNase (Ambion, Austin, TX, USA) according to the manufacturer's instructions. To analyze the 28S and 18S rRNA bands, the total RNA was electrophoresed in a 1.0% (m/v) agarose gel, stained with GelRed nucleic acid gel stain (Biotium, Hayward, CA, USA) and visualized with an E-gel Imager Camera Hood (Life Technologies, Neve Yamin, Israel). The RNA quantity (ng/μL) and purity (260/280 and 260/230) were assessed using a spectrophotometer (DeNovix DS-11 Spectrophotometer, USA) at

260 nm. Complementary DNA (cDNA) synthesis was performed using the HighCapacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions, and samples were stored at −20 °C.

Reverse transcription qPCR (RT-qPCR) was performed on an Eppendorf Realplex system (Eppendorf, Hamburg, Germany) with a SYBR Green detection system (Applied Biosystems, Foster City, CA, USA). The RT-qPCR reactions were performed as the following protocol: 50°C for 2 min, followed by 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The RT-qPCR analyses of each studied gene were performed using cDNA from biological replicates, with two technical replicates per biological replicate. The β-actin (*ACTB*; NM_173979) and prefoldin-like chaperone (*UXT*; NM_001037471) were used as the housekeeping genes (Die et al., 2017). Relative expression levels were calculated according to the method described by Pfaffl (2001).

2.3. Statistical Analyses

Data were analyzed through the mixed models methodology (procedure MIXED of SAS 9.2, SAS Inst. Inc., Cary, NC), considering PS (pregnant or non-pregnant) and DOP as fixed effects and animal as the random effect. When appropriate, initial BW was included as a covariate in the model. Once repeated measurements were taken within the same animal (for DOP), the subject animal nested treatment was included as the error term in the repeated measurement statement. For every DOP, the PS effect on the measured variable was estimated using the “estimate statement” of SAS. Prior to the final analyses, extreme data were removed when Studentized residuals were not within ± 3 standard deviations, and normality (P-value > 0.10) was assessed

using Shapiro-Wilk's test. As expected, the gene expression data was not normal and it was transformed using the RANK procedure of SAS 9.2. The value of 0.10 was adopted as a critical level of probability for the occurrence of Type I error.

3. Results and Discussion

3.1. Respiratory and heart rates

Regardless of evaluation time, pregnant and non-pregnant heifers had similar RR through gestation (Table 2). However, a DOP effect ($P = 0.01$) was detected four hours after feeding. Respiratory rate was reduced over time in pregnant and non-pregnant heifers. Variations in RR are mainly linked to environmental fluctuations (Hansen, 2004). However, some studies have shown that zebu animals maintain a stable respiratory rate regardless of environmental temperatures and reinforce their adaptive potential (Melo Costa et al., 2018; Lima et al., 2020). Additionally, although previous data have shown that visceral organ mass can be influenced by both pregnancy and nutrient intake in pregnant cows (Meyer et al., 2010; Wood et al., 2013; Rotta et al., 2015), no differences were found in the lungs or diaphragm weights.

Before morning feedings, a DOP × PS interaction effect ($P = 0.08$) was observed on HR (Fig. 2). Heart rate was similar until 206 DOP but was greater at late gestation (242, 266, and 286 DOP) in pregnant than in non-pregnant heifers. Similarly, four hours after morning feedings, HR was greater ($P < 0.01$) in pregnant than in non-pregnant heifers. Also, HR increased ($P < 0.01$) through gestation in pregnant, but not in non-pregnant heifers (Fig. 2). This increase can be justified by the high demand for nutrients and oxygen in maternal tissues and, mainly, uteroplacental (Reynolds et al., 1986). Likewise, HR of pregnant heifers gradually increased over time [83 ± 3 at 14 weeks and 97 ± 4 beats/min at 1 week before calving (Trenk et al., 2015)]. According to Brosh

et al. (2002), HR allows estimating energy expenditure in cattle, and the increase toward the end of gestation reflects increasing energy demands of the growing fetus.

3.2. Metabolic profile

In late gestation, conceptus glucose requirements increase by approximately 50% (Bell, 1995). Despite the importance of glucose for fetal metabolism across mammalian species, ruminants exhibit difficulty in increasing its circulatory levels, thus making the use of amino acids as precursors for gluconeogenesis as the most straightforward path to meet the high uterine glucose demand (McNeill et al., 1997). For these reasons, the mobilization of body tissues is compulsory, and consequently, the metabolic profile during pregnancy is altered (Linden et al., 2014; Lopes et al., 2020). Weight and body condition results are presented in detail elsewhere (Moreira et al., 2020). Briefly, despite of no change in body condition score, maternal body weight and shrunk body weight (subtracting gravid uterus weight and udder accretion) were lower in pregnant compared with non-pregnant heifers at late gestation, indicating tissue mobilization at this stage.

