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ANIMAL REPRODUCTION

Impact of maternal protein supplementation during mid or late gestation on skeletal muscle energy metabolism of beef calves

Letícia Maria Pereira Sanglard[1] David Esteban Contreras Marquez[2] Walmir Silva[3] Marta Maria dos Santos[3,4] Thaís Correia Costa[3,4] Dante Teixeira Valente Junior[3,4] Mateus Pies Gionbelli[5] Nick Vergara Lopes Serão[1] Claudia Batista Sampaio[3] Marcio de Souza Duarte[6*]

[1]Department of Animal Science, Iowa State University (ISU), Ames, IA, United States of America.
[2]Facultad de Ciencias Agropecuarias, Universidad de Cundinamarca, Cundinamarca, Colombia.
[3]Departamento de Zootecnia, Universidade Federal de Viçosa (UFV), Viçosa, MG, Brasil.
[4]Laboratório de Biologia Muscular & Nutrigenômica, Universidade Federal de Viçosa (UFV), Viçosa, MG, Brasil.
[5]Departamento de Zootecnia, Universidade Federal de Lavras (UFLA), Lavras, MG, Brasil.
[6]Department of Animal Biosciences, University of Guelph (UOG), N1G-2W1, Guelph, ON, Canada. E-mail: mduarte@uoguelph.ca.
*Corresponding author.

ABSTRACT: This study evaluated the effect of maternal protein supplementation during mid or late gestation on energy metabolism of the skeletal muscle of beef calves. Sixteen pregnant cows were divided into 3 groups: CTRL (not supplemented); MID (supplemented from 30 to 180 days of gestation); and LATE (supplemented from 181 to 281 days of gestation). The supplement contained 30% crude protein. Thirty days after birth, blood and muscle samples of the calves were collected for analyses of gene expression, proteins, and metabolites. No differences ($P \geq 0.15$) in birth weight, performance at weaning, or muscle expression of the genes evaluated ($P \geq 0.21$) were observed. Calves born to CTRL cows had a lower ratio ($P = 0.03$) of p-AMPK/AMPK protein in the skeletal muscle. Calves born to MID cows had lower ($P = 0.04$) glucose concentration than those born to LATE cows. Changes in p-AMPK/AMPK protein, indicated a possible metabolic inflexibility in the skeletal muscle of calves born to CTRL cows. These results indicated that lack of protein supplementation in pregnant cows alter the energy metabolism of their calves and reflect in a metabolic inflexibility.
Key words: beef calves, energy metabolism, fetal programming, maternal nutrition, gene expression, protein expression.

Impacto da suplementação proteica materna durante o terço médio ou terço final da gestação sobre o metabolismo energético do tecido muscular de bezerros de corte

RESUMO: O objetivo deste estudo foi avaliar o efeito da suplementação proteica materna sobre o metabolismo energético do músculo esquelético de bezerros de corte. Dezesseis matrizes gestantes foram divididas em três grupos: CONTROLE (não suplementado); MÉDIO (suplementados entre 30 e 180 dias de gestação); e FINAL (suplementado entre 181 e 281 dias de gestação). O suplemento continha 30% de proteína bruta e foi fornecido em quantidades totais iguais aos tratamentos. Trinta dias após o nascimento, amostras de sangue e músculo dos bezerros foram coletadas para análises de expressão gênica, abundância de proteínas e metabólitos. Não foram observadas diferenças ($P \geq 0,15$) no peso ao nascimento ou parâmetros de desempenho ao desmame, bem como na expressão dos genes avaliados ($P \geq 0,21$). Os bezerros nascidos de matrizes do tratamento CONTROLE apresentaram menor proporção ($P = 0,03$) de proteína p-AMPK/AMPK no músculo esquelético. Os bezerros nascidos de matrizes do tratamento MÉDIO apresentaram concentração de glicose menor ($P = 0,04$) do que aqueles nascidos de matrizes do tratamento FINAL. Os resultados observados indicam que a ausência de suplementação proteica em matrizes gestantes pode alterar o metabolismo energético da progênie e refletir em uma inflexibilidade metabólica, a qual pode ocasionar limitações quanto à eficiência energética do tecido muscular esquelético e consequentemente, limitar o desempenho da progênie ao longo da fase pós-natal.
Palavras-chave: bezerros de corte, metabolismo energético, programação fetal, nutrição materna, expressão gênica, expressão proteica.

INTRODUCTION

Maternal restricted nutrition causing limited fetal growth has been reported as one of the major problems in livestock production (DU et al., 2010; WU et al., 2006). The quantity and quality of forage in beef cattle grazing systems in tropical areas are not consistent during all periods of the year due to the rainfall seasonality. Thus, pregnant cows commonly experience nutrient restriction

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during mid to late gestation, which usually overlaps with the dry season in beef cattle production areas (PAULINO & DUARTE, 2014). Protein is the main limiting nutrient for grazing pregnant cows during gestation in tropical areas as the crude protein content of the forage substantially decreases during the dry season (POPPI et al., 2018). As such, to address this limiting factor, it was used different strategies of protein supplementation for pregnant cows . These strategies are usually focused on the final third of gestation, which is considered the main period when the maternal nutrient restriction can affect the fetal development (FERRELL et al., 1976).

