UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ZOOTECNIA E ENGENHARIA DE ALIMENTOS

ROBERTA CAVALCANTE CRACCO

Effects of fetal programming under offspring development in beef cattle

Pirassununga

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Effects of fetal programming under offspring development in beef cattle

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 Ciências do programa de Pós-graduação em Zootecnia.

Área de Concentração: Qualidade e Produtividade Animal

Orientador: 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

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DEDICATÓRIA

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RESUMO

A produção de bovinos de corte, em sua maioria, ocorre de maneira extensiva, onde os animais dependem quase que exclusivamente da disponibilidade de forragens em termos de qualidade e quantidade. Neste cenário, o feto na matriz gestante enfrenta o grande desafio de depender da mãe para sua nutrição e desenvolvimento, sofrendo muitas vezes o impacto da subnutrição materna. Por sua vez, a nutrição materna tem grande impacto na vida do indivíduo em formação, sendo capaz de causar alterações à longo prazo em diversos âmbitos, e à isso é dado o nome de programação fetal. Com isso, o objetivo desta dissertação foi avaliar o desempenho corporal e desenvolvimento muscular em novilhos por toda a vida, e o desenvolvimento reprodutivo de novilhas na recria. Para isso, 126 vacas Nelore foram inseminadas e divididas em três planos nutricionais: NP (controle) - Não Programado, sem suplementação proteico-energética; PP - Programação Parcial, suplementação proteico-energética no terço final de gestação; e PC - Programação Completa, suplementação proteico-energética durante toda a gestação. Desde o nascimento os animais tiveram seu peso e medidas ultrassonográficas coletadas. Ao todo, foram utilizados 63 machos e 55 fêmeas. Os machos passaram por biopsia do musculo *Longissimus* aos 15 e 22 meses para a realização da transcriptômica. Durante a recria foi feito o acompanhamento reprodutivo das novilhas para avaliar os impactos da programação fetal no desenvolvimento de seu trato reprodutivo. No primeiro capítulo desta dissertação, foi avaliado o desempenho corporal dos machos desde o nascimento até o abate, aos 22 meses, utilizando medidas de peso, área de olho em lombo e espessuras de gordura, tanto subcutânea quanto na picanha, bem como o ganho dessas medidas nos períodos de cria, recria e confinamento. Não foram encontradas diferenças significativas, porém tendencias à diferença surgiram no peso à desmama, ganho de área de olho em lombo e ganho médio diário no confinamento, e ganho de espessura de gordura na picanha durante a cria foram detectadas, sugerindo que as diferenças entre os tratamentos não foram tão grandes quanto poderia ser. No segundo capitulo, foi avaliado os efeitos da suplementação materna sobre a epigenética dos animais utilizando dados de RNA-Seq do musculo *Longissimus*. Nele, não foram observadas diferenças na expressão em genes relacionados à modificações em histonas e cromatina, porém houve diferenças entre os tratamentos e o controle olhado a regulação feita por RNAs longo não codificantes (lncRNA). Genes regulados por lncRNA se mostraram envolvidos no desenvolvimento embrionário uterino, diferenciação de células adiposas, vasculogênese, fator de crescimento transformador beta e via de sinalização canônica do Wnt, entre outros. Por fim, no

terceiro e último capítulo, foi abordado o desenvolvimento reprodutivo das fêmeas durante a recria, correlação entre traços reprodutivos e de desempenho, e interações genótipo ambiente. Houve diferenças no peso ao longo do tempo, porém nenhum efeito da suplementação materna foi detectado nas outras características. Nas análises de correlação, houve alta correlação entre medidas de área de olho em lombo e peso. No estudo de associação genômica exploratório foi identificado um SNP por tratamento. Em suma, os efeitos da programação fetal são variados e dispersos na literatura, e essa dissertação traz grandes contribuições para este campo de estudo, desde a descoberta de mecanismos moleculares à pequenas alterações fenotípicas.

Palavras-chave: Desempenho, epigenética, Nelore, nutrição materna, nutrigenética.

ABSTRACT

The production of beef cattle, for the most part, occurs in an extensive way, where the animals depend almost exclusively on the availability of forage in terms of quality and quantity. In this scenario, the fetus in the pregnant mother faces the great challenge of depending on the mother for its nutrition and development, often suffering the impact of maternal malnutrition. In turn, maternal nutrition has a great impact on the life of the individual in formation, being able to cause long-term changes in several areas, a process called fetal programming. With that, the objective of this dissertation was to evaluate the corporal performance and muscular development in steers throughout life, and the reproductive development of heifers in the rearing. For this, 126 Nellore cows were inseminated and divided into three nutritional plans: NP (control) - Not Programmed, without protein-energy supplementation; PP - Partial Programmed, protein-energy supplementation in the final third of pregnancy; and FP - Full Programmed, protein-energy supplementation during all gestation. From birth, the animals had their weight and ultrasound measurements collected. In total, 63 males and 55 females were used. The males underwent biopsy of the *Longissimus* muscle at 15 and 22 months for transcriptomics. During rearing, heifers were monitored for reproduction to assess the impacts of fetal programming on the development of their reproductive tract. In the first chapter of this dissertation, the growth performance of males was evaluated from birth to slaughter, at 22 months, using measurements of weight, ribeye area and backfat and rumpfat thickness, as well as the gain of these measures in the calving, rearing and feedlot periods. No significant differences were found, but trends to the difference appeared in weaning weight, ribeye area gain and average daily gain during feedlot, and gain in rumpfat thickness during calving were detected, suggesting that differences between the treatments was not as great as it could have been. In the second chapter, the effects of maternal supplementation on the epigenetics of animals were evaluated using RNA-Seq data from the *Longissimus* muscle. In it, no differences were observed in the expression of genes related to changes in histones and chromatin, but there were differences between the treatments and the control when looking at the regulation made by long non-coding RNAs (lncRNA). Genes regulated by lncRNA have been shown to be involved in uterine embryonic development, adipose cell differentiation, vasculogenesis, transforming growth factor beta and canonical Wnt signaling pathway, among others. Finally, in the third and last chapter, the reproductive development of females during rearing, correlation between reproductive and performance traits, and genotype-environment

interactions was addressed. There were differences in weight over time, but no effect of maternal supplementation was detected on the other traits. In the correlation analyses, there was a high correlation between measurements of loin eye area and weight. In the exploratory genomic association study, one SNP per treatment was identified. In summary, the effects of fetal programming are varied and dispersed in the literature, and this dissertation brings great contributions to this field of study, from the discovery of molecular mechanisms to small phenotypic changes.

Keywords: Epigenetics, maternal nutrition, Nellore, nutrigenetics, performance.

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LIST OF ABBREVIATIONS AND ACRONYMS

ABIEC – Associação Brasileira Das Indústrias Exportadoras De Carnes

ADG – Average Daily Gain

AFC – Antral Follicular Count

BCS – Body Condition Score

BFT – Backfat Thickness

BFTg – Backfat Thickness Gain

BLAST – Basic Local Alignment Search Tool

BW – Body Weight

CL – *Corpus luteum*

CP – Crude Protein

CPC2 – Coding Potential Calculator 2

DE – Differential Expression

DM – Dry Matter

DMI – Dry Matter Intake

DNA – Deoxyribonucleic Acid

FAO – Food and Agriculture Organization

FDR – False Discovery Rate

FP – Full Programmed

FTAI - Fixed Time Artificially Inseminated

FZEA – Faculdade de Zootecnia e Engenharia de Alimentos

GWAS – Genome-Wide Association Study

LM – Linear Model

lncRNA – Long Non-Coding RNA

mRNA – Messenger RNA

ncRNA – Non-Coding RNA

NDF – Neutral Detergent Fiber

NP – Not Programmed

ORF – Open Reading Frame

PC – Principal Components

PCIT – Partial Correlation and Information Theory

PELT – Protein-Energy Last Trimester

PEWG – Protein-Energy Whole Gestation

PP – Partial Programming

REA – Ribeye Area

REAg – Ribeye Area Gain

RFT – Rumpfat Thickness

RFTg – Rumpfat Thickness Gain

RIF – Regulatory Impact Factor

RIN – RNA Integrity Number

RNA – Ribonucleic Acid

SAS – Statistical Analysis System

SNP – Single Nucleotide Polymorphism

TDN – Total Digestible Nutrients

TGF-β – Transforming Growth Factor Beta

SUMMARY

1. Introduction

Following trends in global population growth, the annual population increase is approximately 79 million, so that by 2050 the world population is expected to reach the 10 billion mark, boosting the demand for food. Also, the increase in income in emerging countries would cause a transition in the diet of the population, increasing the consumption of meat, fruits, and vegetables when compared to the consumption of cereals, requiring proportional changes in agricultural production (FAO, 2017). In this way, making beef production more efficient, and increasing muscle deposition in animals can be an approach to producing more food.

Brazil has great prominence when it comes to beef production, as it is the largest exporter of this commodity and the second largest producer (after the USA), in addition to having the largest cattle herd in the world (ABIEC, 2022). In this systematic, the most used production method is characterized by the Cerrado-Urochloa-Nellore trinomial. Although Nellore cattle are robust and well adapted to hot climates, the environment that the trinomial offers have several challenges, among them, soils with low fertility, long periods of drought, and high temperatures (DA SILVA; NASCIMENTO JÚNIOR, 2007). These seasonal factors end up altering both the availability and the quality of available forage, nutritionally affecting cattle.

The uterine environment has great importance during the prenatal development of the progeny, after all, it reflects the conditions that this new individual will have to face to perpetuate the species after birth. Several factors that may occur during pregnancy, whether stimulants or stressors, are capable of altering fetal growth and development, modifying organ structure and/or function, and generating long-term changes in the progeny (MEYER; CATON, 2016; REYNOLDS). et al., 2019). Changes that occur in the animal due to events during the fetal period are called fetal programming, a term that originated with epidemiologist David Barker (BARKER, 1990, 1995). Many epidemiological studies were subsequently conducted confirming the observations found, along with the identification of risk factors (REYNOLDS et al., 2019).

Several studies have already shown the effects of fetal programming on progeny, in which the impacts are perceived in various organs, changes occur in body composition and development, hormonal balance, and metabolic functions (LONG et al., 2009; WU et al., 2006), and even the placenta is able to respond to nutritional changes (VAN EETVELDE et al., 2016).

The effects of maternal nutrition can also be observed on the female reproductive system, with supplementation having positive effects on sexual precocity and pregnancy rate of heifers (FUNSTON et al., 2010; MARTIN et al., 2007). Effects of maternal nutrition on maturation (RAE et al., 2001) and quality of oocytes (MURDOCH et al., 2003) were also observed, where malnutrition promoted oxidative lesions in cells.

In terms of muscle and adipose tissue development, maternal undernutrition has more aggressive effects, limiting the formation of myocytes and adipocytes (DU et al., 2010; DU; FORD; ZHU, 2017; YAN et al., 2013). However, these effects seem to be overcome in postnatal life with the compensatory gain in hypertrophy (LONG et al., 2010, 2012).

However, the information found in the literature still presents contradictory results. This may be related to the different diets, gestational periods and breeds evaluated (ZAGO; CANOZZI; BARCELLOS, 2019), showing the need of more extensive studies in the area.

As seen, the condition of maternal nutrition is the biggest factor that impacts the event of fetal programming in production animals. In this way, different strategies of protein-energy supplementation to the mother were the method thought to overcome the effects of low quality and quantity of food on pasture, and thus avoid any loss in development that the progeny may have a posteriori.

In this dissertation, three chapters were prepared in the form of articles. The first chapter relates maternal nutrition to growth performance (weight, average daily gain, ribeye area, backfat and rumpfat thickness and its gains) in male progeny throughout their lifetime. In the second chapter, the involvement of maternal nutrition and epigenetic in the muscle development of the progeny is addressed, where epigenetic mechanisms were investigated through the differential expression of related genes and the search for long non-coding RNAs regulating processes within the treatments. Finally, in the last chapter, the reproductive development of the female progeny during the growing period was evaluated, as well as the genotype-environment interaction between the traits. In summary, this dissertation has as main objective to better understand the phenotypic and molecular effects of maternal nutrition with regard to performance, muscular and reproductive development in beef cattle.

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2. Chapter 1: Evaluation of fetal programming effects in lifelong performance of Nellore bulls

Abstract

Improper maternal nutrition impacts fetal development, which can affect the growth performance and health of the progeny in the long term. Thus, the objective of this study was to evaluate the impact of different protein-energy supplementation strategies during pregnancy on the growth performance of the progeny throughout its life. A herd of 126 Nellore cows were fixed time artificially inseminated (FTAI) and were separated into three treatments at day 30, when pregnancy diagnosis occurred: NP (control) - Not Programmed, without protein-energy supplementation; PP - Partial Programmed, protein-energy supplementation in the final third of pregnancy; and FP - Full Programmed, protein-energy supplementation during all gestation. Protein-energy supplementation was at the level of 0.3% live weight. A total of 63 male offspring were used in this study. The animals were followed throughout the calving phase, rearing and feedlot finishing in which weight, ribeye area (REA), backfat (BFT) and rumpfat thickness (RFT) were collected. Gains in traits (ADG, REAg, BFTg, RFTg) were calculated for each period. Within time, weight, REA, BFT and RFT were similar between treatments ($p>0.05$), except for weaning weight where NP tended to be lighter than others ($p=0.08$). When carried out over time analysis, a trend in time x treatment interaction was shown for RFT ($p=0.06$) and weight ($p=0.07$). In terms of gain, PP group tended to have a major increase in RFT at calving ($p=0.06$), a minor grow of REA in feedlot($p=0.09$) and higher ADG in the same period ($p=0.09$). RFTg also tended to difference over time. Besides that, no differences were found for BFTg at all times, nor for REAg, RFTg and ADG at other times. In addition, all treatments were similar when weight, REAg and BFT was analyzed in repeated measures. Maternal energy protein supplementation had little or no effect on the animal's postnatal growth performance for the traits evaluated, but trends to differences between groups appeared, suggesting that the nutritional difference between treatments was not sufficient.

Keywords: cattle; fetal programming; growth; performance; long-term.

2.1. Introduction

Maternal nutritional status is of great importance during pregnancy, as stimuli or insults during this period can lead to phenotypic and genetic changes in the developing individual (BARKER, 1990; MEYER; ZHANG, 2007; REYNOLDS et al., 2019). During embryo formation, the partition of nutrients from the mother prioritizes vital organs, like heart, brain, kidneys, and with this, tissues such as skeletal muscle and adipose, two of the main constituents of meat, are susceptible to the availability of nutrients offered (DU et al., 2010; ZHU et al., 2006). Particularly beef cattle, when raised on pasture, are more subject to nutritional insults due to seasonal changes in forage quality.

Maternal malnutrition can cause neonatal mortality, changes in body composition and growth, hormonal imbalance, changes in the development, functioning of organs and metabolic functions. (LONG et al., 2009; WU et al., 2006). The effects of malnutrition in early pregnancy have been reported for both muscle fibers (LONG et al., 2010), as for adipose tissue (LONG et al., 2012). Furthermore, maternal restriction at the end of pregnancy can lead to lower birth weight (MICKE et al., 2010), and thus impact the performance of the progeny, once birth weight is related to the growth rate and weight throughout life (RAINFORTH, 2019). At the same time, supplementation in the final third has positive effects, such as greater weight at birth and weaning, greater weight gain until weaning, and with that, greater weight at the beginning of the rearing period. (BOHNERT et al., 2013).

