UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ZOOTECNIA E ENGENHARIA DE ALIMENTOS

FERNANDO JOSÉ SCHALCH JÚNIOR

Fetal programming in beef cattle: isotopic, metabolomic and phenotypic effects

Pirassununga
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Dissertação apresentada à Faculdade de Zootecnia e Engenharia de Alimentos da Universidade de São Paulo, como parte dos requisitos para a obtenção do título de Mestre em Zootecnia.

Área de Concentração: Qualidade e Produtividade Animal

Orientador: Prof. Dr. Arlindo Saran Netto

Coorientador: Prof. Dr. Miguel Henrique de Almeida Santana

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UNIVERSIDADE DE SÃO PAULO
Faculdade de Zootecnia e Engenharia de Alimentos
Comitê de Ética em Pesquisa da FZEA

CERTIFICADO

Certificamos que a proposta intitulada "Fenômica da Programação Fetal na Produção de Bovinos de Corte: Uma Abordagem Sistêmica ", protocolada sob o CEUA nº 1843241117, sob a responsabilidade de **Miguel Henrique de Almeida Santana** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Zootecnia e Engenharia de Alimentos da Universidade de São Paulo - FZEA/USP (CEUA/FZEA) na reunião de 09/03/2018.

We certify that the proposal "Fenomics of Fetal Programming Effects on Beef Cattle Production: A Systemic Approach ", utilizing 250 Bovines (males and females), protocol number CEUA 1843241117, under the responsibility of **Miguel Henrique de Almeida Santana** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Animal Science and Food Engineering - (São Paulo University) (CEUA/FZEA) in the meeting of 03/09/2018.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 12/2017 a 07/2020 Área: Bovinocultura de Corte

Origem:	Prefeitura do Campus da FZEA da USP						
Espécie:	Bovinos	sexo:	Machos e Fêmeas	Idade:	0 a 6 anos	N:	250
Linhagem:	Nelore			Peso:	30 a 550 kg		

Resumo: Indicações de que perturbações em fases críticas da vida pré e pós-natal causam efeitos permanentes na saúde e produtividade na vida dos mamíferos, conhecido como programação fetal (PF), não são novidade na literatura científica. Porém, ainda há muito a estudar quanto aos mecanismos desses estímulos/deficiências nutricionais durante a gestação, em especial a importância quantitativa do fenômeno em relação aos seus efeitos na eficiência produtiva dos bovinos. No caso específico do Brasil, onde predomina a raça Nelore, a partir do terço médio da gestação, ocorre um déficit nutricional nesta época porque as pastagens já não suprem quali-quantitativamente os nutrientes necessários para a vaca. Adicionalmente, no terço final da gestação, quando há uma demanda maior do feto, o déficit nutricional é agravado pela estação seca. No presente projeto serão estudados os efeitos fisiológicos, morfológicos e econômicos do estímulo nutricional durante a gestação de vacas Nelore sobre a reprodução, desempenho, ingestão, eficiência alimentar, características de carcaça e qualidade de carne de suas progênies de forma sistêmica. Serão usadas 150 vacas gestantes de um único touro. Durante a gestação ? das matrizes receberão suplementação proteico-energética por toda gestação, outro ? das matrizes somente no terço final e ? não receberão esse estímulo nutricional. As vacas e suas crias serão avaliadas nas fases de cria, recria e terminação pela biologia de sistema via diversas avaliações fisiológicas, morfológicas e por abordagens ômicas (nutrigenômica, genômica, transcriptômica, proteômica e metabolômica). Complementarmente, a viabilidade econômica da PF será avaliada na venda do bezerro à desmama e na renda obtida ao abate. Os resultados desse projeto ajudarão a determinar os mecanismos fisiológicos da PF por todo ciclo de produção de bovinos de corte, além fomentar o desenvolvimento de novas abordagens de avaliação que poderão impactar positivamente em todas as esferas dessa cadeia produtiva.

Local do experimento: Piquetes de pastagem, Confinamento e Abatedouro

Pirassununga, 10 de março de 2018

Profa. Dra. Daniele dos Santos Martins
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DEDICATÓRIA

Desenvolver o mestrado sempre foi um sonho. Desenvolver esse sonho e ao mesmo tempo manter a rotina de trabalho só foi possível graças ao apoio de minha família, dos meus colgas de trabalho e dos amigos da universidade. Portanto, este trabalho é dedicado à minha esposa Nadia Obrownick Okamoto Schalch e aos meus filhos Carlos Eduardo Okamoto Schalch e Maria Fernanda Okamoto Schalch, em nome de todos que de maneira direta e indireta contribuíram para sua realização.

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“Os grandes navegadores devem sua reputação aos temporais e tempestades”. (Epicuro)

RESUMO

SCHALCH JUNIOR, F. J. **Programação fetal em gado de corte: efeitos isotópicos, metabolômicos e fenotípicos**. 2022. 74 f. Dissertação (Mestrado) – Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga/SP, 2022.

Com o crescente aumento populacional, os avanços tecnológicos e produtivos são pré-requisitos para que a oferta de alimentos consiga suprir a demanda. Neste contexto, abordagens avaliando os efeitos nutricionais pré-natais em bovinos sobre o desempenho produtivo e reprodutivo, metabolismo, produção de carne e desenvolvimento muscular têm sido estudadas com o intuito de otimizar o ciclo produtivo da espécie. Dessa forma, esta dissertação teve por objetivo avaliar os fenótipos, fracionamento isotópico de nitrogênio e o metaboloma do plasma sanguíneo das vacas e bezerros submetidos à diferentes estímulos nutricionais pré-natais. Para a realização destes experimentos foram utilizadas 126 matrizes da raça Nelore que foram divididas igualmente em três diferentes abordagens nutricionais durante a gestação (NP, PP e FP). O tratamento NP (controle) recebeu apenas a suplementação mineral (0,03% do peso corporal) durante toda a gestação; o tratamento PP recebeu a suplementação mineral (0,03% do peso corporal) durante o primeiro e segundo terço da gestação e suplementação mineral proteico-energética durante o terço final da gestação (0,3% do peso corporal); e o tratamento FP recebeu a suplementação mineral proteico-energética (0,3% do peso corporal) durante toda a gestação. A partir do nascimento dos animais, os tratamentos foram cessados e todos os animais (vacas e bezerros) foram submetidos às mesmas condições ambientais, nutricionais e sanitárias. Durante o período gestacional e após o nascimento dos bezerros, os fenótipos e amostras de sangue do par vaca-bezerro foram coletados para analisar os efeitos das diferentes estratégias nutricionais aplicadas. No primeiro capítulo foi realizado a avaliação dos efeitos fenotípicos causados pela nutrição pré-natal nas vacas (peso corporal, escore de condição corporal, espessura de gordura subcutânea) e nos bezerros (peso corporal de machos e fêmeas). Neste capítulo foi possível observar que a nutrição alterou o peso corporal, escore de condição corporal e espessura de gordura das vacas e apresentou influência sobre o peso dos bezerros machos ao nascimento e ao longo do tempo. Entretanto, os diferentes estímulos

maternais não tiveram efeito sobre o peso corporal das progênies fêmeas. No capítulo 2 foi avaliado o fracionamento de nitrogênio (15N/14N) no plasma sanguíneo de 15 vacas (selecionadas aleatoriamente) no início, no final da gestação e no pós-parto e da progênie aos 30 e 180 dias de idade. Inicialmente as vacas não apresentaram diferenças na abundância de 15N, entretanto após receberem os diferentes tratamentos foram observadas diferenças no fracionamento isotópico de nitrogênio entre os grupos no pré-parto e pós-parto. Aos 30 dias de idade dos bezerros foi observado efeitos da nutrição pré-natal sobre os níveis de nitrogênio isotópico, mas isso não se repetiu aos 180 dias de idade. No terceiro capítulo, foi avaliado o metaboloma do plasma sanguíneo das matrizes no início da gestação e no pré-parto, e o metaboloma plasmático dos bezerros aos 30 dias de idade. Encontramos efeitos metabólicos nas vacas em todas as coletas analisadas e alguns metabólitos diferencialmente expressos nos bezerros. Além disso, também encontramos indícios de efeitos epigenéticos relacionados ao metabolismo de histidina e de beta-alanina. Em suma, esta dissertação traz novas abordagens e resultados para o campo da nutrição pré-natal em gado de corte, o que pode contribuir significativamente para o entendimento de parte das respostas fenotípicas e moleculares ainda discutidas na literatura.

Palavras-chave: Fenótipos. Gado de corte. Isótopos de nitrogênio. Metabolômica. Progênie. Suplementação pré-natal.

ABSTRACT

SCHALCH JUNIOR, F. J. **Fetal programming in beef cattle: isotopic, metabolomic and phenotypic effects**. 2022. 74 f. Dissertation (Master's degree) – Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga/SP, 2022.

With the growing population, technological and productive advances are prerequisites for the supply of food to be able to meet the demand. In this context, approaches evaluating the prenatal nutritional effects on productive and reproductive performance, metabolism, meat production and muscle development in cattle have been studied in order to optimize the productive cycle of the species. Thus, this thesis aimed to evaluate the phenotypes, isotopic nitrogen fractionation and the blood plasma metabolome of cows and calves submitted to different prenatal nutritional stimuli. In order to carry out these experiments, 126 Nellore cows were used, which were divided equally into three different nutritional approaches during pregnancy (NP, PP and FP). The NP treatment (control) received only mineral supplementation (0.03% of body weight) throughout pregnancy; the PP treatment received mineral supplementation (0.03% of body weight) during the first and second third of pregnancy and protein-energy mineral supplementation during the final third of pregnancy (0.3% of body weight); and the FP treatment received protein-energy mineral supplementation (0.3% of body weight) throughout pregnancy. From calving, the treatments were stopped and all the animals (cows and calves) were submitted to the same environmental, nutritional and sanitary conditions. During the gestational period and after the calving, the phenotypes and blood samples of the cow-calf pair were collected to analyze the effects of the different nutritional strategies applied. In the first chapter, the evaluation of the phenotypic effects caused by prenatal nutrition in cows (body weight, body condition score, subcutaneous fat thickness) and in calves (body weight of males and females) was carried out. In this chapter it was possible to observe that nutrition altered body weight, body condition score and fat thickness of cows and had an influence on the weight of male calves at birth and over time. However, the different maternal stimuli had no effect on the body weight of female offspring. In chapter 2, the fractionation of nitrogen (15N/14N) in the blood plasma of 15 cows (randomly selected) was evaluated at the beginning, at the end of gestation and postpartum and in the progeny at 30 and 180 days

of age. Initially, the cows did not show differences in the abundance of 15N, however, after receiving the different treatments, differences in the isotopic nitrogen fractionation were observed between the groups in the pre-calving and post-calving periods. At 30 days of age of calves, effects of prenatal nutrition on isotopic nitrogen levels were observed, but this was not repeated at 180 days of age. In the third chapter, the blood plasma metabolome of the dams at the beginning of pregnancy and prepartum, and the plasma metabolome of the calves at 30 days of age were evaluated. We found metabolic effects in cows in all analyzed collections and some metabolites differentially expressed in calves. In addition, we also found evidence of epigenetic effects related to histidine and beta-alanine metabolism. In short, this thesis brings new approaches and results to the field of prenatal nutrition in beef cattle, which can significantly contribute to the understanding of part of the phenotypic and molecular responses still discussed in the literature.

Keywords: Beef cattle. Metabolomics. Nitrogen isotopes. Phenotypes. Prenatal supplementation. Progeny.

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LIST OF ABBREVIATIONS AND ACRONYMS

ADG – Average daily gain

ATP – Adenosine triphosphate

BCS – Body condition score

BFT – Backfat thickness

BW – Body weight

Cis-eQTL – Variants located within 1Mb of the associated gene

CP – Crude protein

DNA – Deoxyribonucleic acid

FDR – False discovery rate

FP – Full programming

FTAI – Fixed-time artificial insemination

IGF-I – Insulin-like growth fator-1

MCA – Muscle cell area

NCREA – Number of cells in ribeye area

NDF – Neutral detergent fiber

NP – Not programmed

PP – Partial programming

REA – Ribeye area

RFT – Rump fat thickness

SNP – Single nucleotide polymorphism

TDN – Total digestible nutrients

Trans-eQTL – Variants located farther than 1Mb from the associated gene

UGC – Ultrasound Guidelines Council

SUMMARY

1 Introduction

The demand for food is constantly increasing, especially in emerging countries such as Brazil. Since 2009, FAO has been warning about the need to increase production in line with demand. This increase in food consumption could reach 70% by the year 2050 and raise the demand for meat from 200 million to 470 million tons per year (FAO, 2009). Thus, the need to develop new technologies to optimize agricultural and livestock productivity is crucial for the nutrition of the next generations. In the year 2021, Brazil showed an increase in the average carcass weight of 1.43% in males and 2.63% in females compared to 2020. Although it showed a 1.1% decrease in productivity in the year 2021, Brazil is still remains the largest exporter and second largest producer in the world (ABIEC, 2022). In addition, it has the second largest herd in the world with 264.1 million head (USDA, 2022). Of this herd, about 80% are *Bos indicus* animals and of these, 90% are Nellore or crossbred (ABCZ, 2022). Most of these animals are on pasture, in extensive production. In this system, the animals stay in large areas of pastures throughout the year, and thus the climatic effects (lack of rain, high temperatures) have a direct influence on the productivity of the animals (SILVA; NASCIMENTO JÚNIOR, 2007). These factors interfere in the development of forages, leading to a seasonal variation in the edible portion of the pasture and affecting the Brazilian beef cattle production system (PAULINO, 2004). The direct impact on the nutrition of cattle present in this environment is relevant mainly in pregnant cows that have low biological efficiency and productivity (CALEGARE; ALBERTINI; LANNA, 2010).

