ELISA BARBOSA DE CARVALHO

TRANSCRIPTOME PROFILE IN THE SKELETAL MUSCLE OF PROGENY FROM DAMS PROTEIN-SUPPLEMENTED DURING MID-GESTATION

Lavras – MG
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Thesis presented to the Federal University of Lavras, as part of the Animal Science Graduate Program requirements, area of Ruminant Production and Nutrition, to obtain the Ph.D. title in Animal Science.

Prof. Mateus Pies Gionbelli
Advisor

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Aprovada em 30 de agosto de 2021

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“Science is a way of thinking much more than it is a body of knowledge”

Carl Sagan

ABSTRACT

Maternal undernutrition and overnutrition during gestation can cause different effects on muscle development and offspring performance. This study aimed to evaluate the differentially expressed genes (DEG), weighed genes co-expression networks (WGCNA), and their biological functions in skeletal muscle of offspring from dams supplemented or not with protein during mid-gestation. From 100 to 200 days of gestation, ten Tabapuã beef cows pregnant of male calves were assigned to one of two treatments: Control [(**CON**) - supply of basal diet achieving 5.5% of crude protein (CP); n = 6]; or Supplemented [(**SUP**) - basal diet plus a supplement with 40% of CP provided at the level of 3.5 g/kg of body weight; n = 4]. Muscle samples were collected from the progeny at the beginning of feedlot phase (260d of postnatal age) to RNA-seq, WGCNA and Gene Ontology (GO) enrichment analysis. A total of 310 DEG (q-value < 0.05) were identified for the effect of diet, being 187 and 123 genes down-regulated and up-regulated in the SUP diet, respectively. Within the SUP up-regulated genes, were genes related to insulin receptor signaling pathway *(LOC107131843)*; apoptosis regulation *(KRT18, KRT8 and KRT19)*; negative regulation of lipoprotein lipase activity *(ANGPTL4)* and potassium ion transmembrane transport *(KCNH3).* The *ENSBTAG00000032057* gene, involved in the ATP synthesis coupled proton transport, were down-regulated in SUP diet. In WGCNA, within hub genes identified in the module negatively associated with diet were *ANGPTL4* and *ANKRD6* (associated with Wnt signaling pathway); whereas in the modules positively correlated was *KLHL10* (homeostasis of number of cells within a tissue). The GO analysis showed that DEG were mainly enriched in potassium ion transmembrane transport, microtubule polymerization or depolymerization and positive regulation of cellular component biogenesis. In conclusion, maternal protein supplementation during mid-gestation modulates the expression of genes involved in important biological process related to myogenesis, lipogenesis, and muscle and adipose tissues metabolism.

Keywords: Fetal programming, gene expression, maternal nutrition, muscle metabolism.

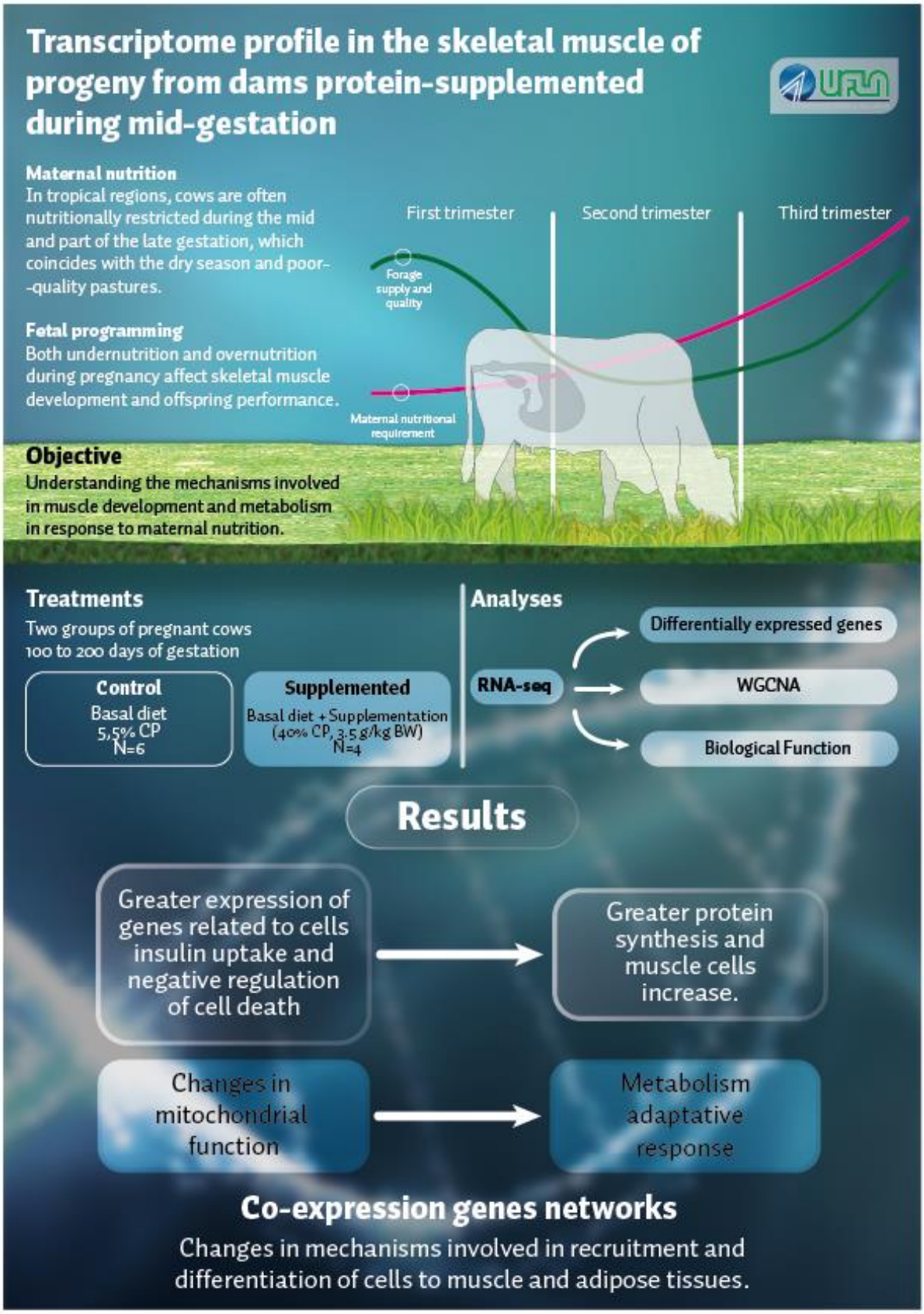
RESUMO

A subnutrição e supernutrição maternas durante a gestação podem causar diferentes efeitos no desenvolvimento muscular e no desempenho da progênie. Este estudo teve como objetivo avaliar os genes diferencialmente expressos (DEG), redes de co-expressão gênica (WGCNA) e suas funções biológicas no músculo esquelético da progênie de vacas suplementadas ou não com proteína durante o terço médio da gestação. De 100 a 200 dias de gestação, dez vacas Tabapuã prenhes de bezerros machos foram alocadas em um de dois tratamentos: Controle [(CON) - fornecimento de dieta basal atingindo 5,5% de proteína bruta (PB); n = 6]; ou Suplementado [(SUP) - dieta basal mais suplemento com 40% de PB fornecido ao nível de 3,5 g / kg de peso corporal; n = 4]. Amostras de tecido muscular foram coletadas da progênie no início da fase de confinamento (260d de vida pós-natal) para análise de RNA-seq, WGCNA e processos biológicos (GO terms) enriquecidos. Um total de 310 DEG (q-valor <0,05) foram identificados para o efeito da dieta, sendo 187 e 123 genes regulados negativamente e regulados positivamente na dieta SUP, respectivamente. Entre os genes mais expressos no grupo SUP, estavam genes relacionados à via de sinalização do receptor de insulina *(LOC107131843)*; regulação da apoptose *(KRT18, KRT8 e KRT19)*; regulação negativa da atividade da lipoproteína lipase *(ANGPTL4)* e transporte transmembrana de íons potássio *(KCNH3)*. O gene *ENSBTAG00000032057*, envolvido na síntese de ATP acoplado ao transporte de prótons, foi regulado negativamente no grupo SUP. Na análise de WGCNA, entre os genes identificados no módulo negativamente associado à dieta, foram encontrados *ANGPTL4* e *ANKRD6* (associado à via de sinalização da Wnt); enquanto nos módulos relacionados positivamente à dieta, foi encontrado o gene *KLHL10* (homeostase do número de células de um tecido). A análise dos GO terms mostrou que os DEG foram enriquecidos principalmente no transporte transmembrana de íons de potássio, polimerização ou despolimerização de microtúbulos e regulação positiva da biogênese de componentes celulares. Em conclusão, a suplementação materna com proteína durante o terço médio da gestação modula a expressão de genes envolvidos em importantes processos biológicos relacionados à miogênese, lipogênese e metabolismo dos tecidos muscular e adiposo.