Before morning feeding, glucose levels were similar during all collection periods with an exception at 286 DOP (DOP × PS interaction; $P = 0.05$) when glucose was lower in pregnant than non-pregnant heifers (Table 3 and Fig. 3). Four hours after feeding, no differences were found between PS and DOP. A tendency ($P = 0.11$) for a PS × DOP interaction effect was detected on NEFA levels. At 286 DOP, NEFA concentrations were lower in non-pregnant heifers, whilst similar throughout gestation in pregnant heifers. Serum BHB concentration was not affected by PS or DOP. Urea concentration (DOP × PS interaction; $P = 0.03$) tended to be lower at 206 DOP (24.3

vs. 21.0 mg/dL) and were greater at 266 DOP (20.4 *vs.*15.7 mg/dL) in pregnant compared to non-pregnant heifers, respectively.

When increased feed intake is not possible, changes in the production and utilization of glucose by tissues can be observed (Wood et al., 2013). In the present study, feed intake decreased as gestation length increased (Moreira et al., 2020). Therefore, high fetal demand for glucose, coupled with low nutrient intake, could be the causes of the lower circulating glucose concentration observed at 286 DOP.

Non-esterified fatty acid and BHB are typical markers of fat tissue mobilization (Wood et al., 2013). However, since NEFA and BHB concentration, as well as the body condition score, did not change during pregnancy, it is suggested that the tissue mobilization observed in this study was a consequence of muscle tissue catabolism. The mechanisms regulating protein mobilization in late gestation of cows are still not clear. Recently, Lopes et al. (2020) found that cows not supplemented during pregnancy tended to have greater total circulating amino acids and concentrations of circulating glycogenic amino acids than supplemented cows. The authors suggested that non-supplemented cows alter their metabolism to meet increasing fetal nutrient demands, increasing amino acid mobilization from skeletal muscle tissue for gluconeogenesis.

3.3. Gene expression in ruminal papillae

Pregnant heifers had lower proliferating cell nuclear antigen (*PCNA*) mRNA expression than non-pregnant. The protein encoded by this gene is essential for DNA replication and can be used as an indicator of cell proliferation. Similarly, cell proliferation in the jejunum decreased due to pregnancy in cows (Scheaffer et al., 2003), suggesting that the visceral tissue use less energy during pregnancy. On the

other hand, the abundance of proteins encoded by *PCNA* was not affected by pregnancy in the rumen papillae (Wood et al., 2013). With a reduced remodeling of the ruminal epithelium, it is expected that cell apoptosis will be also reduced (Bach et al., 2019). In fact, in the present study, a downregulation in the expression of caspase 3 (*CASP3*) was verified in pregnant heifers at 272 DOP. The CASP3 encodes a protease that plays a central role in cell apoptosis. Consequently, the decreased cell proliferation (based on *PCNA*) and apoptosis (based on *CASP3*) could be indicative of reduced rumen epithelium activity at late gestation.

Recent studies have shown that the kallikrein-related peptidases (*KLK*) genes are present in the bovine ruminal epithelium and they are related to feed intake, weight gain, feed efficiency, and VFA ruminal concentration (Baldwin et al., 2012; Veerkamp et al., 2012; Kern et al., 2016a; Kern et al., 2016b). The KLK are serine proteases that are involved in epidermal processes such as tumor development, regulation of inflammation, desquamation of skin cells, and wound healing (Kantyka et al., 2011). It is known that the energetic cost of an immune response is high for the animal (Kvidera et al., 2017; Reynolds et al., 2017). Thus, a reduction in immune and inflammatory responses may allow more energy for maternal and fetal growth. In the study of Kern et al. (2016a), the kallikrein-related peptidase 10 (*KLK10)* was found to be upregulated in steers with low feed efficiency compared to more efficient animals. In the current study, pregnant heifers had lower expression of kallikrein-related peptidase 9 (*KLK9)* and *KLK10* at 272 DOP compared to non-pregnant heifers. These results strengthen the concept that the ruminal epithelium enters into an energy-saving state at late gestation, steadily providing energy for the maintenance of pregnancy.