Thus, fetal programming is of great importance in the meat industry, since its impacts can influence the animal's physiology in several aspects. (FUNSTON; LARSON; VONNAHME, 2010). In cattle, primary myogenesis begins within the first 2 months after conception, while secondary myogenesis occurs between the second and seventh or eighth months of gestation. (RUSSELL; OTERUELO, 1981), being an exclusive event of fetal life and not occurring after that period. Few muscle fibers are formed in primary myogenesis, and thus, maternal nutrition in early development has negligible effects on skeletal muscle development in this period. However, the vast majority of muscle fibers are formed during the second myogenesis, and a decrease in the number of fibers formed has irreversible effects on the progeny (DU et al., 2010; DU; FORD; ZHU, 2017).

Adipose tissue, in turn, is formed primarily during fetal life and early postnatal life, ending its development in puberty (SPALDING et al., 2008). The onset of adipogenesis occurs

concomitantly with the second myogenesis, but the greatest formation of adipocytes occurs between late pregnancy and weaning (DU; FORD; ZHU, 2017) That said, maternal nutrition during these developmental windows will affect the development of adipose tissue (DU et al., 2013)

Related to this, several studies have already shown the effects of maternal nutrition on muscle and adipose tissue development (UNDERWOOD et al., 2010; ZHANG et al., 2021), as well as on body weight (LONG et al., 2021; VALIENTE et al., 2021). However, the data found in the literature on maternal nutrition affecting the body performance of the progeny are sparse, evidencing the need for more studies in the area.

In this study, it was hypothesized that different protein-energy supplementation strategies during pregnancy in beef cattle alters the growth performance of their progeny. Thus, the objective of this study was to evaluate whether the different nutritional plans produced impacts on the muscular, adipose and body development of the animals throughout their life, from the calving period to the finishing in feedlot.

2.2. Material and methods

2.2.1. Ethics statement

This study was approved by the Research Ethics Committee of the Faculty of Food Engineering and Animal Sciences from the University of São Paulo under protocol No. 1843241117, according to the National Council for the Control of Animal Experimentation guidelines.

2.2.2. Experimental design

A total of 126 Nellore cows were fixed time artificially inseminated (FTAI) with semen of four bulls. At day 30 after FTAI pregnancy diagnosis occurred, and animals were separated into three treatments: NP (control) - Not Programmed, without protein-energy supplementation; PP - Partial Programmed, protein-energy supplementation in the final third of pregnancy; and FP - Full Programmed, protein-energy supplementation during all gestation. Protein-energy supplementation was at the level of 0.3% live weight. All groups received 0.03% live weight mineral supplementation, included in protein-energy supplementation. More details about the groups and supplementation can be found in Schalch Jr. et al. (2022).

Cows were blocked based on their age, body weight and body condition score, and kept in pasture paddocks of *Urochloa brizantha* cv. Marandu with access to the supplement and water ad libitum. After calving, protein-energy supplementation ceased and all animals remained together until weaning (average 220 days old). Offspring received the same sanitary, vaccination, and feeding protocols already implemented on the Faculty farm. After weaning, the animals were divided by sex, regardless of the treatment, and placed in separate pastures, remaining there throughout the rearing phase. A total of 63 male offspring were used in this study. More details about rearing phase can be found in Polizel et al. (2021).

At 19 months, young bulls initiated the finishing phase in feedlot system lasting 106 days (15 of adaptation period, and 91 of effective feedlot). There was no difference of weight between treatments when animals entered finishing phase (NP = 452 ± 6, PP = 450 ± 7, FP = 463 ± 7 kg; P value > 0.1). During this period, they received three different diets: an adaptation diet provided in the first 15 days (Dry matter (DM) = 48.1%; TDN = 71.0%; CP = 15.0%; NDF = 36.5%; Fat = 3.2%; Dry matter intake (DMI) = 2.21% of BW); a second diet during 35 days (DM = 53.6%; TDN = 73.6%; CP = 14.0%; NDF = 31.1%; Fat = 3.4%; DMI = 2.20% of BW); and a third diet for 56 days (DM = 60.6%; TDN = 76.2%; CP = 13.0%; NDF = 25.8%; Fat = 3.7%; DMI = 2.04% of BW).

2.2.3. Ultrassound evaluation

Ribeye area (REA), backfat thickness (BFT), and rump fat thickness (RFT) was measured by ultrasound using an Aloka SSD-500 ultrasound equipped with a 17-cm linear transducer at 3.5 MHz frequency (Aloka Co. Ltd., Wallingford, CT, USA), and vegetable oil as coupling to optimize the contact of the transducer with the skin of the animals. Images in sections of the *Longissimus thoracis* muscle, between the 12th and 13th ribs, to measure REA and BFT, while the RFT was measured by positioning the transducer in the final portion of the ileum, between the junction of the *Biceps femoris* and the middle gluteal muscle (SANTANA et al., 2015). The images were captured using the Lince software and later analyzed by a certified technician. Data was collected throughout the calving, rearing, and finishing phases. For the purpose of this study, data was collected at 30 days old (only REA), 6 months, weaning, 12 months, 15 months, 18 months, and the feedlot (days 0, 35, 57, and 70).

Regarding the ribeye area gain (REAg), backfat thickness gain (BFTg), and rump fat thickness gain (RFTg), the differences were calculated individually and inside each period, i.e. gain in the rearing phase was obtained by subtracting the last measure collected in rearing phase from last measured in calving.

2.2.4. Weighting and average daily gain

Weights were obtained using an electronic scale from Coimma (Coimma Scales, Dracena, São Paulo State, Brazil) coupled to the trunk, where regularly from birth to slaughter animals got their weight recorded. To obtain the average daily gain (ADG), a linear regression between age in days and weight was performed individually for the calving, rearing, and finishing phases.

2.2.5. Statistical Analysis

All procedures were performed using the MIXED procedure of the statistical package SAS® version 9.4 (SAS Institute Inc, NC, USA). From data obtained, residues were submitted to the Shapiro-Wilk test for normality implemented at UNIVARIATE procedure, where measurements that did not follow normality were transformed using log (i.e., ln(trait + 1)). The homoscedasticity of residuals for principal effects was tested in the groups using Levene's test. To evaluate the effects of treatments on phenotypes, analysis of variance was used and the means compared by Tukey-Kramer test, with differences between treatments considered significant when $p \leq 0.05$. Age of animal, age of the cow and sire were also considered in the linear model. Concerning to the repeated measures, the same variables than linear model were considered and the time of data collection was also included. The covariance structure for the residuals was tested for each variable and chosen based on Akaike information criterion (AIC). The variables REA, REAg, BFT, BFTg, RFTg and weight used an unestructured (un), while ADG used first-order factor analytic (fa(1)). For those analysis the model was as follows:

$$y_{ijkl} = \mu + \beta_1 Age_{m_l} + Sire_i + Treat_j + Time_k + (Treat \times Time)_{jk} + e_{ijkl}, \quad (1)$$

Where: y_{ijkl} is the observed variable from l^{th} animal, son of ith sire, recorded on j^{th} treatment at k^{th} time of measurement (weaning, 12, 15, 18 and/or 24 months of age); μ is just a constant; β_1 is the regression coefficient of covariate mother's age; Age_{m_l} is the observed value for mother's age of lth animal; $Sire_i$ is the fixed effect of ith sire; $Treat_j$ is the fixed effect of jth treatment; $Time_k$ is the fixed effect of kth time of measurement; $(Treat \times Time)_{jk}$ is the fixed interaction between

treatment and time; and e_{ijkl} is the residual random term, which was assumed normally distributed with covariance structure as presented above. It must be noticed that when the analysis was performed within time, this effect (and also the treatment by time interaction) was removed from the model.

2.3. Results

Relating to weight, there was a trend of difference at weaning ($p = 0.08$), where the NP group tended to be different from others, and in the other periods, all treatments were similar ($p > 0.05$). When analyzing repeated measures over time, a trend in time x treatment interaction was shown ($p = 0.07$). In terms of ADG, a trend in the difference between treatments was identified for the finishing phase ($p = 0.09$), but there were no impacts of supplementation kind over time or any other period ($p > 0.05$; Table 1).

Table 1. Lifelong weight and weight gain of Nellore young bulls under fetal programming

Trait	Time	NP	PP	FP	p-value[1]	p-value[2]
Weight (Kg)	30 days	68.42 ± 2.71	68.81 ± 2.20	73.28 ± 2.89	0.35	0.30
	6 months	190.22 ± 4.92	199.09 ± 3.79	198.99 ± 5.08	0.27	
	Weaning	216.61^{b} ± 5.20	231.84^{a} ± 5.01	232.9^{a} ± 4.62	0.08	
	12 months	293.75 ± 5.03	296.88 ± 5.95	301.70 ± 5.54	0.46	
	15 months	370.01 ± 5.43	370.78 ± 6.02	382.99 ± 5.48	0.12	
	18 months	430.70 ± 4.79	429.78 ± 6.12	439.92 ± 4.34	0.13	
	Feedlot_D0	452.07 ± 6.02	448.99 ± 7.17	464.01 ± 7.20	0.16	
	Feedlot_D35	512.01 ± 6.73	518.97 ± 8.11	528.27 ± 8.04	0.21	
	Feedlot_D57	556.52 ± 7.04	568.70 ± 9.29	568.94 ± 9.55	0.34	
	Feedlot_D70	576.25 ± 8.11	590.92 ± 10.01	587.02 ± 10.19	0.39	
ADG	Calving	0.85 ± 0.020	0.91 ± 0.017	0.92 ± 0.015	0.27	0.21
	Rearing	0.58 ± 0.011	0.56 ± 0.011	0.60 ± 0.013	0.34	
	Feedlot	1.70^{b} ± 0.034	1.87^{a} ± 0.048	1.71^{b} ± 0.042	0.09	

The data are expressed as means of the characteristics ± standard error of the mean. [1] – P-value between groups on the same age; [2] – P-value on repeated measures over time. NP - without protein-energy supplementation, PP – Protein-Energy Last Trimester (0,3%BW protein-energy supplementation in the final third of pregnancy), FP – Protein-Energy Whole Gestation (0,3%BW protein-energy supplementation upon pregnancy confirmation).

The REA was similar between the treatments both within and over time, with no significant differences being found ($p > 0.05$). When the REAg was analyzed, there was a trend for difference

at feedlot phase (p = 0.09), however, no differences appeared at other periods or when it was carried over time (Table 2).

Table 2. Ribeye area throughout life of Nellore young bulls submitted to fetal programming

Trait	Time	NP	PP	FP	p-value[1]	p-value[2]
REA (cm^2)	30 days	19.4 ± 0.9	19.85 ± 0.64	20.95 ± 1.01	0.78	0.62
	6 months	39.95 ± 1.32	43.41 ± 1.08	44.60 ± 1.16	0.35	
	Weaning	45.22 ± 1.03	47.51 ± 1.11	47.21 ± 0.94	0.62	
	12 months	58.78 ± 1.02	57.40 ± 1.05	59.32 ± 1.11	0.75	
	15 months	68.1 ± 1.08	67.99 ± 0.95	68.05 ± 0.89	0.96	
	18 months	77.18 ± 1.09	78.67 ± 1.01	78.60 ± 1.15	0.72	
	Feedlot_D0	84.45 ± 1.22	86.90 ± 0.75	84.71 ± 1.06	0.35	
	Feedlot_D35	92.75 ± 1.21	91.30 ± 1.16	94.89 ± 1.20	0.33	
	Feedlot_D57	97.78 ± 1.22	102.33 ± 1.35	101.95 ± 1.24	0.31	
	Feedlot_D70	97.50 ± 1.01	98.76 ± 1.22	98.26 ± 0.98	0.77	
REAg	Calving	26.01 ± 1.41	27.20 ± 1.20	25.71 ± 1.17	0.88	0.81
	Rearing	18.21 ± 0.82	20.88 ± 1.39	19.94 ± 0.88	0.45	
	Feedlot	13.52^a ± 0.85	11.01^b ± 1.12	13.10^a ± 1.01	0.09	

No differences between treatments were found for fat deposition in this area (p > 0.05) in all periods evaluated, for repeated measures over time and for BFTg, showing a BFT similar for all three groups in a lifelong period (Table 3).

Table 3. Lifelong backfat thickness of Nellore young bulls submitted to fetal programming

Trait	Time	NP	PP	FP	p-value[1]	p-value[2]
BFT (mm)	6 months	1.20 ± 0.18	1.15 ± 0.16	1.47 ± 0.17	0.32	0.72
	Weaning	1.65 ± 0.15	1.55 ± 0.17	1.69 ± 0.13	0.90	
	12 months	0.64 ± 0.12	0.40 ± 0.10	0.45 ± 0.10	0.39	
	15 months	0.64 ± 0.16	0.51 ± 0.11	0.55 ± 0.12	0.84	
	18 months	1.52 ± 0.14	1.78 ± 0.18	1.33 ± 0.12	0.69	
	Feedlot_D0	1.82 ± 0.15	1.83 ± 0.20	2.39 ± 0.17	0.32	
	Feedlot_D35	4.93 ± 0.16	4.87 ± 0.21	5.18 ± 0.18	0.69	
	Feedlot_D57	6.32 ± 0.23	6.27 ± 0.26	6.33 ± 0.24	0.91	
	Feedlot_D70	7.5 ± 0.20	8.47 ± 0.27	8.45 ± 0.26	0.23	
BFTg	Calving	0.41 ± 0.12	0.45 ± 0.16	0.30 ± 0.15	0.82	0.42
	Rearing	0.84 ± 0.24	1.42 ± 0.19	0.95 ± 0.17	0.39	
	Feedlot	5.99 ± 0.19	6.67 ± 0.28	6.20 ± 0.25	0.32	

All treatments had similar RFT when data was analyzed within time ($p > 0.05$). When the analysis was carried over time, a trend in time x treatment interaction appeared ($p = 0.06$) i.e, the effect of time may depend on which treatment animals received. Relating to RFTg, there was a trend at calving phase ($p = 0.06$), where PP group tended to be different from NP and FP, and over time ($p = 0.09$), but at rearing phase and feedlot all treatments were similar (Table 4).

Table 4. Rumpfat thickness throughout life of Nellore young bulls under fetal programming

Trait	Time	NP	PP	FP	p-value[1]	p-value[2]
RFT (mm)	6 months	2.23 ± 0.15	2.15 ± 0.18	2.50 ± 0.16	0.66	0.59
	Weaning	2.36 ± 0.17	2.84 ± 0.15	2.45 ± 0.20	0.39	
	12 months	1.04 ± 0.14	0.82 ± 0.16	1.02 ± 0.13	0.44	
	15 months	1.47 ± 0.16	1.56 ± 0.17	1.89 ± 0.13	0.46	
	18 months	2.94 ± 0.17	3.18 ± 0.19	3.29 ± 0.19	0.56	
	Feedlot_D0	3.41 ± 0.15	3.14 ± 0.23	3.6 ± 0.21	0.65	
	Feedlot_D35	6.10 ± 0.19	6.35 ± 0.27	6.79 ± 0.22	0.43	
	Feedlot_D57	7.41 ± 0.20	7.06 ± 0.31	7.65 ± 0.25	0.40	
	Feedlot_D70	8.51 ± 0.27	9.19 ± 0.39	9.72 ± 0.23	0.49	
RFTg	Calving	0.09^{b} ± 0.14	0.69^{a} ± 0.15	0.0^{b} ± 0.17	0.06	0.09
	Rearing	1.90 ± 0.19	2.35 ± 0.17	2.29 ± 0.20	0.27	
	Feedlot	5.20 ± 0.17	6.09 ± 0.32	6.10 ± 0.24	0.35	

2.4. Discussion

This work followed the performance throughout the life of 63 Nellore bulls that underwent fetal programming During the 22 months of collection (age at slaughter), no significant differences were found between treatments, but trends towards difference appeared at interesting points evaluated, indicating the presence of processes that can be studied further.