Nutrient restriction during pregnancy may impair the ability of the skeletal muscle metabolism to adapt in response to nutrient intake alterations (ARAGÃO et al., 2014). This phenomenon is known as metabolic inflexibility and has been associated with insulin resistance, obesity, and diabetes (KELLEY et al., 1999). Indeed, offspring born to dams that were protein-restricted during gestation developed insulin resistance later in life (OZANNE et al., 2003). Another study has shown that offspring born to protein-restricted dams exhibited changes in the gene expression of the skeletal muscle and the energy metabolism, favoring the transport of long-chain fatty acids into the mitochondrial matrix and switching from carbohydrate to lipid oxidation (ARAGÃO et al., 2014). It is noteworthy that the studies mentioned above were performed with rodent models other than livestock animals. Thus, it is important to highlight the need to investigate the impacts of protein restriction during beef cattle gestation, which may negatively affect the production and carcass deposition of the offspring (MARQUEZ et al., 2017).

Therefore, we hypothesized that protein supplementation of dams at different periods of gestation differently affect the metabolic flexibility of the skeletal muscle of beef calves. This study evaluated the effect of maternal protein supplementation during mid or late gestation on energy metabolism of skeletal muscle of beef offspring during postnatal phase.

MATERIALS AND METHODS

Animals and diet

The complete description of the experiment is in MARQUEZ et al. (2017). The experiment was held at the Department of Animal Science at the Federal University of Viçosa (Viçosa, Brazil). Twenty-seven Nellore cows with 490±12.82 kg of initial body weight (BW) were artificially inseminated at a fixed time using semen from the same bull. The cows used in this trial were from the same contemporary group, with five years of age and two parturitions prior to the experimental trial.

A completely randomized design was used to allocate the animals in three groups as follow: CTRL, control group which did not received supplementation during gestation; MID, group supplemented from 30 to 180 days of gestation; and LATE, group supplemented from 181 to 281 days of gestation. The transrectal ultrasound determined the fetus sex at 60-days of gestation to obtain a homogeneous treatment. Only 16 cows pregnant with male fetuses were kept in the experiment, being four animals in the CTRL group, six animals in MID group, and six animals in LATE group.

All animals were kept on 6-ha paddocks per treatment with pasture evenly covered with *Urochloa decumbens* grass and equipped with a drinker and feeders. To minimize possible paddock effects, all groups were rotated every 7 days. Table 1 shows the composition of pastures .

Supplement provided for MID and LATE groups contained 30% of crude protein (CP), and each individual received 150 kg during the total period of supplementation. Thus, MID cows had a daily consumption of 1 kg per animal and LATE cows, 1.50 kg per animal, besides the mineral mix *ad libitum*. The CTRL group received only mineral *ad libitum* during the gestation.

After birth, all calves (born to MID, LATE, and CTRL cows) were kept on the same conditions for 8 months (until weaning) receiving 6 g/kg of BW of a supplement formulated to contain 25% of CP, along with *ad libitum* mineral. The composition of the supplement and mineral provided to the calves is shown in table 2.

The supplement was provided daily in the morning, at a single feeder, sized to allow access only to the calves. To minimize possible paddock effects, the animals were rotated between the paddocks every seven days in a way that every group remained the same amount of time in each paddock under the same environmental conditions during the experiment. Pastures samples were collected via manual grazing simulation, every 15 days, dried in a forced-air circulation oven (Ar SL – 102; SOLAB®, Piracicaba, São Paulo, Brasil) at 55 °C to 60 °C for 72 h and then grounded with 2 and 1mm knife mill type Wiley (TE-680, SOLAB®, Piracicaba, São Paulo, Brazil).

It was evaluated the nutritional characteristics and digestibility of diet in a trial for 9 days at both 100 and 230 days. Fecal samples were collected immediately after defecation or directly from

Table 1 - Composition of the pasture offered to the cows during early-mid gestation (MID), late gestation (LATE), and to the calves from birth to weaning (Growing).