Ruminal epithelial proliferation has been positively associated with the increased surface area for nutrient absorption (Baldwin, 1999). Additionally, an

increase in ruminal epithelium inflammation could reduce the absorption of nutrients due to papillae swelling (Kern et al., 2016b). Therefore, the current results suggest that pregnant heifers have less surface area for absorption. However, reduced papillae swelling can compensate for this diminished absorptive capacity of the ruminal epithelium. It is noteworthy that these considerations are based only on gene expression since papillae morphology was not assessed.

The mRNA expressions of monocarboxylic acid transporter 1 (*SLC16A1*/MCT1), sodium–hydrogen antiporter 1 (*SLC9A1*/NHE1), down-regulated in adenoma (*SLC26A3*/DRA), putative anion transporter 1 (*SLC26A6*/PAT1), and sodium/glucose cotransporter 1 (*SLC5A1*/SLGT1) were affected by a PS × DOP interaction ($P \leq 0.09$). The mRNA expression of these genes at 272 DOP were decreased in pregnant compared to non-pregnant heifers. Also, the monocarboxylic acid transporter 4 (*SLC16A3*/MCT4) mRNA expression was lower in pregnant than in non-pregnant heifers. These genes are well known as markers for the regulation of VFA, H^+, HCO_3^-, and glucose epithelial transport processes (Connor et al., 2010). The MCT4 is responsible for VFA transport from the lumen into epithelial cells. In contrast, MCT1 is accountable for transporting VFA from the epithelium into the bloodstream (Connor et al., 2010). The mechanism controlling the expression of these genes is still unclear. It is suggested that ruminal VFA concentration, especially butyrate, and pH can regulate MCT expression (Laarman et al., 2013). However, no difference was found in the ruminal VFA profile and pH between the animals in this study (Moreira et al., 2020). These results suggest that rumen absorption of VFA by late-pregnant heifers is reduced, and other metabolic factors besides rumen fermentation products control gene expression in the rumen wall.

Ruminal VFA absorption increases H^+ concentrations in the cytosol (Laarman et al., 2016). Thus, the main function of NHE1 is recycling H^+ back to the ruminal lumen and importing Na^+ (Connor et al., 2010). On the other hand, the major role of DRA and PAT1 in the rumen epithelium is to transport HCO_3^- from inside the cell to the lumen (Connor et al., 2010). The downregulation of these genes suggests that the ruminal epithelium reduces the absorption of VFA in late-pregnant heifers. Zhang et al. (2013) observed that short-term feed restriction, as occurring in late gestation, has a negative effect on acetate absorption. The authors speculated that this acetate absorption impairment is a mechanism to reduce the energy required by the ruminal epithelium for synthesis of transport proteins when the energy supply is low.

4. Conclusion

The heart rate increased throughout gestation in heifers. Also, based on the circulating metabolite profiles and body weight loss during the gestational period, it can be concluded that pregnant heifers mobilized tissues, especially skeletal muscle. Lastly, the current results indicate that the ruminal epithelium of late-pregnant cows converts into an energy-saving state, reducing its remodeling and absorptive capacity. The current results increase the understanding of the homeorhetic alterations observed during the gestation of zebu beef heifers to benefit fetal development.

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Table 1

Sequences (5′ to 3′) and efficiencies of the primers used in quantitative real-time PCR.

Gene	Symbol	Forward (F) and reverse (R)	Access number	Amplicon size (bp)	R^2	Efficiency
PCNA	PCNA	F: GCTACACTTTCCTCAGTCCTTC R: GCCTCCAGCACTTTCTTCA	XM_019973361.1	105	0.97	1.00
CASP3	CASP3	F: GAGACGGGTTGAGGACAATAAG R: TGACAGAAGAGCCCTTTAGATATTC	XM_019953295.1	96	0.99	1.03
KLK10	KLK10	F: GGGTGGTGAACTCTGACTAAAT R: GCAAAGGGTGGTTAGGATTAGA	XM_019978443.1	100	0.99	1.03
KLK9	KLK9	F: TCTCTGAGTCACCAGGAACT R: GGGAAGCACCTGAAGCTATT	XM_019978450.1	97	0.93	1.04
SLC16A1	MCT1	F: TGTGGGACTGAAGGGTAAATG R: CCTGGTATGATTCCCACAGAAA	XM_019956796.1	114	0.98	1.00
SLC16A3	MCT4	F: CTGGTGCTGGGTAACTTCTT R: GTTCTTCTCAGGCTCTGTCTTC	XM_019980902.1	138	0.98	0.93
SLC26A3	DRA	F: GGATTTCTCTTGGAGCCTCTAC R: CTTTCGCCACAATCTTCGTATTT	XM_019958684.1	108	0.99	0.97
SLC26A6	PAT1	F: GGGACTGAGCTAGAGGATACA R: CAGGATGAGGGTGTGGAAAT	NM_001076852.2	114	0.97	1.03
SLC9A1	NHE1	F: CCCATTCTATTCCTTCCTCTGTC R: AGAGGGACCAGGACCTATTT	XM_019980388.1	124	0.99	0.99
SLC5A1	SGLT1	F: TCCTGACTGGGTTTGCTTTC R: TGACGGTGGTGTTTCCATAAG	XM_019977410.1	105	0.98	0.98