Regarding weight, we observed a trend at weaning, where the control group had lower weight compared to the others, however, no other trend was observed throughout life. In some studies, where the cows passed the pregnancy to pasture, but received protein supplementation, the calves did not show differences in weight at birth, however at weaning there was an increase in weight when compared to the non-supplemented group. (LARSON et al., 2009; MARTIN et al., 2007; STALKER et al., 2006) Additionally, similar results were observed by Marques et al. (2016),

where cows that had an increase in the body conditioning score during the second and final third of gestation weaned heavier animals.

Although there were no differences for ADG between treatments, a trend was observed in the feedlot, where the PP group tended to have a weight increase above the others (Figure 1). When searching for studies in the literature, once again scattered results were found. Some studies suggest there is no difference between treatments for ADG in feedlot (BLOCK et al., 2020; WILSON; FAULKNER; SHIKE, 2016) while in Mulliniks et al. (2015), animals whose mothers were able to maintain or gain weight during late pregnancy had higher ADG in feedlot.

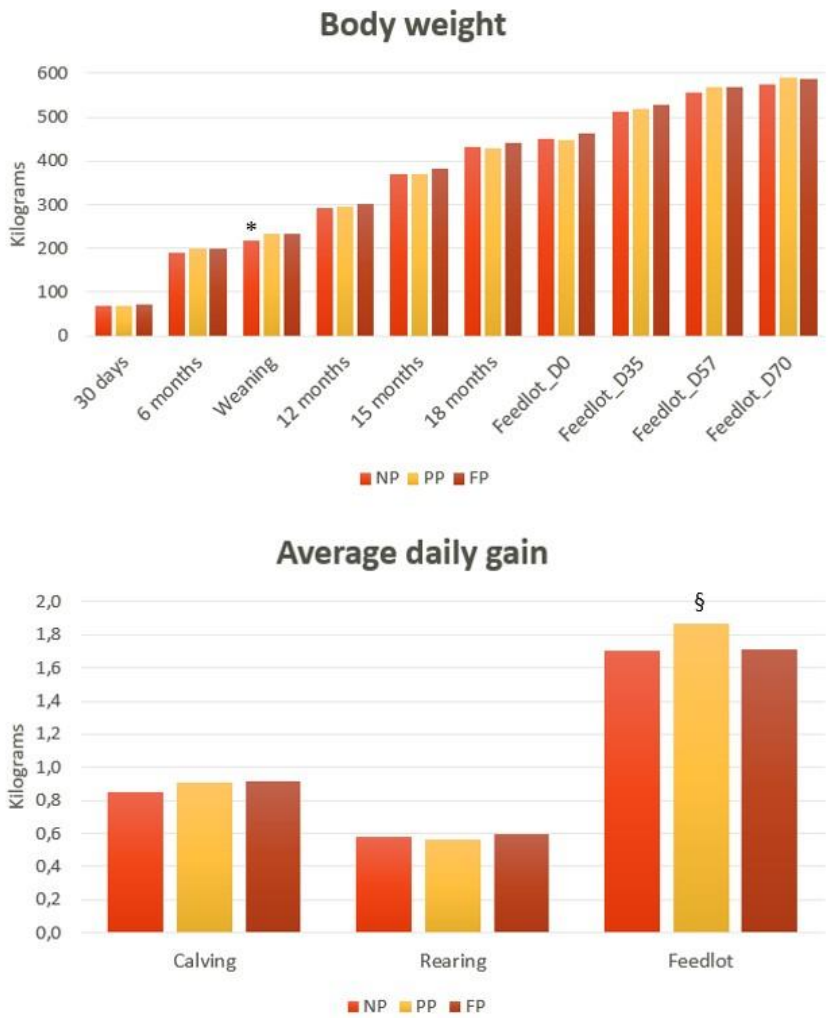

Figure 1. Body weight and average daily gain throughout life in fetal programmed young bulls. * NP tended to be lighter than others (p=0.08); § ADG tended to be higher in PP (p=0.09).

During pregnancy, the nutrients ingested by the mother and sent to the fetus prioritize the formation of vital organs, such as the heart, brain and kidneys (DU et al., 2010), leaving tissues

considered secondary, such as skeletal muscle, at the mercy of the availability of nutrients (ZHU et al., 2006). Thus, it is expected that maternal energy restriction causes changes in the muscle development of the offspring, as in some studies in sheep (REED et al., 2014) and cattle (COSTA et al., 2021; MARESCA et al., 2019b) have already demonstrated. Although there are indications of the impact of fetal programming on muscle, other studies, similarly to ours, also did not observe differences in the REA (PIAGGIO et al., 2018; QUARNBERG et al., 2016) when the mothers underwent energy restriction. It is possible that this absence of differences in REA throughout life is due to a compensatory gain after birth.

Following this idea, we also measured the gains that occurred between the periods, in an attempt to detect if there was a compensatory gain in postnatal life. A trend towards a difference in REAg during confinement was identified (Figure 2), where the PP treatment, which received protein-energy supplementation only in the final third, had a lower increase in REA compared to the others. Despite being only a trend in treatment effect, important questions can be raised for future investigations.

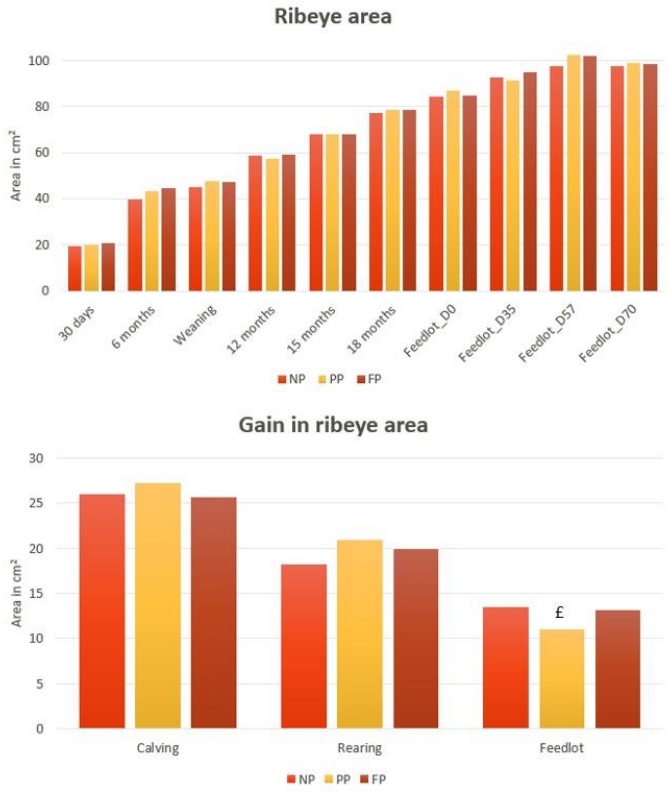

Figure 2. Ribeye area (REA) and ribeye area gain throughout life in young bulls under fetal programming. £ PP tended to have a minor grow in REA (p=0.09).

The FP group may have received good levels of nutrition during their fetal formation and thus managed to maintain satisfactory muscle development. While the NP group, considered our control and a good representative of beef cattle that undergo an extensive system in Brazil, tolerates maintaining the same levels of muscle development as the FP since they are zebu animals, a naturally more rustic species, and that perhaps, after generations developing in Brazilian production, it has adapted and is able to overcome the adversities of this system. Still following this thought, and taking into account the primary window of muscle fiber formation that occurs only from the first third to the middle of the second third of pregnancy (DU et al., 2010), it is possible to question that the supplementation received by the PP only in the final third of gestation was not able to interfere with the constitution of muscle fibers given the formation window, and adding to this, it may have annulled any previous adaptation of the species due to the “thrifty” phenotype (HALES; BARKER, 1992).

The similarity between treatments for REA was also observed by Mohrhauser et al. (2015) and Ramírez et al. (2020), however, differences in BFT measurements were reported in these studies. Results involving fat thickness and fetal programming are well dispersed in the literature. There are studies that reported an increase in thickness in the groups that underwent restriction, such as the two presented above, which probably happen due to a compensatory growth in adipose tissue (DU; FORD; ZHU, 2017). However, others studies did not observe differences in fat thickness, but observed differences in REA (BLOCK et al., 2020; MARESCA et al., 2019a, 2019b). At the same time, other works, such as this one, did not find differences in any of the two characteristics, as in Mulliniks et al. (2015) and Wilson et al. (2016).

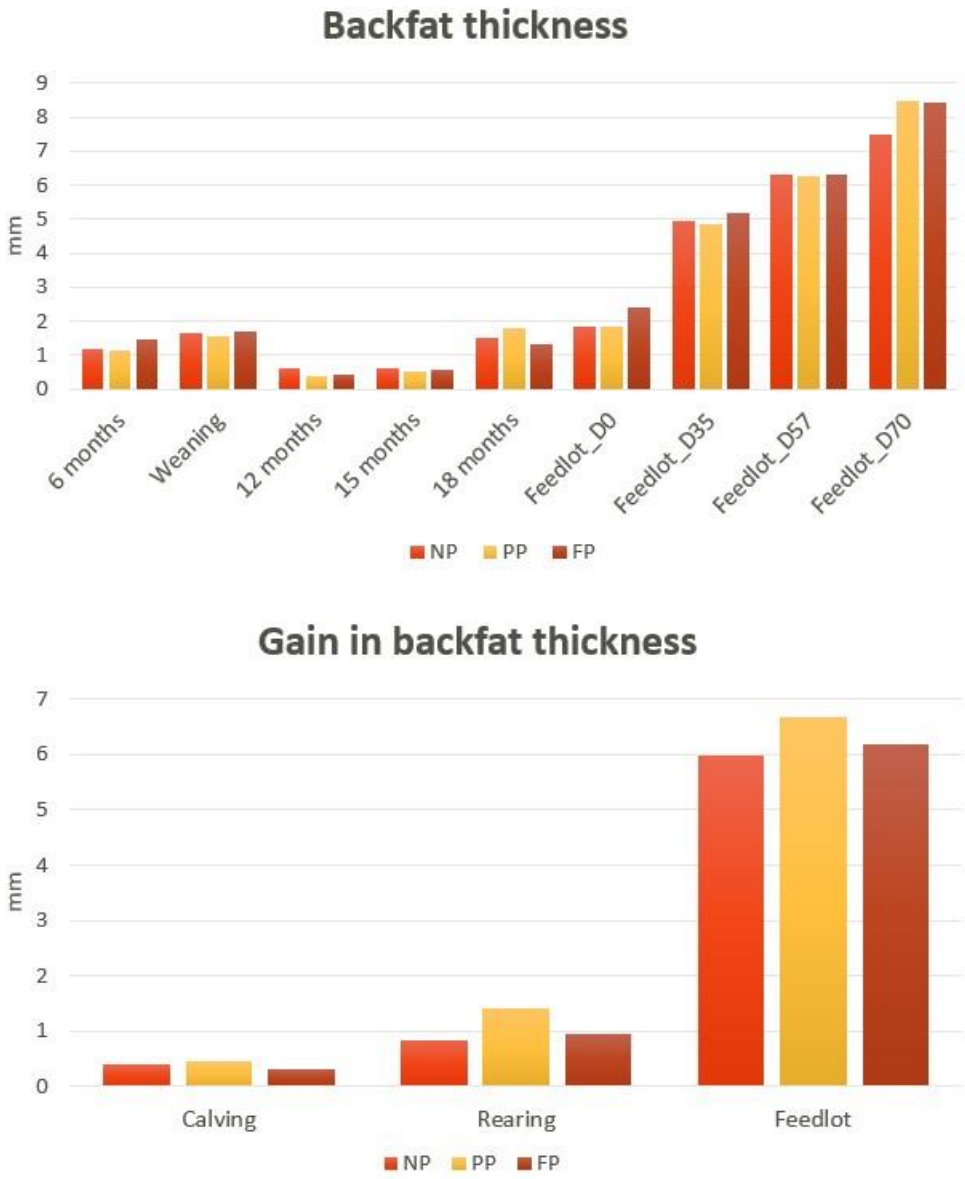

Figure 3. Backfat thickness and gain throughout life in fetal programmed young bulls.

There was no difference in BFT (Figure 3), but some trends emerged in RFT and its gain (Figure 4). There was a trend in the interaction time x treatment, and when analyzing the RFTg, there was a trend in the calving period, where the PP group was superior to the others, and also over time. This trend towards a greater gain in fat thickness for the PP, coupled with the increase observed in some studies in groups that underwent restriction, reinforces a little more the idea presented for the treatments previously when we talk about REAg.

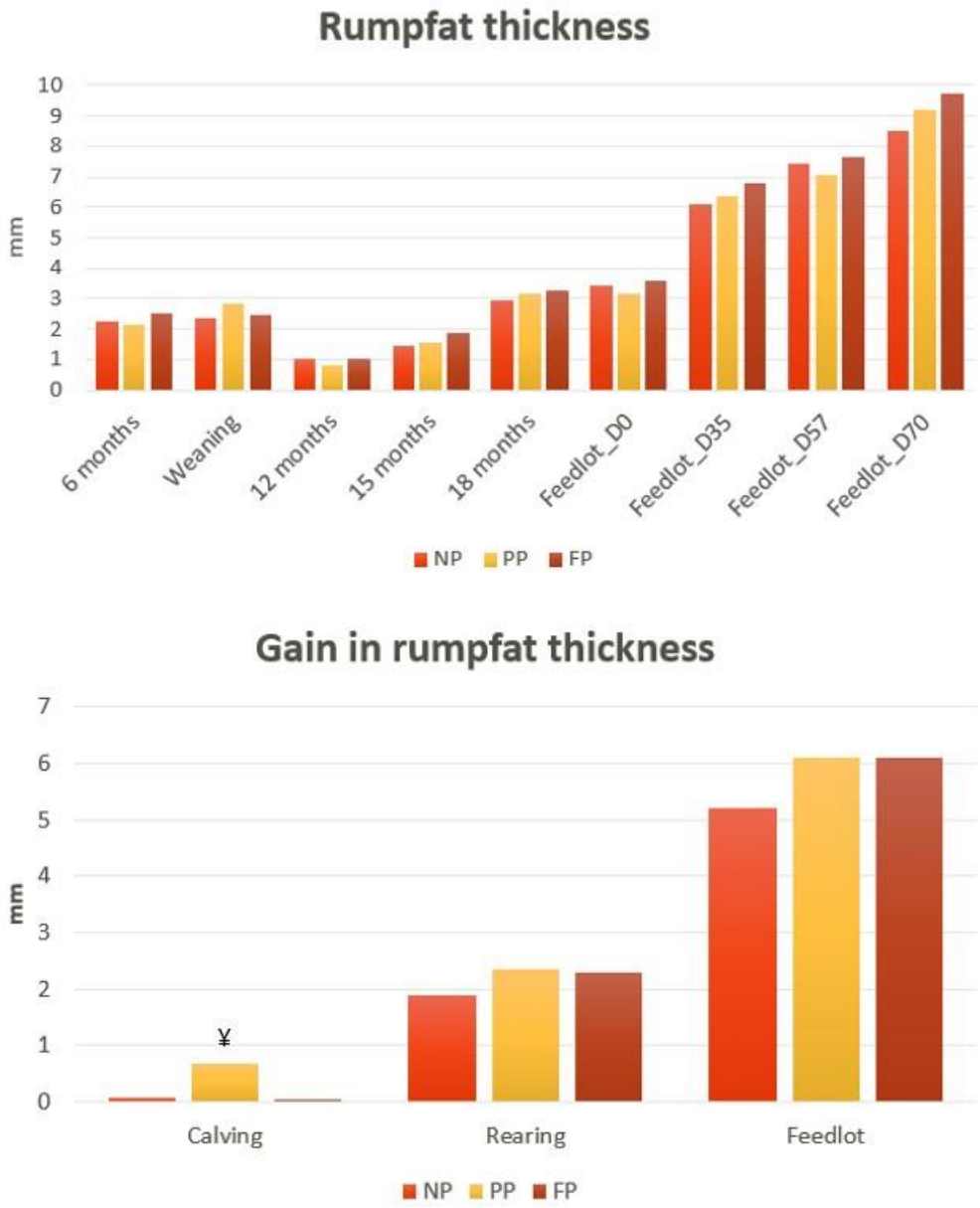

Figure 4. Rumpfat thickness and rumpfat thickness gain throughout life in young bulls under fetal programming. ¥ PP tended to have a higher gain (p=0.06).