According to Moreira et al. (2019), fetal programming is a concept that encompasses adaptation mechanisms in which nutrition during the gestational period and the uterine environment influence fetal development, which can lead to permanent phenotypic changes in the progeny. However, the concept of fetal programming goes beyond just nutritional stimuli, being pointed out in several studies as any process that has an influence on the pregnant female and can have consequences for the fetus and in the long term in the progeny (BARKER et al., 1993; LUCAS; FEWTRELL; COLE, 1999; ABRUZZESE et al., 2018).

According to Long et al. (2009) and Wu et al. (2006), maternal malnutrition in ruminants can lead to some consequences such as: increased neonatal mortality rate, change in body composition and growth, hormonal balance, modification in the

development and functioning of organs, including the respiratory, cardiovascular and intestinal systems and metabolic functions. Du et al. (2011) showed in cattle that there is a relationship between maternal nutritional deficiency and poor fetal development and growth. In addition, Funston e Summers (2013) pointed out that the adequate supply of nutrients to the pregnant cow results in the adequate formation of the placenta, and thus, there is transfer of nutrients to the fetus. However, even if the cow is receiving a high quality diet, some genetic, metabolic and autoimmune diseases can hinder the passage of nutrients from the placenta to the conceptus, resulting in fetal underdevelopment (CETIN et al., 2004). Still, according to Martin et al. (2007) maternal nutrition can also influence the reproductive performance of the progeny, showing that heifers from cows supplemented at the end of pregnancy had higher post-weaning weight and fertility compared to females from cows that did not have supplementation during pregnancy. Other researches have also demonstrated the reproductive and productive effects on progenies (male and female) arising from different prenatal nutritional levels (BOLLWEIN; JANETT; KASKE, 2016; MOSSA et al., 2018; JOHNSON et al., 2019; CRACCO et al., 2021; POLIZEL et al., 2021a, 2021b; SILVA et al., 2021).

Currently, there is a large amount of work being published related to fetal programming in beef cattle, but most of them are concentrated in experiments developed in North America and presenting inconclusive results between studies (ZAGO; CANOZZI; BARCELLOS, 2019). The same authors also state that these divergences may be related to the different diets, breed and gestational periods evaluated, allowing new studies to be carried out so that convergent results are obtained and, consequently, the molecular mechanisms behind fetal programming are determined.

In this dissertation we have elaborated three chapters in article form. The first shows the effects of different prenatal supplementation strategies on cow performance (body weight, subcutaneous fat thickness and body condition score) and progeny body weight according to sex. In the second chapter, we discuss the effect of fetal programming on the isotopic fractionation of nitrogen in the blood plasma of cows (early gestation, pre-partum and postpartum) and their respective progenies (30 days and 180 days of age). The third chapter evaluated the effects of different prenatal nutrition strategies on the metabolome of cows (beginning of pregnancy and in pre-delivery) and calves (30 days of age). In summary, this dissertation contributes

significantly to a better understanding of the phenotypic and molecular effects of prenatal nutrition in beef cattle.

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2 Chapter 1: Different prenatal supplementation strategies: Impacts on performance of dams and body weight of their offspring

Abstract

The effects of maternal nutrition on beef cows' performance and the long-term consequences for the offspring in tropical regions are still not well understood. The literature still presents conflicting results and more studies that seek to assess the effects of fetal programming are needed. This study evaluated the effects of three levels of supplementations (NP, PP and FP) on Nellore cows and its consequences on the offspring. The dams were evaluated for body weight (BW), subcutaneous fat thickness (SFT), and body condition score (BCS) upon pregnancy diagnosis, middle third of pregnancy, pre-delivery, and in the postpartum period. Calves were evaluated for birth weight, at 30, 120, 180, and 210 days of age on average. All phenotypes were subjected to an analysis of variance. The BW, SFT, and BCS showed significant differences in the middle third of pregnancy and pre-delivery. In the postpartum period, only SFT showed difference between the groups. The birth weight and from birth-to-weaning in male calves was responsive to the different prenatal treatments; however, female progenies showed no difference in BW between the groups. These results show that under the same prenatal stimulus, male calves and female calves show different responses. In addition, more studies should be done to unravel the mechanisms that involve this different type of responses between sexes.

Keywords: Body condition score. Body weight. Fetal programming. Maternal nutrition. Subcutaneous fat thickness.

2.1 Introduction

The most important environmental factor for efficiency in livestock production is nutrition, which can determine the productive success of the cow-calf pair (DA SILVA et al., 2017). Seasonal factors in tropical regions influence pasture production and thus affect beef cattle, mainly pregnant cows, which often spend the middle and final thirds of gestation in the dry season (FERREIRA et al., 2020; SAMPAIO et al., 2010). Therefore, the concept of fetal programming presents as a challenge for

ruminants during a critical time window, which can influence their development (NATHANIELSZ et al., 2007).

Several physiological adaptations occur during pregnancy. The maternal cardiovascular system changes during pregnancy, decreasing blood pressure and vascular resistance, and increasing cardiac output, heart rate, heart stroke volume, and blood volume (MAGNESS, 1998). Adequate blood supply is necessary to protect the dam and the fetus from the deleterious effects of a reduction in venous blood return and cardiac output (TORGERSEN; CURRAN, 2006). Placenta in cows progressively develops throughout pregnancy (FUNSTON et al., 2010), with an increase in the cotyledonary tissue weight of approximately 650% and caruncular tissue of 530% until the pregnancy end (VONNAHME; LEMLEY, 2012). These changes allow fetus development and may be influenced by environmental factors, such as nutrition.

Mulliniks et al. (2013) demonstrated that protein supply to dams at the pregnancy end positively affected progeny health in the finishing phase. The reproductive performance of the offspring also improves in terms of weight, muscular growth, marbling, meat, and fetal development (FUNSTON; SUMMERS, 2013). Furthermore, nutritional stimulation during gestation can affect the cow, especially primiparous females, as they require nutrients for their maintenance, reproduction, lactation, and growth (SPITZER et al., 1995). This study investigated the effects of protein-energy mineral supplementation at different pregnancy stages on the body condition score (BCS), body weight (BW) and subcutaneous fat thickness (SFT) of Nellore cows and the offspring BW. Thus, we hypothesized that these different maternal nutrition approaches can impact on the dams' performance and on the offspring BW.

2.2 Material and methods

2.2.1 Experimental design

The study comprised Nellore cows (n = 126) and their progenies. The dams were fixed time artificially inseminated (FTAI) with semen from four breeders with known genetic values and had their pregnancy diagnosis confirmed 30 days later.

The cows were divided into three groups of 42 animals based on age, BW and BCS (group selection criteria). All groups were allocated to pasture paddocks of *Urochloa brizantha* (Syn. *Brachiaria brizantha*) cv. Marandu, equipped with a trough

to supply food supplement and water. The nutritional plans were: NP – Not Programmed, PP – Partial Programming, and FP – Full Programming. All treatments received mineral supplementation (0.03% of BW), but only PP and FP received protein-energy mineral supplementation equivalent to 0.3% of the average BW per day during pregnancy. The PP group was submitted to this nutritional protocol in the final third of gestation, whereas dams in FP had supplementation upon pregnancy confirmation until calving (Table 1).

Table 1 - Ingredients and nutrients content of the dams' supplement

Ingredients/Nutrients	**Mineral supplement**	**Protein-energy supplement**
Corn (%)	35.00	60.00
Soybean meal (%)	-	30.00
Dicalcium phosphate (%)	10.00	-
Urea 45% (%)	-	2.50
Salt (%)	30.00	5.00
Minerthal 160 MD (%)*	25.00	2.50
Total digestible nutrients (%)	26.76	67.55
Crude protein (%)	2.79	24.78
Non-protein nitrogen (%)	-	7.03
Acid detergent fiber (%)	1.25	4.76
Neutral detergent fiber (%)	4.29	11.24
Fat (%)	1.26	2.61
Calcium (g/kg)	74.11	6.20
Phosphorus (g/kg)	59.38	7.24

*Mineral premix composition (Minerthal company): Calcium = 200 g/kg; Cobalt = 160 mg/kg; Copper = 2,700 mg/kg; Sulfur = 60 g/kg; Fluorine = 1,600 mg/kg; Phosphorus = 160 g/kg; Iodine = 135 mg/kg; Manganese = 2,700 mg/kg; Selenium = 80 mg/kg; Zinc = 8,100 mg/kg; Sodium monensin = 4,000 mg/kg.

After calving, protein-energy mineral supplementation ceased and all progenies (regardless of the nutritional prenatal treatment and sex) were subjected to the same health and vaccination protocols and nutritional managements, remained together until weaning (210 ± 28 days). During this period (calving to weaning), the cows received the same mineral supplement (0.03% of BW) that have received during pregnancy period.

2.2.2 Bromatological analysis of pastures

Forages were sampled by collecting five areas of 1 m² in each paddock at random, avoiding areas with feces and invasive plants. The five samples were homogenized and a single 300-gram sample was obtained. Samples to determine dry matter (DM) were oven dried by forced air ventilation at 65 °C for 72 h (SILVA; QUEIROZ, 2009) and later ground in a 2-mm sieve for the bromatological and mineral analyses. The pasture morphological composition was determined by separating leaf material, stem, and dead material, which were dried afterward. This allowed estimating feed consumption and characterizing pasture conditions of each treatment (Table 2).

Table 2 - Availability and botanical composition of forage for dams during all gestational period (mean ± standard deviation)

Forage availability	**NP**	**PP**	**FP**
Pasture Availability (kg DM/ha)	3,476 ± 1,594	4,597 ± 1,189	5,578 ± 2,049
Leaf Availability (kg DM/ha)	573 ± 340	569 ± 485	727 ± 643
Thatch Availability (kg DM/ha)	562 ± 396	799 ± 545	1,347 ± 1,038
Dead material availability (kg DM/ha)	2,340 ± 1,275	3,229 ± 973	3,503 ± 1,410
Stocking Rate (AU/ha)	2.19 ± 1.02	1.74 ± 0.56	2.26 ± 0.99
Leaf supply for animal unit (KG/DM)	316 ± 216	359 ± 245	366 ± 306

NP – not programmed; PP – partial programming; FP – full programming.

Table 3 - Nutrients in pastures consumed by dams in the different groups (mean ± standard deviation)

Forage nutrients	**NP**	**PP**	**FP**
CP % (crude protein)	7.38 ± 1.72	7.82 ± 2.28	7.40 ± 2.30
TDN % (total digestible nutrients)	63.1 ± 1.45	64.1 ± 2.33	61.4 ± 2.12
NDF % (neutral detergent fiber)	59.0 ± 3.67	61.4 ± 5.05	58.4 ± 4.11
Ca % (calcium)	0.38 ± 0.11	0.35 ± 0.05	0.39 ± 0.08
P % (phosphorus)	0.19 ± 0.03	0.19 ± 0.03	0.17 ± 0.03

NP – not programmed; PP – partial programming; FP – full programming

The bromatological and mineral analyses (Table 3) were conducted at the bromatology and mineral laboratory at the University. Crude protein was determined by

the methodology of Silva and Queiroz (2009), neutral detergent fiber (NDF) by Van Soest (1995, 1967), and abundance of minerals was determined by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES), according to Sindirações (2013).

2.2.3 Phenotypic data from the cows

Collection of phenotypic data started shortly after pregnancy diagnosis (FTAI) of the dams. The dams were monthly contained in trunks equipped with electronic scales for weighing and ultrasound evaluation of the animals. The SFT was assessed at the intersection of the Biceps femoris muscle with the Gluteus medius between the ileum and ischial bones using an Aloka SSD-500 ultrasound equipped with a 17 cm linear transducer at a frequency of 3.5 MHz (Aloka Co. Ltd., Wallingford, CT, USA). Vegetable oil was used as a coupling to optimize contact of the transducer with the skin of the animal. The body condition score (BCS) was performed in the FTAI and pre-delivery (initial and final, respectively) on a scale with values from 1 to 9, according to Richards et al. (1986).