PCNA = Proliferating cell nuclear antigen; *CASP3* = Caspase 3; *KLK10* = Kallikrein-related peptidase 10; *KLK9* = Kallikrein-related peptidase 9; *SLC16A1* (MCT1) = Monocarboxylate transporter 1; *SLC16A3* (MCT4) = Monocarboxylate transporter 4; *SLC26A3*

(DRA) = Down-regulated in adenoma; *SLC26A6* (PAT1) = Putative anion transporter 1; *SLC9A1* (NHE1) = Sodium-hydrogen antiporter 1; *SLC5A1* (SGLT1) = Sodium/glucose cotransporter 1.

Table 2

Effect of physiological status (PS) and days of pregnancy (DOP) on respiratory (RR; breaths/min) and heart (HR; beats/min) rates of beef heifers.

Item	Physiological status		SEM	*P*-value		
	Non-pregnant	Pregnant		PS	DOP	PS×DOP
Before morning feeding						
RR	23.2	24.6	1.1	0.35	0.44	0.79
HR	70.2	74.2	1.1	0.01	0.04	0.08
Four hours after morning feeding						
RR	30.6	32.1	1.6	0.47	0.01	0.96
HR	74.8	82.0	1.8	<0.01	<0.01	0.63

PS×DOP = interaction between physiological status and days of pregnancy.

Table 3

Effect of physiological status (PS) and days of pregnancy (DOP) on blood metabolites levels of beef heifers.

Item	Physiological status		SEM	*P*-value		
	Non-pregnant	Pregnant		PS	DOP	PS×DOP
Before morning feeding						
NEFA, µmol/L	202	211	54	0.82	<0.01	0.11
BHB, µmol/L	348	364	42	0.77	0.24	0.67
Urea, mg/dL	21.1	21.5	2.1	0.78	<0.01	0.03
Glucose (mg/dL	100	96.6	3.7	0.41	0.03	0.05
Four hours after morning feeding						
Glucose, mg/dL	97.2	96.5	2.7	0.82	0.20	0.17

NEFA = non-esterified fatty acids; BHB = beta-hydroxybutyrate; PS×DOP = interaction between physiological status and days of pregnancy.