Given the results found in this work and in a search in the literature, it is possible to perceive the need for further studies on the effects of fetal programming, being it the restriction or supplementation of mothers. Still, some of our results tended to differ, which may become significant if the experiment is repeated and the conditions of the extensive system cause a more severe restriction in animals that do not receive protein-energy supplementation.

2.5. Conclusion

Maternal energy protein supplementation had little or no effect on the animal's postnatal growth performance for the traits evaluated. However, there were trends toward the difference between treatments in the characteristics of REA, RFT and body weight, suggesting that the nutritional difference between treatments was not sufficient.

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3. Chapter 2: Fetal programming affect epigenetics regulation in *Longissimus thoracis* muscle of Nellore young Bulls

Abstract

Maternal nutrition has the ability of influence critical process in fetal life, including muscle development. Also in this period, epigenetic sensitivity to external stimuli is higher and produces long lasting effects. Thus, the aim of this study was to investigate epigenetic mechanisms, including the identification and characterization of long non-coding RNAs (lncRNA), in RNA-Seq data from animals that underwent different strategies of prenatal supplementation. A group of Nellore cows (n=126) were fixed time artificially inseminated (FTAI) and were separated into three nutritional plans at day 30, when pregnancy diagnosis occurred: NP (control) - Not Programmed, without protein-energy supplementation; PP - Partial Programmed, protein-energy supplementation in the final third of pregnancy; and FP - Full Programmed, protein-energy supplementation during all gestation. A total of 63 male offspring were used in this study, of which 15 (5 per treatment) had *Longissimus thoracis* muscle biopsy at 15 and 22 months (slaughter). Data of weight, average daily gain (ADG), ribeye area (REA), backfat (BFT) and rumpfat thicknesses (RFT) were collect. Biopsy samples had their RNA extracted and sequenced. Differencial expression (DE) of remodeling factors and chromatin-modifying enzymes genes were performed. For the identification and characterization of lncRNA, a series of size filters and protein coding potential were performed. The lncRNAs identified had differential expression and regulatory potential tested. All groups were similar for phenotypic traits ($p>0.05$). Regarding DE of epigenetic mechanisms, no differentially expressed gene was found ($p>0.1$). Identification of potential lncRNA was successful, identifying 1823 transcripts at 15 months and 1533 at 22 months. Of those, 4 were considered DE between treatments at 15 months and 6 were DE at 22 months. Yet, when testing regulatory potential, 13 lncRNA were considered key regulators in PP group, and 17 in FP group. PP group lncRNAs possibly regulate fat cell differentiation, in utero embryonic development, and transforming growth factor beta receptor, whereas lncRNA in FP group regulates in utero embryonic development, fat cell differentiation and vasculogenesis. Maternal nutrition had no effect on differential expression of epigenetic mechanisms, however it seems to impair lncRNA regulation of epigenetics.

Keywords: cattle; epigenetics; fetal programming; gene expression; muscle.

3.1. Introduction

The main product in beef cattle production is meat. In this setting, muscle development is highlighted and it is of global interest to find out more about the mechanisms that act on it and that can be handled in order to produce meat in a more efficient way. However, poor maternal nutrition is a common scenario in beef cattle production, once the cow is usually managed under an extensive production system, depending only on pastures for feed availability (NOYA et al., 2022). In this case, maternal dietary intake can influence critical processes in fetal and embryonic development, even though the nutrient requirement for the conceptus is negligible in the earliest stages of gestation (VELAZQUEZ, 2015). These processes can predispose offspring to altered endocrine regulation of growth and maintenance, associated with other metabolic dysregulations later in life, as a long-term consequence of fetal programming (BARKER, 1995; WU et al., 2006).

During fetal development, the conceptus depends on the nutrients sent by the mother. However, given the priority in the development of vital organs such as the heart and brain, fetal skeletal muscle is subject to the availability of nutrients (BAUMAN; EISEMANN; CURRIE, 1982; CLOSE; PETTIGREW, 1990; ZHU et al., 2006). However, the intrauterine period is crucial in the development of skeletal muscle, since there is no net increase in the number of muscle fibers after birth (GLORE; LAYMAN, 1983; GREENWOOD et al., 2000; ZHU et al., 2004).

Epigenetics is defined as a set of heritable changes in gene expression, without any change in the genetic code, which can be altered by environmental factors, and are the primary mechanisms through which the effects of fetal programming are carried out (REYNOLDS et al., 2019). There is growing evidence that nutritional conditions can alter genome activity through epigenetic modifications (BOLLATI; BACCARELLI, 2010; BORDONI; GABBIANELLI, 2019; ŞANLI; KABARAN, 2019). Epigenetic modifications include DNA methylation, histone modifications, and non-coding RNA, such as long non-coding RNA (lncRNA) and microRNA (BERNSTEIN; MEISSNER; LANDER, 2007; GOYAL et al., 2019).

Although epigenetic sensitivity persists throughout life, there are periods when it is higher and produces longer lasting effects. (THAYER; RUTHERFORD; KUZAWA, 2020). Many of these critical periods, particularly in mammals, overlap with the periods when resource transfer between mother and progeny occurs, either through the placenta or breast milk (KUZAWA, 2005).

The lncRNA molecules are characterized by having a size greater than 200 nucleotides, having a very low coding potential, being poorly conserved between species, and also not having a specific pattern in their sequence, which makes them difficult to categorize and predict their function. (DENIZ; ERMAN, 2017). The majority of lncRNA that has already been characterized is generated by the same transcriptional machinery as other messenger RNAs (mRNA; GUTTMAN et al., 2009). Also, these transcripts have a 5' terminal methylguanosine cap and are polyadenylated (MERCER; MATTICK, 2013). Its regulatory role in epigenetics is linked to chromatin-modifying proteins and recruits them to specific sites in the genome, to modulate chromatin state and impair gene expressions (MERCER; MATTICK, 2013).

Finally, we previously reported similarities between treatments regarding growth performance (CRACCO et al., unpublished data). However, there are reports in the literature that maternal nutrition can impact fetal development even without notable phenotypic differences (PARADIS et al., 2017). Thus, the hypothesis of this work is that there are epigenetic mechanisms acting silently in the muscular development of cattle that underwent different nutritional strategies during fetal life. Thus, the objectives of this work were (1) to test the differential expression of genes related to epigenetic mechanisms and (2) to identify and characterize lncRNA using RNA-Seq data from animals that underwent different strategies of prenatal supplementation.

3.2. Material and methods

3.2.1. Ethics statement

This study was approved by the Research Ethics Committee of FZEA / USP, under protocol No. 1843241117, according to the guidelines of the National Council for the Control of Animal Experimentation (CONCEA).

3.2.2. Experimental design

A group of 126 Nellore dams were fixed time artificially inseminated (FTAI) with semen of four bulls with known genetic value. Pregnancy diagnosis was taken at 30 days after FTAI, then animals were separated into three treatments: NP - Not Programmed, without protein-energy supplementation (control); PP - Partial Programmed, protein-energy supplementation in the final third of pregnancy; and FP - Full Programmed, protein-energy supplementation during all gestation. All groups received a 0.03% live weight mineral supplementation; PP and FP animal's received protein-energy supplementation at the level of 0.3% live weight and mineral supplement was already included in it. Dams were blocked in the groups based on age, body weight, and body condition score. Animals were allocated to pasture paddocks of Brachiaria brizantha cv. Marandu with access to the supplement and water ad libitum. More details about the groups and supplementation can be found in Schalch Jr. et al. (2022).

After calving, all animals remained together until weaning (average 220 days old), regardless of the treatment, and protein-energy supplementation ceased. The animals were subjected to the same sanitary, vaccination, and feeding protocols already implemented on the farm where the experiment was conducted. After weaning, the animals were divided by sex, regardless of treatment, and placed in separate pastures, where they remained throughout the rearing phase. More details about the rearing phase, including management, evaluations and sample collection, can be found in Polizel et al. (2021). Young bulls remained on the pasture until the beginning of the finishing phase, at 19 months.

Young bulls were finished in feedlot system for 106 days (15 of adaptation period, and 91 of effective feedlot). More details about finishing phase can be found in Polizel et al. (2022). At the end of the finishing phase, animals were slaughtered at the FZEA/USP school slaughterhouse, located approximately 500 meters from the feedlot installations. The slaughter and processing of the carcasses was carried out in accordance with the procedures required by the Ministry of Agriculture, Livestock and Supply of Brazil (MAPA, Normative Instruction No. 9 of 2004). This trial comprised of 63 young bulls, which were evaluated for its muscle development using an epigenetic approach.

3.2.3. Sample collection, RNA extraction and sequencing

At slaughter (676 ± 28 days of age), samples of approximately 2 cm3 were collected from *Longissimus* muscle (between 9 and 10th ribs), cut into smaller pieces using a scalpel and rapidly stored in liquid nitrogen till the moment of RNA extraction. Samples of 5 progenies from the same sire were randomly selected within each treatment for both 15 and 22 months of age sequencing (totaling 30 samples). About 80 milligrams of each sample was macerated with crucible and pestle in nitrogen, and extraction was made using TRIzol (Invitrogen, Carlsbad, CA), following manufacturer's protocol. The concentration and quality obtained at the end of the extraction were evaluated using a spectrophotometer (NanoDrop 2000, ThermoScientific, USA), analyzing the ratios A260/280 and A260/230. The samples that showed undesirable parameters were re-extracted.

The construction of the libraries and RNA sequencing was carried out by the company NGS Soluções Genômicas. RNA integrity (RIN) was obtained using the Bioanalyzer 2100 equipment with Labchips RNA 6000 Nano, following the manufacturer's guidelines (Agilent Technologies Ireland, Dublin, Ireland), where all samples had an RIN value greater than 7.0. For the construction of the RNA libraries, the TruSeq™ RNA Sample Prep kit (Illumina, USA, 2012, Part # 15026495 Rev. D) was used according to the instructions TruSeq® RNA Sample Preparation v2. The libraries were sequenced on the Illumina HiSeq 2500 instrument using the TruSeq PE Cluster Kit and TruSeq SBS Kit (2x100bp).

To determine the quality of the sequencing, FastQC 4.1 software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used. Then, adapters inserted during library formation were removed by Seqyclean v1.9.10 software (ZHBANNIKOV et al., 2017). The alignment of the samples to the Bos taurus reference genome (ARS-UCD1.2.95) was performed by the STAR v020201 software (DOBIN et al., 2013) with default parameters, using an annotation file (ARS-UCD1.2.95), and generated a file with the amount of reads paired to each gene (counts).

3.2.4. Expression of genes related to epigenetic mechanisms

We selected 164 genes related to remodeling factors and chromatin-modifying enzymes. After aligning the samples to the genome and obtaining the read counts for the genes of interest, we

performed the analysis of differentially expressed epigenetic genes by contrasting each group to the others. EdgeR v3.32.0 (ROBINSON; MCCARTHY; SMYTH, 2010) and Limma v3.46.0 (RITCHIE et al., 2015) packages were used, in the R statistical environment. The read counts file of the genes of interest and a file containing factors for normalization (sample number, treatment, age of dam, age of animal) were used to assemble the comparison matrix. Afterward, the steps were followed as presented by (LAW et al., 2018).

3.2.5. lncRNA differential expression

After aligning the reads of each sample to the reference genome (ARS-UCD1.2), the Cufflinks software (TRAPNELL et al., 2013) was used to generate an annotation file for each sample using the reference annotation. Individual annotation files and the bovine reference annotation were then merged into one using Cuffmerge. Through the genomic position of the transcripts, it was possible to select those with potential to be lncRNA. Only transcripts from class codes “i” (intron transcripts, “j” (new isoforms), “o” (generic overlap with known exon), “u” (intergenic transcripts) and “x” (overlap with known gene on the opposite strand) were selected. From there, a FASTA file was generated containing the sequence of transcripts that passed through size filters (>200 base pairs [bp]) and open reading frame size (ORF; <300bp), using GetOrf software. Absence of protein homology was tested through BLASTx (ALTSCHUL et al., 1990) and coding potential using CPC2 (KANG et al., 2017). Transcripts that pass through the filters were considered new lncRNAs. In order to generate the reads counts table for the new lncRNA, FeatureCounts (LIAO; SMYTH; SHI, 2014) was used. The edgeR package was used in the R environment in order to test the differential expression between the three treatments in the identified lncRNAs, where those with Q value < 0.05 were considered differentially expressed. To characterize the differentially expressed lncRNAs, a search for homology was performed using BLAST+ (CAMACHO et al., 2009) in the NONCODE database (FANG et al., 2018), where homologies with an E value > 10 -6 were considered significant, as described by (ALEXANDRE et al., 2020).

3.2.6. Regulatory potential and co-expression networks

To identify regulatory genes related to fetal programming and generate a co-expression network, from 14,125 expressed genes in muscle across all the samples, 1222 were selected for having gene ontology (BLAKE et al., 2015) terms associated with skeletal muscle (GO:0048641,

GO:0048630, GO:0048631, GO:0048741, GO:0003009, GO:0003010, GO:0003011, GO:0043501, GO:0043503, GO:0043403, GO:0007519, GO:0035914, GO:0014856, GO:0014734, GO:0014732, GO:1904204, GO:0014816, GO:0048644, GO:0048634). These genes were considered targets in a regulatory impact factor (RIF; REVERTER et al., 2010) analysis which tested the potential for the lncRNA to be key regulators of epigenetic moduling, contrasting treatments with control. This algorithm assumes that master regulators in a network contribute to altering gene expression by changing their behavior in different biological conditions. To try to predict the role of lncRNA in muscle development in animals that underwent fetal programming, co-expression networks were constructed for each treatment using 1222 mRNA and 394 lncRNA using the partial correlation and information theory algorithm was used (PCIT; (REVERTER; CHAN, 2008). After the execution of the PCIT, the filtering between the groups was performed for: connections that appeared only in the PP and not in the NP, relations exclusive to the PP, relations from the FP that did not appear in the NP, relations exclusive to the FP and relations that appeared only in the PP and FP, but not in NP. The Cytoscape software (SHANNON et al., 2003) was used to build the co-expression networks and DAVID (SHERMAN et al., 2022) for functional enrichment.

3.3. Results

No gene related to epigenetic mechanism were differentially expressed between treatments in any time. All had p-value > 0.1.

Regarding identification of lncRNA, after selecting transcripts through class code, 68,316 new transcripts were identified at 15 months of age and 62,573 new transcripts at 22 months of age with the potential to be new lncRNA. Of these, 88.1% and 89.7% of transcripts (15 and 22 months of age, respectively) belonged to class code “j”, followed by 7.6% and 6.4% of transcripts (in their respective ages) belonging to the class code “u”.