Palavras-chave: Programação fetal, expressão gênica, nutrição materna, metabolismo muscular.

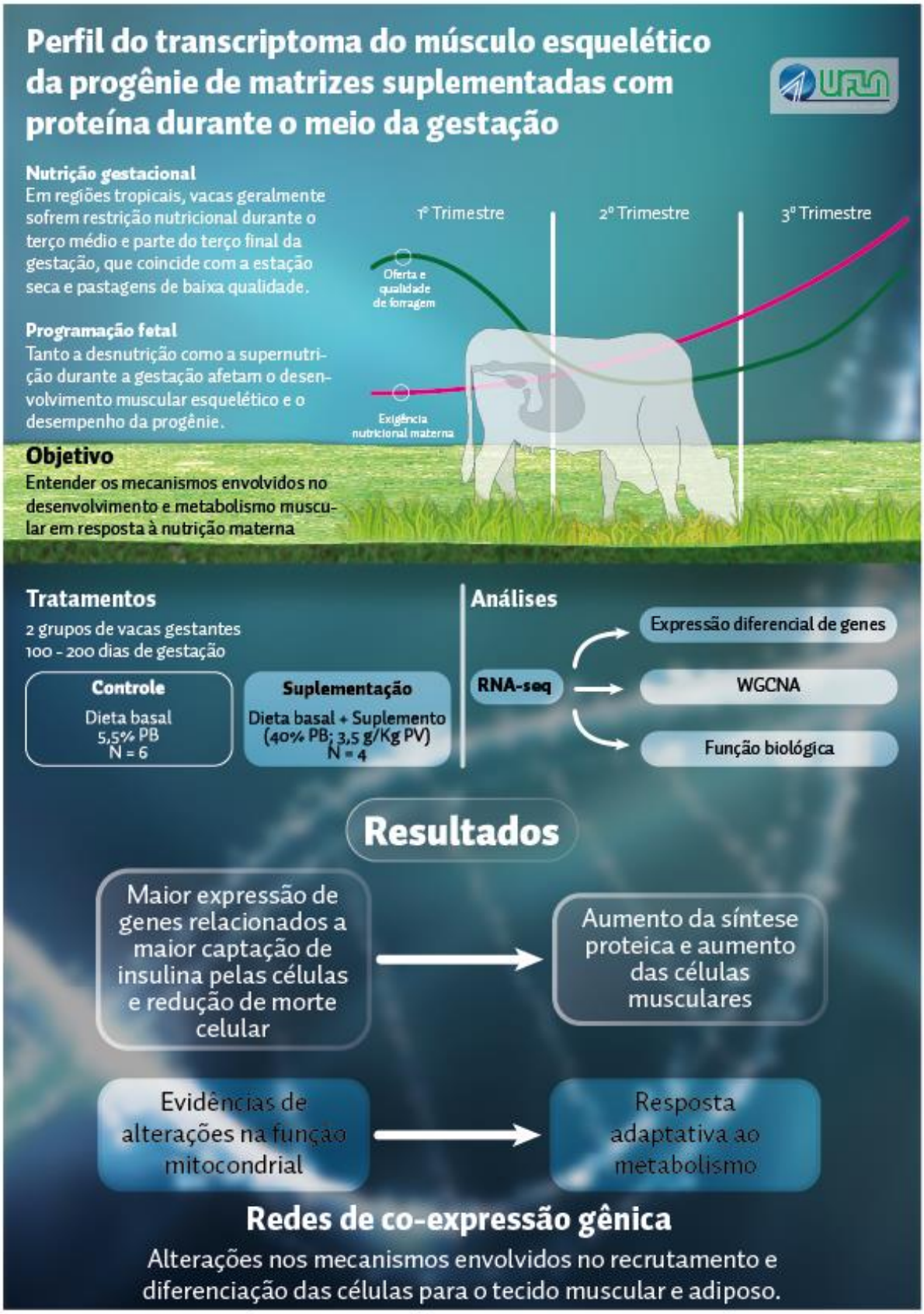
Inform Graphic

Created by Elisa Barbosa de Carvalho and supervised by Mateus Pies Gionbelli



Info Gráfico

Criado por Elisa Barbosa de Carvalho e supervisionado por Mateus Pies Gionbelli



SUMMARY

FIRST CHAPTER - BACKGROUND

1 INTRODUCTION

In tropical regions, due to seasonal variations of dry and rainy period, beef cattle reproduction and lactation are synchronized within the rainy season in order to maximize the intake of high-quality forages. Nonetheless, in crucial stages of gestation, such as, mid-gestation, the quantity and quality of the pastures are limited (DUARTE; PAULINO; DU, 2012).

Livestock animals are raised for their skeletal muscle growth. The fetal stage is crucial for skeletal muscle development in mammalian livestock and nutrient fluctuations during this stage affects muscle development, which may have long-term impacts on the production efficiency and quality of meat (DU et al., 2015).

Strategies of maternal supplementation during critical periods of gestation or offspring's post-natal supplementation are adopted to minimize the damages caused by maternal restriction during gestation. However, since the number of muscle fibers are fixed at birth, offspring's supplementation may be limited in improving beef cattle potential for muscle growth in the postnatal stages of life (COSTA et al., 2021).

Skeletal muscle is composed by myocytes, adipocytes, and fibroblasts, which are all derived from a common pool of mesenchymal stem cells (DU et al., 2015) and their commitment to one of those lineages may be altered by maternal nutrition during pregnancy. Meat quality is a multifactorial parameter, affected by extrinsic and intrinsic factors, such as composition of muscle fibers, intramuscular fat (marbling level) and connective tissue structure.

There are several studies on fetal programming in the literature evaluating the gene expression of targeted genes in offspring's muscle, however, few uses the whole-transcriptome profiling to investigate the underlying mechanisms for the changes observed in these animals. We hypothesized that maternal protein supplementation during mid-gestation would cause changes in the expression of genes related to muscle development in beef calves. Therefore, this study aimed to evaluate the effects of protein supplementation at gestation on the skeletal muscle transcriptome of the offspring.

2 BACKGROUND

2.1 Fetal Programming in Beef Cattle

Fetal programming, also called developmental programming or fetal developmental programming, is the response to a specific challenge during a critical developmental time window that alters the trajectory of development qualitatively and/or quantitatively with resulting persistent effects (BARKER et al., 2002; NATHANIELSZ; POSTON; TAYLOR, 2007).

The concept of fetal programming was originally developed from human epidemiological data linking low birth weight and poor maternal nutrition to an increased incidence of adult diseases, such as coronary heart diseases, stroke, diabetes, and hypertension (BARKER et al., 2002). Poor nutrition, environmental temperature, oxygen availability and overnutrition all have been shown to significantly affect intrauterine development (OPSOMER et al., 2017).

In livestock production, undernutrition can often occur during gestation, particularly during the first two trimesters (VONNAHME, 2007). In Brazil, considering that the breeding season in most of grazing production systems occurs between November and January, pregnant cows usually experience feed restriction during the mid-gestation period, which overlaps with the dry season in most of the beef cattle production areas (DUARTE; PAULINO; DU, 2012).

Undernutrition of the dam during the initial stages of fetal development may appear to be less important because of the limited nutrient requirements of the fetus for growth and development during the first half of gestation. This is accentuated by the fact that 75% of the growth of the ruminant fetus occurs during the last two months of gestation (ROBINSON et al., 1977). However, it is during this early phase of fetal development that maximal placental growth, differentiation, and vascularization occurs, as well as fetal organogenesis, all of which are critical events for normal conceptus development (VONNAHME, 2007).

Fetal stage is crucial for skeletal muscle development since there is no increase in muscle fibers numbers after birth (DU et al., 2011). Furthermore, development of vital organs has higher priority in nutrients portioning than skeletal muscle, which makes the muscular tissue more susceptible to variations of maternal nutrition during pregnancy (DUARTE; PAULINO; DU, 2012). Studies linking fetal programming to animal performance in ruminants and other species

have shown that both undernutrition and overnutrition during gestation affect skeletal muscle development and offspring performance (DU et al., 2010).

The thrifty phenotype hypothesis says that metabolic adaptations by the undernourished fetus may lead to adverse consequences later in life under conditions of both adequate nutrition and overnutrition (HALES; BARKER, 2001; PARK et al., 2004).

Historically, birth weight has been used as a marker to identify individuals who have suffered adverse effects from fetal programming. However, this feature is a poor indicator of nutritional programming, since it provides little information about body composition, adiposity and potentially altered body functions (KHANAL; NIELSEN, 2017). Besides that, previous studies have demonstrated that the differential prenatal nutritional status can impact fetal development without noticeable phenotypic differences (PARADIS et al., 2017). Thus, the study of gene expression associated with fetal programming is important to better understand the underlying mechanisms.