Figures

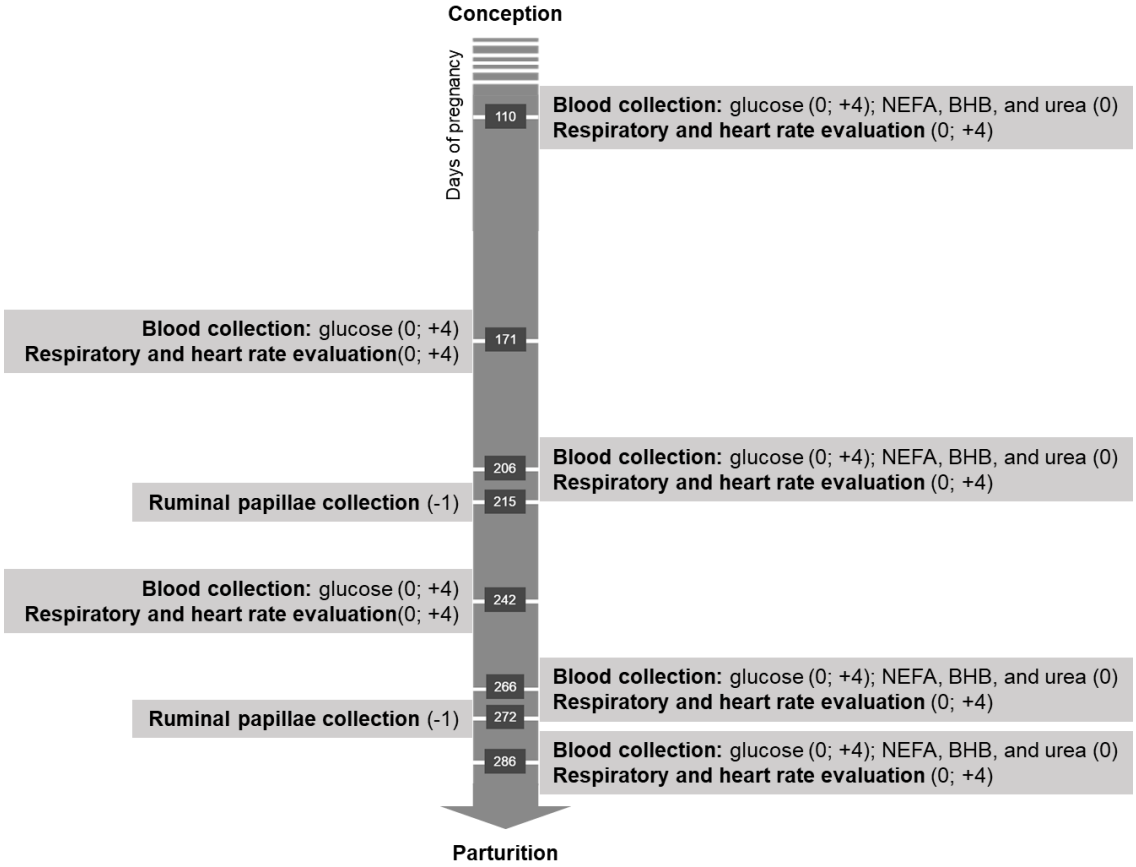


Fig. 1. Timeline of data collections during pregnancy of beef heifers. Collection times: where -1: one hour before the morning feeding, 0: immediately before the morning feeding, and +4: four hours after the morning feeding. NEFA = non-esterified fatty acids; BHB = beta-hydroxybutyrate.