When applying the sequential filters, 99.9% of the transcripts in both ages were larger than 200 nucleotides. The next filter, which required transcripts to have an ORF smaller than 300 bp, was the one that excluded the most, leaving only 7.4% and 7.0% (15m and 22m, respectively) of the initial transcripts. After that, 1.7% of the initial transcripts were excluded at both ages because of similarity to the UniProt database, and 15 transcripts at 15 months and 19 at 22 months were excluded because of their coding potential according to CPC2. Finally, an exon filter was applied,

excluding 3.0% and 2.8% of the initial transcripts, leaving only 1823 transcripts (2.7% of the initial amount) at 15 months of age and 1533 transcripts (2.5%) at 22 months.

When looking at the adjusted p-value, only one transcript was considered differentially expressed between the groups at both times (TCONS_00092235, at 22 months for NP vs FP contrast). However, given the exploratory nature of the study, transcripts with p-value < 0.01 were also considered, totaling 10 transcripts, 4 of which appeared at 15 months, and 6 transcripts at 22 months. Of these 10 total transcripts, 1 appeared in more than one contrast, and none of them was repeated at both times. The complete list of transcripts can be found in Table 1.

When searching for homology with previously described non-coding RNAs for cattle using the NONCODE database (ZHAO et al., 2021), 3 of the 4 transcripts in the 15 months had already been identified (TCONS_00038113 as NONBTAT030133.1, TCONS_00044746 as NONBTAT029274.1 and TCONS_00057377 as NONBTAT031951.1) and in the 22 months, of the 6 transcripts, only 2 were identified (TCONS_00039302 as NONBTAT031112.1 and TCONS_00052474 as NONBTAT027406.1). All of these identifications had over 80 percent of identical matches.

Table 1. Differentially expressed long noncoding RNAs

Period	Contrast	Transcript	Identification	P-value	Adj. p-value
15m	NP.vs.PP	TCONS_00030990		0.0048	0.99
	FP.vs.PP	TCONS_00038113	NONBTAT030133.1	0.0017	0.99
		TCONS_00044746	NONBTAT029274.1	0.0052	0.99
		TCONS_00057377	NONBTAT031951.1	0.0077	0.99
22m	NP.vs.FP	TCONS_00092235		2.26×10^{-7}	0.0001
	NP.vs.PP	TCONS_00092235		0.0004	0.19
	NP.vs.FP	TCONS_00052474	NONBTAT027406.1	0.0030	0.80
		TCONS_00073566		0.0044	0.80
		TCONS_00007180		0.0062	0.83
	FP.vs.PP	TCONS_00030818		0.0085	0.99
		TCONS_00039302	NONBTAT031112.1	0.0037	0.99

Regulatory impact factors were used to identify lncRNAs that could be modulating the expression of genes related to muscle tissue. Using this, 25 (6.3%) of the 394 lncRNA were

identified as potential modulators of the expression of these genes. The comparison between NP and PP showed 13 lncRNAs, of which 8 were exclusive, while the comparison of NP and FP treatments revealed 17 transcripts, 12 of which were exclusive, and 5 lncRNAs shared between the two contrasts (table 2).

Table 2. lncRNA with possible key regulation of muscle development and its connections on the coexpression network

Treatment	lncRNA	Identification	Connections
PP	TCONS_00107245	NONBTAT031978.1	247
	TCONS_00105083	-	167
	TCONS_00031013	NONBTAT028263.1	131
	TCONS_00008937	-	74
	TCONS_00074879	NONBTAT028969.1	55
	TCONS_00132830	NONBTAT031353.1	44
	TCONS_00050716	NONBTAT028732.1	42
	TCONS_00125019	-	41
	TCONS_00119425	NONBTAT026662.2	39
	TCONS_00118957	NONBTAT021767.2	34
	TCONS_00126574	NONBTAT031687.1	28
	TCONS_00122572	-	24
	TCONS_00132533	NONBTAT031349.1	-
FP	TCONS_00105330	NONBTAT030235.1	108
	TCONS_00113158	NONBTAT030355.1	87
	TCONS_00078394	-	85
	TCONS_00028261	NONBTAT026662.2	71
	TCONS_00074879	NONBTAT028969.1	68
	TCONS_00118957	NONBTAT021767.2	50
	TCONS_00106901	NONBTAT019405.2	48
	TCONS_00022335	-	47
	TCONS_00031681	-	46
	TCONS_00105083	-	45
	TCONS_00122572	-	43
	TCONS_00017335	-	42
	TCONS_00053837	-	42
	TCONS_00050716	NONBTAT028732.1	42
	TCONS_00063942	NONBTAT028058.1	35
	TCONS_00050901	NONBTAT028721.1	24
	TCONS_00108094	NONBTAT027378.1	24

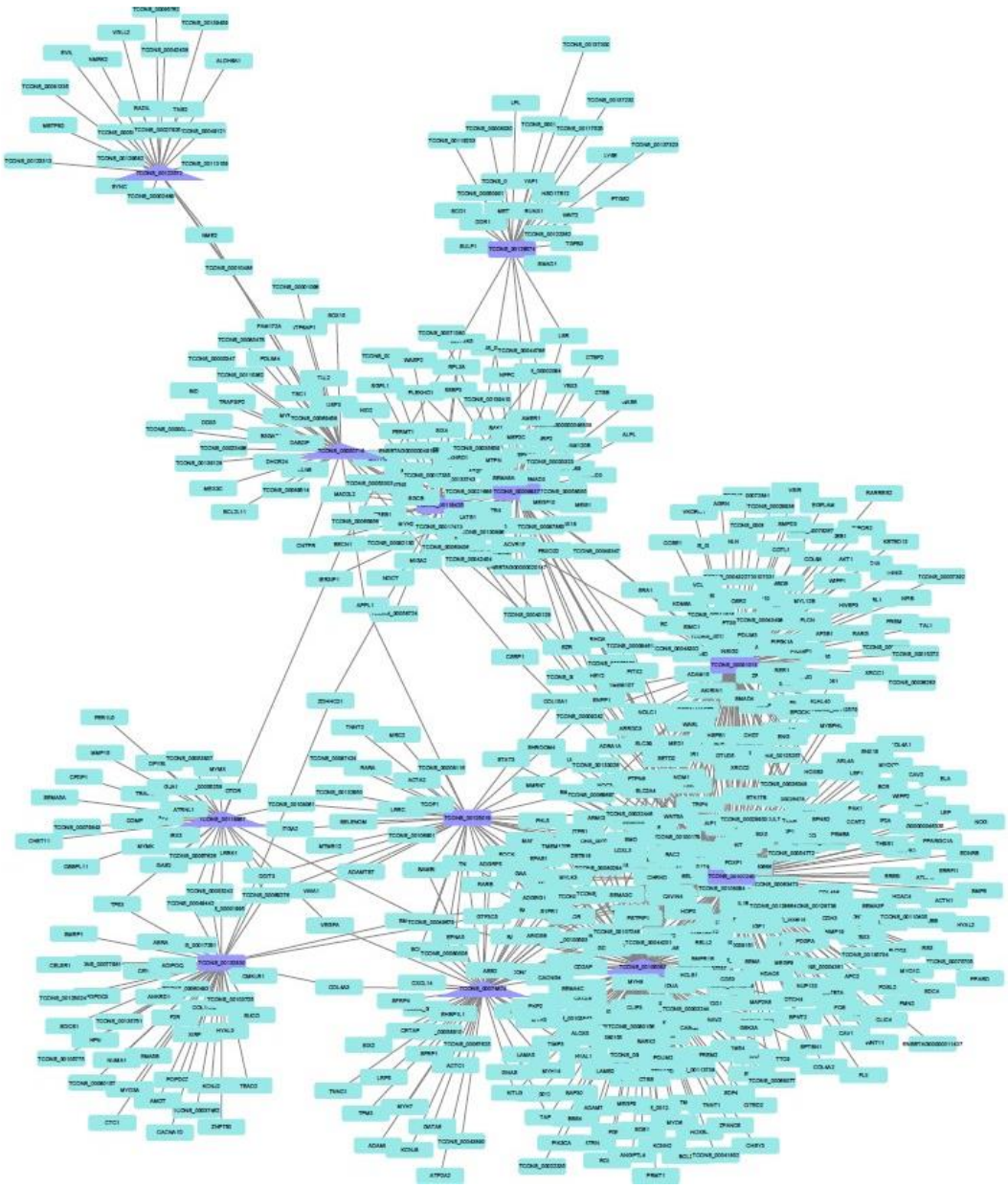

Figure 1. Co-expression network of potential key lncRNA regulating PP group.

When building the co-expression networks for each treatment based on the lncRNA key regulators, the PP group network had lncRNA connections with 478 mRNA (figure 1). When functional enrichment was performed, the involvement of lncRNA in fat cell differentiation, in utero embryonic development, transforming growth factor beta (TGF-β) receptor signaling pathway, semaphorin-plexin signaling pathway and skeletal muscle tissue development processes was observed. When looking at the network built by the lncRNA key regulators of the FP group,

they connected with 495 mRNA (figure 2). These mRNAs were identified as involved in in utero embryonic development, positive regulation of fat cell differentiation, vasculogenesis, positive regulation of epithelial to mesenchymal transition and negative regulation of canonical Wnt signaling pathway.

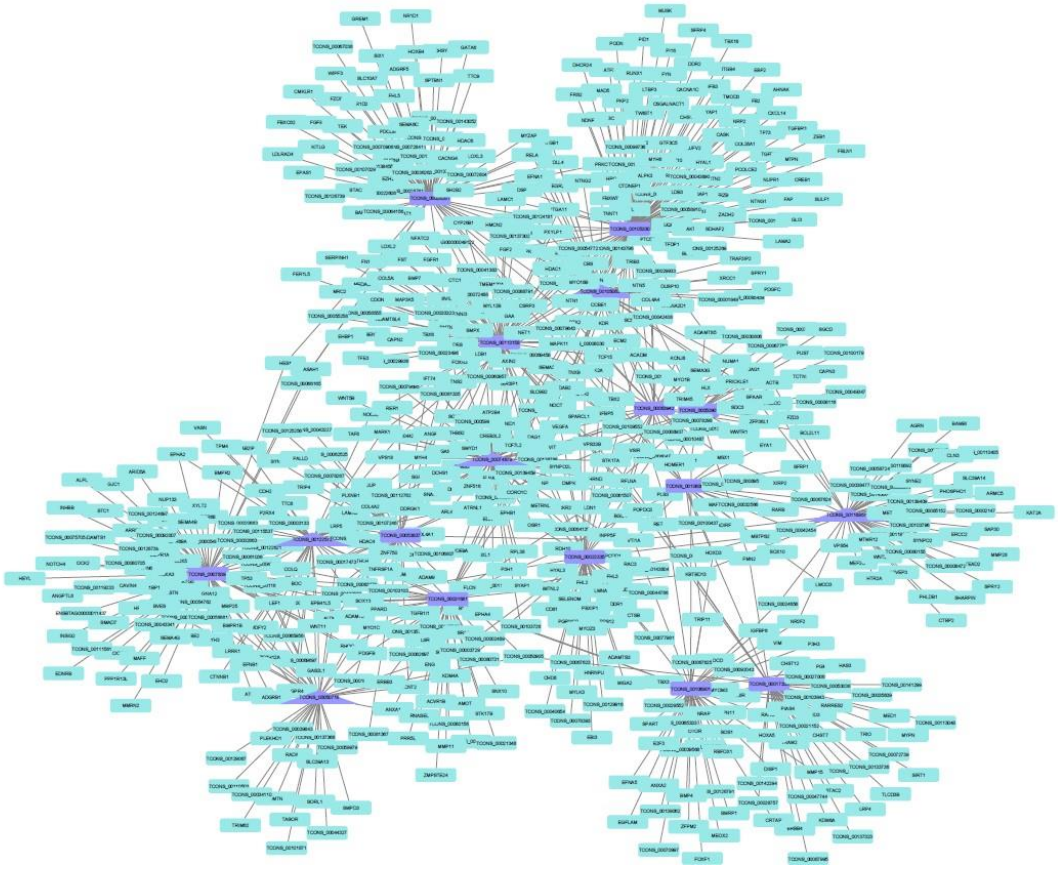

Figure 2. Co-expression network of key lncRNA with potential to regulate FP group.

3.4. Discussion

In this study, we used transcriptomic data from 15 Nellore young bulls that underwent fetal programming to investigate epigenetic mechanisms that could be acting on muscle development during the rearing and termination phases. The findings of this research suggest that, although there were no statistically significant differences in phenotypes related to muscle development directly (REA) or indirectly (weight and ADG), or in gene expression, there was an action of epigenetic regulators of the lncRNA type.

Although we did not find phenotypic differences that indicate changes in muscle development caused by fetal programming, it has already been shown that even without phenotypic differences, there may be changes in gene expression (PARADIS et al., 2017). This occurs because for there to be phenotypic differences, a priori a change in gene expression is necessary. Thus, the primary mechanisms through which fetal programming probably begins to show its effects is through epigenetic modifications (JIRTLE; SKINNER, 2007; REYNOLDS et al., 2019). There is growing evidence that nutritional conditions can alter genome activity through epigenetic modifications (BOLLATI; BACCARELLI, 2010; THOMPSON; AL-HASAN, 2012), and several studies with fetal programming have already demonstrated its effects on various organs (DUARTE et al., 2014; LAN et al., 2013), including skeletal muscle (COSTA; GIONBELLI; DUARTE, 2021; DU et al., 2015; YAN et al., 2013).

Among the epigenetic mechanisms, three of the main ones are histone modifications, DNA methylation and gene regulation caused by non-coding RNA (AL ABOUD; TUPPER; JIALAL, 2018). In this sense, there are studies showing the relationship between changes in histone and maternal nutrition (BLIN et al., 2020; GLENDINING; JASONI, 2019; YANG et al., 2012), and also its effects on methylation (BEKDASH, 2021; KELEHER et al., 2018; LECORGUILLÉ; TEO; PHILLIPS, 2021). Although we did not find genes related to epigenetic mechanisms being differentially expressed, it is possible that their action did not occur in an exacerbated way to the point of being detected by RNA-Seq data, although another work has already used this technique (LIEW; SINGH; BHALLA, 2013).

Another way to search for epigenetic changes would be through lncRNA. The search for this category of ncRNA using data obtained by RNA-Seq is already consolidated in the literature. (ALEXANDRE et al., 2020; ILOTT; PONTING, 2013; SCOTT et al., 2017), where a series of filters are applied in order to identify them. However, it is worth mentioning that part of the lncRNA transcripts are lost when the RNA-Seq library is assembled using poly-A tail selection (ZHAO et al., 2014). It is possible to say that our search for new lncRNA using RNA-Seq was successful, since when passing through CPC2, less than 20 transcripts (approximately 0.03% of the initial amount) were excluded.

With the new lncRNAs identified, it was possible to perform the differential expression analysis. At the FDR level, only one transcript was found to be differentially expressed.

TCONS_00092235 was identified in the contrast between NP and FP treatments for the 22-month analysis. This lncRNA is a transcript located on chromosome 6 in an intergenic region (class code "u"), on the + strand, and has 3 exons. TCONS_00030990 is an intergenic region transcript on chromosome 16, which has 2 exons and is on the - strand. TCONS_00073566 also has 2 exons, and is in the intergenic region of chromosome 29. TCONS_00007180 is located in the intergenic region of chromosome 10, is on the strand - and has 2 exons. Finally, TCONS_00030818 has 2 exons, and is in a region that overlaps the IGFN1 gene on strand - of chromosome 16. The lncRNAs that had already been identified by NONCODE (table 1) were found in a study that searched for new lncRNA in bovine skin transcriptome (WEIKARD; HADLICH; KUEHN, 2013).