2.2.4 Measuring offspring BW

Male (n = 63) and female (n = 58) animals were evaluated. Collection of phenotypic data on birth weight began within 24 hours after the calves were born and were obtained using an iron rod steelyard still in the maternity paddock. To evaluate the BW at 30, 120, 180, and 210 days of age on average, the animals were taken to a corral and weighed on an electronic scale (Coimma®, Brazil), allowing for individual BW.

2.2.5 Statistical analysis

The data were subjected to an analysis of variance (ANOVA) in the MIXED procedure of the SAS® OnDemand for Academics software. The residues were tested for normality (Shapiro-Wilk test) and for homogeneity of variance (Levene's test). For the cows' phenotypes, their ages and the treatments were considered in the linear model. For the BW in calves, the sires, the ages of dams, the treatments and the calves' age were considered in the linear model. The analysis and the differences between treatments were considered significant when P value ≤ 0.05 and tendency when $0.05 <$

P value ≤ 0.10 by the Tukey Kramer test. Data on progenies (males and females) were analyzed separately to assess the effects of fetal programming on BW in different sexes and throughout time (repeated measures over time; covariance structure).

2.3 Results

2.3.1 Phenotypic traits of dams

The initial BW did not show any significant difference (Table 4). However, BW in the middle third of gestation and the pre-delivery showed a significant difference (P value < 0.01), with BW values in the FP group higher than in the others. Postpartum BW had no difference between the groups (P value = 0.13). There were no differences in SFT in the early and middle third of gestation (Table 4) and pre-partum FP presented a higher SFT than the other treatments (P value < 0.01). In the postpartum period, FP showed a higher SFT than NP (P value = 0.04); however, PP did not show any difference from the others. The initial BCS was not different between treatments (P value = 0.34; Table 4). In the pre-delivery period, FP obtained a higher BCS than the NP (P value = 0.04) and the PP group did not show any difference between the other two treatments.

Table 4 - Average of body weight, subcutaneous fat thickness and body condition score ± standard deviation of Nellore cows submitted to fetal programming with their respective *P* values (*P*)

Traits	**NP**	**PP**	**FP**	***P***
Body weight (kg)				
Initial	461±45	451±61	454±57	0.85
Middle third	490±55[a]	493±64[a]	516±73[b]	<0.01
Pre-delivery	508±47[a]	524±59[a]	541±66[b]	<0.01
Postpartum	503±45	502±59	518±62	0.13
Subcutaneous fat thickness (mm)				
Initial	4.28±4.0	4.31±4.0	4.33±4.0	0.92
Middle third	6.33±4.6[a]	6.87±4.3[a]	9.35±5.7[b]	<0.01
Pre-delivery	7.23±4.3[a]	9.24±4.4[a]	12.5±6.4[b]	<0.01
Postpartum	9.77±5.4[a]	11.4±5.5[ab]	12.6±6.5[b]	0.04
Body condition score				
Initial	4.5±0.6	4.6±0.8	4.5±0.6	0.34
Pre-delivery	5.4±0.9[a]	5.6±0.9[ab]	5.9±0.9[b]	0.04

NP – not programmed; PP – partial programming; FP – full programming

2.3.2 Progeny' performance

The BW of female progenies showed no significant differences between the different prenatal treatments received in all evaluations (birth to weaning) (Table 5). On the other hand, male animals presented a significant difference of birth weight between treatments FP and NP (P value = 0.05). The analysis of repeated measurements over time showed a significant difference between FP and PP in relation to NP (P value < 0.01), presenting greater BW in progeny males from cows that received protein-energy mineral supplementation during pregnancy (Table 5).

Table 5 - Average body weight (BW; kg) ± standard error of calves (male and female) from different prenatal treatments (NP, PP and FP) with the respective *P* values (P^1 and P^2)

Body weight (kg)	Female					Male				
	NP	**PP**	**FP**	***P*¹**	***P*²**	**NP^A**	**PP^B**	**FP^B**	***P*¹**	***P*²**
Birth	35.9±1.1	34.1±1.0	36.0±0.8	0.72		35.1±0.8^a	37.0±0.5^{ab}	37.1±0.6^b	0.05	
30 days	69.8±3.6	69.6±3.6	65.6±3.1	0.53		68.4±3.3	68.8±2.6	73.3±3.5	0.49	
120 days	148±3.6	144±3.6	141±4.5	0.71	0.37	145±4.2	156±3.7	154±4.8	0.15	<0.01
180 days	190±4.9	184±4.4	183±5.4	0.89		190±5.3	199±4.1	198±5.5	0.33	
210 days	216±4.4	210±4.7	208±5.4	0.78		219±5.8	231±5.3	231±4.9	0.15	

The different capital letters in superscripts in the male treatments correspond to the significant contrasts presented by P^2 (*P* value corresponding to the contrasts between the different treatments regardless of the collection; repeated measures over time). The different lowercase letters represent the significant contrasts between treatments in the same evaluation (P^1).

2.4 Discussion

Stalker et al. (2006) and Warner et al. (2011) found differences on performance between animals that received prenatal supplementation and those that did not, similar to the results for animals in the FP group in our study that increased BW, BCS, and SFT throughout pregnancy in relation to the other treatments.

According to Melo (2018), supplementation during pregnancy can increase cow BW, but with no effect in the postpartum period. However, regarding SFT, the postpartum analysis showed that the FP cows managed to maintain an energy reserve higher than those the NP, favoring thus growth, maintenance, reproduction, and breastfeeding of primiparous dams (SPITZER et al., 1995) and possibly shortening the interval between deliveries (FORDYCE et al., 1997). Differences observed in BCS and SFT can positively affect fetal development, the reproductive capacity of dams for the following breeding season and have long-term effects on the offspring (DU et al., 2011), especially on tropical regions.

Some recent studies have evaluated the effects of energy-protein mineral supplementation in pregnant Nellore cows (BRASIL et al., 2021; FERREIRA et al., 2020; MOURA et al., 2020); however, due to the large number of variables (supplementation period and level, pasture quality and other environmental characteristics) that may influence animal responses, the results show inconsistent effects. In addition, this is the first study to evaluate these three approaches of prenatal supplementation (NP, PP and FP) on cows and the effect of sex on their progenies.

Many authors have reported differences of birth weight of calves from cows subjected to different prenatal stimuli (CORAH et al., 1975; FREETLY et al., 2000; FUNSTON et al., 2010; LONG et al., 2009; MICKE, SULLIVAN, SOARES MAGALHAES, et al., 2010; SPITZER et al., 1995); nevertheless, different responses between offspring sexes are not well understood and still poorly reported.

In our study, females and males born from cows submitted to the same prenatal nutritional stimulus presented no different responses in BW at birth and in the period between birth and weaning. According to Palou et al. (2010), rats subjected to different caloric levels during pregnancy demonstrated differences in the feeding behavior and circulating leptin levels between genders when compared to control. Thone-Reineke et al. (2006) reported that blood pressure, feed efficiency, and BW had different responses between the sexes of rats subjected to high protein intake during pregnancy and

lactation. Dearden et al. (2018) concluded that most of the phenotypic/metabolic differences that occur between human progenies sex are related to the greater susceptibility of the placenta of the male progeny to undernutrition or overnutrition. Some recent studies have been trying to understand the specific sex responses presented by progenies submitted to the same prenatal stimulus (DIAS-ROCHA et al., 2018; PAYEN et al., 2021; RIVERA et al., 2022; ROBB et al., 2017; SAOI et al., 2020). However, few studies have been conducted on ruminants, which may have different effects, as their placental anatomy is different from that of monogastric animals.

In cattle, offspring sex responds to the prenatal nutritional stimulus differently in relation to BW, height and carcass traits (MICKE, SULLIVAN, GATFORD, et al., 2010). In addition, metabolic and epigenetic differences occur in the placenta between different sexes under the same gestational stimuli (BATISTEL et al., 2019), which possibly explains partly the effect of offspring sex on their performance. According to Rodríguez-Rodríguez et al. (2015), female progenies are more resilient to oxidative stress caused by prenatal undernutrition, with less impact from gestational insult. These differences may be attributed to the role of sex hormones and other innate sexual differences (OJEDA et al., 2014); however, they have not yet been fully elucidated in the literature.

We have assessed the different BW responses between the sexes of the progenies, however the molecular mechanisms need to be clarified. We encourage more studies to find the epigenetic mechanisms related to placentation and sex specific susceptibility in ruminants.

2.5 Conclusion

The supplementation during all gestational period improved the BW and pre-partum BCS of cows and SFT in the pre and post-partum. Female offspring showed no difference in BW, while males showed a response to the different prenatal supplementation strategies in birth weight and in the birth-to-weaning period. In summary, our results show that response to maternal nutrition in beef cattle varies according to progeny sex.

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3 Chapter 2: Maternal nutrition affects nitrogen isotopic fractionation in blood plasma of beef cattle dams and their offspring

Abstract

This study evaluated the effects of gestational supplementation strategy on nitrogen isotopic fractionation in blood plasma of beef cows and their progeny. The study comprised 126 pregnant Nellore cows divided into three different supplementation protocols: non-programmed group (NP) did not receive protein-energy mineral supplement; partially programmed group (PP) received protein-energy mineral supplement only in the final third of pregnancy; and full programmed group (FP) supplemented with mineral, protein and energy during all the gestational period (from confirmation of pregnancy to calving). We collected blood plasma from 15 dams randomly at the beginning of gestation, in the pre-partum, and post-partum periods as well as from their calves at 30 and 180 days of age for the analysis of stable isotope ratios 15N/14N. The analysis of variance was performed for the traits above and measurements were repeated over time. Abundance of 15N isotope (δ15N) from the dams did not differ significantly at the beginning of pregnancy. However, PP and FP values differed from NP ($p<0.05$) at the pre-partum and all three groups showed significant differences ($p<0.05$) in the postpartum period. The δ15N values of calves at 30 days of age differed significantly between the NP group and PP and FP groups ($p<0.05$). No difference was found between the groups ($p>0.05$) at 180 days. The different gestational supplementation strategies influenced isotopic fractionation of nutrients of cows and their calves after birth, indicating effects on nutritional metabolism and cumulative behavior on isotope abundance related to consumption during gestation.

Keywords: Fetal programming. Molecular effects. Nellore. Prenatal nutrition. Stable isotopes.

3.1 Introduction

In Brazil, beef cattle are usually produced in extensive systems, mainly the cow-calf phase, an important factor due to the large territorial extension and annual

seasonality of climate and environment. Therefore, changes in rainfall and temperatures along the year directly affect production and nutritional quality of forages (EUCLIDES FILHO et al., 2010).

In this shifting scenario of food supply, dams can suffer from variations in forage production and, throughout pregnancy, have different nutritional needs. The middle and late thirds of the pregnancy period usually coincide with dry season in Brazil and this phase accounts for 75% of fetal growth (ROBINSON et al., 1977).

Nutrient deficiency during pregnancy can hinder fetal development and growth, delay progeny growth, reduce feeding efficiency, and affect body composition, reducing meat quality (WU et al., 2006; DU et al., 2010; DUARTE JÚNIOR et al., 2016). Fetal programming is a process of stimuli, negative or positive, for pregnant cows that influences nutrition of the fetus and its postnatal life (BARKER, 1990; FUNSTON; SUMMERS 2013).

The relationship of isotopic values of tissues, blood, and others allows demonstrating food uptake variation in animals. Ingested foods have a higher 15N/14N ratio than feces, showing that 14N is more eliminated in the digestive process of mammals. Therefore, animal tissues tend to be rich in 15N values than the diets (STEELE; DANIEL 1978; SUTOH et al., 1987).

Studies on stable isotopes use wild species to assess their eating habits and even compare the feeding habits of offspring with their mothers (ELORRIAGA-VERPLANCKEN et al., 2016; FRANKEL et al., 2012; JENKINS et al., 2001; POLISCHUK et al., 2001). Feed uptake of offspring reflects the maternal habit, since progenies are dependent consumers and need breastfeeding. This progeny-mother relationship showed that δ15N in tissues of offspring simulated a predator (progeny) - prey (mother) relationship, closely related to newborns that feed almost exclusively on breast milk. (AURIOLES et al., 2006; AURIOLES-GAMBOA et al., 2009; ELORRIAGA-VERPLANCKEN et al., 2013; NEWSOME et al., 2006).