Ingredients [A]	MID	LATE	Growing
DM (g/kg fresh matter)	877.2	886.7	887.8
MO (g/kg DM)	907.4	906.9	910.8
CP (g/kg DM)	92.5	74.8	82.9
EE (g/kg DM)	49.5	41.7	16.2
NDFap (g/kg DM)	565.7	587	565.7
NFC (g/kg DM)	199.7	203.3	245.9
iNDF (g/kg DM)	191.3	207.5	191.8

[A]DM = dry matter; MO = matter organic; EE = ether extract; NDFap = neutral detergent fiber essayed with heat stable amylase expressed exclusive of residual ash and protein; NFC = non-fibrous carbohydrates; iNDF = indigestible NDF.
Mineral mix – CaHPO4 = 500 g/kg; NaCl = 471.5 g/kg; ZnSO4 = 15 g/kg; Cu2SO4 = 7.5 g/kg; CoSO4 = 0.5 g/kg; KIO3 = 0.5 g/kg and MnSO4 = 0.5 g/kg.

the rectum of the animals. These samples were used to estimate the fecal excretion and individual supplement intake of the animals. The faecal excretion of animals, estimated by Chromic oxide (Cr_2O_3) was wrapped in paper cartridges in an amount of 20 g/animal per day for cows and 10 g/animal per day for calves. It was administered with a metal probe via the oesophagus at 10 h. Titanium dioxide (TiO2) estimated the individual supplement intake, provided via supplement at the proportion of 10 g/kg of supplement (TITGEMEYER et al., 2001; MARQUEZ et al., 2017). Indigestible neutral detergent fiber was used to estimate the pasture dry matter intake (DMI; DETMANN et al., 2001). The complete description of these methods can be seen in MARQUEZ et al. (2017). There were no differences ($P > 0.18$) between total DMI, DMI from 30 to 180 days and pasture dry matter from 30 to 180 days, while cows receiving supplement during late gestation had greater ($P \leq 0.04$) DMI and pasture dry matter from 181 to 281 days. There was no difference ($P > 0.57$) on the dry matter intake, dry matter supplement, dry matter milk, and crude protein consumed by the progeny. The complete results for dry matter and nutrient intake of dietary treatments (cows and progeny) and cow performance can be seen in MARQUEZ et al. (2017).

The experiment was carried out for 16 months, corresponding to the last 8 months of gestation (after pregnancy diagnosis that occurred 30 days after insemination) and 8 months of postnatal growth of the offspring.

Table 2 - Composition of the supplement offered to the cows according to the groups: supplemented at the second third of gestation (MID), supplemented at the final third of gestation (LATE), and control group (CTRL), and to the calves from birth to weaning (Growing).

Ingredients	MID	LATE	CTRL	Growing
Corn meal	26.25	26.25	0	30
Wheat bran	26.25	26.25	0	30
Soybean meal	47.50	47.50	0	35
Molasses	-	-	-	5
Mineral Mix[A]	*ad libitum*	*ad libitum*	*ad libitum*	*ad libitum*

[A]composition: CaHPO4 = 500 g/kg; NaCl = 471.5 g/kg; ZnSO4 = 15 g/kg; Cu2SO4 = 7.5 g/kg; CoSO4 = 0.5 g/kg; KIO3 = 0.5 g/kg and MnSO4 = 0.5 g/kg.

Collection of skeletal muscle and blood tissues

At 30 days after birth, skeletal muscle tissue biopsies from calves were collected to obtain samples of *Longissimus lumborum* muscle as described by ARRIGONI et al. (2004). Upon collection, samples of skeletal muscle tissue were snap-frozen and kept in liquid nitrogen for further mRNA and protein extraction. At same time, blood samples were collected via jugular venipuncture using vacuum tubes with a coagulation accelerator gel (BD Vacutainer®, SST II Advance, Franklin Lakes, NJ) to measure serum glucose, triglycerides, insulin, and leptin concentrations. Glucose (K082, Bioclin® Quibasa, Belo Horizonte, Brazil), and triglycerides (K117, Bioclin® Quibasa, Belo Horizonte, Brazil) were quantified by enzymatic-colorimetric method, and total protein (K031, Bioclin® Quibasa, Belo Horizonte, Brazil). Insulin concentration was measured with a commercial kit (Ref. 10995628, Atellica IM Insulin, Siemens Healthcare, Tarrytown, United States) and the Atellica™ analyzer (Siemens Healthcare, Tarrytown, United States) by following the manufacturer's instructions. Leptin concentration was determined with a commercial kit (ref. ab179884, Abcam, Cambridge, UK) and a ThermoFisher Multiskan spectrum spectrophotometer (Thermofischer, Waltham, United States) by following the manufacturer's instructions.