We tried to predict the function of lncRNA key regulators through co-expression networks. In the PP treatment network, function in the TGF-β receptor pathway was identified. This family of proteins is related to the induction of signals that regulate growth, regeneration, differentiation, transformation and cell death in skeletal muscle (IIZUKA; MACHIDA; HIRAFUJI, 2014). Another identified pathway was the semaphorin-plexin signaling pathway, where 11 semaphorin genes were related in this network. Semaphorin-plexins are related to synaptic signaling, and indirectly to muscle excitation (ORR; FETTER; DAVIS, 2017). Regarding the in utero embryonic development pathway, Ma et al. (2020) considered this pathway significantly enriched when comparing lncRNA differentially expressed in muscle samples collected at different stages of animal development. On the other hand, the enrichment of the fat cell differentiation and skeletal muscle tissue development pathways was expected since, when performing the analysis, there was a pre-selection of genes related to this tissue.

As for the network of the FP group, the vasculogenesis pathway was enriched. Vasculogenesis occurs when new blood vessels are formed (MARÍN-GARCÍA, 2014), which may mean the greater need for vascularization in skeletal muscle by the animals in this treatment. Regarding negative regulation of the canonical Wnt signaling pathway, is an important pathway in skeletal muscle, both in the fetal stage and in adults. Canonical Wnt is associated in adulthood with the differentiation of muscle stem cells (VON MALTZAHN et al., 2012), and the negative regulation of this pathway may indicate the absence of need in the recruitment of these cells.

In this work, several methodologies were tested, and although we did not find great effects of maternal supplementation, we know that the differences can be subtle. Perhaps these differences

are not so expressive in terms of generating differential gene expression, but these changes may occur in the relationship of genes to each other, depending on the treatment. Given this, we made the co-expression networks, to try to extract some difference between the treatments that were not noticed in any other analysis.

3.5. Conclusion

In search of epigenetic modifications that could be regulating muscle development in cattle, treatments were similar when the search was made for epigenetic mechanisms that act directly on histone modifications and chromatin methylation. Despite this, interesting results were found that suggest that protein-energy supplementation in the prenatal period can influence muscle development through regulation by lncRNA.

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4. Chapter 3: Effects of maternal nutrition on female offspring weight gain and sexual development

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Abstract

Maternal nutrition during pregnancy influences postnatal life of animals; nevertheless, few studies have investigated its effects on the productive performance and reproductive development of heifers. This study evaluated the performance, reproductive development, and correlation between reproduction x fat thickness and performance x ribeye area (REA) traits of heifers. We also performed an exploratory genomic association during the rearing period in heifers submitted to fetal programming. The study comprised 55 Nellore heifers born to dams exposed to one of the following nutritional planes: Control, without protein-energy supplementation; PELT – Protein-energy last trimester, protein-energy supplementation offered in the final third of pregnancy; and PEWG - Protein-Energy Whole Gestation, protein-energy supplementation upon pregnancy confirmation. Protein-energy supplementation occurred at the level of 0.3% live weight. After weaning, heifers were submitted to periodic evaluations of weight and body composition by ultrasonography. From 12 to 18 months, we evaluated the reproductive tract of heifers to monitor its development for sexual precocity and ovarian follicle population. The treatments had no effect ($p > 0.05$) on average daily gain; however, the weight of the animals showed a significant difference over time ($p = 0.017$). No differences were found between treatments for ribeye area, backfat and rump fat thickness, nor for puberty age, antral follicular count, and other traits related to reproductive tract development ($p > 0.05$). The correlation analysis between performance traits and REA showed high correlations ($r > 0.37$) between REA at weaning and year versus weight from weaning until yearling; however, no correlation was found for reproductive development traits versus fat thickness ($p > 0.05$). The exploratory genomic association study showed one SNP for each treatment on an intergenic region for control and PEWG, and the one for PELT on an

intronic region of RAPGEF1 gene. Maternal nutrition affected only the weight of the animals throughout the rearing period.

Key words: beef heifer, fetal programming, Nellore, nutrigenetic, performance, reproduction.

4.1. Introduction

The concept of fetal programming has emerged in recent decades and is used to explain metabolic and systemic changes due to events during fetal life (Barker, 1990). Nutritional changes, such as over- or under-nutrition, may occur with the mother and result in fetal programming, as reported by several studies (Wu et al., 2006; Long et al., 2009; Duarte et al., 2014). Systemic changes due to maternal nutrition include low birth weight, hormonal imbalances, and changes in organ development and functionality (Long et al., 2009; Micke et al., 2010a).

Moreover, studies show that fetal programming affects the reproductive system of both genders (Funston and Summers, 2013; Mossa et al., 2013, 2017; Polizel et al., 2021). Other studies (Martin et al., 2007; Funston et al., 2010a) report the effects of nutrition during pregnancy on sexual precocity and pregnancy rate in heifers as well as on the reproductive potential of heifers in ovarian follicular reserve, even without changing other phenotypic characteristics (Mossa et al., 2013). Puberty is one of the most important periods for heifers, since it directly affects their productive, reproductive, and economic efficiency (Monteiro et al., 2013). The production of precocious animals is desirable, mainly to reduce the use of resources.

In Brazil, dams commonly undergo nutritional restriction due to the dry season present in tropical and sub-tropical conditions, especially during the second and third trimester of pregnancy; therefore, investigations of undernutrition effects on progenies are needed to seek viable alternatives to overcome nutrient restriction during dry periods. In addition, few studies have investigated the effects of fetal programming on the performance of heifers in the rearing phase (Micke et al., 2010a; Long et al., 2012, 2021; Noya et al., 2019).

The post-weaning period is crucial for the reproductive development of heifers, since the animal needs to reach adequate body weight to attain puberty rapidly and then become replacement heifers or ready for finishing and slaughter. This study assessed the performance, reproductive

development, and correlation between traits of heifers. We also performed an exploratory genomic association study during the rearing period in heifers submitted to different planes of maternal nutrition, with the objective of showing possible genotype-environment interactions, evaluating how individual genetic variants respond to nutritional stimuli. Therefore, our hypothesis is that different prenatal supplementation strategies influence weight gain, reproductive traits, and that genetic variants influence the nutritional response in female offspring of Nellore dams.

4.2. Material and methods

4.2.1. Ethics statement

This study was approved by the Research Ethics Committee of FZEA / USP, under protocol No. 1843241117, according to the guidelines of the National Council for the Control of Animal Experimentation (CONCEA).

4.2.2. Experimental design

The study comprised 126 Nellore dams, which were fixed time artificially inseminated (FTAI) with semen of four bulls with known genetic value, representing the majority of national Nellore animals. After confirming pregnancy at 30 days after FTAI, the animals were separated into three treatments: Control, without protein-energy supplementation; PELT – Protein-Energy Last Trimester, protein-energy supplementation in the final third of pregnancy; and PEWG – Protein-Energy Whole Gestation, protein-energy supplementation upon pregnancy confirmation. The 126 animals were homogenized in the groups based on age (3-8 years), parity, body weight, and body condition score (Table 1), in order to make the groups as homogeneous as possible. Animals were allocated to pasture paddocks of Brachiaria brizantha cv. Marandu with access to the supplement (0.03% live weight for control, and 0.3% live weight for PELT and PEWG) (Table 2) and water ad libitum. More details can be found on Polizel et al. (2021).

Table 1. Weight and Body Conditioning Score (BCS) of dams on the beginning and end of gestation.

Traits	Time	CONTROL	PELT	PEWG	P-value
	Initial	457 ± 9	453 ± 12	439 ± 16	0.96

		Min =	385	324	349	
		Max =	524	542	602	
Weight (kg)	Pre-delivery		501 ± 10	523 ± 13	521 ± 18	
		Min =	410	380	428	0.20
		Max =	575	620	692	
	Postpartum		501 ± 10	502 ± 13	495 ± 16	
		Min =	429	350	398	0.91
		Max =	582	604	650	
BCS	Initial		4.5 ± 0.1	4.6 ± 0.1	4.4 ± 0.1	
		Min =	4	3	3	0.43
		Max =	5	5	5	
	Pre-delivery		5.4 ± 0.2	5.6 ± 0.2	5.5 ± 0.3	
		Min =	4	4	4	0.55
		Max =	7	7	7	
Age (years)			4.7 ± 0.3	4.5 ± 0.3	4.1 ± 0.3	
		Min =	2.3	2.4	2.2	0.48
		Max =	6.2	7.2	6.2	
Parity		Primiparous	17%	17%	19%	
		Multiparous	83%	83%	81%	

The data are expressed as means of the characteristics ± standard error of the mean. BCS – Body conditioning score. CONTROL - without protein-energy supplementation, PELT – Protein-Energy Last Trimester (0,3%BW protein-energy supplementation in the final third of pregnancy), PEWG – Protein-Energy Whole Gestation (0,3%BW protein-energy supplementation upon pregnancy confirmation)

After calving, protein-energy supplementation ceased and all animals remained together until weaning (average 220 days old), regardless of the treatment. The animals were subjected to the same sanitary, vaccination, and feeding protocols already implemented on the farm where the experiment was conducted. After weaning, the animals were divided by sex, regardless of treatment, and placed in separate pastures, where they remained throughout the breeding. The females remained on the pasture until the beginning of the reproductive season at 24 months. This trial comprised 55 heifers (control = 19, PELT = 22, PEWG = 14), which were evaluated for reproductive development and performance regularly.

Table 2. Ingredients and nutrients content of the dams supplement

Ingredients	Mineral supplement	Energetic-protein supplement
Corn (%)	35	60
Soybean meal (%)	-	30
Dicalcium phosphate (%)	10	-
Urea 45% (%)	-	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 pre-mix composition (guarantee levels per 25 kilograms): Calcium – 200-300g; Cobalt – 160mg; Copper – 2700mg; Sulfur – 60g; Fluorine – 1600mg; Phosphor – 160g; Iodine – 135mg; Manganese – 2700mg; Selenium – 80mg; Zinc – 8100mg; Sodium monensin – 4000mg.

4.2.3. Reproductive tract assessment

The females were evaluated to determine the stage of reproductive development every 30 days from 12 months of age onward. Puberty was characterized based on the presence of *corpus luteum* (CL) and puberty age referred to the age in days of the animal of the first CL. A single specialized operator used an ultrasound machine equipped with a transrectal transducer (Mindray Z5 VET; Shenzhen Mindray Bio-Medical Electronics Co, Shenzhen, Guangdong, China) to qualify the CL presence. Antral follicle count (AFC) was performed to estimate the ovarian reserve at the same time that the presence of CL occurred, where a single operator visually enumerated the antral follicles ≥ 3.00 mm. Each ovary was investigated exhaustively throughout to standardize the count, identifying the positions of the antral follicles and capturing images of different sections of the

organ. The size of each ovary was measured using its largest diameter and the average size between the two ovaries was considered for each animal for statistical purposes. The thickness of the endometrial wall was also measured right after the corneal bifurcation during ultrasound, as described by Souza et al. (2011). The tonus and uterine sizes were also accessed through transrectal palpation, assigning scores (tonus = flaccid, minimal tonus or medium tonus; uterine size = infant, small, medium or developed) according to the perception of the evaluator and as proposed by Holm et al. (2009). For the statistical analysis, we used assessments at 12, 15, and 18 months, when evaluations of the reproductive tract ended.

4.2.4. Performance evaluation

The performance of animals was evaluated in the periods of weaning, year (12 months), yearling (18 months) and 24 months, measuring weight and average daily gain (ADG). The ultrasound was used to measure ribeye area (REA), backfat thickness (BFT), and rump fat thickness (RFT). Weights were obtained regularly during the rearing period using an electronic scale from Coimma (Coimma Scales, Dracena, São Paulo State, Brazil) coupled to the trunk. The linear regression was performed using all collections between weaning and 24 months, totaling seven collections of weight, to obtain the ADG.

The body composition was evaluated by ultrasound using an Aloka SSD-500 ultrasound equipped with a 17-cm linear transducer at 3.5 MHz frequency (Aloka Co. Ltd., Wallingford, CT, USA). Vegetable oil was used as coupling to optimize the contact of the transducer with the skin of the animals. The REA and BFT were measured by images in sections of the *Longissimus dorsi* muscle, between the 12th and 13th ribs, while the RFT was measured by positioning the transducer in the final portion of ileum, between the junction of the *biceps femoris* and the middle gluteal muscle. The images were captured using the Lince software and later analyzed by a certified technician.

4.2.5. Nutrigenetic evaluation

The DNA material was obtained from tail hair bulb; DNA extraction from the bulb of these hairs was performed by MICRO LAB ID STARlet® automated robot (Hamilton Company, Reno, Nevada, USA) using the NucleoSpin® 96 extraction kit (Macherey-Nagel, Düren, Germany). The

55 Nellore heifers were genotyped with the low-density panel GeneSeek® Genomic Profiler Bos Indicus GGP Nellore LD BeadChip containing 35,339 markers, and before the imputation process, all SNP arrays had their maps converted to the new ARS UCD 1.2 reference genome. Imputation procedure was implemented using the FIMPUTE 2.2 software (Sargolzaei et al., 2014) and all genotypes were imputed to a panel containing 735,965 markers. A reference population with 2,502 sires and dams genotyped with the Illumina BovineHD BeadChip (Illumina Inc., San Diego, CA, USA) containing 777,962 markers, was used. This population contains important and representative sires and dams within the Nellore breed, whose genetic material is widely used in breeding programs. Prior imputation, samples were edited for call rate (<90%) for the genotyped and the reference populations. SNPs unassigned to any chromosome and those assigned to sexual chromosomes were removed from the dataset. After imputation, accuracy obtained was a mean (standard deviation [SD]) of 0.93 (0.02), and genotypes presenting less than 0.90 of imputation accuracy were not considered in further analysis. The relationship degree between the target and reference population, was, on average (SD) of 0.08 (0.01).

We performed the genomic association analysis to understand the nutrigenetic effects of fetal programming, using the imputed SNP panel (35K) for reproductive characteristics (12, 15, and 18 months) and performance (weaning, year, yearling and 24 months). Statistical information on models used can be found in section 2.6.2. We used the packages SNPStats (Solé et al., 2006), gdata (Warnes et al., 2017), qvalue, data.table, ggplot2 (Wickham, 2011) and qqman (Turner, 2018). Concerning quality control (QC) for genomic data, all markers on sexual chromosome were removed from analysis, as well as markers with call rate < 0.95 (0); with minor allele frequency < 0.01 (209,831); p-value from Hardy-Weinberg equilibrium < 1 x 10-10 (26); and monomorphic (86,070). In addition, individuals with call rate < 0.90 were also removed. Thus, the final genotypic data was left after QC with 55 individuals and 440,038 markers.

4.2.6. Statistical analysis

4.2.6.1. Phenotypes

All procedures were performed using the MIXED procedure of the statistical package SAS® version 9.4 (SAS Institute Inc, NC, USA). From data obtained, residues were submitted to the Shapiro-Wilk test for normality implemented at UNIVARIATE procedure, where measurements

that did not follow normality were transformed using log (i.e., ln(trait + 1)). The homocedasticity of residuals for principal effects was tested in the groups using the Levene's test. Then, the effects of treatments (control, PELT, and PEWG) on phenotypes were evaluated using the analysis of variance and the means compared by the Tukey-Kramer test, with contrasts considered significant when $p < 0.05$ and trend when $p < 0.10$. The age of the animals, age of the dams and sire were also considered in the linear model. Regarding to the repeated measures, the same variables than linear model were considered, and time of data collection was also included in analysis of variance. The covariance structure for the residuals was tested for each variable and chosen based on the Bayesian information criterion (BIC) criteria. They were different for each variable as follows: AFC used variance components (vc); weight used an autoregressive of first order (ar); REA used a compound symmetry structure for the residuals (cs); BFT used a heterogenous compound symmetry (csh); and RFT used an autoregressive structure with moving average (arma).