The stable isotope analyses should take into account physiological and biochemical effects, as they influence isotopic values between tissue types and individuals (GANNES et al., 1998; VIEIRA JÚNIOR et al., 2012). Environmental conditions, such as fetal programming, can cause variability in the isotopic N composition in animals within the same population (BAHAR et al., 2005), especially ruminants (TIESZEN, 1991; VIEIRA JÚNIOR et al., 2012). This study assessed the

effect of different prenatal supplementation strategies on nitrogen isotopic fractionation in blood plasma of Nellore dams and their progenies.

3.2 Material and methods

3.2.1 Experimental design

The experiment was conducted at the Department of Animal Science, College of Animal Science and Food Engineering, University of São Paulo (FZEA/USP), in Pirassununga/SP. The study comprised 126 Nellore cows fixed time artificially inseminated (FTAI) with semen from four breeders with known genetic values. The cows were divided into three groups of 42 animals based on age, body weight, and body condition scores (group selection criteria). The cows entered the breeding season and were separated into three gestational supplementary treatments, allocated in paddocks with *Urochloa brizantha* (Syn. *Brachiaria brizantha*) cv. Marandu. The treatments were offered from the confirmation of pregnancy at 30 days. The three groups received mineral supplementation (0.03% of body weight) during the entire period. The treatments comprised: NP - Non-Programmed, no protein-energy mineral supplementation during pregnancy; PP - Partially Programmed, protein-energy mineral supplementation in the final third of pregnancy (estimated consumption of 0.3% body weight); and FP - Full Programmed, protein-energy mineral supplementation throughout pregnancy (estimated consumption of 0.3% body weight). After calving, the cows and their progenies had their protein-energy mineral supplement ceased and all groups were kept under the same sanitary and nutritional conditions. Five cows per group with their respective progenies (NP, PP and FP) were randomly selected and totaling 30 animals to evaluate the effect of maternal supplementation strategies on nitrogen isotopic fractionation of dams and calves.

3.2.2 Bromatological analysis of supplements and pastures

Supplements offered to the dams during the gestational period were evaluated for their chemical composition (Table 1) and the mineral contents are shown in Table 2.

Table 1 - Contents of ingredients and nutrients of supplement for dams

Ingredients	**Mineral supplement**	**Energetic-proteic supplement**
Ground corn (%)	35	60
Soybean meal (%)	-	30
Dicalcium phosphate (%)	10	-
Urea (%)	-	2.5
Salt (%)	30	5
Minerthal 160 MD (%)*	25	2.5
Total digestible nutrients (%)	26.76	67.55
Crude protein (%)	2.79	24.78
Non-protein nitrogen (%)	-	7.03
Acid detergent fiber (%)	1.25	4.76
Neutral detergent fiber (%)	4.29	11.24
Fat (%)	1.26	2.61
Calcium (g/kg)	74.11	6.2
Phosphate (g/kg)	59.38	7.24

*Mineral premix

Table 2 - Mineral premix content of supplement for dams

Minerals	**Guarantee levels (kg)**
Calcium (Ca)	200 - 230 g
Cobalt (Co)	160 mg
Copper (Cu)	2,700 mg
Sulfur (S)	60 g
Fluorine (F)	1,600 mg
Phosphor (P)	160 g
Iodine (I)	135 mg
Manganese (Mn)	2,700 mg
Selenium (Se)	80 mg
Zinc (Zn)	8,100 mg
Sodium monensin	4,000 mg

Forages were sampled by collecting five areas of 1 m² in each paddock at random, avoiding areas with feces and invasive plants. The five samples were homogenized and a single 300-gram sample was obtained. Samples to determine dry matter (DM) were oven dried by forced air ventilation at 65°C for 72 h (SILVA; QUEIROZ 2009) and later ground in a 2-mm sieve for the bromatological and mineral analyses. The pasture morphological composition was determined by separating leaf material, stem, and dead material, which were dried afterward. This allowed estimating feed consumption and characterizing pasture conditions of each treatment (Table 3).

Table 3 - Availability and botanical composition of forage for dams during all gestational period (mean ± standard deviation)

Forage availability	**NP**	**PP**	**FP**
Pasture Availability (kg DM/ha)	3,476.24 ± 1594.40	4,597.35 ± 1189.80	5,578.03 ± 2049.37
Leaf Availability (kg DM/ha)	573.59 ± 340.56	569.13 ± 485.76	727.49 ± 643.30
Thatch Availability (kg DM/ha)	562.46 ± 396.97	799.05 ± 545.80	1,347.44 ± 1038.42
Dead material availability (kg DM/ha)	2,340.07 ± 1275.00	3,229.43 ± 973.34	3,503.07 ± 1410.43
Stocking Rate (AU/ha)	2.19 ± 1.02	1.74 ± 0.56	2.26 ± 0.99
Leaf supply for animal unit (KG/DM)	316.64 ± 216.96	359.93 ± 245.10	366.59 ± 306.58

*NP - Non-Programmed, PP - Partially Programmed, FP - Full Programmed

The bromatological and mineral analyses (Table 4) were conducted at the bromatology and mineral laboratory at the university. Crude protein was determined by the methodology of Silva and Queiroz (2009), neutral detergent fiber (NDF) by Van Soest (1995, 1967), and abundance of minerals was determined by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES), according to Sindirações (2013).

Table 4 - Nutrients in pastures consumed by dams in the different groups (mean ± standard deviation)

Forage nutrients	**NP**	**PP**	**FP**
CP % (crude protein)	7.38 ± 1.72	7.82 ± 2.28	7.40 ± 2.30
TDN % (total digestible nutrients)	63.07 ± 1.45	64.10 ± 2.33	61.43 ± 2.12
NDF % (neutral detergent fiber)	59.03 ± 3.67	61.43 ± 5.05	58.49 ± 4.11
Ca % (calcium)	0.38 ± 0.11	0.35 ± 0.05	0.39 ± 0.08
P % (phosphor)	0.19 ± 0.03	0.19 ± 0.03	0.17 ± 0.03

*NP - Non-Programmed, PP - Partially Programmed, FP - Full Programmed

3.2.3 Processing of forage and supplements for the isotopic analysis

To evaluate isotopic fractionation of the different treatments on dams, samples of supplements offered during pregnancy (mineral and protein-energetic) were packed in sterilized plastic pots with approximately 30 g of material, transported to the laboratory and ground for the analysis of Δ15N (15N/14N), under the protocol of CENA/ESALQ-USP.

The two supplement samples (mineral and protein-energetic) and picket leaves samples were ground in a 4-blade Macro “Willer” mill to avoid atom accumulation due to sample contamination. The grinding protocol consisted of mill aspiration at the beginning and end of each grinding procedure, followed by a jet of compressed air to remove gross remaining solids, washing with deionized water and subsequent cleaning with 70° liquid alcohol. After these procedures, the mill was dried using compressed air and the samples were ground. From the ground content, a homogeneous sample was generated in a 23-ml sterilized acrylic tube and subjected for the analysis of nitrogen isotopic fractionation (Δ15N).

3.2.4 Blood plasma collection and processing

Blood samples were collected by venipuncture of the jugular vein in vacuum tubes containing EDTA (anticoagulant) at 30 and 240 days of gestation and at 30 days postpartum for the evaluation of isotopes of dams, and at 30 and 180 days of age of calves. The material was identified and placed on ice until processing in a centrifugation laboratory to obtain blood plasma (15 min at 3000 rpm and 4 °C). After pipetting the supernatant with 1,000 µl pipettes in 2 ml microtubes, the plasma was kept in an ultra-freezer (-80 °C). For the isotopic analysis, microtubes were thawed and 30 µl of sample were pipetted into sterile Eppendorf tubes and shipped with ice to the Center of Nuclear Energy in Agriculture/USP (CENA) at the Stable Isotope Laboratory.

3.2.5 Isotopic analysis of Delta 15N (15N/14N)

The Δ15N analysis to evaluate nitrogen isotopic fractionation was carried out at the CENA/USP, Piracicaba, São Paulo, Brazil.

Plasma samples were pipetted to obtain 10 µl for storage in tin (Sn) capsules, inserted in the analyzer. The solids (supplements and ground leaves) were stored in the tin capsules. An IRMS spectrometer (Hydra 20-20, SerCon Co., UK) was used, interfaced with an automatic N and C analyzer (ANCA-GSL, SerCon Co., UK) coupled to an automatic sampler (222 XL Liquid Handler, Gilson). According to the equipment manual, accuracy of the analysis for natural abundance is 1.23 ‰ (delta per thousand 15N) for a mass of 10 µg of N in sediments.

Inside the ANCA-GSL, the gas passed through a column containing Mg (ClO4)2 to remove water vapor and then through a column containing Carbosorb to eliminate CO2. A chromatographic column (500x6.35x4 mm), filled with Carbosieve G (stationary phase) and heated to 80ºC, separated N2 from possible contaminants. Due to its nonpolar character, N2 flowed first, 80 seconds after the sample injection, and went to the IRMS after crossing a reduction column (CuO wires at 650ºC) that removed eluted O2. The O2 removal prevented the NO formation in the ion source by the reaction between O2 and N2 that could generate a false signal mass: charge (m:z). N2O eluted 80s after N2 passing through the oven containing CuO, reducing to N2. The IRMS Hydra 20-20 has three collectors that integrate the ion streams of m:z 28, 29, and 30. The atoms of 14N and 15N contained in N2 form molecules 14N14N, 14N15N, and 15N15N, written as 28N2, 29N2 and 30N2. Separation of these N2 molecules after

ionization and their quantification in IRMS allow to calculate the contribution of the source marked with 15N to the total amount of gas produced (MILAGRES, 2014).

The atmospheric air (78% by volume of N2 and 0.3663% in atoms of 15N) was used as N2 standard, considering density of N2 equals to 1.25 µg µL-1 with an analytical error of 0.2 ‰ (per thousand) through the dimensionless expression:

$$\delta 15N=[(R_{sample}-R_{standard})/R_{standard}]*1000$$

Where: *R*sample is the isotopic ratio (15N/14N) measured in the sample and *R*standard the same ratio as a standard, in this case, atmospheric air. As the numerical values of the differences between the isotopic ratios (r) are small, it is usual to multiply the expression by 1000, obtaining the terminology in delta per thousand (δ ‰).

3.2.6 Statistical analysis

The effects of treatments (NP, PP, and FP) and time on traits were evaluated by the analysis of variance. The residues were tested for normality utilizing Shapiro Wilk test. The age of dams and paternity were used in the linear model. The results with a significant difference ($p < 0.05$) had the means compared by the Tukey test at 5% level of significance. All statistical analyses were performed using the GLM procedure from SAS 9.3 statistical package (SAS Institute Inc, 2011).

3.3 Results

3.3.1 Isotopic evaluation of forages and supplements

All pastures where cows were kept until delivery and where the dam-offspring group remained after birth showed negative δ15N, indicating that pastures were richer in 14N isotope than in heavy isotope 15N. Pre-calving pasture of PP and FP treatments had δ15N of -1.39‰, while pre-calving pasture had δ15N of -3.07‰ in NP treatment, the lowest abundance presented. Pasture where all animals remained during the entire period from birth to weaning had δ15N of -2.93‰. Mineral supplement offered to all treatments had δ15N of 1.89‰, while the protein-energy diet provided only to PP and FP treatments had δ15N equals to 0.55‰.

3.3.2 Isotopic evaluation of blood plasma of dams

In the prepartum period of dams, PP and FP treatments had similar values of δ15N, differing only from NP group (Table 5). At the beginning of pregnancy, cows showed statistically similar values for both concentration and abundance of 15N isotope (δ15N). The 15N isotope concentration showed a significant difference ($p < 0.05$) only between the periods in NP treatment ($p = 0.037$), with a trend for a difference between the treatments in the prepartum period ($p = 0.064$). Abundance showed a statistical difference between treatments in the prepartum ($p < 0.001$) and postpartum ($p < 0.001$) periods. In the prepartum period, FP and PP treatments differed from NP, while all treatments differed from each other in postpartum. For the periods, all treatments showed differences, indicating that each pregnancy stage has a different abundance ($p < 0.05$; Table 5).

Table 5 - Mean of isotopic fractionation in blood plasma of dams from different treatments and periods with their respective standard errors and p values

Traits	Period	NP	PP	FP	p value
Abundance	Initial	2.002 ± 0.086^{A}	2.107 ± 0.151^{A}	2.160 ± 0.078^{A}	0.341
	Pre	2.412 ± 0.104^{Ba}	3.390 ± 0.135^{Bb}	3.796 ± 0.157^{Bb}	<0.001
	Post	2.156 ± 0.073^{ABa}	2.722 ± 0.087^{Cb}	3.094 ± 0.090^{Cc}	<0.001
	p value	0.033	<0.001	<0.001	
Concentration	Initial	1.204 ± 0.034^{AB}	1.192 ± 0.027	1.190 ± 0.073	0.986
	Pre	1.120 ± 0.022^{A}	1.270 ± 0.080	1.246 ± 0.060	0.064
	Post	1.254 ± 0.038^{B}	1.280 ± 0.049	1.338 ± 0.056	0.207
	p value	0.037	0.518	0.275	

*NP - Non-Programmed, PP - Partially Programmed, FP - Full Programmed

Capital letters ($^{A, B, C}$) represent significant contrasts between periods and lowercase letters ($^{a, b, c}$) represent significant contrasts between treatments.