Total RNA extraction and PCR analysis in real-time

Total RNA was extracted from 0.1 g of frozen muscle samples powdered in liquid nitrogen. Trizol® (InvitrogenTM, Thermo Fisher Scientific®, OR, USA) was used for RNA extraction by following the manufacturer's recommendations. RNA 6000 Nano kit and 2100 Bioanalyzer System (Agilent Technologies, CA, USA) was used for evaluation of the RNA integrity and quantity through capillary electrophoresis. GoScriptTM Reverse Transcription System Kit (Promega Corporation, Madison, WI, USA) were used for reverse transcription of the RNA into cDNA. PrimerQuest software (www.idtdna.com/Scitools/Applications/PrimerQuest) was used for designing the primers (Table 3) with sequences obtained using GenBank (www.ncbi.nlm.nih.gov) for amplification of target and endogenous genes. The thermal cycler ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) was used for real-time quantitative PCR using the detection method SYBR Green (Applied Biosystems, Foster City, CA, USA) and GoTaq® qPCR Master Mix kit (Promega Corporation, Madison, WI, USA). For that, the cycle parameters were: 95 °C for 2 minutes, 40 cycles at 95 °C for 15 seconds and 60 °C for 60 seconds. This study determined if maternal supplementation at mid and late gestation would shift the commitment of mesenchymal stem cells towards myogenesis or adipogenesis and alter the skeletal muscle energy metabolism of beef calves during postnatal phase. Therefore, the genes evaluated in this study were associated with energy metabolism (ACC1, CD36, CPT1B, INSR, LEPR, MLYCD, NRF1, PFKFB2, SREBP1C, and TBC1D1), myogenesis (MEF2A and MYH3), and adipogenesis (ADIPOR1, FASN, HSL, LPL, and PPARGC1A). Gene expression values were calculated and expressed relatively to the housekeeping gene, GAPDH, as described by (LIVAK & SCHMITTGEN, 2001).

Total protein extraction and protein abundance determination by western blot

Frozen muscle samples powdered (0.5 g) in liquid nitrogen were also used for extraction of protein (*p*-AMPK and AMPK) in 1 mL of Lysis Buffer [10 mM Tris HCl, 100 mM of NaCl, 0.5 mM of DDT (dithiothreitol), 2.5 mM of MgCl2, 0.5% triton X-100, and 1% protease inhibitor cocktail (Sigma-Aldrich®)]. The quantification was done by Bradford Method (BioRad, Hercules, CA), and the protein integrity was analyzed by gel polyacrylamide electrophoresis. Samples were stored in ultra-freezer (-80 ºC) for posterior western-blot analyzes. AMPK is an enzyme involved in cellular energy homeostasis whose activation increases glucose and fatty acid uptake and oxidation when cellular energy is low. Thus, AMPK was analyzed to evaluate if protein supplementation during mid and late gestation caused an alternation in the energy metabolism of the calves.

After extracted, proteins were separated using electrophoresis on sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis gel (acrylamide/bisacrylamide 29:1) 12% prepared in buffer containing 1.5 M Tris-HCl pH 8.8 and 0.4% SDS stacking gel and 3% in buffer 0.5 M tris-HCl pH 6.8 and 0.4% SDS. The electrophoresis buffer composition was 25 mM Tris-HCl pH 8.3, 200 mM glycine and 1% SDS. Electrophoresis of proteins was held at 100 Volts gel 1 cm x 16 cm for 2 hours.

After electrophoresis, the proteins were transferred to a nitrocellulose membrane using a buffer containing 25 mM Tris, 193 mM glycine, and 20% methanol. Nitrocellulose membranes with the proteins were blocked for 2 hours with 3% Bovine Serum Albumin (BSA, Sigma-Aldrich®, St. Louis, MO, USA) in Tris-Buffered Saline (TBS) 1x for

Table 3 - Gene sequences and NCBI codes used for primer construction.

Gene	Abbreviation	Forward sequence	Reverse sequence	NCBI code
Acetyl-CoA carboxylase 1	ACC1	GCACGCCAGGTTCT TATT	CATGTCAATGGCGGATAGG	NM_174224.2
Adiponectin receptor 1	ADIPOR1	AGGACAACGACTAC CTACTG	GTGTGGATGCGGAAGATG	NM_001034055.1
CD36 molecule	CD36	GAGGCAGACACAAC AAGAG	CAGTGGTAACCAGTTGG AAG	NM_001278621.1
Carnitine palmitoyl transferase 1B	CPT1B	GTCCCTTCCCTTGCT CTA	GGACAGCAGAGACCCATA	NM_001034349.2
Fatty acid synthase	FASN	ATCGCTGGCTACTC CTAC	GCCGTCAAACAGGAAGAG	NM_001012669.1
Lipase, hormone-sensitive	HSL	GAGGGTGATGAGAG GGTAAT	GATGGCAGGTGTGAACTG	EF140760.1
Insulin receptor	INSR	TCCAGGGTGAAGGA TTGT	CTGCAGACCCATATCCA AAG	XM_005208817.2
Leptin receptor	LEPR	TCCTGGGTCTTCGTA TGG	GTAAGAAGGGCACTCCA ATC	XM_010803430.1
Lipoprotein lipase	LPL	CAGACAGGATTACA GGAGGA	GGAATGAGGTGGCAAGTG	NM_001075120.1
Malonyl-CoA decarboxylase	MLYCD	CACTTCCACCTGCA GAAC	CCTCTAGGAAGTAGCGG TAG	NM_001098946.1
Myocyte enhancer factor 2A	MEF2A	CCACCTCAAGCCAC ATTAC	CTGAAGTGCTCAACATCCC	NM_001083638.2
Myosin heavy chain 3, skeletal muscle, embryonic	MYH3	GGATGCACTCGTTT CTAG	GGCACTCTTGGCCTTTATC	NM_001101835.1
Nuclear respiratory factor 1	NRF1	GGTGACTCTGTCCC TGTAT	GTGAGGGCTGATTACAA GAC	NM_001098002.2
6-phosphofructo-2-kinase/fructose-2.6-biphosphatase 2	PFKFB2	GTCCCTTCCCTTGCT CTA	GGACAGCAGAGACCCATA	NM_174812.4
Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	PPARGC1A	ACCTCCACCATCCA AGAA	CTGTGCGTACAACTCAGAC	NM_177945.3
Sterol regulatory element binding transcription factor 1	SREBP1C	CTCGTCTTCCTCTGT CTCTC	GTTGATGCTGGTGGTGTC	NM_001113302.1
TBC1 domain family member 1	TBC1D1	GTTTCGCCCTCTGG ATTG	GTGAACTCGTTGAGGCTT AC	NM_001166524.1