For those analysis the model was as follows:

$$y_{ijkl} = \mu + \beta_1 \mathrm{Age}_{m_l} + \mathrm{Sire}_i + \mathrm{Treat}_j + \mathrm{Time}_k + (\mathrm{Treat} \times \mathrm{Time})_{jk} + e_{ijkl}, \quad (1)$$

Where: y_{ijkl} is the observed variable from l^{th} animal, daughter of ith sire, recorded on j^{th} treatment at k^{th} time of measurement (weaning, 12, 15, 18 and/or 24 months of age); μ is just a constant; β_1 is the regression coefficient of covariate mother's age; Age_{m_l} is the observed value for mother's age of lth animal; Sire_i is the fixed effect of ith sire; Treat_j is the fixed effect of jth treatment; Time_k is the fixed effect of kth time of measurement; $(\mathrm{Treat} \times \mathrm{Time})_{jk}$ is the fixed interaction between treatment and time; and e_{ijkl} is the residual random term, which was assumed normally distributed with covariance structure as presented above. It must be noticed that when the analysis was performed within time, this effect (and also the treatment by time interaction) was removed from the model. Finally, for AFC it was included the weight at puberty as a covariate in the model.

The Kruskal-Wallis test was performed due to the scalar nature of the data collected for the characteristics of tonus and uterine sizes.

4.2.6.2. Nutrigenetics

For nutrigenetics, two models were implemented through the "LM" function in R to correct the phenotype for the fixed effects, as follows:

$$y_{ijk} = \mu + \beta_1 Age_{m_k} + Sire_i + Treat_j + \varepsilon_{ijk}, \quad (2)$$

$$y_{ijk} = \mu + \beta_1 Age_{m_k} + \beta_2 Weight_k + Sire_i + Treat_j + \varepsilon_{ijk}, \quad (3)$$

Where: y_{ijk} is the observed phenotype of kth animal, daughter of ith sire, on j^{th} treatment; μ is just a constant; β_1 is the regression coefficient of covariate mother's age; β_2 is the regression coefficient of covariate weight at puberty (only for age at puberty); Age_{m_k} is the observed value for mother's age of kth animal; $Weight_k$ is the observed puberty weight of kth animal; $Sire_i$ is the fixed effect of ith sire; $Treat_j$ is the fixed effect of jth treatment; and ε_{ijk} are random residual terms. The AFC observed values were transformed on log scale as: $\ln(AFC + 1)$ and when the analysis was performed within each treatment, the treatment effect was not included in the model.

Under matrix notation, the models can be written as:

$$\mathbf{y} = \mathbf{X\beta} + \boldsymbol{\varepsilon}, \quad (4)$$

Where: $\mathbf{y}$ is the phenotype vector; $\mathbf{X}$ is the incidence matrix for the fixed effects; $\boldsymbol{\beta}$ is the vector of solutions for the fixed effects; and $\boldsymbol{\varepsilon}$ is the vector of residual random terms. It was assumed that: $E[\mathbf{y}] = \mathbf{X\beta}$; $\boldsymbol{\varepsilon} \sim N(0, \mathbf{I}\sigma_\varepsilon^2)$; and thus $Var(\mathbf{y}) = Var(\boldsymbol{\varepsilon}) = \mathbf{I}\sigma_\varepsilon^2$. Under our assumptions, the residual can be re-written as (Searle, 1997): $\boldsymbol{\varepsilon} = \mathbf{Zu} + \mathbf{e}$; where: $\mathbf{Z}$ is the incidence matrix for the animal additive effect; $\mathbf{u}$ is the solution vector for animal additive effect, and $\mathbf{e}$ is the vector of true residuals.

After obtaining solutions for $\boldsymbol{\beta}$, phenotypes we used $\boldsymbol{\varepsilon}$ as pseudo phenotypes for a genome-wide association analysis (GWAS) through the approach SNP by SNP, that is, each marker was fitted once. The adjusted phenotypes can be calculated as: $\hat{\boldsymbol{\varepsilon}} = \mathbf{y} - \mathbf{X}\widehat{\boldsymbol{\beta}}$, where $\widehat{\boldsymbol{\beta}}$ is the empirical BLUE for the fixed effects. Thus, the GWAS was performed by the following model:

$$\hat{\boldsymbol{\varepsilon}} = \beta_0 + \beta_1 PC1_k + \beta_2 PC2_k + \beta_3 SNP_{k_i} + e_i = \mathbf{X\theta} + \mathbf{e}, \quad (4)$$

Where: $\hat{\boldsymbol{\varepsilon}}$ was the same as above; β_0 is the intercept; β_1 and β_2 are regression coefficients of the first and second principal components (PC) from the genomic relationship matrix (VanRaden, 2008); $PC1_k$ is the observed value of the first PC on k^{th} animal; $PC2_k$ is the observed value of the second PC on k^{th} animal; β_3 is the regression coefficient of the marker effect (SNP effect); SNP_{k_i} is the scaled genomic content of kth animal on i^{th} marker; e_i is the residual; $\boldsymbol{\theta}$ is the vector of solutions for β_0, β_1, β_2 and β_3; and $\mathbf{e}$ is the vector of residual terms which was assumed $\mathbf{e} \sim N(0, \mathbf{I}\sigma_e^2)$. For the GWAS, we also used the "LM" function within a loop for i from 1 to the total number of markers (440,038). After estimation of marker p-values, those were corrected for

multiple testing by Bonferroni correction; i.e., the threshold for significance was set to 0.05/440,038.

4.2.6.3. Correlation between performance, body and reproductive characteristics

The Pearson correlation analysis was performed using the LM function of the statistical environment R to elucidate the relationship between the variables of weight, ADG, REA, BFT, RFT, age at puberty, AFC, ovary size and endometrium thickness.

4.3. Results

On repeated measures, the interaction between time and treatment was not statistically significant for all studied traits ($p > 0.05$), but time was significant ($p < 0.05$) in all of them.

4.3.1. Weight and average daily gain/ Performance at rearing phase

The heifer's weight in the three treatments was similar in all periods evaluated ($p > 0.05$); however, when an analysis was carried out over time, a significant difference occurred among the treatments ($p = 0.017$), where control was heavier and differed from the others. The ADG of the period showed no statistical difference between treatments ($p > 0.05$) with homogeneous weight gain (Table 3).

4.3.2. Fat thickness and ribeye area

The body traits measured by ultrasound had no differences in the periods ($p > 0.05$), not even when the analysis was performed for repeated measurements over time, showing homogeneous fat and muscle deposition in these locations. However, in the RFT measurements, a difference related to a sire effect was found ($p < 0.01$) (Table 3).

Table 3. Performance traits of Nellore heifers submitted to fetal programming.

Traits	Age	CONTROL A*	PELT B*	PEWG B*	P value 1	P value 2
	Weaning	216.4 ± 4.4	210.1 ± 4.7	208.3 ± 5.4	0.78	0.017

Weight (kg)	Year	271.2 ± 4.1	263.9 ± 5.1	257.5 ± 5.4	0.37	
	Yearling	398.5 ± 5.5	384.9 ± 7.5	384.2 ± 7.3	0.39	
	24 months	428.7 ± 4.5	410.0 ±6.6	410.7 ± 7.2	0.13	
Ribeye area (cm^2)	Weaning	42.7 ± 1.2	43.5 ± 1.1	43.3 ± 1.5	0.92	0.929
	Year	55.7 ± 1.7	57.5 ± 1.1	56.3 ± 1.5	0.63	
	Yearling	73.5 ± 1.2	73.30 ± 1.5	75.0 ± 1.7	0.46	
	24 months	70.8 ± 1.1	70.8 ± 1.1	70.8 ± 1.5	0.83	
Backfat thickness (mm)	Weaning	2.85 ± 0.4	3.28 ± 0.4	2.72 ± 0.4	0.55	0.115
	Year	1.91 ± 0.3	1.94 ± 0.4	1.15 ± 0.4	0.19	
	Yearling	6.52 ± 0.6	7.41 ± 0.5	6.55 ± 0.6	0.27	
	24 months	5.70 ± 0.7	6.27 ± 0.5	6.20 ± 0.6	0.87	
Rump fat thickness (mm)[§]	Weaning	4.90 ± 0.4	4.46 ± 0.4	4.35 ± 0.3	0.53	0.373
	Year	3.69 ± 0.4	3.36 ± 0.4	3.07 ± 0.5	0.51	
	Yearling	10.1 ± 0.7	10.2 ± 0.5	10.3 ± 0.7	0.95	
	24 months	8.81 ± 0.7	8.25 ± 0.5	8.99 ± 0.7	0.72	

The data are expressed as means of the characteristics ± standard error of the mean. * - Refers to contrasts on weight characteristic; [1] – P value between groups on the same age; [2] – P value on repeated measures over time; [§] - Sire effect found on repeated measures over time ($p < 0.05$). CONTROL - without protein-energy supplementation, PELT – Protein-Energy Last Trimester (0,3%BW protein-energy supplementation in the final third of pregnancy), PEWG – Protein-Energy Whole Gestation (0,3%BW protein-energy supplementation upon pregnancy confirmation)

4.3.3. Reproduction traits

Puberty age and AFC of the treatments showed no significant differences between periods or over time ($p > 0.05$). There was no significant difference for ovary size and endometrial thickness in the periods and in the repeated measurements over time. However, ovary size had a significant difference for the age of animals (12, 15 and 18 months)($p < 0.05$). The uterine size and tonus classificatory variables also displayed no differences between treatments ($p > 0.05$) (Table 4).

Table 4. Maternal nutritional effect on reproductive traits of Nellore offspring heifers.

Traits	Age	CONTROL	PELT	PEWG	P value [1]	P value [2]
Ovary Size	15 months	22.7 ± 0.6	22.0 ± 0.6	21.0 ± 0.6	0.32	0.37
	18 months	23.6 ± 0.6	24.6 ± 0.5	23.8 ± 0.5	0.34	

Endometrium Thickness	15 months	6.2 ± 0.1	6.2 ± 0.2	6.2 ± 0.1	0.85	0.53
	18 months	5.6 ± 0.1	5.6 ± 0.1	5.3 ± 0.1	0.33	
AFC	15 months	15.1 ± 1.4	16.4 ± 1.0	17.1 ± 1.4	0.18	0.31
	18 months	15.6 ± 0.1	16.0 ± 1.2	16.4 ± 1.9	0.92	
Age at Puberty		475.76	474.94	475.33	0.87	

The data are expressed as means of the characteristics ± standard error of the mean. [1] – P value between groups on the same age; [2] – P value on repeated measures over time.

4.3.4. Phenotypic correlations

The correlation analysis between performance characteristics and REA showed a positive high correlation in weight at weaning vs REA at weaning (r = 0.63), weight at year vs REA at weaning (r = 0.55), weight at yearling vs REA at weaning (r = 0.45), weight at weaning vs REA at year (r = 0.41), weight at year vs REA at year (0.53), weight at yearling vs REA at year (r = 0.49), and weight at 24 months vs REA at year (r = 0.37; p <0.01). A positive moderate correlation was shown between weaning weight vs REA at yearling (r = 0.30), weight at 24 months vs REA at weaning (r = 0.28), weight at 24 months vs REA at 24 months (r = 0.26), and ADG vs REA at 24 months (r = 0.30; p < 0.05)(Table 5). When relating reproduction characteristics and fat thickness, no significant correlations were found between puberty ages, AFC and BFT, and RFT for the periods analyzed (p > 0.05) (Table 6).

Table 5. Pearson's correlation between performance traits and ribeye area (REA).

Performance vs REA	REAWE	REA12	REA18	REA24
WWE	0.63**	0.41**	0.30*	0.02
W12	0.55**	0.53**	0.38	0.17
W18	0.45**	0.49**	0.36	0.19
W24	0.28*	0.37**	0.23	0.26*
ADG	-0.02	0.09	0.19	0.30*

* P value < 0.05; ** P value < 0.01; WE - weaning; 12 - year (12 months); 18 - yearling (18 months); 24 - 24 months.

Table 6. Pearson's correlation between reproductive traits and fat thickness.

Reproduction vs Fat Thickness	BFTWE	BFT12	BFT15	BFT18	BFT24	RFTWE	RFT12	RFT15	RFT18	RFT24
Age at puberty	-0.05	-0.03	0.06	-0.05	-0.01	-0.12	0,00	0.13	0.06	-0.02
AFC12	-0.19	-0.2	-0.1	-0.07	-0.16	-0.08	-0.18	-0.03	-0.12	-0.2
AFC15	-0.02	-0.21	0.01	0.05	-0.08	0.04	0.02	0.11	0.19	0.07
AFC18	-0.08	-0.2	0.02	0.05	-0.15	-0.02	0.02	0.02	-0.05	-0.15

* P value < 0.05; ** P value < 0.01; WE - weaning; 12 - year (12 months); 15 – 15 months; 18 - yearling (18 months); 24 - 24 months.

4.3.5. Exploratory genomic association study

When all animals were analyzed, no SNP had significance for any of the characteristics. However, when performing the analysis within each group, a significant SNP was identified for each treatment, with control in the trait AFC at yearling, PELT for weight at yearling, and for PEWG, BFT at year (Figures 1 and 2). The SNPs of the control and PEWG treatments are in the intergenic region, and the one of the PELT treatment is an intron variant of the RAPGEF1 gene (Table 7). For the SNPs in intergenic region, we considered candidate genes the ones within a window of 1Mb around the marker. Only one gene, in AFC at yearling's SNP (GFRA2), was as close as 100 Kb from the marker.

Table 7. SNPs highlighted by the exploratory genomic association analysis.

Characteristic	Treatment	SNP	$-\log_{10}P$	Gene Associated
AFC at yearling	CONTROL	rs135063035 Location 8:68964611	9.51×10^{-8}	*GFRA2* (d) *XPO7* (d) *DOK2* (d) *NPM2* (d) *FGF17* (d)

				DMTN (d) *HR* (d) *FAM160B2* (d) *NUDT18* (d)
Weight at yearling	PELT	rs110561890 Location 11:101799978	8.52×10^{-8}	*RAPGEF1*
BFT at year	PEWG	rs137051110 Location 17:58480895	2.82×10^{-9}	*SPRING1/C12ORF49* (u) *RNFT2* (u) *FBXW8* (u) *ENSBTAG00000037415* (u) *ENSBTAG00000053074* (u) *MED13L* (d)

(d) or (u) – refers to whether the gene is up (u) or downstream (d) their related SNP

4.4. Discussion

This study investigated the potential effects of protein and energy supplementation on dams during the entire gestation and in the final third of gestation as well as on cows that did not receive supplementation, regarding performance in the rearing season and reproductive tract development. To date, few studies have related the rearing phase of beef heifers to fetal programming. Here, we did not find differences in sexual development; however, there were differences in body weight throughout the rearing period, showing the contribution of this study to this research field.

Some studies have shown that energy restriction during fetal life can negatively affect growth and performance in postnatal life, including body composition (DANIEL et al., 2007; DU et al., 2010); nevetheless, few studies report its effects on females, especially on Bos indicus heifers.