3.3.3 Isotopic evaluation of blood plasma from calves

In calves, two different periods were evaluated at 30 and 180 days of age. Concentration showed no statistical difference in any interaction ($p > 0.05$). The δ15N values showed a difference between the treatments during 30 days of age ($p < 0.01$), in which PP and FP differed from NP. However, the same was not observed at 180 days, in which all treatments displayed the same abundance ($p = 0.878$). For the δ15N analysis between the periods, only FP showed difference in isotope abundance in blood concentration ($p = 0.023$; Table 6).

At 30 days of age (postpartum period of dams), calves showed enrichment (Δ15N) of δ15N of 0.86‰ in NP and FP treatments and 1.19 ‰ in PP in relation to their dams, where Δ15N = δ15Nprogeny – δ15Ndam.

Table 6 - Mean of isotopic fractionation in blood plasma of calves from different treatments and periods with their respective standard errors and p values

Traits	Age	NP	PP	FP	p value
Abundance	30 days	3.022 ± 0.149[a]	3.910 ± 0.095[b]	3.954 ± 0.150[b]	0.002
	180 days	3.028 ± 0.228	3.385 ± 0,221	3.134 ± 0.223	0.878
	p value	1.000	0.368	0.023	
Concentration	30 days	1.082 ± 0.048	1.105 ± 0,022	1.180 ± 0.042	0.479
	180 days	1.090 ± 0.022	1.137 ± 0,049	1.178 ± 0.025	0.386
	p value	1.000	0.991	1.000	

*NP - Non-Programmed, PP - Partially Programmed, FP - Full Programmed

Lowercase letters ([a, b, c]) represent significant contrasts between treatments.

3.4 Discussion

To our knowledge and based on a literature search, this is the first study to evaluate stable isotopes in animals submitted to different prenatal nutrition approaches, demonstrating an innovation in this field.

In ruminants, stable isotopes in fluids or tissues vary by ingested food and water, inhaled gases, and are influenced by other environmental conditions and the physiological phase of the animals (VIEIRA JÚNIOR et al., 2012). Therefore, similarity between the two groups (PP and FP) can be attributed to the protein-energy mineral supplementation, as both groups consumed supplements for at least 90 days. The NP treatment showed a slight enrichment between the beginning and the end of pregnancy (pre-delivery), due to the positive isotopic abundance (δ15N of 1.89‰) in the mineral supplementation, as the pasture in this treatment was poor in δ15N. Both PP and FP treatments showed differences between the periods. The FP treatment was richer than the PP between the beginning and the end of pregnancy, possibly due to the longer supply of protein-energy mineral supplementation (FP = nine months; PP = three months).

In the postpartum period (30 days after birth), cows of all treatments were consuming the same diet since delivery; thus, no significant differences were expected. The slight differences could be explained by a cumulative effect on the abundance of isotopes related to consumption during pregnancy. According to Jenkins et al. (2001), isotopic signature in the plasma reflects the last 7-10 days; therefore, differences between treatments in the postpartum period are not expected. In our study, we used two animal categories (dams and calves), supporting that this time interval actually has a wider species-specific range. Cantalapiedra-Hijar et al. (2020) found that plasma isotopic turnover in Charolais bulls is five months on average. The turnover time of about five months for proteins in the plasma (CANTALAPIEDRA-HIJAR et al., 2020) also helps explain the difference between treatments in calves only at 30 days. At this time, isotopic abundance in the plasma still reflected the abundance of fetal life, unlike the findings at 180 days, when all values were similar, because the calves started grazing at the same source and consumed milk produced by their mothers that also fed on the same pasture.

The significant difference found between the periods in FP calves showed greater reduction of δ15N. A significant reduction was expected in PP calves, whose dams received protein-energy mineral supplementation for a shorter time. However, animals that fed a protein-rich diet showed lower values of abundance in plasma proteins in 15N than animals fed with normo-protein diets (CANTALAPIEDRA-HIJAR et al., 2020). While in fetal life, the progeny reflects the maternal metabolism, justifying the significant reduction in 15N levels of FP calves. Dams from PP and FP groups

supplied with nutritional protein during some time in pregnancy also displayed a reduction in δ15N blood plasma levels in the postpartum period.

The δ15N values in plasma in all treatments were higher in newborn calves with 30 days of life (Table 2) than in the respective mothers during the postpartum period, indicating fetal enrichment, similar to other findings in the literature. Jenkins et al. (2001) reported offspring plasma with an average Δ15N of 0.9 ± 0.8‰ above the maternal plasma, regarded as breastfeeding by the authors. Barboza and Parker (2006) suggested that offspring enrichment in 15N in relation to the dams is due to the fetal protein origin from maternal reserves and not directly from the diet consumed by the cow. These results corroborate our findings on isotopic evaluations of calves at 30 days of life (Table 2).

Additionally, isotopic fractionation and feed efficiency have previously been associated. Wheadon et al. (2014) evaluated feed efficiency (FE) in growing heifers and found a negative association between δ15N and FE and a positive association between δ15N and body weight. Nevertheless, no relationship occurred between δ15N and residual feed intake, main measurement for feed efficiency. In addition, Cantalapiedra-Hijar et al. (2018) carried out a meta-analysis and identified a negative relationship between 15N abundance and N efficiency, another important measurement for feed efficiency, which relates how well animals can convert N in their diets into their products (milk and meat).

3.5 Conclusion

Different prenatal supplementation strategies of Nellore dams caused differences on fractionation of stable N isotopes in blood plasma of cows and their calves, which are indicative of an effect of protein-supplement supply and cumulative behavior on isotope abundance related to consumption during gestation.

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4 Chapter 3: Prenatal supplementation in beef cattle and its effects on plasma metabolome of dams and calves

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Abstract

This study investigated the effect of different prenatal nutrition on plasma metabolome of Nellore dams and their offspring. For that purpose, three nutritional treatments were used in 126 cows during pregnancy: NP – (control) only mineral supplementation; PP – protein-energy mineral supplementation in the final third; and FP – protein-energy mineral supplementation during the entire pregnancy. Targeted metabolomics were analyzed in plasma at the beginning of pregnancy and in pre-delivery of cows (n=27) as well as in calves (n=27, 30±9.6 days of age). Data were analyzed by the analysis of variance, partial least squares discriminant analysis, and the principal component analysis (PCA). The PCA showed a clear clustering in the periods investigated only in cows (early gestation and pre-delivery). We found significant metabolites in both

supervised analyses ($p<0.05$ and VIP score>1) for cows (Taurine, Glutamic acid, Histidine and PC aa C42:2) and for calves (Carnosine, Alanine and PC aa C26:0). The enrichment analysis revealed biological processes ($p<0.1$) common among cows and calves (histidine metabolism and beta-alanine metabolism), which may be indicative of transgenerational epigenetic changes. In general, fetal programming affected mainly the metabolism of amino acids.

Keywords: Beef cattle. Fetal programming. Mass spectrometry. Maternal nutrition. Metabolites.

4.1 Introduction

Maternal nutrition during pregnancy has received great prominence in livestock studies because of its effects on production traits of dams and long-term consequences on the offspring (PARADIS et al., 2017; REYNOLDS et al., 2017; VAN EMON et al., 2020; VONNAHME et al., 2018). Undernutrition or overnutrition during the gestational period can cause physiological and metabolic changes to the fetus that can last throughout its productive life (CHAVATTE-PALMER, et al., 2016; LONG et al., 2009). Several studies have reported the phenotypic effects on offspring due to different prenatal stimuli in beef cattle from extensive production systems (CRACCO et al., 2021; MORIEL et al., 2020; POLIZEL et al., 2021a; POLIZEL et al., 2021b; RAMÍREZ et al., 2020; SILVA et al., 2021). Many of these phenotypic consequences are related to carcass characteristics, meat quality, body composition, weight gain, body weight, and reproductive performance. However, the molecular mechanisms involved need further elucidation.

With recent advances of “omics” technologies, holistic molecular approaches, such as metabolomics, are more accessible and can contribute to a better understanding of the molecular mechanisms involved with the phenotype of interest. Metabolome is defined as a complete collection of metabolites in a specific organ, tissue, cell, organelle, or biofluid (WISHART et al., 2007). Metabolites can be sensitive indicators of changes in the genome, transcriptome and/or proteome, as they are the product of complex interactions between different molecular levels (GOLDANSAZ et al., 2017). Thus, metabolomics approach shows great potential to analyze the metabolic and physiological effects related to a phenotype (HELLMUTH et al., 2019).

Some studies have been using metabolomics perspective to assess environmental effects and phenotypes on livestock (ABARGHUEI et al., 2014; KARISA et al., 2014; NOVAIS et al., 2019; SALEEM et al., 2012). However, few studies have investigated metabolomics approaches in experiments that evaluate maternal nutrition and its effects on off-spring in beef cattle (MUROYA et al., 2021; MUROYA et al., 2022).

We hypothesize that different protein-energy mineral supplementation strategies could change the metabolic profile of cows during pregnancy and thus of their progenies. Here, we evaluated whether the different prenatal nutritional conditions affect the metabolic profile (amino acid, biogenic amines, hexose, acylcarnitines, lysophosphatidylcholines, phos-phatidylcholines, and sphingolipids) of the blood plasma of cows and their respective offspring using the targeted metabolomics approach.

4.2 Material and methods

4.2.1 Experimental design

The study comprised 126 Nellore cows and their progenies. The dams were fixed time artificially inseminated (FTAI) with semen from four sires and had their pregnancy diagnosis confirmed 30 days later.

The cows were blocked into three groups of 42 animals based on age, body weight (BW) and body condition score (BCS). The three groups were allocated in pasture paddocks of *Urochloa brizantha* (Syn. *Brachiaria brizantha*) cv. Marandu, equipped with a trough to supply feed supplement and water. The different prenatal nutrition strategies were: NP (control) – Not Programmed, PP – Partial Programming, and FP – Full Programming. All treatments received mineral supplementation (0.03% of BW), but only PP and FP received protein-energy mineral supplementation equivalent to 0.3% of the average BW per day during pregnancy. The PP group was submitted to this nutritional protocol only in the final third of pregnancy, whereas dams in FP had supplementation upon pregnancy confirmation (30 days after FTAI) until calving (Tables 1 and 2). Table 4 shows the supplements used and Table 5 presents the nutrients of the pastures.

Forages were sampled by collecting five areas of 1 m² in each paddock at random, avoiding areas with feces and invasive plants. The five samples were

homogenized and a single 300-gram sample was obtained. Samples to determine dry matter (DM) were oven dried by forced air ventilation at 65 °C for 72 h (SILVA; QUEIROZ, 2009) and later ground in a 2-mm sieve for the bromatological and mineral analyses.

The bromatological and mineral analyses were conducted at the bromatology and mineral laboratory at the university. Crude protein (SILVA; QUEIROZ, 2009), neutral detergent fiber (NDF; VAN SOEST, 1967; VAN SOEST, 1995), and abundance of minerals were determined by Inductively Coupled Plasma Op-tical Emission Spectrometry (ICP-OES; SINDIRAÇÕES, 2013).

Table 1 - Ingredients and nutrients content of the supplement for dams

Ingredients/Nutrients	**Mineral supplement**	**Protein-energy mineral supplement**
Corn (%)	35.00	60.00
Soybean meal (%)	-	30.00
Dicalcium phosphate (%)	10.00	-
Urea (%)	-	2.50
Salt (%)	30.00	5.00
Minerthal 160 MD (%)*	25.00	2.50
Total digestible nutrients (%)	26.76	67.55
Crude protein (%)	2.79	24.78
Non-protein nitrogen (%)	-	7.03
Acid detergent fiber (%)	1.25	4.76
Neutral detergent fiber (%)	4.29	11.24
Fat (%)	1.26	2.61
Calcium (g/kg)	74.11	6.20
Phosphorus (g/kg)	59.38	7.24

*Mineral premix composition (Minerthal company): Calcium = 200 g/kg; Cobalt = 160 mg/kg; Copper = 2,700 mg/kg; Sulfur = 60 g/kg; Fluorine = 1,600 mg/kg; Phosphorus = 160 g/kg; Iodine = 135 mg/kg; Manganese = 2,700 mg/kg; Selenium = 80 mg/kg; Zinc = 8,100 mg/kg; Sodium monensin = 4,000 mg/kg (Polizel et al., 2021a).