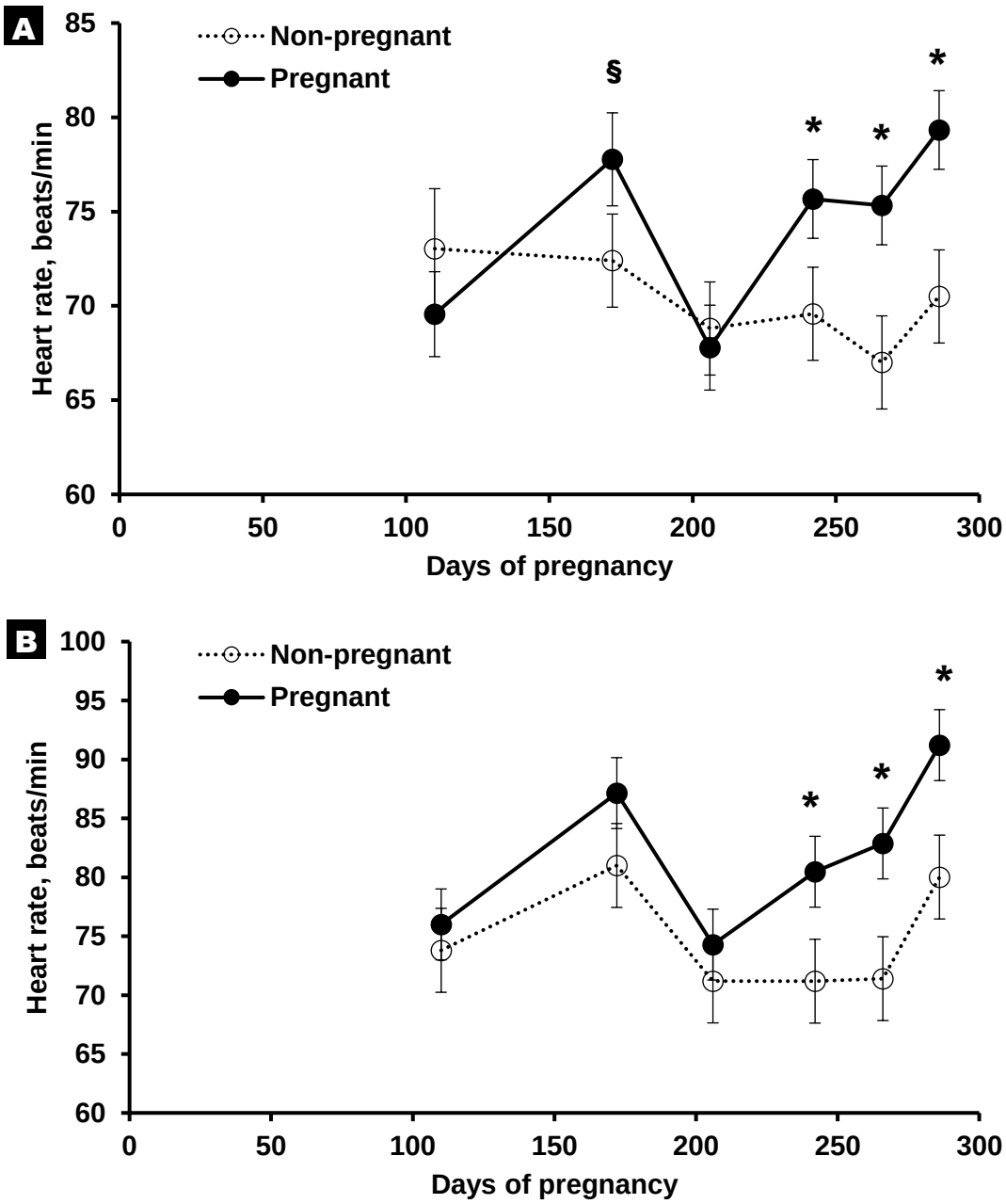


Fig. 2 Effect of physiological status and days of pregnancy on the heart rate of beef heifers. (**A**) Heart rates of pregnant and non-pregnant beef heifers (beats/min) over days assessed just before morning feeding. (**B**) Heart rates of pregnant and non-pregnant beef heifers (beats/min) over days assessed four hours after the morning feeding. § $P < 0.15$; * $P \leq 0.10$.

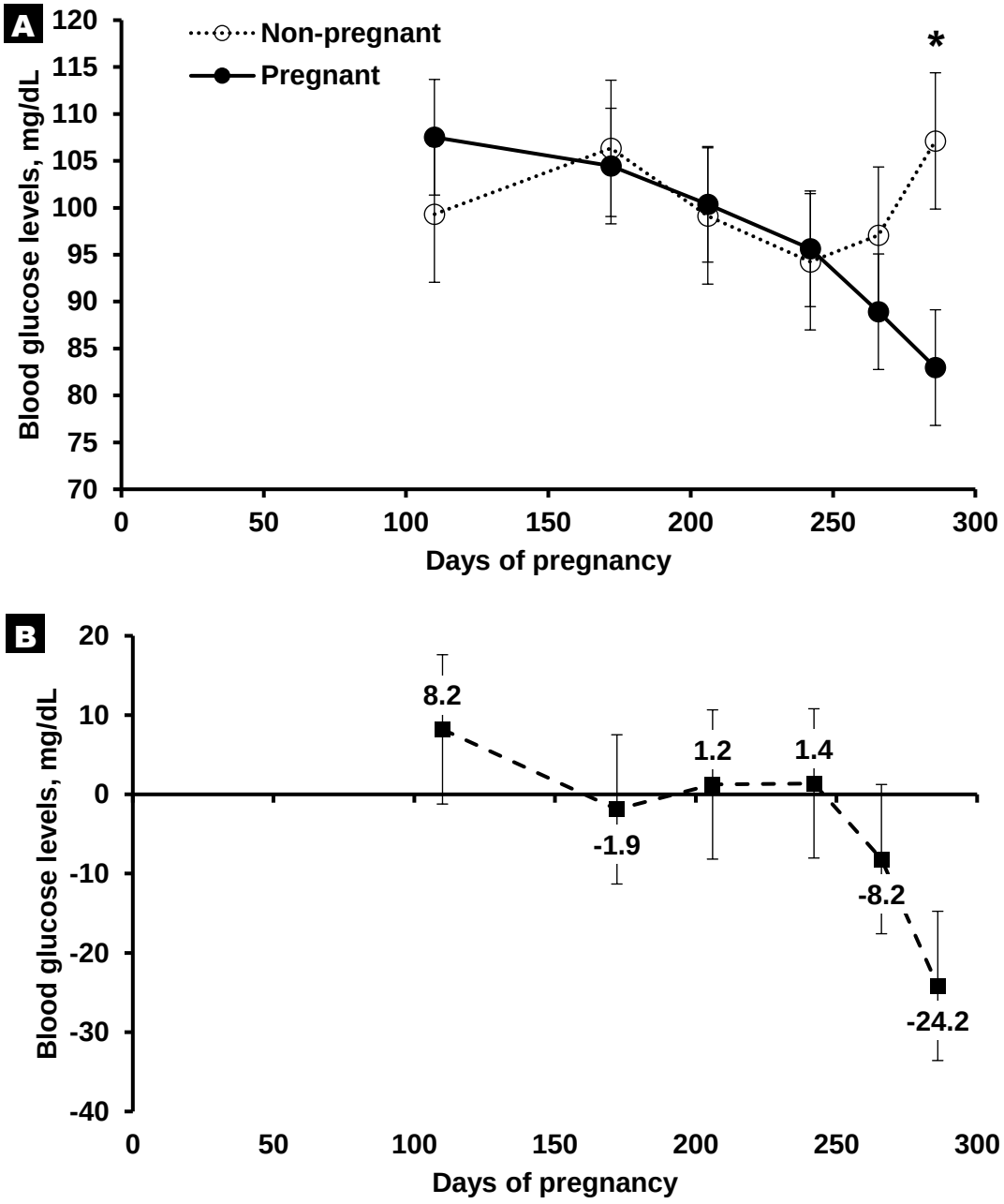
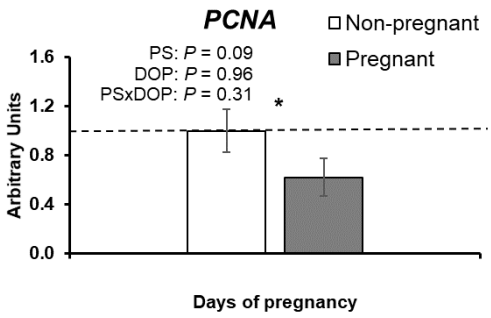
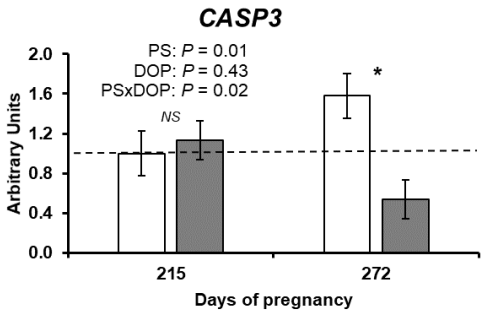
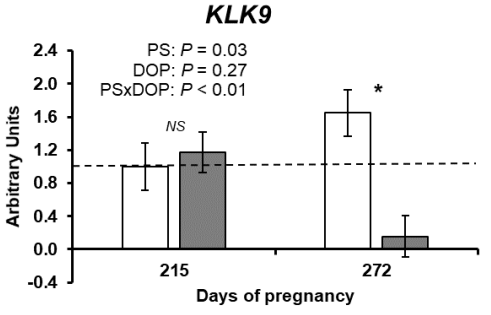
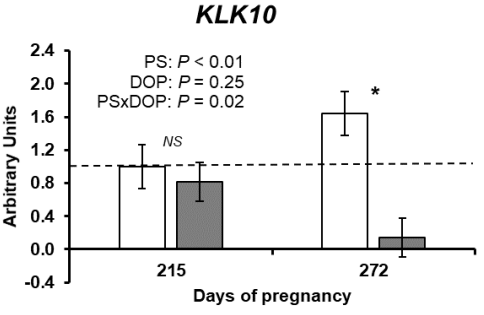