2.2 Gene regulation of myogenesis, adipogenesis, and fibrogenesis

Mesenchymal stem cells (MSCs) are multipotent stem cells that are capable of self-renewing and differentiating into functional cell types. During the differentiation of MSCs toward a specific cell type, various stimuli and inhibitors play important roles in initial commitment and later stages of differentiation, such cytokines, growth factors, extra cellular matrix molecules, and transcription factors (ALMALKI; AGRAWAL, 2016).

Fetal and neonatal skeletal muscle development involve myogenesis, adipogenesis and fibrogenesis, all derived mainly from mesenchymal stem cells (Figure 1). Therefore, the commitment of MSCs to myogenic, adipogenic or fibrogenic lineages can be considered a competitive process, and is "shaped" by numerous inductive regulators (REHFELDT; FIEDLER; STICKLAND, 2004; YAN et al., 2013).

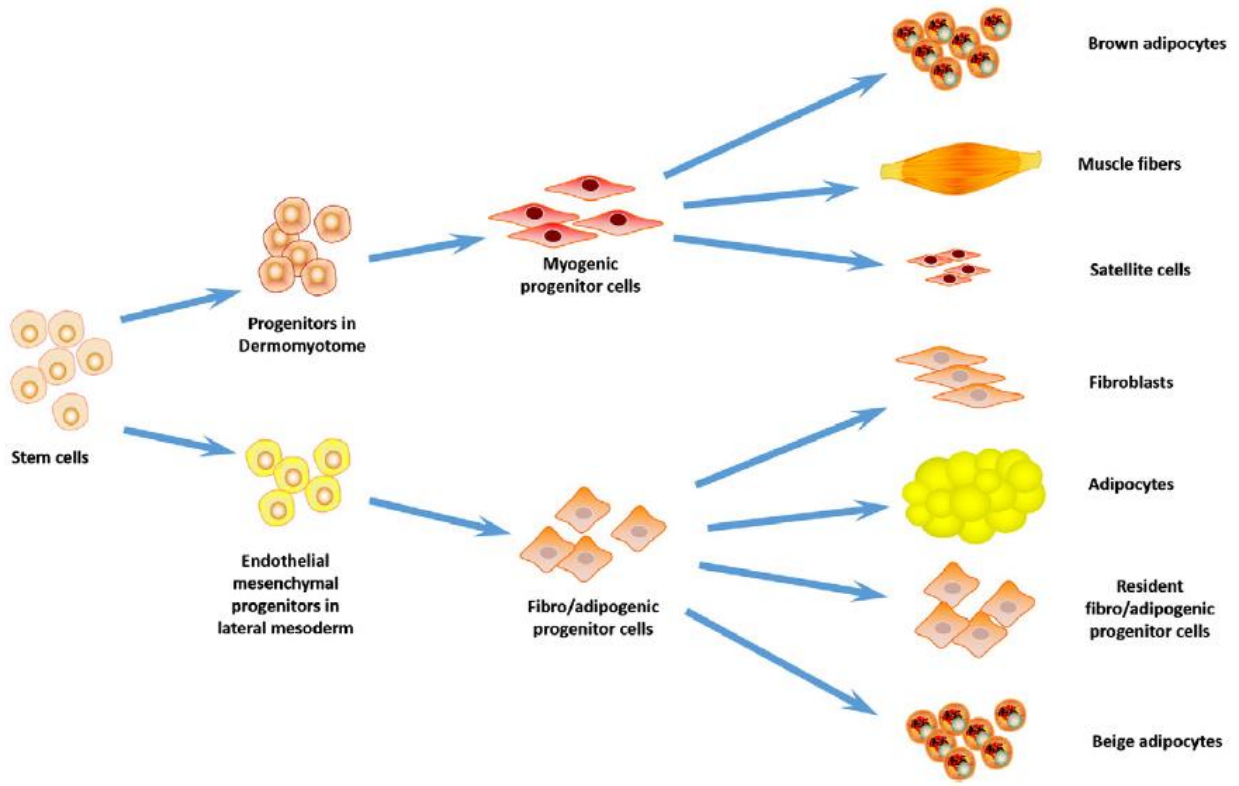


Figure 1. Early mesoderm development and the commitment of mesenchymal progenitor cells into myogenic and fibro-adipogenic cell lineages during fetal muscle development (DU et al., 2015).

The Wingless and Int (Wnt) pathway, a β-catenin-dependent signaling pathway called the Wnt/β-catenin signaling pathway, is what determines which tissue will be formed. Upregulation of Wnt/β-catenin promotes myogenesis, and downregulation enhances adipogenesis (Figure 2).

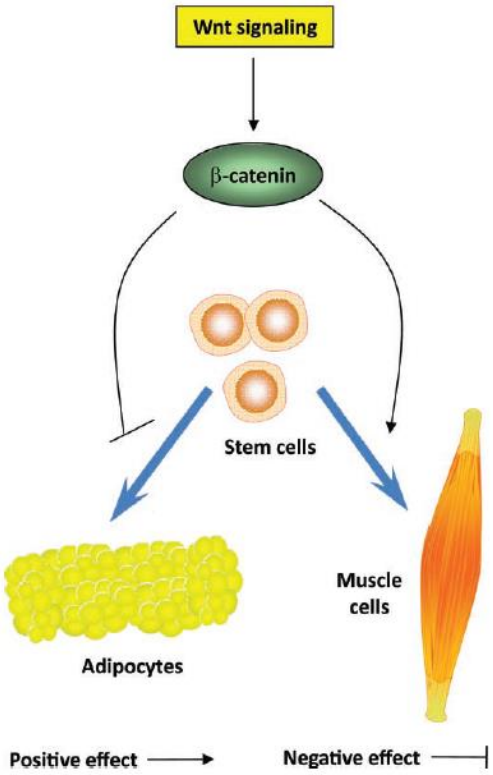


Figure 2. Wingless and Int (Wnt) signaling and fetal skeletal muscle development (DU et al., 2010).

Wnt and Sonic hedgehog (Shh) regulate the expression of paired box (Pax) 3, Pax7, and Glioma-associated oncogene homolog 1 (Gli) which then initiate expression of myogenic regulatory factors (CHARGÉ; RUDNICKI, 2004; DU et al., 2010). During the expression of Pax3, cells migrate through the dorsomedial lip of the dermomyotome to form the myotome and promote myogenic differentiation. The overexpression of Pax3 in MSCs promotes the myogenic differentiation and blocks the adipogenic, osteogenic, and chondrogenic differentiation of MSCs (ALMALKI; AGRAWAL, 2016). Pax 7 plays an important role in the biology of satellite cells (BRAND-SABERI, 2005).

The muscle regulatory factors (MRFs) of the basic helixloop- helix (bHLH) type are key regulators of myogenesis. Four genes for MRFs have been identified altogether: MyoD, Myf5, Myogenin and MRF4. Myf5 and MyoD, in a redundant fashion, act genetically upstream of myogenin and MRF4 to specify myoblasts for terminal differentiation. Myogenin and MRF4 are more directly involved in the differentiation process and trigger the expression of myotube specific genes (BENTZINGER; WANG; RUDNICKI, 2012).

Beside MRFs and Pax proteins, another important group of factors involved in the positive regulation of muscle differentiation are the myocyte enhancer factors (MEFs) which belong to the group of proteins related to serum response factors (RSRFs). MEF2 factors can increase the efficiency of myogenic conversion of non-muscle cells in combination with MRFs (BRAND-SABERI, 2005).

Myogenic differentiation can be induced or inhibited by signaling factors. Insulin-like growth factor-II (IGF-II) induces the myogenic differentiation through the insulin-like growth factor receptor-1, which targets coregulators of important cofactors for MyoD. Tumor necrosis factor- α (TNF-α) also has a regulatory role in the differentiation of MSCs into myocytes. It down-regulates the expression of MyoD and inhibits myogenic differentiation through NF-κB activation and reduction of IGF-1 signaling pathway. Similarly, Smad3, which belongs to receptor-regulated Smad family, suppresses myogenic differentiation through its association with myogenic transcription factors. TGF-β-activated Smad3 directly suppresses the transcription activity of MyoD and myogenin (ALMALKI; AGRAWAL, 2016).

The PPARs (peroxisome proliferator-activated receptors) are members of the nuclear hormone receptor superfamily of ligand-dependent transcription factors (TFs). Three PPAR isoforms, called α, β/δ and γ, have been identified (GRIMALDI, 2003; LEFTEROVA et al., 2014). PPARγ is required for adipocyte differentiation, regulation of insulin sensitivity, lipogenesis, and adipocyte survival and function (LEFTEROVA et al., 2014).