Table 2 - Nutrients in pastures consumed by dams in the different groups (mean ± standard error of the mean)

Forage nutrients	**NP**	**PP**	**FP**
CP % (crude protein)	7.38 ± 0.70	7.82 ± 0.93	7.40 ± 0.93
TDN % (total digestible nutrients)	63.1 ± 0.59	64.1 ± 0.95	61.4 ± 0.86
NDF % (neutral detergent fiber)	59.0 ± 1.49	61.4 ± 2.06	58.4 ± 1.67
Ca % (calcium)	0.38 ± 0.04	0.35 ± 0.02	0.39 ± 0.03
P % (phosphorus)	0.19 ± 0.01	0.19 ± 0.01	0.17 ± 0.01

NP – not programmed; PP – partial programming; FP – full programming.

Table 3 - Average of BW (kg), subcutaneous fat thickness (SFT; mm) and body condition score (BCS) ± standard error of the mean of Nellore cows submitted to different maternal nutrition approaches (NP, PP and FP) with their respective p values

Traits	**NP**	**PP**	**FP**	**p value**
Initial BW (kg)	461.1 ± 6.90	451.2 ± 9.38	454.1 ± 8.76	0.85
Pre-delivery BW (kg)	508.0 ± 7.23^{a}	524.2 ± 9.07^{a}	541.2 ± 10.1^{b}	<0.01
Initial SFT (mm)	4.28 ± 0.61	4.31 ± 0.61	4.33 ± 0.61	0.92
Pre-delivery SFT (mm)	7.23 ± 0.66^{a}	9.24 ± 0.67^{a}	12.5 ± 0.98^{b}	<0.01
Initial BCS	4.50 ± 0.09	4.60 ± 0.12	4.50 ± 0.09	0.34
Pre-delivery BCS	5.40 ± 0.13^{a}	5.60 ± 0.13^{ab}	5.90 ± 0.13^{b}	0.04

NP – not programmed; PP – partial programming; FP – full programming.

Table 6 shows the phenotypic effect of the different prenatal supplementation strategies on dams. After calving, protein-energy mineral supplementation ceased and all progenies (regardless of the nutritional prenatal treatment) were subjected to the same health protocols and nutritional managements, remaining together until weaning

(210 ± 28 days). During this period (calving to weaning), the cows received the same mineral supplement (0.03% of BW) that they received during pregnancy period and remained in extensive pasture system (paddocks of *Urochloa brizantha* (Syn. *Brachiaria brizantha*) cv. Marandu, as well as during the pregnancy period).

4.2.3 Plasma sample collection and preparation

From the 126 cows, we selected 27 (9 per treatment randomly) and their respective offspring for plasma the metabolomics analysis. The cows were analyzed at early gestation (30 days of pregnancy; before receiving the nutritional treatments) and at pre-delivery (9 months of pregnancy; after receiving the nutritional treatments). Blood samples from calves were collected at an average of 30 ± 9.6 days of age. The blood was collected into EDTA-coated tubes (BD Vacutainer, São Paulo, SP, Brazil) from the jugular vein (conditioned on ice until the samples were processed in the laboratory). Blood samples were centrifuged for 10 min at 3000× g and 4 °C within 1 hour after sampling. Plasma supernatants were transferred into fresh collection tubes, immediately snap frozen using dry ice and stored at −80 °C until use.

4.2.4 Targeted metabolomics

The metabolomics analysis was carried out by Apex Science Company (Campinas, São Paulo, Brazil). Metabolites were quantified using AbsoluteIDQ® p180 Kit (Biocrates Life Sciences AG, Innsbruck, Austria). The kit covers 188 metabolites of which 21 are amino acids, 21 biogenic amines, 40 acylcarnitines (Cx:y), 14 lysophosphatidylcholines (lysoPC), 76 phosphatidylcholines (PC), and 15 sphingolipids (SMx:y). The placeholders x and y in these formulas represent the number of carbons and double bonds of all chains, respectively. The analysis uses two different mass spectrometric methods with isotope labeled and other internal standards for absolute quantification of metabolites. Mass detection and compound identification were performed by multiple reaction monitoring. The lysophosphatidylcholines, phosphatidylcholines, acylcarnitines, and the hexose were performed by flow injection analysis-tandem mass spectrometry (FIA-MS/MS). The amino acids and biogenic amines were derivatized using phenylisothiocyanate reagent (PITC; 5%) and analyzed by liquid chromatography tandem-mass spectrometry (quantified by stable isotopes;

HPLC-MS/MS) in a positive mode using an AB SCIEX 4000 QTrap mass spectrometer (AB SCIEX, Darmstadt, Germany) with electrospray ionization. More specifically, the analyses of amino acids and biogenic amines were based on PITC derivatisation, separation of metabolites on a Waters Acquity UHPLC BEH18 C18 reversed-phase column (Waters, Vienna, Austria) using water and acetonitrile with 0.1% formic acid as mobile phases, and quantification on a Triple-Stage Quadrupole tandem mass spectrometer with electrospray ionization in the presence of internal standards. The analyses were performed in 96-well-plate format, allowing measurement of batches of 81 samples at one time.

Data analysis for metabolite quantification and quality assessment was performed using MetIDQ® software, which is part of the AbsoluteIDQ® p180 kit. Metabolite concentrations were calculated using internal standards. The concentration of each metabolite was measured in μM. The metabolite-specific limits of detection (LOD), lower limits of quantification and upper limits of quantification of the assay were experimentally deter-mined by Biocrates (Table 1).

4.2.5 Statistical analysis

Data processing and the univariate analysis (analysis of variance; ANOVA) were performed using the R software environment (version 4.1.2). Metabolites with more than 70% of samples below LOD were removed from the dataset (Early gestation (dams) = 129 metabolites remaining; Pre-delivery (dams) = 128 metabolites remaining; Calves = 124 metabolites remaining). The LOD values that remained in the metabolome after filtering were replaced by the mean of each variable. Two models were implemented through the “LM” function in R.

The statistical model used in the metabolomics analysis of dams:

$$Y_{jk} = \mu + \beta_1 Age_{d1} + Treat_j + e_{jk} \qquad (1)$$

The statistical model used in the metabolomics analysis of offspring:

$$Y_{ijk} = \mu + \beta_1 Age_{c1} + Treat_j + Sex_i + e_{ijk} \qquad (2)$$

Where: Yijk and Yjk are the observed metabolite from kth animal, recorded on jth treatment of sex ith (only in the model for offspring); μ is only a constant; β1 is the regression coefficient of covariate for animal age; Aged1 is the observed value for the age of dams of kth animal; Agec1 is the observed value for the age of calves of kth animal; Treatj is the fixed effect of jth treatment; and eijk is the residual random term. The residuals were tested for normality (Shapiro-Wilk test) and for homoscedasticity (Levene's test) and the differences between treatments were considered significant when p value was ≤ 0.05 by the Tukey Kramer test. Data that did not meet these prerequisites were transformed into log10, square root or cubic root, depending on which transformation met the requirements (homoscedasticity and normality of the residuals). The evaluation of initial metabolome of dams was performed to investigate if the initial metabolomics profiles among treatments were at the same levels, since initial phenotypic traits were similar (Table 3).

In addition, the metabolite concentration table was uploaded to MetaboAnalyst 5.0 (PANG et al., 2021) and the data were Auto-scaled (mean-centered and divided by the standard deviation of each variable) before the analysis. The supervised method (Partial least squares discriminant analysis (PLS-DA)) and the unsupervised method (Principal component analysis (PCA)) were performed. The PLS-DA, PCA and the enrichment analysis were performed only for dams in pre-delivery stage and for calves, since the aim of these analyses was to find variables and processes related to a group, treatment, or phenotype. However, we carried out a PCA for time to evaluate the clustering between dams without the treatment (early gestation) and after receiving the treatments (pre-delivery). Cross-validation was performed for PLS-DA using the leave-one-out cross validation method (LOOCV) and the performance measure "accuracy" (dams: R2 = 0.98, accuracy = 0.70; calves: R2 = 0.88, accuracy = 0.41). The variable importance in the projection (VIP) plot was used to rank the metabolites based on their importance in the treatments. Metabolites with the highest VIP values were the most powerful group discriminators. The VIP values > 1 were significant, while the VIP values > 2 were highly significant. The enrichment analysis was performed to identify the most relevant biological processes associated to differentially expressed metabolites (identified in univariate analysis) using MetaboAnalyst 5.0 (based on the Kyoto Encyclopedia of Genes and Genomes database). Biological processes with p value < 0.1 were considered significant.

4.3 Results

4.3.1 Univariate analysis of metabolome

Initially (early gestation), cows had 5 differentially expressed metabolites between prenatal treatments (PC aa C30:0; Histidine; PC ae C30:2; PC ae C30:0; PC ae C30:1; Table 4). In the evaluation of pre-delivery metabolomics, the cows presented 16 differentially ex-pressed metabolites between the groups (Symmetric dimethylarginine (SDMA); PC aa C34:4; PC aa C38:3; PC aa C40:3; Taurine; Glutamic acid; PC ae C38:1; PC ae C34:3; PC aa C40:4; PC aa C42:4; PC aa C42:2; PC ae C42:4; PC aa C42:6; Histidine; Carnosine; PC aa C36:2; Table 5). Regarding the metabolomes of calves, 6 differentially expressed metabolites between the treatments (PC aa C42:6; PC ae C38:4; Carnosine; Alanine; PC aa C26:0; PC ae C40:4; Table 6). The tables with all metabolites (significant and not significant) and respective p values can be found in Supplementary material (Tables 4, 5, and 6).

Table 4 - Mean concentration of significant metabolites ± standard error of the mean for cows in early gestation (initial period, before receiving the treatments) with their respective p values

Metabolites (µM)	**NP**	**PP**	**FP**	**p value**
PC aa C30:0	1.44 ± 0.05^{ab}	1.26 ± 0.04^{a}	1.58 ± 0.06^{b}	< 0.001
Histidine	61.3 ± 4.03^{a}	43.1 ± 3.31^{b}	57.5 ± 2.83^{a}	0.003
PC ae C30:2	254.1 ± 16.0^{ab}	235.2 ± 8.11^{a}	289.3 ± 6.17^{b}	0.004
PC ae C30:0	110.3 ± 5.80^{ab}	101.0 ± 3.76^{a}	127.0 ± 7.51^{b}	0.009
PC ae C30:1	413.2 ± 37.3^{a}	419.1 ± 21.4^{ab}	505.1 ± 19.9^{b}	0.044

The small letters overwritten represent the significant contrasts. NP – not programmed; PP – partial programming; FP – full programming.

Table 5 - Mean concentration of significant metabolites ± standard error of the mean for cows in pre-delivery period (after receiving the prenatal nutritional treatments) with their respective p values

Metabolites (µM)	**NP**	**PP**	**FP**	**p value**
SDMA	0.86 ± 0.03^{a}	0.65 ± 0.03^{b}	0.67 ± 0.04^{b}	0.001
PC aa C34:4	10.5 ± 1.21^{a}	17.9 ± 2.09^{b}	17.0 ± 1.82^{b}	0.002
PC aa C38:3	61.9 ± 4.35^{a}	89.6 ± 6.73^{b}	97.8 ± 10.6^{b}	0.003
PC aa C40:3	4.14 ± 0.38^{a}	6.71 ± 0.59^{b}	7.35 ± 0.84^{b}	0.005
Taurine	16.2 ± 2.03^{a}	30.7 ± 4.54^{b}	23.0 ± 1.86^{ab}	0.005
Glutamic acid	48.0 ± 3.34^{a}	55.3 ± 2.66^{ab}	70.2 ± 6.06^{b}	0.005
PC ae C38:1	12.8 ± 1.00^{a}	14.4 ± 2.02^{b}	14.4 ± 2.31^{b}	0.009
PC ae C34:3	30.6 ± 3.60^{a}	51.7 ± 5.92^{b}	44.1 ± 4.29^{ab}	0.014
PC aa C40:4	11.9 ± 1.01^{a}	14.8 ± 1.09^{ab}	17.0 ± 1.32^{b}	0.015
PC aa C42:4	60.7 ± 5.83^{a}	79.3 ± 8.37^{ab}	92.0 ± 8.31^{b}	0.030
PC aa C42:2	51.0 ± 5.67^{a}	69.0 ± 5.75^{b}	55.1 ± 4.22^{ab}	0.035
PC ae C42:4	0.55 ± 0.05^{a}	0.75 ± 0.06^{b}	0.73 ± 0.06^{ab}	0.037
PC aa C42:6	0.92 ± 0.06^{a}	0.81 ± 0.06^{ab}	0.70 ± 0.05^{b}	0.040
Histidine	74.2 ± 4.17^{ab}	70.3 ± 4.91^{a}	88.2 ± 5.36^{b}	0.042
Carnosine	25.4 ± 2.03^{a}	31.1 ± 2.39^{ab}	33.7 ± 2.80^{b}	0.044
PC aa C36:2	289.1 ± 41.1^{a}	457.2 ± 54.3^{b}	414.0 ± 48.31^{ab}	0.048

The small letters overwritten represent the significant contrasts. NP – not programmed; PP – partial programming; FP – full programming.