Micke et al. (2010b) reported that supplemented heifers were heavier than non supplemented ones. However, Long et al. (2012) found no difference between treatments. That study used heifers from dams with or without nutritional restriction during the final third of gestation and identified no differences between the groups for weight, ADG, and REA in the analyzed periods. In the same study, the authors reported that the progeny of dams that underwent undernutrition in the final third of gestation had greater deposition of internal fat, which may help explain why the control group was heavier than the others over time. Daniel et al. (2007) observed that ewes nutrient-restricted during pregnancy showed no effect of maternal nutrition on the deposition rate of muscle and fat in the progeny, corroborating our results of REA, BFT and RFT. Reis et al. (2015) studied calves that underwent or not to creep-feeding during the nursing period and reported no differences for BFT between treatments. The contradictory results of several studies reinforce the need to further investigate the mechanisms of fetal programming.

Puberty in heifers is defined as the age when the animal experiences its first ovulation accompanied by visual signs of estrus and normal luteal function (MORAN et al., 1989), an important characteristic, as pregnancy success during the breeding season is associated to the number of heifers that reached puberty before the season (SHORT AND BELLOWS, 1971). Weight is the most important factor for puberty onset, since puberty is achieved when the animal is between 55% and 60% of its mature body weight, regardless of the breed (FREETLY et al., 2011). Studies on fetal programming show the effects of supplementation during pregnancy on puberty age in heifers (GUZMÁN et al., 2006; FUNSTON et al., 2010b; HARVEY et al., 2021). Other investigations show no effects (CUSHMAN et al., 2014; GUNN et al., 2015; NEPOMUCENO et al., 2017), corroborating the lack of difference between treatments. Previous studies have indicated that maternal nutrition during pregnancy can interact with nutrition in early postnatal life to determine the puberty age in heifers (CARDOSO et al., 2020). Furthermore, although postnatal nutrition has more significant effects than maternal nutrition, heifers from mothers that underwent nutritional restriction were more sensitive to the negative effects of limited postnatal growth (O'NEIL et al., 2019). Therefore, it is justifiable that the heifers used in our study do not show differences of puberty ages when they start receiving the same environmental conditions, regardless of maternal treatment groups, and did not undergo nutritional restrictions that could limit their postnatal growth in their first months of life, since the animals were born in the rainy season. Moreover, although there was no difference between groups, the mean age at

puberty (16 months) was earlier than the Nellore mean, between 22-36 months (NOGUEIRA, 2004), also the body weight of the animals in this study was greater than literature reports for Nellore females (BOLIGON AND ALBUQUERQUE, 2011). A point to be reinforced is that up to 24 months animals received an excellent nutritional management, which contributed to body development in general. However, the effect under more restricted conditions can produce different results and needs to be evaluated in future research.

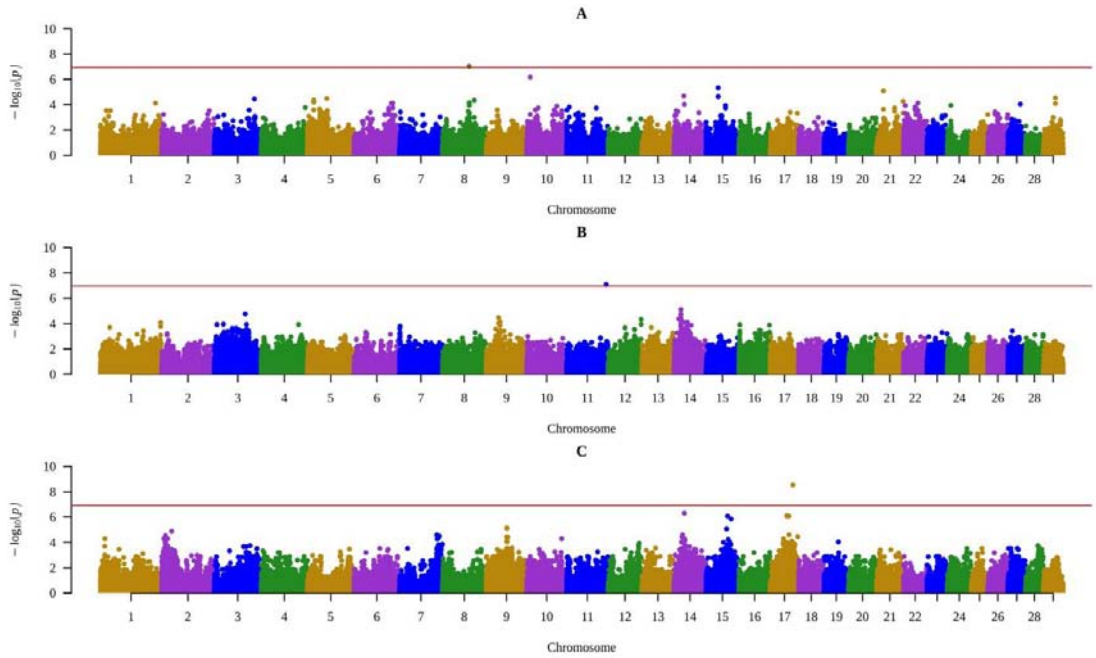

Figure 1. Manhattan-plots of traits with SNPs highlighted by the exploratory genomic association analysis in each treatment. **(A)** AFC at Yearling in control group; **(B)** Weight at Yearling in PELT group; **(C)** BFT at Year in PEWG group.

The AFC is an important marker of ovarian follicle reserve (MOSSA et al., 2012) and thus of the animal reproductive efficiency. Studies associate maternal malnutrition during pregnancy to a low AFC (MOSSA et al., 2013). The formation of primordial cells, precursors of follicles, begins between the 90th and 140th days of fetal life in cattle (YANG; FORTUNE, 2008); therefore, in our study, the treatments without supplementation in this period (second third of gestation; control and PELT) may have suffered the undernutrition effects due to the dry season. Nevertheless, undernutrition was possibly not severe enough. In our study, we used cows, which possibly affected these results. On the other hand, Mossa et al. (2013) used heifers, which are still growing

in addition to having to spend energy for their maintenance and gestation, as hypothesized by Cushman et al. (2014).

One way to assess pubertal status indirectly is through palpation of the reproductive tract (HOLM et al., 2009). Andersen et al. (1991) developed a standard method for the reproductive tract score, a tool to access the animal proximity to puberty by the uterine size and tonus sizes of ovary and structures in the organ. In our study, we used these characteristics to investigate effects of maternal nutrition in the gestational period on the development of the reproductive tract of heifers. However, with the absence of statistical differences in these characteristics and in the puberty ages between treatments, we can suggest that maternal nutrition did not affect the offspring's reproductive tract development.

The correlation between the phenotypes shows the association degree between them, or a measurement of the joint variation degree. According to the results in Table 3, REA at weaning and at one year of age showed a positive correlation with weights between weaning and 24 months. This correlation can be explained by animal growth, since heavier heifers have greater REA (MINICK et al., 2002). At 18 months, the correlation between REA and weight is practically nonexistent, possibly because the animals entered puberty and decreased muscle deposition, switching it for fat deposition (OWENS et al., 1995). Since the phenotypes were corrected for the fixed effects before calculating the correlation, part of this coefficient takes into account the genetic value of the animals. Therefore, despite the small size of the database, it is still plausible to assume that part of this coefficient is a good approximation for the genetic correlation. Studies show a correlation between weight and REA (LAMB; ROBISON; TESS, 1990; SPLAN; CUNDIFF; VAN VLECK, 1998) and between ADG and REA (MAHMOOD et al., 2016). The correlation between reproductive tract and fat thicknesses showed a possible association between the characteristics, since RFT was already related to the reproductive tract and became more significant, as the heifers became more mature (Minick et al., 2002). Another factor with a great effect on the expected result is the relationship between leptin and puberty onset. Leptin is a hormone produced by adipose tissue and has a direct action on the hypothalamus-pituitary-ovary axis, causing an increase in peaks of GnRH (DUITTOZ et al., 2016; PERRY, 2016; ZAMBRANO et al., 2006).

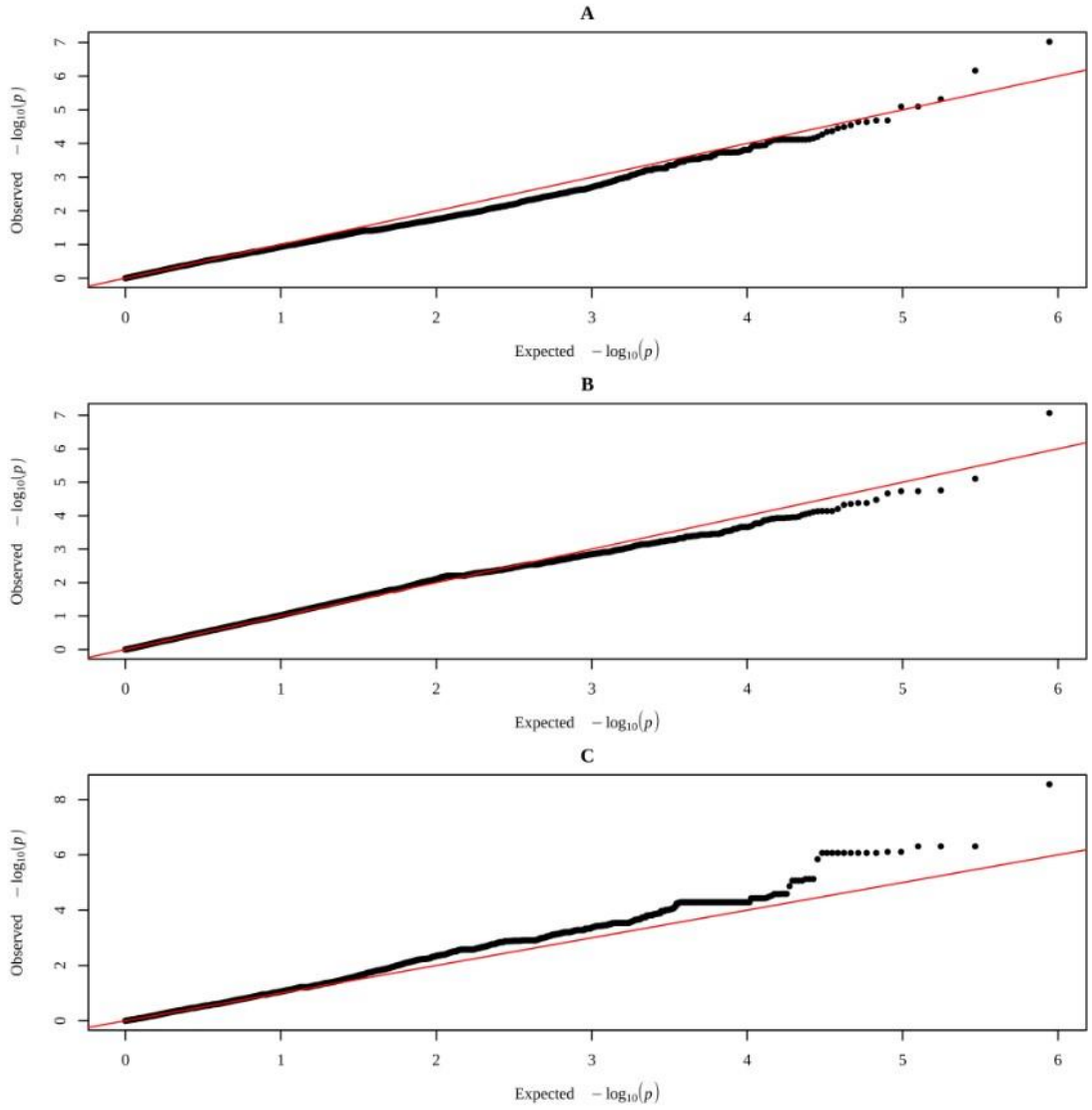

Figure 2. QQ-plots of traits with SNPs highlighted by the exploratory genomic association analysis in each treatment. **(A)** AFC at Yearling in control group; **(B)** Weight at Yearling in PELT group; **(C)** BFT at Year in PEWG group.

In addition, we conducted an exploratory study on the genomic association that resulted in a significant SNP for each treatment. SNPs related to control and PEWG treatments are located in intergenic regions and the affected genes are often difficult to determine (BRODIE; AZARIA; OFRAN, 2016). However, some genes found near the intergenic regions of SNPs have functions related to the characteristic in which it was identified. The SNP related to the control treatment is

linked to the characteristic AFC at yearling. The NPM2 gene relates to the function of the ovarian and reproductive tract and encodes an oocyte-specific nuclear protein, with great importance in early embryonic development (LINGENFELTER et al., 2011). The FGF17 gene plays a role in the differentiation of granulosa cells (MACHADO et al., 2009) and also in hypogonadism (MIRAOUI et al., 2013). On the other hand, the GFRA2 gene, the only gene within 100Kb from the marker, has an important role in the differentiation of stem cells in the pituitary (PRADILLA et al., 2021), important organ related to reproductive development. The gene XPO7 have been linked to ovarian cancer ((Cáceres-Gorriti et al., 2014)) and the DOK2 gene was related to fetal programming, having its gene expression reduced in offspring of animals that underwent uteroplacental insufficiency (MASTER et al., 2015).

Several genes related to lipid metabolism were found close to the SNP for BFT at year, which is related to the PEWG treatment. The SPRING1 gene, also known as C12orf49, is an important regulator of lipid metabolism homeostasis (AREGGER et al., 2020; GIRARDI; SUPERTI-FURGA, 2020). Studies on characteristics of buffalo milk link the RNFT2 gene to the production of fat, proteins, and milk in (DU et al., 2019; VENTURINI et al., 2014). The FBXW8 gene was associated to fetal programming in a study that analyzed intrauterine growth restriction (GASCOIN-LACHAMBRE et al., 2010). The MED13L gene is associated to heart development in humans (NAPOLI; SCHIANO; SORICELLI, 2019). RAPGEF1, the significant gene for the PELT treatment and is studies relate it to persistence of lactation (DO et al., 2017) and mastitis in dairy cows (CHEN et al., 2015) also to the Lipomatous Myopathy disease in Piedmontese cattle (PELETTO et al., 2017). Nevertheless, these results need to be considered with great parsimony, since the sample size used in the analysis is small, which can lead to false-positive results. Our work investigated possible genotype-environment interactions in animals submitted to fetal programming. This is an innovative study, since no studies evaluated animals under these conditions and phenotypes. Furthermore, the data presented will attain greater accuracy in future studies, as the fetal programming database increases its amount of information.

4.5. Conclusion

Protein-energy supplementation at different gestation periods in Nellore cows did not affect the reproductive tract development or body composition and ADG during the rearing period of their daughters. However, the treatment affected weight over time and the animals of the control group

were heavier. The exploratory genomic association study showed one SNP on an intergenic region for control and PEWG, and one for PELT in an intronic region of RAPGEF1 gene. Our study provided insights into the effects of fetal programming on Nellore heifers, showing that protein-energy supplementation may not affect their sexual development. However, this field requires further studies, once the results found in literature are still contradictory, and research with exploratory genome-wide association studies in animals that have undergone fetal programming are still scarce.

4.6. Conflict of interest

The authors declare absence of any type of conflict of interest.

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5. General Conclusions

The results found in the present thesis are of great importance to the enrichment of research on body performance, muscular development and female reproduction system development in fetal programming. Thus, this thesis has much to contribute to studies in the areas of fetal programming and development. However, it is still inevitable to deepen the research with regard to fetal programming, making more studies necessary relating distinct strategies of stimuli and insults in different gestational periods.