PPARγ and CCAAT-enhancer-binding proteins (C/EBPs) are crucial intracellular factors controlling adipogenesis (ROSEN; SPIEGELMAN, 2001). In early adipogenesis, activation of PPARγ and C/EBPα is induced by the expression of CEBPβ e CEBPδ factors. In the terminal stages of adipogenesis, PPARγ activates the expression of C/EBP-α, which, in response, also induces PPARγ gene expression through binding to the same DNA sites in the PPARγ promoter that are induced by C/EBP-β, and -δ. Thus there is a positive feedback loop between PPARγ and C/EBP-α process that subsequently induces the expression of genes related to adipocyte differentiation and maturation (CHRISTODOULIDES et al., 2009). PPARγ is required for differentiation, whereas C/EBPα plays a more ancillary role by promoting full insulin sensitivity and specific gene expression (ROSEN; SPIEGELMAN, 2001; LEONARDINI et al., 2009).

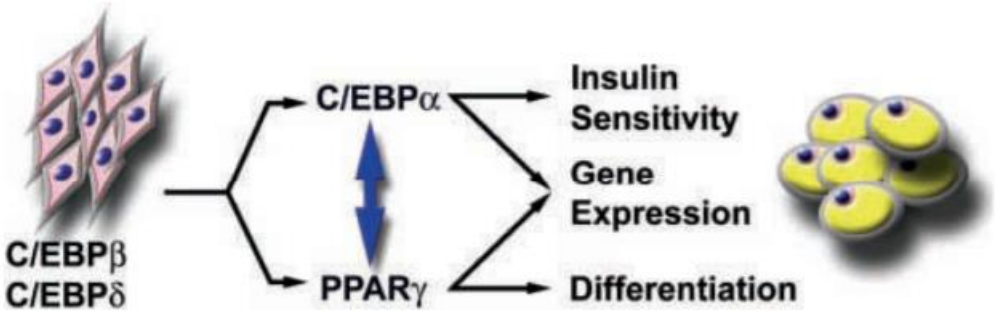


Figure 3. PPARγ plays a critical role in the adipogenic transcriptional cascade. As preadipocytes begin to differentiate they express C/EBPβ and C/EBPδ, which in turn activate both PPARγ and C/EBPα. These two proteins potently induce each other's expression (ROSEN; SPIEGELMAN, 2001).

The formation of intramuscular fat deposits, which is considered a specialized connective tissue, and fibrogenesis are correlated events that derive from a unique pool of fibro-adipogenic progenitor cells (FAP) present in the stromal-vascular fraction within skeletal muscle (DU et al., 2013). The development of adipogenic and fibrogenic lineages within the vascular stroma are initially defined by the expression of the zinc finger protein (ZFP423) and the transforming growth factor (TGF-β), respectively (DU et al., 2015).

Fibrogenesis is mainly mediated by the TGF-β signaling pathway, which promotes fibrosis via activation of the Smad signaling pathway, specifically through phosphorylation of Smad2 and Smad3, which then oligomerize with Smad4 and translocate into the nucleus to initiate transcription of TGF-β target genes, including fibronectin and type I collagen that are crucial components of connective tissue (DU et al., 2011). In addition, TGF-β1 prevents adipogenic differentiation through cooperation with the Wnt10B pathway and suppressing C/EBPα and PPARγ (ZAMANI; BROWN, 2011).

2.3 Gene regulation of energy metabolism of skeletal muscle adipose tissue

A remarkable attribute of skeletal muscle is its metabolic plasticity. Whereas acute alterations in skeletal muscle metabolism are largely mediated by changes in substrate flux and activation of signaling pathways that together coordinate the balance between substrate availability and metabolic demand, chronic alterations are regulated by changes in gene and subsequent protein

expression that ultimately result in metabolic reprogramming (HOWLETT; MCGEE, 2016). The high plasticity in the responsiveness of muscle is exemplified by myosin isoform transitions, mitochondrial division, and alterations in gene expression of enzymes involved in oxidative and glycolytic pathways (JANSSEN et al., 2003).

Energy in skeletal muscle is derived mostly from glucose and fatty acids. It is also stored in significant amounts as glycogen and triglycerides, respectively, in the muscle fibers. The chemical energy trapped within the bounds of the carbohydrate, lipid, and protein molecules is extracted as adenosine triphosphate (ATP), an immediate source of energy (ATALAY; HÄNNINEN, 2010).

In muscle and fat cells, the clearance of circulating glucose depends on the insulin-stimulated translocation of the glucose transporter GLUT4 isoform to the cell surface. Insulin also profoundly affects lipid metabolism, increasing lipid synthesis in fat cells, and attenuating fatty acid release from triglycerides in fat and muscle (PESSIN; SALTIEL, 2000).

Skeletal muscle fibers are classified into three types: type I, type IIa, and type IIb. Slow-twitch type I and fast-twitch type IIa fibers contain more mitochondria and exhibit relatively higher rates of oxidative metabolism. In contrast, type IIb fibers have fewer mitochondria and are metabolically glycolytic (LIANG; WARD, 2006).

Oxidative metabolism of glucose and lipids in muscle fibers is regulated by transcriptional factors of metabolic genes, such as peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) and Succinate dehydrogenase (SDH). Succinate dehydrogenase is one of the mitochondrial oxidative enzymes in skeletal muscle and controls transcription of metabolism-related genes, promoting metabolism of glucose and lipids. The reduction in mRNA expression levels of PGC-1α and SDH inhibits oxidative metabolism of skeletal muscle (ADACHI et al., 2007).

The PGC-1α stimulates the conversion of muscle fiber type toward more oxidative type I and IIa fibers, favoring fatty acid oxidative metabolism, activates mitochondria biogenesis and increases mitochondrial function. All PPARs isoforms (PPARα, PPARγ, and PPAR β/δ) are subject to transcriptional coactivation by PGC-1α (LIANG; WARD, 2006).

Peroxisome proliferator-activated receptors (PPARs) are ligand activated transcription factors that regulate genes important in cell differentiation and various metabolic processes, especially lipid and glucose homeostasis (GRYGIEL-GÓRNIAK, 2014). Through their distinct

functions and tissue distribution, the PPARs regulate many aspects of energy metabolism at the transcriptional level (WANG, 2010).

The PPARα is predominantly expressed in the liver and skeletal muscles, participating in fatty-acids oxidation and its activation lowers lipid levels, while PPARγ is mostly involved in the regulation of the adipogenesis, energy balance, and lipid biosynthesis (LEONARDINI et al., 2009; GRYGIEL-GÓRNIAK, 2014). In white adipose tissue (WAT), PPARγ regulates the expression of a number of genes involved in lipid metabolism, including lipoprotein lipase (LAPLANTE et al., 2003). PPARβ/δ participates in fatty acid oxidation, mostly in skeletal and cardiac muscles, but it also regulates blood glucose and cholesterol levels (GRYGIEL-GÓRNIAK, 2014).

In muscle cells, PPARβ/δ increases fatty acid uptake and catabolism via β-oxidation, switching energy production from glycolysis to fatty acid oxidation as an alternative energy source; increases succinate-dehydrogenase-positive muscle fibers with enhanced fatty acid oxidative capabilities and leads to an overall decrease in body fat. Similarly, PPARβ/δ regulates downstream target genes required for fatty acid transport, β-oxidation of fatty acid, and mitochondrial respiration (LIU et al., 2018).

The PPARβ/δ is required for the formation and maintenance of oxidative muscle fibers. Deletion of PPARβ/δ decreases the expression of many genes for fatty acid β-oxidation and mitochondrial respiration function and increases the number of muscle fibers with lower oxidative capacity, leading to an increased body weight gain and insulin resistance (WANG, 2010).

Adipose PPARγ protects non-adipose tissues against excessive lipid overload and maintains normal organ function (liver, skeletal muscle). Activated PPARγ in adipocytes guarantees a balanced and adequate secretion of adipocytokines (adiponectin and leptin) that are mediators of insulin action in peripheral tissues. In consequence, the insulin sensitivity of the whole body is maintained. It is also important in lipid metabolism and regulates the genes participating in the release, transport, and storage of fatty acids such as lipoprotein lipase (LPL) and the fatty acid transporter CD36 (GRYGIEL-GÓRNIAK, 2014).

The lipoprotein lipase (LPL) is a rate limiting enzyme for triglyceride (TG) hydrolysis that has a central role in several aspects of lipid metabolism, regulating the concentration of plasma TG and the entry of fatty acids into adipose tissues and muscles (KAGEYAMA et al., 2003). LPL influences the partitioning of triglyceride-derived fatty acid uptake between different tissues,

plasma cholesterol metabolism and the subsequent downstream intracellular effects related to lipid availability (BEY; HAMILTON, 2003).

2.4 Effects of maternal nutrition during mid-gestation on skeletal muscle and adipose tissue development

The fetal period is crucial for skeletal muscle development, because no net increase in the number of muscle fibers occurs after birth. Postnatal muscle growth in mammals relies largely on protein accretion leading to muscle fiber hypertrophy (BRAMELD, 2004). Skeletal muscle has a lower priority in nutrient partitioning compared with the brain and heart in response to the challenges the fetus faces during development, rendering it particularly vulnerable to nutrient deficiency (ZHU et al., 2006).

The nutrients requirements of beef cows increases throughout gestation (LARSON et al., 2009). When this demands are not met, alterations in the metabolism of the offspring may occur and impact the performance of the adult animal.