Table 6 - Mean concentration of significant metabolites ± standard error of the mean for offspring at 30 days of age submitted to different prenatal nutrition approaches (NP, PP and FP) with their respective p values

Metabolites (µM)	**NP**	**PP**	**FP**	**p value**
PC aa C42:6	1.45 ± 0.15^{a}	1.24 ± 0.07^{ab}	0.96 ± 0.06^{b}	0.010
PC ae C38:4	6.14 ± 0.42^{a}	8.43 ± 0.59^{b}	7.50 ± 0.51^{ab}	0.011
Carnosine	31.8 ± 3.20^{a}	30.3 ± 3.46^{ab}	20.6 ± 1.88^{b}	0.030
Alanine	261.0 ± 17.8^{ab}	279.2 ± 14.8^{a}	226.0 ± 8.40^{b}	0.048
PC aa C26:0	7.64 ± 0.24^{ab}	9.07 ± 0.85^{a}	6.57 ± 0.72^{b}	0.049
PC ae C40:4	1.56 ± 0.12^{a}	2.09 ± 0.16^{b}	1.94 ± 0.21^{ab}	0.049

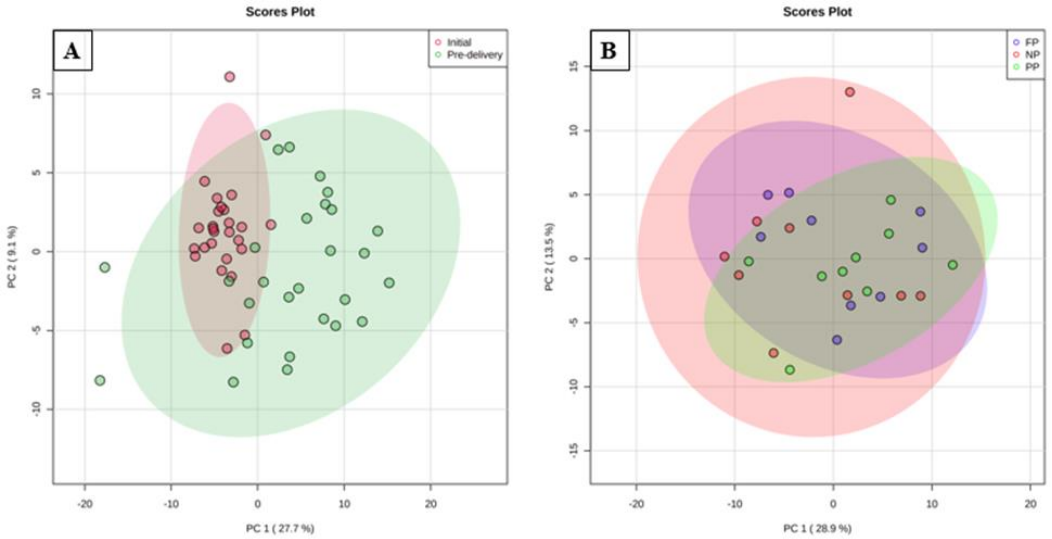
The small letters overwritten represent the significant contrasts. NP – not programmed; PP – partial programming; FP – full programming.

4.3.2 Principal component analysis (PCA)

The PCA results of the different periods analyzed for dams (Figure 1A) showed that data distribution presented a clustering for the initial period and another for the pre-delivery period. Thus, the data from the initial period were more homogeneous, indi-cating more similar metabolic levels than in the pre-delivery period (after receiving nutri-tional treatments). The two principal components together explained 36.8% of the total variance (PC1 = 27.7%; PC2 = 9.1%).

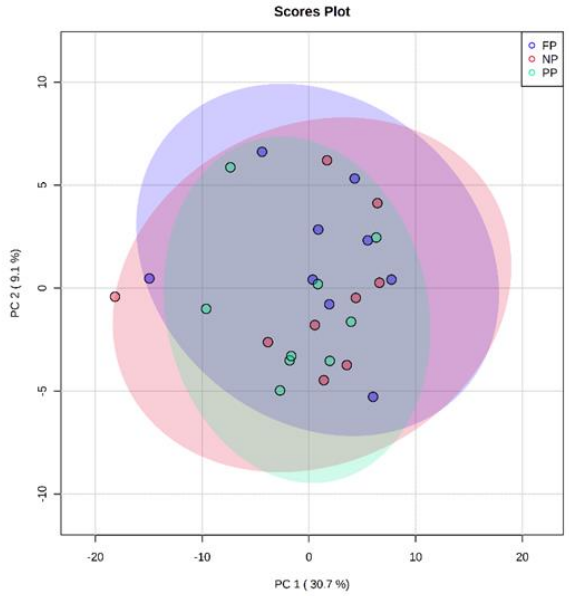
The PCA of treatments in pre-delivery of dams (Figure 1B) showed data distribution with a partial overlap between all groups. This may indicate that the metabolite profile did not present a large number of differentially expressed variables between the treat-ments. The two principal components together explained 42.4% of the total variance (PC1 = 28.9%; PC2 = 13.5%).

Figure 1 - Principal component analysis (PCA) scores plot of metabolome distribution of plasma of dams between the times (A; initial and pre-delivery) and between the nutritional treatments in pre-delivery period (B; NP, PP and FP)



Source: own authorship.

Figure 2 - Principal component analysis (PCA) scores plot of metabolome distribution of plasma of offspring between the prenatal nutritional treatments at 30 days of age (NP, PP, and FP)



Source: own authorship.

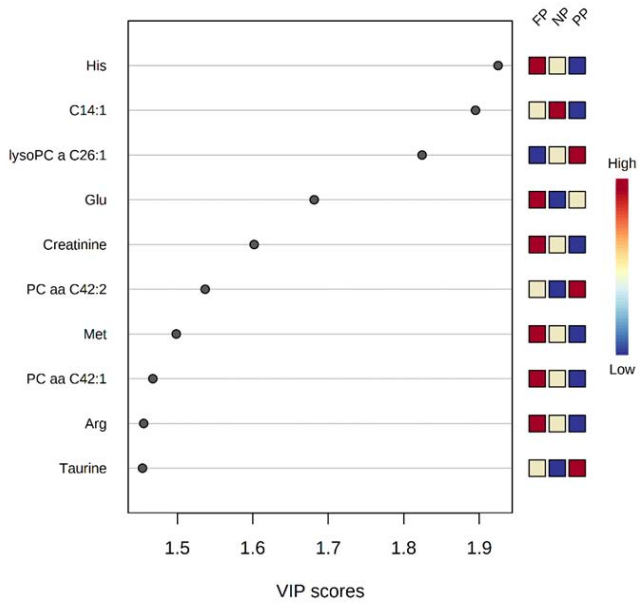
The PCA of treatments in calves at 30 days of age (Figure 2) showed data distribution with an overlap between all groups; however, it was not possible to observe a clustering between the treatments. This may indicate that the metabolite profile

presented only a few or no differentially expressed variables between the treatments. The two principal com-ponents together explained 39.8% of the total variance (PC1 = 30.7%; PC2 = 9.1%).

4.3.3 Partial least squares discriminant analysis (PLS-DA)

Based on variable importance in the projection (VIP) scores, compounds that contributed to a higher percentage of the residuals explained in the PLS-DA plot between the prenatal nutritional treatments in dams (pre-delivery; Figure 3) were: His (Histidine; VIP = 1.925), C14:1 (VIP = 1.895), lysoPC a C26:1 (VIP = 1.824), Glu (Glutamic acid; VIP = 1.681), Creatinine (VIP = 1.601), PC aa C42:2 (VIP = 1.536), Met (Methionine; VIP = 1.498), PC aa C42:1 (VIP = 1.467), Arg (Arginine; VIP = 1.455), and Taurine (VIP = 1.453).

Figure 3 - Top 10 metabolites (VIP scores) of dams in the pre-delivery stage associated to the different prenatal treatments (NP, PP and FP)

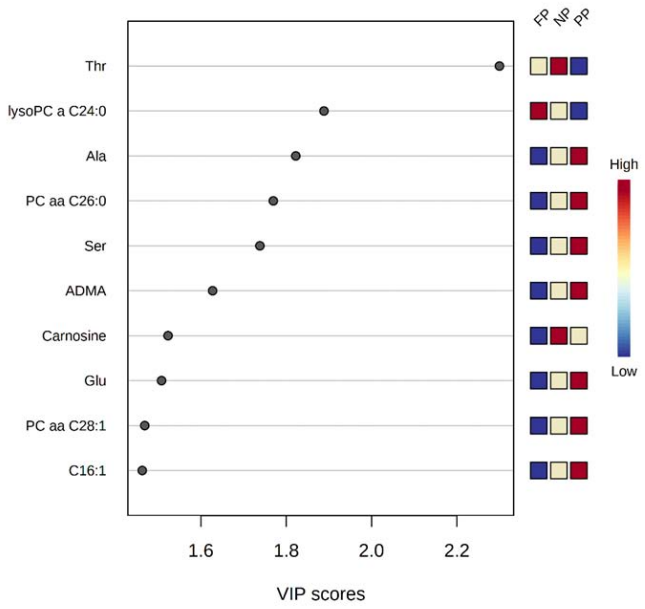


Source: own authorship.

The top 10 metabolites, based on VIP scores, of calves at 30 days of age submitted to different prenatal nutritional treatments (Figure 4) were: Thr (Threonine; VIP = 2.299), lysoPC a C24:0 (VIP = 1.888), Ala (Alanine; VIP = 1.822), PC aa C26:0 (VIP = 1.769), Ser (Serine; VIP = 1.738), ADMA (Asymmetric dimethylarginine; VIP

= 1.627), Carnosine (VIP = 1.522), Glu (VIP = 1.507), PC aa C28:1 (VIP = 1.468), and C16:1 (VIP = 1.462).

Figure 4 - Top 10 metabolites (VIP scores) of calves at 30 days of age associated to the different prenatal treatments (NP, PP, and FP)

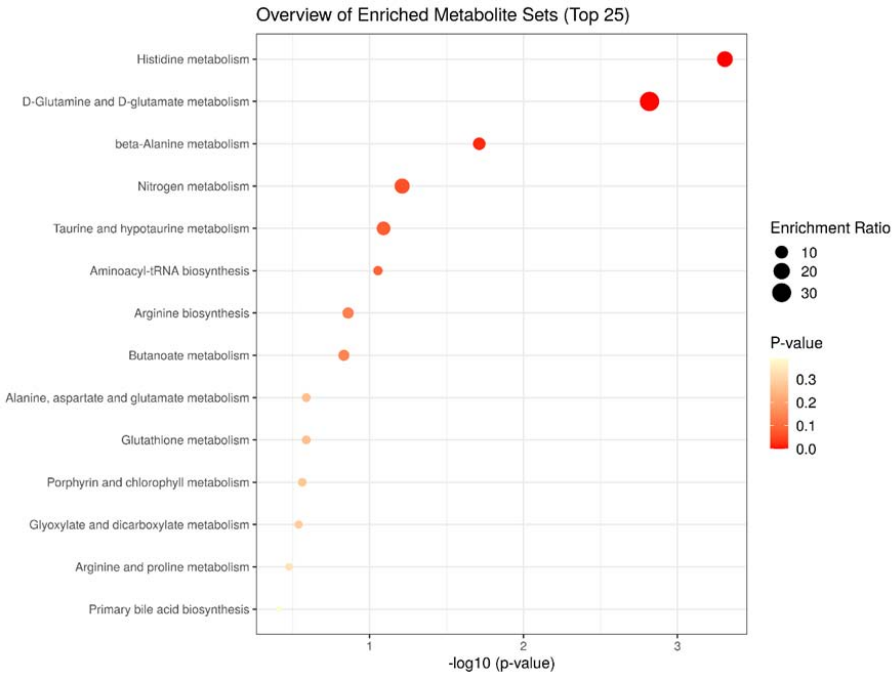


Source: own authorship.