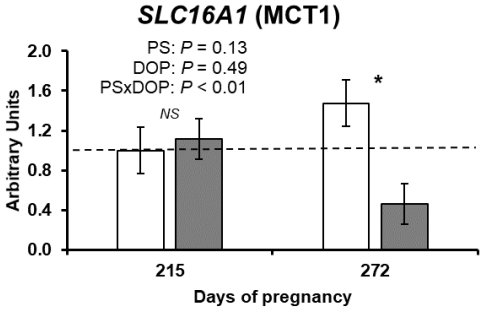
Fig. 3 Effect of physiological status and days of pregnancy on blood glucose concentration of beef heifers. (**A**) Blood glucose concentration in pregnant and non-pregnant beef heifers (mg/dL) over days. (**B**) Estimated blood glucose concentration (mg/dL) between pregnant and non-pregnant beef heifers over days. * $P \leq 0.10$.

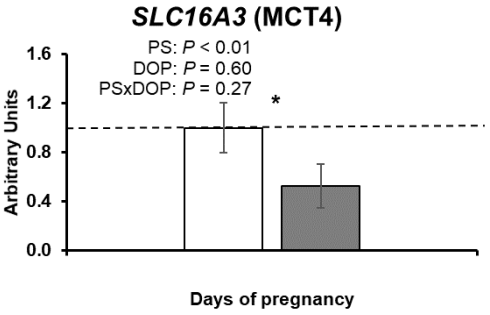

PCNA
Non-pregnant
Pregnant
PS: P = 0.09
DOP: P = 0.96
PSxDOP: P = 0.31
*
Arbitrary Units
Days of pregnancy