p-AMPK and 3% nonfat dry milk in TBS1x for AMPK at room temperature. Then the membranes were incubated for 12 h at 4 °C with the primary antibodies. Both primary antibodies were diluted 1:1000 in BSA 3% (*p*-AMPK, n. 4188S, Cell Signaling Technology, Danvers, MA, USA) or skim powdered milk 2.5% (AMPK, n.2532. Cell Signaling Technology, Danvers, MA, USA) in TBS-T buffer for 16 hours at 4 °C. After this period, the membrane was washed 3 times (15 minutes each) in TBS-T buffer 0.2% under gentle stirring at room temperature. Further, the membrane was incubated with the secondary antibody anti-rabbit IgG produced in goat (Santa Cruz Biotechnology Inc., Dallas, TX, USA) diluted 1:5000 in BSA 3% or skim powdered milk 2.5% in TBS-T buffer for 1 hour at room temperature under gentle shaking and followed by 3 washes (5 minutes each rinse) in TBS-T buffer.

ClarityTM substrate (Bio-Rad, Hercules, CA, USA) was used for revealing the membrane by ECL, the c-Digit® Blot device (Licor Biosciences,

Nebraska, USA) was used for generating the images, and the software Image Studio Digits Lite Version 5.2 (LI-COR Biosciences, Lincoln, NE, USA) was used for quantification of the bands by densitometry. All samples could fit on the same gel thus each SDS gel included protein extracted from all treatments and the internal control for signal normalization (CRUZEN et al., 2014).

Carcass traits assessment at weaning

Carcass traits of the calves were evaluated at the end of the suckling phase. The measurements were obtained from the right side of the animals and evaluated by ultrasound (model: SSD 500v, with a linearprobe of 18 cm; Aloka, Wallingford, CT, USA). Images for the ribeye area were obtained from a transversal section of the muscle (between the T12 and T13 thoracic vertebrae). The images for subcutaneous fat thickness were obtained from the same position where the images of *Longissimus dorsi* were taken and from the pelvic region, between the ischium and pubis. This trait was analyzed as an average of two measurements. Images were analyzed in the BioSoft Toolbox® II for Beef software (Biotronics Inc., Ames, IA, USA).

Statistical analyses

Performance data were analyzed using a linear model including the fixed effects of treatment (levels: CTRL, MID, and LATE), total DMI (covariate), birth weight (covariate), and weaning age (covariate). For gene and protein expressions and metabolites concentrations, weaning age was not included in the model because the samples were taken prior to weaning. Normality assumption of the residuals was checked based on Shapiro-Wilk's statistic ($P \leq 0.01$) and visual assessment of the residual variance. For protein expression, two outliers were removed. For gene expression, the data were analyzed using ΔCt values, represented by the difference between the genes of interest (Table 3) and the housekeeping gene. Gene expression results are shown as $\log_2$ fold change (FC). Two pre-defined orthogonal contrasts were tested. These two contrasts represented the difference between CTRL and the average of MID and LATE (which represented "supplemented" animals; SUP), and the difference within SUP (i.e., between MID and LATE). Significant results were considered at $P \leq 0.05$ and tendency to significance was considered at $P \leq 0.10$. Analyses were performed using the GLIMMIX procedure of SAS 9.4 (Statistical Analysis System; Cary, NC, USA).

RESULTS

mRNA expression in the skeletal muscle of the offspring

Results for gene expression can be seen in table 4. There were no differences between groups ($P \geq 0.21$) for the genes analyzed.