4.3.4 Enrichment analysis

The enrichment analysis of dams in the pre-delivery period showed 6 significant biological processes related to differentially expressed metabolites between the prenatal treatments (Figure 5). The significant metabolic processes were: Histidine metabolism (p = 4.92E-4), D-Glutamine and D-glutamate metabolism (p = 1.52E-3), beta-Alanine metabolism (p = 0.02), Nitrogen metabolism (p = 0.06), Taurine and hypotaurine metabolism (p = 0.08), and Aminoacyl-tRNA biosynthesis (p = 0.08).

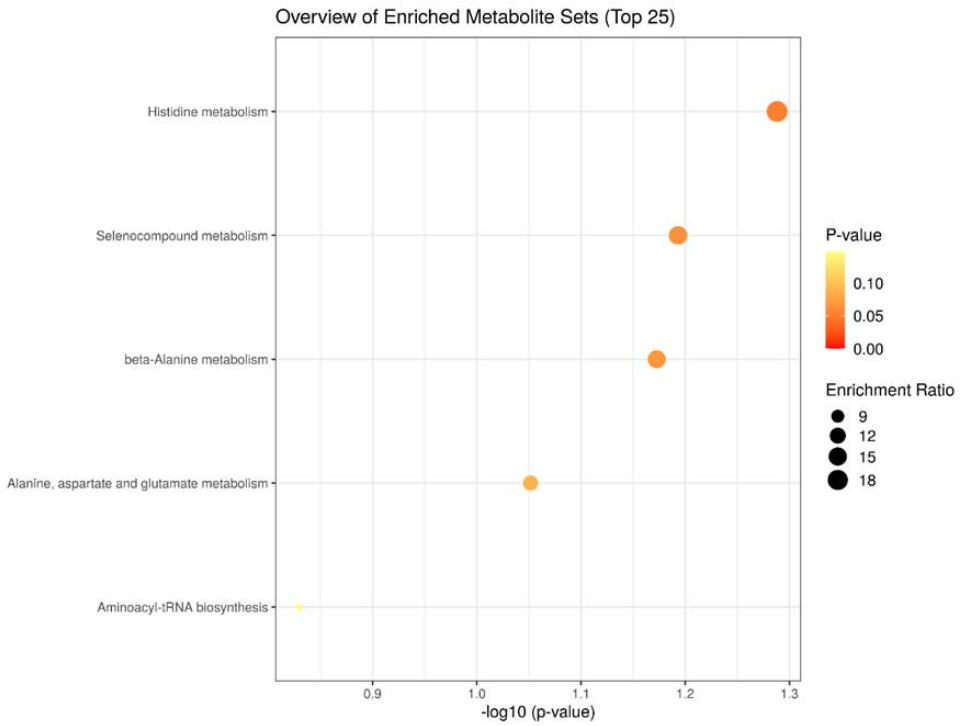
Figure 5 - Biological processes involved with significant plasma metabolites of the dams in the pre-delivery stage after receiving the three nutritional treatments (NP, PP, and FP)



Source: own authorship.

The enrichment analysis of calves on the average of 30 days of age showed 4 significant biological processes related to differentially expressed metabolites between the pre-natal treatments (Figure 6). The significant metabolic processes were: Histidine metabolism (p= 0.05), Selenocompound metabolism (p = 0.06), beta-Alanine metabolism (p = 0.06), Alanine, aspartate, and glutamate metabolism (p = 0.09).

Figure 6 - Biological processes involved with significant plasma metabolites of calves at 30 days of age submitted to the three prenatal nutritional treatments (NP, PP and FP)



Source: own authorship.

4.4 Discussion

To the best our knowledge and based on a literature search, this is the first study that assessed the impact of three prenatal supplementation approaches on plasma metabolome of beef cattle dams and their progenies.

The results showed that the initial period of dams had 5 differentially expressed metabolites between the groups. During this period, the animals showed the same initial phenotypic levels (see Table 3 in the methodology section) and clustering variables than the pre-delivery period in PCA (Figure 1A). This demonstrated that the different maternal nutrition approaches affected plasma metabolome of the animals in the pre-delivery stage.

We selected the significant metabolites for discussion found in both supervised analyses (ANOVA and PLS-DA) and related to significant biological processes. Thus, as they were significant in both analyses, these metabolites were considered the main plasma metabolites involved in the different prenatal nutrition strategies in Nellore cattle. In pre-delivery of dams, the following metabolites were selected: Taurine,

Glutamic acid, Histidine, and PC aa C42:2. In calves, the following were selected: Carnosine, Alanine, and PC aa C26:0.

Taurine is a sulfur containing amino acid (non-essential amino acid) that can be found in plasma of mammals after birth. This amino acid is synthesized in the liver and in the white adipose tissue. Low Taurine levels can be associated to alterations in glucose metabolism, insulin sensitivity, as well as to the function and number of β-cells (ARANY et al., 2017). In a previous study, no difference was found in blood levels of Taurine between groups of cows (pre-delivery) with higher and lower body condition score (BCS) (GHAFFARI et al., 2019) In our study, Taurine levels showed differences between the NP and PP groups, which did not show differences in BCS in the pre-delivery period, but these groups received different protein and energy inputs. Protein-energy mineral supplementation only in the final third of pregnancy (PP) showed higher Taurine levels in pre-delivery compared to dams that did not receive this nutritional stimulus in any gestational period (NP). Low levels of maternal Taurine may reduce the antioxidant activity, impairing progeny growth and perinatal development of the central nervous system and of the endocrine pancreas (AERTS et al., 2002; JUNG et al., 2019). Glutamic acid is a functional amino acid that plays several roles in the intestinal tract (WU et al., 2014), such as immune responses and function barrier (CAROPRESE, et al., 2012; REN et al., 2013; RUTH et al., 2013). It also plays specific roles as a source of energy for the intestinal mucosa (WATFORD et al., 2008), as a mediator of cell signaling (ZHANG et al., 2013), a regulator of oxidative processes (WU et al., 2004), and a substrate for several metabolic pathways (FENG et al., 2014). In another study, the glutamate metabolic pathway was one of the biological processes found among cows with divergent residual feed intake (low RFI and high RFI), demonstrating an association with feed efficiency in cows (WANG et al., 2019). In our study, one of the significant metabolic pathways found between different prenatal treatments was also related to glutamic acid and glutamate (D-Glutamine and D-glutamate metabolism), showing that different supplementation approaches in cows may also affect this metabolic pathway.

Histidine is an essential amino acid in mammals and must thus be obtained through the diet (WU et al., 2009). Histidine deficiency can reduce the body weight (BW) of animals (MORO et al., 2020). Specifically in cattle, histidine was the first limiting for growth of all amino acids in the duodenum (SCHOOF et al., 2000). The higher histidine levels also affect the cow milk yield, lactose yield, protein milk yield,

fat milk proportion, dry matter intake (DMI), and blood hemoglobin (DOELMAN et al., 2008; GIALLONGO et al., 2017). High histidine levels in the diet may cause the conversion of this amino acid into glucose through the regulation of genes related to the gluconeogenic pathway in bovine hepatocytes (YANG et al., 2021). One of the metabolic pathways related to different prenatal supplementation strategies was the histidine metabolism. The highest histidine levels were found in the FP treatment (cows fed protein-energy mineral supplement throughout the gestational period). These effects may imply a higher milk production and body weight of the dams (Table 6), directly affecting the performance of the offspring during the breastfeeding period.

The PC aa C42:2 and PC aa C26:0 were the phosphatidylcholines in dams and calves, respectively, selected for the discussion. However, more than half of the significant metabolites found for pre-delivery dams (PC aa C34:4, PC aa C38:3, PC aa C40:3, PC ae C38:1, PC ae C34:3, PC aa C40:4, PC aa C42:4, PC aa C42:2, PC ae C42:4, PC aa C42:6 and PC aa C36:2) and calves (PC aa C42:6, PC ae C38:4, PC aa C26:0, PC ae C40:4) between the treatments were from the phosphatidylcholines class. Phosphatidylcholines belong to a major class of lipids: the phospholipids (most abundant lipids in eukaryotic cells; BHAGAVAN et al., 2015; VAN MEER et al., 2008). These lipids are units of functional membranes and their composition determines most proper-ties of the cell membrane (fluidity, permeability, and thermal phase behavior; EDIDIN et al., 2003). In humans, a correlation has been identified between pre-delivery maternal lipid metabolism and DNA methylation in the progeny. This may be responsible partly for health impacts and disease risks throughout the life of progenies (MARCHLEWICZ et al., 2016). The lipidome of cows and calves are similar to each another (KLOPP et al., 2021). In our study, with the exception of PC aa C42:6 (in dams and calves), all other differentially expressed phosphatidylcholines showed higher levels in one of the treatments supplemented with protein and energy (PP or FP). The higher lipid levels found in these treatments can positively influence the fetus development the during pregnancy and the reproductive parameters (ovarian follicle and corpus luteum function; MATTOS et al., 2000) in cows. The different levels of phosphatidylcholines found between pre-natal treatments in calves may also affect their immune and inflammatory responses. This may occur because this class of lipids plays a role in the regulation of the inflammatory reactions, in addition to being related to cell membrane functions (CONTARINI et al., 2013).

Carnosine is a dipeptide (β-alanyl-L-histidine) present in mammals. This metabolite is highly prevalent (about 99%) in skeletal muscle (SALE et al., 2010) and can also be found at low concentrations in the plasma of non-primate mammals (WU et al., 2020). When present in blood plasma, carnosine is transported into extra-intestinal tissues and cells, increasing its concentrations in skeletal muscle, brain, and heart (BOLDYREV et al., 2013). The most important functions of carnosine are related to pH-buffering, activation of muscle ATPase to provide energy, antioxidant capacity, metal-ion (copper, zinc and iron) chelation and homeostasis (BARCA et al., 2019; BOLDYREV et al., 2013). In another study, correlations of carnosine with carcass quality and sensory scores in beef cattle were low, without significantly affecting the meat organoleptic properties (LIU et al., 2011). However, more recently, an association of carnosine with low residual feed intake in Nellore animals was identified (CÔNSOLO et al., 2021). The greatest contrast in carnosine levels was found in plasma of calves between the NP and FP treatments. This shows that progenies of cows that received protein-energy intake throughout pregnancy had lower carnosine levels compared to the control group. Thus, based only on carnosine levels, feed efficiency of calves may be affected by prenatal nutrition (CÔNSOLO et al., 2021).

Alanine is a non-essential amino acid that plays a role as raw material for glucose synthesis in the liver and muscles (FELIG et al., 1970). In cattle, alanine is one of the most abundant metabolites in the *Longissimus* thoracis and semimembranosus muscles (FOROUTAN et al., 2020) and the second most abundant metabolite in the liver (MILES et al., 2015). The effects of alanine on productive traits in ruminants are still little discussed in the literature. However, it was demonstrated that, in an intrauterine environment with low nutrient and oxygen availability, sheep prioritized alanine and glutamine production for fetal organ growth and metabolism (a mechanism to sustain total energy needs; CHANG et al., 2019). Here, we did not observe differences in alanine levels in dams, whereas the supplement of protein energy in the final third of pregnancy (PP) increased the alanine levels in calves compared to the treatment that received the same type of supplementation throughout the entire pregnancy (FP). These levels may be related to the fact that offspring in the PP treatment are more likely to present abnormalities in energy metabolism.

In another study, some biological processes (insulin secretion, PPAR signaling, and biosynthesis of amino acids) were identified due to the inclusion of vitamins and minerals to the diet as well as the rate of maternal weight gain of pregnant Angus cows

(DINIZ et al., 2021). In our study, histidine metabolism and beta-alanine metabolism were the significant biological processes found in both dams and calves. This may be indicative that epigenetic alterations caused by different prenatal supplementation approaches persist in the metabolism of the progenies throughout their life, affecting specific metabolic pathways. Studies on the epigenetic effects caused by maternal nutrition in cows and its impacts on metabolic pathways in the offspring should be carried out to elucidate some gaps still present in the literature.

4.5 Conclusion

Fetal programming altered the metabolome of dams and their offspring, especially in terms of protein metabolism. Significant metabolites affect productive traits of interest in livestock as well as important biological pathways. Two of these pathways (histidine metabolism and beta-alanine metabolism) are common to both dams and progenies in Nellore cattle, which may be indicative of transgenerationally transmitted epigenetic alterations.

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5 General conclusions and perspectives

Throughout the three chapters that comprise this thesis, it was possible to observe the phenotypic and molecular effects caused by different prenatal nutritional stimuli in Nellore cows and calves. These results make significant contributions to the field of maternal nutrition and its effects on offspring in beef cattle of tropical origin. Furthermore, given the scarcity in the literature on the molecular mechanisms involved in fetal programming, this work brings part of the molecular effects that contribute to the phenotypic responses found in cows and calves submitted to different strategies of maternal nutrition.

Thus, this thesis encourages that more molecular studies are carried out in order to fully elucidate the mechanisms involved in the responses to prenatal nutrition in beef cattle.