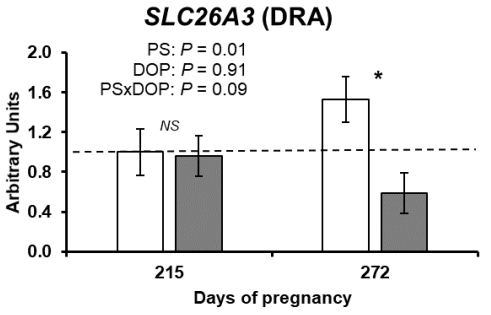

CASP3
PS: P = 0.01
DOP: P = 0.43
PSxDOP: P = 0.02
NS
*
Arbitrary Units
215
272
Days of pregnancy

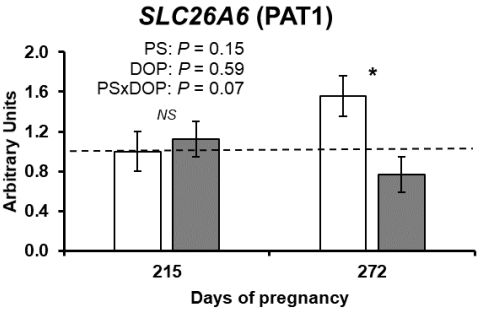

KLK9
PS: P = 0.03
DOP: P = 0.27
PSxDOP: P < 0.01
NS
*
Arbitrary Units
215
272
Days of pregnancy

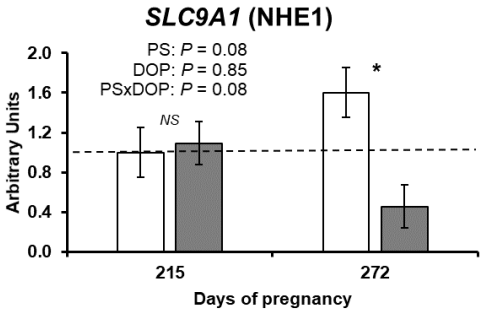

KLK10
PS: P < 0.01
DOP: P = 0.25
PSxDOP: P = 0.02
NS
*
Arbitrary Units
215
272
Days of pregnancy


SLC16A1 (MCT1)
PS: P = 0.13
DOP: P = 0.49
PSxDOP: P < 0.01
NS
*
Arbitrary Units
215
272
Days of pregnancy


SLC16A3 (MCT4)
PS: P < 0.01
DOP: P = 0.60
PSxDOP: P = 0.27
*
Arbitrary Units
Days of pregnancy


SLC26A3 (DRA)
PS: P = 0.01
DOP: P = 0.91
PSxDOP: P = 0.09
NS
*
Arbitrary Units
215
272
Days of pregnancy


SLC26A6 (PAT1)
PS: P = 0.15
DOP: P = 0.59
PSxDOP: P = 0.07
NS
*
Arbitrary Units
215
272
Days of pregnancy


SLC9A1 (NHE1)
PS: P = 0.08
DOP: P = 0.85
PSxDOP: P = 0.08
NS
*
Arbitrary Units
215
272
Days of pregnancy

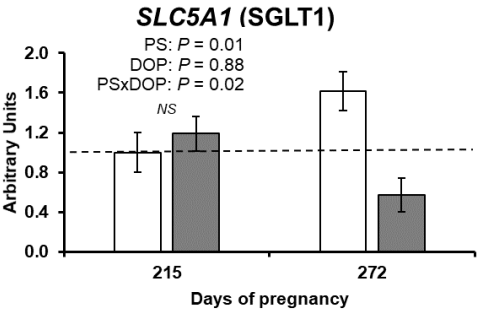

SLC5A1 (SGLT1)
PS: P = 0.01
DOP: P = 0.88
PSxDOP: P = 0.02
NS
*
Arbitrary Units
215
272
Days of pregnancy

Fig. 4 Relative gene expression in ruminal papillae of beef heifers according to physiological status (PS), days of pregnancy (DOP) and their interaction (PS×DOP). Proliferating cell nuclear antigen (*PCNA*), caspase 3 (*CASP3*), kallikrein-related peptidase 9 (*KLK9*), kallikrein-related peptidase 10 (*KLK10*), monocarboxylate transporter 1 (*SLC16A1*/MCT1), monocarboxylate transporter 4 (*SLC16A3*/MCT4), down-regulated in adenoma (*SLC26A3*/DRA), putative anion transporter 1 (*SLC26A6*/PAT1), sodium-hydrogen antiporter 1 (*SLC9A1*/NHE1), sodium/glucose cotransporter 1 (*SLC5A1*/SGLT1), NS = Non-significant. * $P < 0.10$.