Protein expression in the skeletal muscle of the offspring

Results for protein expression can be seen in figure 1. Abundance ratio of *p*-AMPK/AMPK was lower ($P = 0.03$) in calves born to CTRL cows than to SUP cows; however, there was no difference ($P = 0.22$) between calves born to MID and LATE cows.

Blood metabolites concentration in the blood of the offspring

Results for metabolites quantification can be seen in table 5. The concentration of glucose was lower ($P = 0.04$) in calves born to MID than to LATE cows. There were no differences ($P \geq 0.87$) in the concentrations of insulin, leptin, and triglycerides between the groups.

Performance of the offspring

Results for performance and body composition can be seen in table 6. There were no differences ($P \geq 0.15$) between the groups for any of the traits analyzed.

DISCUSSION

In this study, we examined the impact of increasing protein intake in the maternal nutrition during early and late periods of gestation on the performance and expression of energy metabolism markers in the skeletal muscle of the offspring. The findings herein partially supported the hypothesis that protein supplementation at different periods of gestation may differently affect the metabolic flexibility of the skeletal muscle of beef calves. Support was provided by the alteration of the expression of protein associated with energy metabolism in the muscle of calves born from SUP cows compared to CTRL during pregnancy. Conversely, no differences in performance and growth compositions, gene expressions, and blood metabolites concentration were observed between the periods of supplementation during gestation.

We did not find differences in birth weight between the groups. Several other studies found similar results and reported that fetal programming might be happening in consequence of changes in

Table 4 - Effect of maternal diet[A] on the expression of genes[B] related to energy metabolism, myogenesis and adipogenesis in calves.

Gene	P-value[C]	----------CTRL x SUP----------		----------MID x LATE----------	
		Log_2FC [CI[D]]	P-value	Log_2FC [CI[D]]	*P*-value
ACC1	0.38	0.00 [-0.34, 0.34]	0.71	0.17 [-0.38, 0.04]	0.18
ADIPOR1	0.46	0.06 [-0.30, 0.43]	0.99	-0.14 [-0.09, 0.37]	0.23
CD36	0.51	-0.54 [-1.63, 0.55]	0.26	0.04 [-0.71, 0.64]	0.98
CPT1	0.94	0.08 [-0.27, 0.44]	0.76	-0.02 [-0.20, 0.24]	0.91
FASN	0.55	-0.16 [-0.57, 0.25]	0.36	0.07 [-0.33, 0.18]	0.50
HSL	0.83	0.28 [-0.69, 1.26]	0.95	0.14 [-0.74, 0.47]	0.55
INSR	0.87	0.05 [-0.73, 0.82]	0.99	-0.05 [-0.43, 0.53]	0.61
LEPR	0.53	0.18 [-0.15, 0.52]	0.27	-0.01 [-0.20, 0.22]	0.86
LPL	0.63	0.20 [-1.25, 1.66]	0.94	-0.08 [-0.82, 0.98]	0.36
MLYCD	0.74	0.05 [-0.17, 0.27]	0.47	-0.00 [-0.14, 0.13]	0.89
MEF2A	0.21	-0.18 [-0.47, 0.11]	0.09	-0.01 [-0.17, 0.19]	0.95
MYH3	0.24	-0.03 [-0.14, 0.08]	0.52	0.05 [-0.12, 0.01]	0.11
NRF	0.80	0.17 [-0.37, 0.71]	0.72	0.02 [-0.36, 0.31]	0.63
PFK	0.70	0.02 [-0.42, 0.47]	0.77	0.09 [-0.37, 0.18]	0.46
PGC	0.62	-0.50 [-2.48, 1.48]	0.90	-0.21 [-1.02, 1.44]	0.34
SREBP1C	0.72	-0.11 [-0.58, 0.36]	0.45	0.02 [-0.31, 0.27]	0.75
TBC1D1	0.27	0.29 [-0.07, 0.65]	0.11	-0.02 [-0.20, 0.24]	0.73

[A]Non-supplemented (CTRL) or supplemented (SUP) cows. SUP cows received supplementation during middle (MID) or late gestation (LATE);

[B] Expressed in log_2 fold change (FC), with 95% confidence interval of the log_2 FC within brackets. For the pre-defined contrast between CTRL and SUP (CTRL x SUP), negative and positive log_2FC represent upregulation in SUP and CTRL, respectively. For the pre-defined contrast between MID and LATE (MID x LATE), negative and positive log_2FC represent upregulation in LATE and MID, respectively;

[C] P-value for the main effect of treatment;

[D] CI, 95% confidence interval for the log_2 FC.

maternal nutrition even when no difference in birth weight is observed (STALKER et al., 2006; WU et al., 2006; MORIEL et al., 2015; DA SILVA et al., 2017). Although, there was no alteration in birth weight, these studies reported changes in growth performance (MORIEL et al., 2015; STALKER et al., 2006), gene expression (PEÑAGARICANO et al., 2013; SANGLARD et al., 2018), and innate and humoral immune responses (MORIEL et al., 2015) after nutrient restriction or supplementation during pregnancy. This happened likely because changes in cell signaling are still very subtle at birth and may be noted only later in life. In this study, we have not observed differences in growth performance between the groups. This may indicate a compensation of the growth of calves born to cows not receiving supplementation during gestation. These results contrast previous studies, which reported greater weaning weight after maternal supplementation in beef calves (LARSON et al., 2009; STALKER et al., 2006; UNDERWOOD et al., 2010) and ewes (VAN EMON et al., 2014).