The process of skeletal muscle development is initiated during the embryonic stage and can be divided in two distinct muscle fiber formation events. In primary myogenesis, during the first two months of gestation, the primary myotubes are formed, which originate the type 1 fibers and provide a framework for the larger population of smaller secondary fibers. In secondary myogenesis, between the third and seventh month of gestation, occurs the formation of secondary myotubes, which originate type 2 fibers (REHFELDT; FIEDLER; STICKLAND, 2004; DU et al., 2010).

Another population of myoblasts does not form fibers but stays quiescent and close to the myofibers; these are termed satellite cells and are able to divide and serve as the source of new myonuclei during postnatal growth, contributing to growth of the fibers (REHFELDT; FIEDLER; STICKLAND, 2004).

Maternal nutrient restriction during each stage of gestation (early, mid and late) can cause different effects on muscle development and offspring performance (Figure 4).

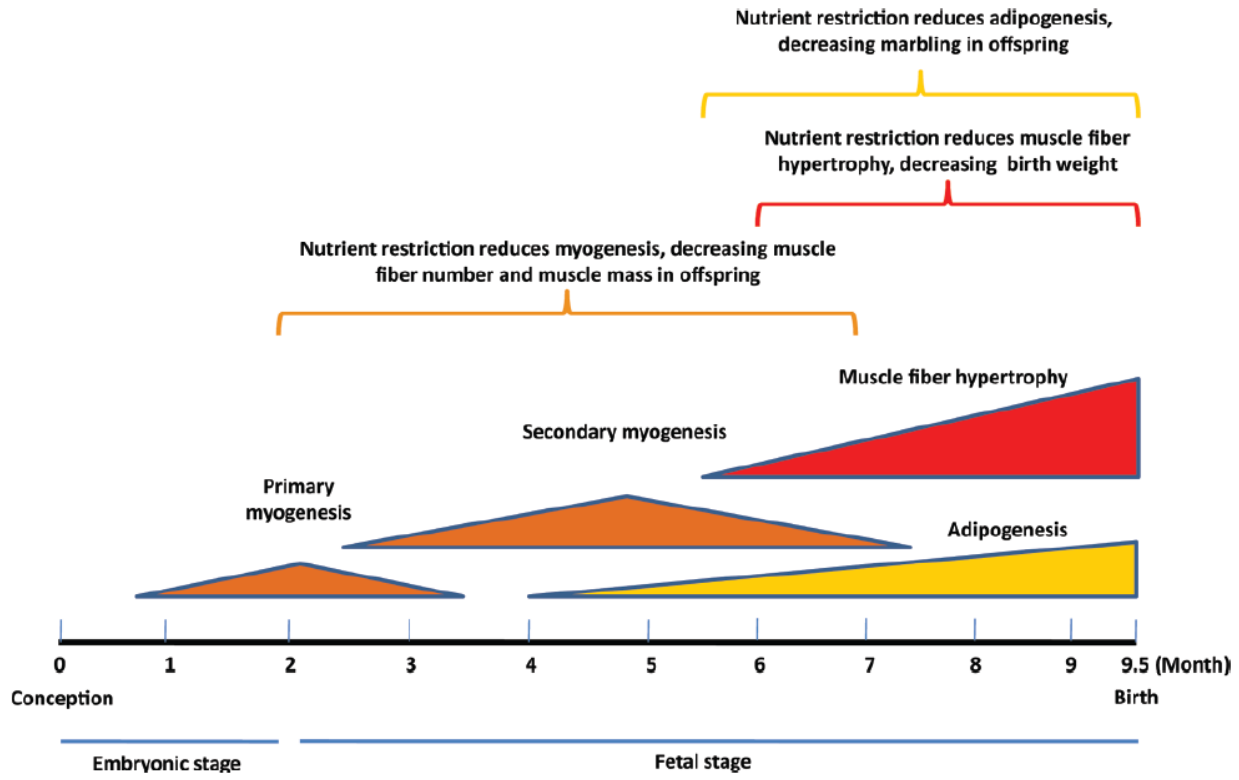


Figure 4. Effects of maternal nutrition on bovine fetal skeletal muscle development (DU et al., 2010).

Mid-gestation is a critical period for skeletal muscle and adipose tissue development (SYMONDS et al., 2004; FORD et al., 2007). During such period, a pool of undifferentiated mesenchymal stem cells is present, and either myocytes or adipocytes can differentiate from this cells (DU et al., 2010). Different nutritional status of the dam during mid-gestation affects fetal development, leading to long-term impacts on the production efficiency and meat quality via epigenetic effects and differential expression of myogenic and adipogenic genes (LILLYCROP et al., 2007; GICQUEL; EL-OSTA; LE BOUC, 2008).

Evaluating skeletal muscle's composition of offspring with or without protein restriction during fetal life, Costa et al. (2021) identified differences in the number of muscle fibers, which were lower in the resulting offspring from maternal protein restriction during mid-gestation and this impairment persisted until the finishing phase. Maternal restriction before the 210d of gestation had a major impact in muscle fibers number, due to myogenesis that begins during the embryonic stage (primary myofibers), persists in fetal stages (secondary myofibers), and slows until late gestation.

Although muscle fiber number cannot increase after birth, a population of quiescent satellite cells may contribute to support postnatal growth and repair. PAX7-positive cells are markers of satellite cells, which indicates the presence of undifferentiated myoblasts that have not reached their terminal differentiation. Under the demand of hypertrophy, these cells may proliferate and fuse with existing muscle fibers. Costa et al. (2021) found a tendence of increase in the expression of PAX7 in the restricted progeny at 30d of age.

Skeletal muscle is the main site for the utilization of glucose and fatty acids. The reduction in skeletal muscle mass during fetal development may therefore have long-lasting irreversible negative physiological consequences for offspring, including predisposing offspring to obesity and diabetes (ZHU et al., 2006).

Another aspect that can be altered by maternal nutrition is the muscle composition in terms of fiber types, which affects the muscle metabolism and the growth potential of offspring. Type I myofibers have greater protein turnover rates and are less efficient for growth, whereas type II myofibers have reduced catabolic rates and exhibit greater growth efficiency. The oxidative capacity and insulin sensitivity of muscle generally follows the order of type I ≥ type IIa ≥ type IIb. GLUT4 is found in larger amounts in type I and IIa muscle fibers than in type IIb and insulin-resistance is associated with an increased number of type IIb muscle fibers (ZHU et al., 2006).

In general, prenatal undernutrition increases the proportion of type I fibers and reduces the proportion of type II fibers in young offspring, due to a reduction in the numbers of secondary fibers formed (MALLINSON et al., 2007).

Aragão et al. (2014), found that, under ad libitum feeding conditions, adult offspring born to protein rstricted dams exhibit a gene expression pattern in skeletal muscle favoring the transport of long-chain fatty acids into the mitochondrial matrix without a concomitant increase in fatty acid oxidation. Moreover, the transcriptional network regulating the metabolic switch from carbohydrate to lipid oxidation during fasting was clearly altered in the protein rats, which otherwise exhibit a normal metabolic state. This observation suggests that impaired metabolic flexibility precedes and may underpin the development of obesity and insulin resistance induced by maternal protein restriction.

Besides muscle fiber formation, adipose and connective tissue are established during the prenatal period. The population of fibro-adipogenic progenitor (FAPs) cells holds the capacity of differentiate into adipocytes and fibroblasts, therefore, both processes may have antagonistic

effects (DU et al., 2013). In cattle, adipogenesis and fibrogenesis initiate concomitantly with the secondary myogenesis during mid-gestation, however, most of adipocytes and fibroblast develops in late gestation (DU et al., 2010). Costa et al. (2021) found that protein restriction during mid-gestation increased the collagen content in the skeletal muscle of the offspring, despite the lack of difference in the expression of fibrogenic genes.

Marbling (intramuscular fat) is crucial for meat palatability and the fetal and neonatal stages are major stages for generation of intramuscular adipocytes, which provide the sites for intramuscular fat accumulation or marbling formation during fattening (DU et al., 2015). Hence, fetal programming also affects the marbling in offspring cattle. In addition, the overall fat accumulation in offspring is also affected by maternal nutrition (ZHU et al., 2006).

Data from Underwood et al. (2010) indicate that gestational plane of nutrition may alter subcutaneous and intramuscular adipose depots of the offspring. Subcutaneous adipose tissue of steers born in better quality forage tended to have a greater number of cells. The mean adipocyte diameter was then examined, and no differences were found between treatments. These results indicate increased fat thickness of these animals may be due to increased number of adipocytes, possibly affected by gestational nutrition.

Rodrigues et al. (2020) investigated the effects of protein supplementation during mid-to-late gestation in grazing beef cows with moderate nutritional restriction on performance and molecular markers in offspring. The protein supplementation of the dams did not affect the expression of myogenic genes or muscle fiber type of the offspring. However, a downregulation of *C/EBPA* and *FABP4* genes was observed in 11-day-old calves from supplemented dams. These findings indicate that the offspring from non-supplemented cows showed an early adipogenic differentiation, therefore, this may impair the proliferation of intramuscular adipocytes.