Although, no differences were observed in the gene expressions and metabolites concentration between the groups, there was a greater expression of p-AMPK/AMPK ratio in the skeletal muscle of calves born to CTRL cows compared to SUP. AMPK needs to be phosphorylated to produce significant activity (THOMSON, 2018). Thus, calves born to CTRL cows had a greater activated AMPK compared to the SUP. AMPK is activated when there is a depletion of ATP (energy) and inhibits anabolic processes, such as protein synthesis (BOLSTER et al., 2002). AMPK also regulates protein synthesis through

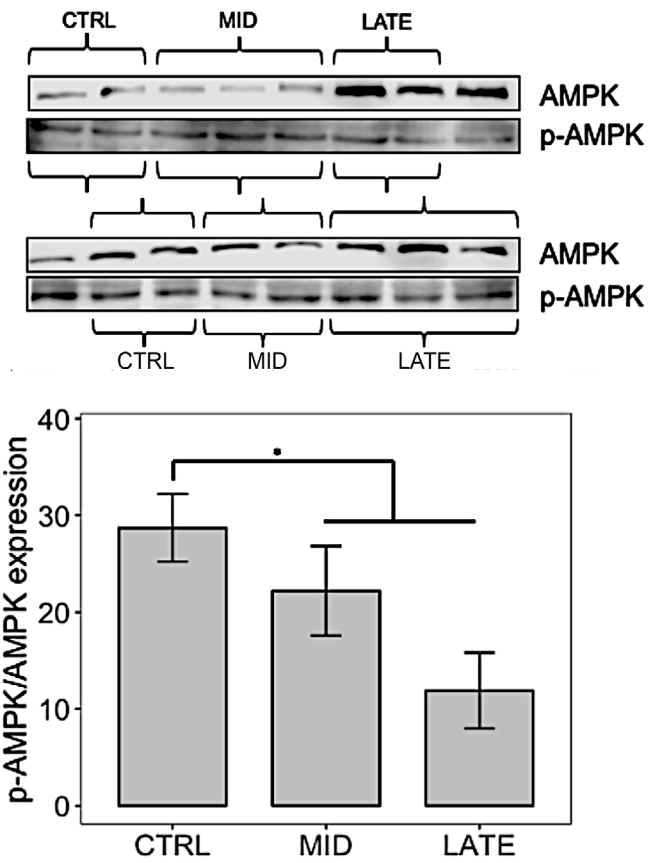

Figure 1 - Western-blot membranes with protein expressions (AMPK and p-AMPK) and rate expression of p-AMPK/AMPK. on muscle of beef calves born from cows under 3 treatments: CTRL (Control not supplemented), MID (supplemented from 3 to 6 months of gestation), and LATE (supplemented from 6 to 9 months of gestation). P-value for main effect of treatment = 0.04, P-value for the comparison of CTRL vs supplemented = 0.03, and P-value for the comparison of MID vs LATE = 0.22. Error bars represent standard error of the mean and the star represent significant difference at $P < 0.05$.

impairing MTORC1 activity (GWINN et al., 2008) and inhibiting eEF2 (MU et al., 2003). Given these general actions, AMPK has a potentially negative effect on skeletal muscle growth (THOMSON, 2018). Furthermore, AMPK phosphorylation in the hypertrophying muscle was associated with decreased muscle hypertrophy, MTOR pathway signaling, and myoblast proliferation in the absence of glucose (THOMSON, 2018; THOMSON & GORDON, 2005). Along with the metabolism inflexibility, these results may indicate that calves born to CTRL cows had less energy storage than calves born to SUP cows postnatally and, thus, had to activate the AMPK signaling pathway to liberate energy. This process may have a negative effect on muscle hypertrophy due to protein degradation. Therefore, the little availability of protein for CTRL cows may have stimulated a switch to type I oxidative fibers on the fetus skeletal muscle to use lipid as an energy source. After birth, all calves were fed protein supplementation which resulted in calves born to CTRL cows transitioning from nutrient restriction to feeding. However, due to the protein deprivation during pregnancy, calves born to CTRL cows may have metabolic inflexibility, reflecting a reduced capacity to switch back from lipid oxidation to glucose utilization after birth. Consequently, the

Table 5 - Blood metabolite levels of male beef calves[A].