In conclusion, restricted maternal nutrition in mid-gestation can modulate the gene expression and metabolism of skeletal and adipose tissue and affect the performance of the adult offspring, which makes important the investigation of its outcomes.

3 REFERENCES

ADACHI, T. et al. Fibre type distribution and gene expression levels of both succinate dehydrogenase and peroxisome proliferator-activated receptor-γ coactivator-1α of fibres in the soleus muscle of Zucker diabetic fatty rats. **Experimental Physiology**, v. 92, n. 2, p. 449–455, 2007.

ALMALKI, S. G.; AGRAWAL, D. K. Key transcription factors in the differentiation of mesenchymal stem cells. **Differentiation**, v. 92, n. 1–2, p. 41–51, 2016. Disponível em: <http://dx.doi.org/10.1016/j.diff.2016.02.005>.

ANDREWS, S. **FastQC A Quality Control tool for High Throughput Sequence Data**. Disponível em: <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>. Acesso em: 28 maio. 2020.

ARAGÃO, R. D. S. et al. Maternal protein restriction impairs the transcriptional metabolic flexibility of skeletal muscle in adult rat offspring. **British Journal of Nutrition**, v. 112, n. 3, p. 328–337, 2014.

ATALAY, M.; HÄNNINEN, O. O. P. Muscle Energy Metabolism. In: HANNINEN, O. O. P. et al. (Ed.). **The Encyclopedia of Life Support Systems**. Oxford: Eolss Publishers Co. Ltd., 2010. p. 26–48.

BARKER, D. J. P. et al. Fetal origins of adult disease: Strength of effects and biological basis. **International Journal of Epidemiology**, v. 31, n. 6, p. 1235–1239, 2002.

BATEMAN, A. UniProt: A worldwide hub of protein knowledge. **Nucleic Acids Research**, v. 47, n. D1, p. D506–D515, 2019.

BENTZINGER, C. F.; WANG, Y. X.; RUDNICKI, M. A. Building muscle: molecular regulation of myogenesis. **Cold Spring Harbor perspectives in biology**, v. 4, n. 2, 2012.

BEY, L.; HAMILTON, M. T. Suppression of skeletal muscle lipoprotein lipase activity during physical inactivity : a molecular reason to maintain daily low-intensity activity. p. 673–682, 2003.

BRACK, A. S. et al. A Temporal Switch from Notch to Wnt Signaling in Muscle Stem Cells Is Necessary for Normal Adult Myogenesis. **Cell Stem Cell**, v. 2, n. 1, p. 50–59, 2008.

BRAMELD, J. M. The influence of undernutrition on skeletal muscle development. **British Journal of Nutrition**, v. 91, p. 327–328, 2004.

BRAND-SABERI, B. Genetic and epigenetic control of skeletal muscle development. **Annals of Anatomy**, v. 187, n. 3, p. 199–207, 2005.

CHARGÉ, S. B. P.; RUDNICKI, M. A. Cellular and Molecular Regulation of Muscle Regeneration. **Physiological Reviews**, v. 84, n. 1, p. 209–238, 2004.

CHRISTODOULIDES, C. et al. Adipogenesis and WNT signalling. **Trends in Endocrinology & Metabolism**, v. 20, n. 1, p. 16–24, 2009.

COSTA, T. C. et al. Skeletal Muscle Development in Postnatal Beef Cattle Resulting from Maternal Protein Restriction during Mid-Gestation Thais. **Animals**, v. 11, 2021.

DI, Y. et al. The NBP negative binomial model for assessing differential gene expression from RNA-Seq. **Statistical Applications in Genetics and Molecular Biology**, v. 10, n. 1, 2011.

DOBIN, A. et al. STAR: Ultrafast universal RNA-seq aligner. **Bioinformatics**, v. 29, n. 1, p. 15–21, 2013.

DONG, Q. M.; LING, C.; ZHAO, L. Immunofluorescence analysis of cytokeratin 8/18 staining is a sensitive assay for the detection of cell apoptosis. **Oncology Letters**, v. 9, n. 3, p. 1227–1230, 2015.

DU, M. et al. Fetal programming of skeletal muscle development in ruminant animals. **Journal of animal science**, v. 88, n. 13 Suppl, 2010.

DU, M. et al. Fetal muscle development, mesenchymal multipotent cell differentiation, and associated signaling pathways. **Journal of Animal Science**, v. 89, n. 2, p. 583–590, 2011.

DU, M. et al. Meat science and muscle Biology Symposium: Manipulating mesenchymal progenitor cell differentiation to optimize performance and carcass value of beef cattle. **Journal of Animal Science**, v. 91, n. 3, p. 1419–1427, 2013.

DU, M. et al. Fetal programming in meat production. **Meat Science**, v. 109, p. 40–47, 2015. Disponível em: <http://dx.doi.org/10.1016/j.meatsci.2015.04.010>.

DUARTE, M. S.; PAULINO, P. V. R.; DU, M. Fetal programiming in beef cattle: How to optimize performance and carcass value in early life stages. **IV Simpoósio Internacional de Produção de Gado de Corte**, p. 123–139, 2012.

FORD, S. P. et al. Maternal undernutrition during early to mid-gestation in the ewe results in altered growth, adiposity, and glucose tolerance in male offspring. **Journal of Animal Science**, v. 85, n. 5, p. 1285–1294, 2007.

GICQUEL, C.; EL-OSTA, A.; LE BOUC, Y. Epigenetic regulation and fetal programming. **Best**

Practice and Research in Clinical Endocrinology and Metabolism, v. 22, n. 1, p. 1–16, 2008.

GIONBELLI, T. R. S. et al. Foetal development of skeletal muscle in bovines as a function of maternal nutrition, foetal sex and gestational age. **Journal of Animal Physiology and Animal Nutrition**, v. 102, n. 2, p. 545–556, 2017.

GLASS, D. J. Signalling pathways that mediate skeletal muscle hypertrophy and atrophy. **Nature Cell Biology**, v. 5, n. February, p. 87–90, 2003.

GRIMALDI, P. A. Roles of PPARδ in the control of muscle development and metabolism. **Biochemical Society Transactions**, v. 31, n. 6, p. 1130–1132, 2003.

GRYGIEL-GÓRNIAK, B. Peroxisome proliferator-activated receptors and their ligands: Nutritional and clinical implications - A review. **Nutrition Journal**, v. 13, n. 1, p. 1–10, 2014.

GUPTA, V. A.; BEGGS, A. H. Kelch proteins: Emerging roles in skeletal muscle development and diseases. **Skeletal Muscle**, v. 4, n. 1, p. 1–12, 2014.

HALES, C. N.; BARKER, D. J. P. The thrifty phenotype hypothesis. **British Medical Bulletin**, v. 60, p. 5–20, 2001.

HOWLETT, K. F.; MCGEE, S. L. Epigenetic regulation of skeletal muscle metabolism. **Clinical Science**, v. 130, n. 13, p. 1051–1063, 2016.

HUNTLEY, R. P. et al. The GOA database: Gene Ontology annotation updates for 2015. **Nucleic Acids Research**, v. 43, n. D1, p. D1057–D1063, 2015.

JANSSEN, E. et al. Adenylate kinase 1 deficiency induces molecular and structural adaptations to support muscle energy metabolism. **Journal of Biological Chemistry**, v. 278, n. 15, p. 12937–12945, 2003.

JARMUSZKIEWICZ, W.; SZEWCZYK, A. Energy-dissipating hub in muscle mitochondria: Potassium channels and uncoupling proteins. **Archives of Biochemistry and Biophysics**, v. 664, n. August 2018, p. 102–109, 2019. Disponível em: <https://doi.org/10.1016/j.abb.2019.01.036>.

JENNINGS, T. D. et al. The influence of maternal nutrition on expression of genes responsible for adipogenesis and myogenesis in the bovine fetus. **Animal**, v. 10, n. 10, p. 1697–1705, 2016.

KAGEYAMA, H. et al. Lipoprotein lipase mRNA in white adipose tissue but not in skeletal muscle is increased by pioglitazone through PPAR-γ. **Biochemical and Biophysical Research Communications**, v. 305, n. 1, p. 22–27, 2003.

KARCZEWSKA-KUPCZEWSKA, M. et al. Wnt signaling genes in adipose tissue and skeletal muscle of humans with different degrees of insulin sensitivity. **Journal of Clinical**

Endocrinology and Metabolism, v. 101, n. 8, p. 3079–3087, 2016.

KHANAL, P.; NIELSEN, M. O. Impacts of prenatal nutrition on animal production and performance: A focus on growth and metabolic and endocrine function in sheep. **Journal of Animal Science and Biotechnology**, v. 8, n. 1, p. 1–14, 2017.

KURODA, K. et al. Canonical Wnt signaling induces BMP-4 to specify slow myofibrogenesis of fetal myoblasts. **Skeletal Muscle**, v. 3, n. 1, p. 5, 2013.

LA PAGLIA, L. et al. Potential Role of ANGPTL4 in the Cross Talk between Metabolism and Cancer through PPAR Signaling Pathway. **PPAR Research**, v. 2017, 2017.

LANGFELDER, P.; HORVATH, S. WGCNA: An R package for weighted correlation network analysis. **BMC Bioinformatics**, v. 9, 2008.

LAPLANTE, M. et al. PPAR-gamma Activation Mediates Adipose Depot - Specific Effects on Gene Expression and Lipoprotein Lipase Activity. **Diabetes**, v. 52, p. 291–299, 2003. Disponível em: <http://diabetes.diabetesjournals.org/content/52/2/291.short>.

LARSON, D. M. et al. Winter grazing system and supplementation during late gestation influence performance of beef cows and steer progeny. **Journal of Animal Science**, v. 87, n. 3, p. 1147–1155, 2009.

LASKOWSKI, M. et al. What do we not know about mitochondrial potassium channels? **Biochimica et Biophysica Acta - Bioenergetics**, v. 1857, n. 8, p. 1247–1257, 2016.

LEE, C.-F. et al. Oxidative Stress–Induced Depolymerization of Microtubules and Alteration of Mitochondrial Mass in Human Cells. In: Annals of New York Academy of Science, **Anais**...2005.

LEFTEROVA, M. I. et al. PPARγ and the global map of adipogenesis and beyond. **Trends in Endocrinology and Metabolism**, v. 25, n. 6, p. 293–302, 2014.

LEONARDINI, A. et al. Cross-Talk between PPARγ and insulin signaling and modulation of Insulin Sensitivity. **PPAR Research**, v. 2009, 2009.

LIANG, H.; WARD, W. F. PGC-1α: a key regulator of energy metabolism. **Advances in Physiology Education**, v. 30, p. 145–151, 2006.

LILLYCROP, K. A. et al. Induction of altered epigenetic regulation of the hepatic glucocorticoid receptor in the offspring of rats fed a protein-restricted diet during pregnancy suggests that reduced DNA methyltransferase-1 expression is involved in impaired DNA methylation and . **British Journal of Nutrition**, v. 97, n. 6, p. 1064–1073, 2007.

LIU, Y. et al. The role of ppar-δ in metabolism, inflammation, and cancer: Many characters of a critical transcription factor. **International Journal of Molecular Sciences**, v. 19, n. 11, 2018.

LONG, Y. C. et al. Insulin Receptor Substrates Irs1 and Irs2 Coordinate Skeletal Muscle Growth and Metabolism via the Akt and AMPK Pathways. **Molecular and Cellular Biology**, v. 31, n. 3, p. 430–441, 2011.

MALLINSON, J. E. et al. Fetal exposure to a maternal low-protein diet during mid-gestation results in muscle-specific effects on fibre type composition in young rats. v. 44, p. 292–299, 2007.

MEGENEY, L. A. et al. MyoD is required for myogenic stem cell function in adult skeletal muscle. **Genes and Development**, v. 10, n. 10, p. 1173–1183, 1996.

MI, H. et al. PANTHER version 11: Expanded annotation data from Gene Ontology and Reactome pathways, and data analysis tool enhancements. **Nucleic Acids Research**, v. 45, n. D1, p. D183–D189, 2017.

MURIEL, J. M. et al. Keratin 18 is an integral part of the intermediate filament network in murine skeletal muscle. **American Journal of Physiology - Cell Physiology**, v. 318, n. 1, p. C215–C224, 2020.

NATHANIELSZ, P. W.; POSTON, L.; TAYLOR, P. D. In Utero Exposure to Maternal Obesity and Diabetes: Animal Models That Identify and Characterize Implications for Future Health. **Obstetrics and Gynecology Clinics of North America**, v. 34, n. 2, p. 201–212, 2007.

OPSOMER, G. et al. Epidemiological evidence for metabolic programming in dairy cattle. **Reproduction, Fertility and Development**, v. 29, n. 1, p. 52–57, 2017.

PARADIS, F. et al. Maternal nutrient restriction in mid-to-late gestation influences fetal mRNA expression in muscle tissues in beef cattle. **BMC Genomics**, v. 18, n. 1, p. 1–14, 2017.

PARK, H. K. et al. Changes of mitochondrial DNA content in the male offspring of protein-malnourished rats. **Annals of the New York Academy of Sciences**, v. 1011, p. 205–216, 2004.

PEÑAGARICANO, F. et al. Maternal nutrition induces gene expression changes in fetal muscle and adipose tissues in sheep. **BMC Genomics**, v. 15, n. 1, p. 1–13, 2014.

PESSIN, J. E.; SALTIEL, A. R. Signaling pathways in insulin action : molecular targets of insulin resistance Find the latest version : Signaling pathways in insulin action : molecular targets. **Journal of Clinical Investigation**, v. 106, n. 2, p. 165–169, 2000.

PIRKMAJER, S.; CHIBALIN, A. V. Na,K-ATPase regulation in skeletal muscle. **American**

Journal of Physiology - Endocrinology and Metabolism, v. 311, n. 1, p. E1–E31, 2016.

QAID, M. M.; ABDELRAHMAN, M. M. Role of insulin and other related hormones in energy metabolism - A review. **Cogent Food & Agriculture**, v. 2, n. 1, p. 1–18, 2016. Disponível em: <http://dx.doi.org/10.1080/23311932.2016.1267691>.

R CORE TEAM. **R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.** Disponível em: <https://www.r-project.org/>. Acesso em: 28 maio. 2020.

REHFELDT, C.; FIEDLER, I.; STICKLAND, N. C. Number and Size of Muscle Fibres in Relation to Meat Production. In: TE PAS, M. F. W.; EVERTS, M. E.; HAAGSMAN, H. P. (Ed.). **Muscle development of livestock animals: physiology, genetics, and meat quality**. Wallingford: CAB International, 2004. p. 267–296.

ROBCIUC, M. R. et al. Serum angiopoietin-like 4 protein levels and expression in adipose tissue are inversely correlated with obesity in monozygotic twins. **Journal of Lipid Research**, v. 52, n. 8, p. 1575–1582, 2011.

ROBINSON, J. et al. Studies on reproduction in prolific ewes: I. Growth of the products of conceptionitle. **The Journal of Agricultural Science**, v. 88, n. 3, p. 539–552, 1977.

ROBINSON, M. D.; OSHLACK, A. A scaling normalization method for differential expression analysis of RNA-seq data. **Genome Biology**, v. 11, p. R25, 2010.

RODRIGUES, L. M. et al. Effects of protein supplementation on Nellore cows ' reproductive performance , growth , myogenesis , lipogenesis and intestine development of the progeny. **Animal Production Science**, 2020.

ROSEN, E. D.; SPIEGELMAN, B. M. PPARγ: A Nuclear Regulator of Metabolism, Differentiation, and Cell Growth. **Journal of Biological Chemistry**, v. 276, n. 41, p. 37731–37734, 2001. Disponível em: <http://dx.doi.org/10.1074/jbc.R100034200>.

ROUSSEL, D.; BOËL, M.; ROMESTAING, C. Fasting enhances mitochondrial efficiency in duckling skeletal muscle by acting on the substrate oxidation system. **Journal of Experimental Biology**, v. 221, n. 4, 2018.

SAFRAN, M. et al. GeneCards Version 3: the human gene integrator. **Database : the journal of biological databases and curation**, v. 2010, p. 1–16, 2010.

SANGLARD, L. P. et al. Impact of energy restriction during late gestation on the muscle and blood transcriptome of beef calves after preconditioning. **BMC Genomics**, v. 19, n. 1, p. 1–18,

2018.

SCHWARZ-ROMOND, T. et al. The ankyrin repeat protein diversin recruits casein kinase Iε to the β-catenin degradation complex and acts in both canonical Wnt and Wnt/JNK signaling. **Genes and Development**, v. 16, n. 16, p. 2073–2084, 2002.

STAIGER, H. et al. Muscle-derived angiopoietin-like protein 4 is induced by fatty acids via peroxisome proliferator-activated receptor (PPAR)-δ and is of metabolic relevance in humans. **Diabetes**, v. 58, n. 3, p. 579–589, 2009.

SYMONDS, M. E. et al. Timing of nutrient restriction and programming of fetal adipose tissue development. **Proceedings of the Nutrition Society**, v. 63, n. 3, p. 397–403, 2004.

TU, A. X. M. et al. Generalized Covariance-Adjusted Discriminants : Perspective and Application. **International Biometric Society**, v. 53, n. 3, p. 900–909, 1997. Disponível em: <https://www.jstor.org/stable/2533551>.

UNDERWOOD, K. R. et al. Nutrition during mid to late gestation affects growth , adipose tissue deposition , and tenderness in cross-bred beef steers. v. 86, p. 588–593, 2010.