Item	CTRL	----------SUP----------		----------P-value[B]----------		
		MID	LATE	Main effect	CTRL x SUP	MID x LATE
Glucose (mg/dL)	104.67 (4.12)	89.17 (4.12)	105.25 (5.04)	0.04	0.18	0.03
Insulin (UI/dL)	1.17 (0.24)	1.17 (0.24)	1.35 (0.34)	0.90	0.79	0.68
Leptin (ng/mL)	0.95 (0.15)	0.87 (0.15)	0.85 (0.18)	0.89	0.63	0.94
Tryglicerides (mg/dL)	29.33 (2.34)	27.67 (2.34)	29.0 (2.86)	0.87	0.74	0.73

[A]Born from non-supplemented (CTRL) or supplemented (SUP) cows. SUP cows received supplementation during middle (MID) or late gestation (LATE);
[B]P-values for the main effect of treatment (Main effect), and the two pre-determined orthogonal contrast: between cows not supplemented and supplemented (CRTL x SUP), and between cows supplemented at mid and late gestation (MID x LATE).
[a-b]Means within a row lacking a common superscript letter differ at $P < 0.05$.

incomplete fatty acid oxidation and accumulation of beta oxidative intermediates resulting from the mismatch between lipid metabolism and energy availability could further generate insulin resistance in calves born to CTRL cows.

Finally, the lack of differences in the insulin concentration suggests a lack of relationship between either glucose or insulin concentrations and circulating leptin concentrations in the control and supplemented cows during early or late gestation. It is consistent with studies of MUHLHAUSLER et al. (2007) in fetus that showed plasma glucose concentrations were greater in the supplemented ewes group; however, there was no effect on plasma leptin concentrations. These findings suggest that glucose was more sensitive than leptin to a moderate increase in maternal nutrient intake. Previous studies demonstrated a significant correlation between fetal leptin concentrations and fetal adiposity (SHEKHAWAT et al., 1998). It could be due to the leptin concentration related to body fat content and current nutritional intake. The serum triglyceride concentrations were also similar ($P \geq 0.10$) between the groups despite the evidence for increased lipid mobilization in calves born to CTRL cows. There was no difference in total dry matter intake, which

Table 6 - Performance of male beef calves[A].

Item	CTRL	Maternal Supplementation (SUP)		----------P-value[B]---------		
		MID	LATE	Main effect	CTRL x SUP	MID x LATE
Birth weight (kg)	37.7 (2.15)	34.7 (2.00)	35.3 (2.62)	0.68	0.38	0.87
Body weight adjusted to 240 days (kg)	256.38 (9.04)	242.56 (6.3)	254.93 (9.17)	0.27	0.59	0.21
Average daily gain (kg/day)	0.92 (0.04)	0.86 (0.03)	0.91 (0.04)	0.35	0.58	0.29
Rib fat thickness (mm)	2.83 (0.55)	2.12 (0.38)	2.64 (0.55)	0.45	0.60	0.37
Rib eye area (cm^2)	43.32 (1.52)	45.24 (1.06)	42.21 (1.54)	0.15	0.87	0.08

[A]Born from non-supplemented (CTRL) or supplemented (SUP) cows. SUP cows received supplementation during middle (MID) or late gestation (LATE);
[B]P-values for the main effect of treatment (Main effect), and the two pre-determined orthogonal contrast: between non-supplemented and supplemented cows (CRTL x SUP), and between cows supplemented at mid or late gestation (MID x LATE).

could justify the lack of differences in blood lipid content.

Altogether, these results indicate differences in the blood and muscle of calves born to SUP and CTRL cows independent of supplementation time. It seems that calves born to CTRL cows had an increase of oxidative metabolism during fetal development to cope with the nutrient restriction. However, these calves had impaired metabolism flexibility to return to glycolytic fibers postnatally when energy was available. This is supported by changes in the expression of protein involved in energy metabolism.

CONCLUSION

In this study, we were able to show changes in the abundance of a protein involved in the skeletal muscle energy metabolism of calves born to cows receiving different level of protein during the pregnancy. These changes are likely associated with a metabolism inflexibility in the skeletal muscle of calves born to the group without supplementation, reflecting the inability of this group to re-adapt to nutritional changes after birth. This study showed the importance of protein supplementation during pregnancy in tropical beef production systems to avoid metabolism disorders.

BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

This study was approved by the Brazilian Ethics Committee on Animal Use (CEUAP/UFV – process no. 26/2014), according to ethical principles of animal experimentation established by the National Council of Animal Experimentation Control (CONCEA).

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

Conceptualization: MSD and MPG; Methodology: L.M.P.S., W.S., and D.E.C.M.; Data Analysis: L.M.P.S. and N.V.L.S.; Resources: M.S.D. and N.V.L.S.; Writing: L.M.P.S.; Review and editing: L.M.P.S., M.M.S.F., T.C.C., N.V.L.S., M.P.G., M.S.D.

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