VON MALTZAHN, J. et al. Wnt Signaling in Myogenesis. **Trends in Cell Biology**, v. 22, n. 11, p. 602–609, 2012. Disponível em: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3624763/pdf/nihms412728.pdf>.

VONNAHME, K. A. Nutrition During Gestation and Fetal Programming. In: The Range Beef Cow Symposium XX, Fort Collins, Colorado. **Anais**... Fort Collins, Colorado: 2007.

WANG, C. et al. Ascl2 inhibits myogenesis by antagonizing the transcriptional activity of myogenic regulatory factors. **Stem Cells and Regeneration**, v. 144, p. 235–247, 2017.

WANG, Y. X. PPARs: Diverse regulators in energy metabolism and metabolic diseases. **Cell Research**, v. 20, n. 2, p. 124–137, 2010.

WENG, Y. R.; CUI, Y.; FANG, J. Y. Biological functions of cytokeratin 18 in cancer. **Molecular Cancer Research**, v. 10, n. 4, p. 485–493, 2012.

WU, G. et al. Maternal Nutrition and Fetal Development. **The Journal of Nutrition**, v. 134, n. 9, p. 2169–2172, 2004.

YAN, X. et al. Developmental Programming of Fetal Skeletal Muscle and Adipose Tissue Development. **Journal of Genomics**, v. 1, p. 29–38, 2013.

YIN, H.; PRICE, F.; RUDNICKI, M. A. Satellite cells and the muscle stem cell niche. **Physiological Reviews**, n. 329, p. 23–67, 2013.

ZAMANI, N.; BROWN, C. W. Emerging roles for the transforming growth factor-β superfamily in regulating adiposity and energy expenditure. **Endocrine Reviews**, v. 32, n. 3, p. 387–403, 2011.

ZHU, M. J. et al. Maternal nutrient restriction affects properties of skeletal muscle in offspring. **Journal of Physiology**, v. 575, n. 1, p. 241–250, 2006.

ZHU, P. et al. Angiopoietin-like 4: A decade of research. **Bioscience Reports**, v. 32, n. 3, p. 211–219, 2012.

SECOND CHAPTER – ARTICLE

Transcriptome profile in the skeletal muscle of progeny from dams protein-supplemented during mid-gestation

Article formatted according to the Animal Genetics journal

Summary

This study aimed to evaluate the differentially expressed genes (DEG), weighed genes co-expression networks (WGCNA), and their biological functions in skeletal muscle of offspring from dams supplemented or not with protein during mid-gestation. From 100 to 200 days of gestation, 10 Tabapuã cows pregnant of males were assigned to one of two treatments: Control [(**CON**) - supply of basal diet achieving 5.5% of crude protein (CP); $n = 6$]; or Supplemented [(**SUP**) - basal diet plus a supplement with 40% of CP provided at the level of 3.5 g/kg of body weight; $n = 4$]. Muscle samples were collected from the progeny at 260d of postnatal age to RNA-seq, WGCNA, and Gene Ontology (GO) enrichment analysis. A total of 310 DEG (q-value < 0.05) was identified for the effect of diet, being 187 and 123 genes down-regulated and up-regulated in the SUP diet, respectively. Within the SUP up-regulated genes, were genes related to insulin receptor signaling pathway (*LOC107131843*); apoptosis regulation (*KRT18*, *KRT8 and KRT19*); negative regulation of lipoprotein lipase activity (*ANGPTL4*) and potassium ion transmembrane transport (*KCNH3*). The *ENSBTAG00000032057* gene, involved in the ATP synthesis coupled proton transport, was down-regulated in SUP. In WGCNA, within hub genes identified in the module negatively associated with diet were *ANGPTL4* and *ANKRD6* (associated with Wnt signaling pathway); whereas in the modules positively correlated was *KLHL10* (homeostasis of number of cells within a tissue). The GO analysis showed that DEG were mainly enriched in potassium ion transmembrane transport, microtubule polymerization or depolymerization and positive regulation of cellular component biogenesis.

Keywords Fetal programming, gene expression, lipogenesis, maternal nutrition, muscle metabolism.

Introduction

Maternal nutrition during different stages of gestation cause significant changes in the structure, physiology, and metabolism of the offspring (Wu *et al.* 2004; Peñagaricano *et al.* 2014). These changes have essential implications on animal production, especially impacting muscle and adipose tissue development (Peñagaricano *et al.* 2014). The nutrients requirements of beef cows increases throughout gestation (Larson *et al.* 2009). When this demands are not met, alterations in the metabolism of the offspring may occur and impact the performance of the adult animal.

Mid-gestation is a critical period for skeletal muscle and adipose tissue development (Symonds *et al.* 2004; Ford *et al.* 2007). During such period, a pool of undifferentiated mesenchymal stem cells is present, and either myocytes or adipocytes can differentiate from this cells (Du *et al.* 2010). Different nutritional status of the dam during mid-gestation affects fetal development, leading to long-term impacts on the production efficiency and meat quality via epigenetic effects and differential expression of myogenic and adipogenic genes (Lillycrop *et al.* 2007; Gicquel *et al.* 2008).

Previous studies have demonstrated that the differential prenatal nutritional status can impact fetal development without noticeable phenotypic differences (Paradis *et al.* 2017). In this sense, gene expression analysis becomes important, as it identifies genes relevant to biological processes and provides insights into the complex regulatory networks in which they are involved (AN *et al*., 2016).

There are several studies on fetal programming in the literature evaluating the gene expression of targeted genes in offspring's muscle (Jennings *et al.* 2016; Gionbelli *et al.* 2017; Paradis *et al.* 2017; Rodrigues *et al.* 2020; Costa *et al.* 2021), however, a few of them (Peñagaricano *et al.* 2014; Sanglard *et al.* 2018) have used the whole-transcriptome profiling to

investigate the underlying mechanisms for the changes observed in these animals. We hypothesized that maternal protein supplementation during mid-gestation would cause changes in the expression of genes related to muscle development in beef calves. Therefore, this study aimed to evaluate the effects of protein supplementation at gestation on the skeletal muscle transcriptome of the offspring.

Materials and methods

Animals and diets

Ten pregnant Tabapuã cows and their male calves were used in this study. Cows had an average body weight (BW) of 502 ± 83 kg and were 5.78 ± 3.80 years old. In the first third of gestation (0 to 100 days) all cows were kept in *Brachiaria decumbens* cv. Marandu pasture, and at day 100 of gestation, were randomly assigned to individual pens until calving.

During mid-gestation, from 100 to 200 days of gestation, the cows were assigned to one of two treatments, with 6 and 4 experimental units from the Control (CON) and Supplemented (SUP) groups, respectively. The CON group received a basal diet (corn silage and sugarcane bagasse) achieving 5.5% crude protein (CP), plus a mineral mixture enough to achieve mineral requirements, whereas the SUP group received a basal diet plus protein supplementation (40% CP offered at the level of 3.5 g/kg BW).

After 200 days of gestation, all animals received a diet containing only corn silage (7% crude protein) and mineral supplement up to parturition. After calving, cow–calf pairs were transferred to a *Brachiaria decumbens* cv. Marandu pasture with creep-feeding supplementation for calves (offered at 3.5 g/kg of BW).

Calves were weaned at 200 days of age and kept in a *Brachiaria* pasture receiving a concentrate supplement to provide a gain of 300 g /day until reaching 240 days of age when they were then transferred to the feedlot. Animals were housed in individual pens and fed the same diet.

The chemical composition of the supplement and basal diet at each phase of gestation are presented in Table 1.

Maternal and progeny performance

Maternal average daily gain (kg/d) and average maternal tissues daily gain (kg/d) were calculated based on the differences of weights at different time points divided by the number of days on evaluation. The fractioning of maternal tissues gain was estimated according Gionbelli *et al*. (2015) considering the gestational compounds and maternal tissues as different units. The progeny average daily gain was calculated considering the difference of weight at the weaning and birth weight divided by the postnatal age. These data were statistical analyzed trough the GLM procedure of SAS 9.4 (Statistical Analysis System Institute, Inc., Cary, NC, USA) considering the maternal nutritional treatment (main effect) and maternal initial body weight (covariate) as the fixed effects. Differences were declared at $P < 0.05$.

Skeletal muscle tissue sampling

Twenty days after the feedlot beginning (at 260d of progeny postnatal age), a biopsy of the skeletal muscle *Longissimus thoracis* was performed in each steer using a Bergstrom biopsy needle (Eskilds Tuna, Sweden) between the 12th and 13th rib for total RNA extracion. Skeletal muscle samples were placed in a 2-mL Cryovial tube containing RNAlater (Ambion Inc., Austin, TX, USA) and stored in a −20°C freezer for subsequent analysis.

Total RNA extraction, sequencing, and bioinformatics

Total RNA was extracted using Trizol® (InvitrogenTM, Thermo Fisher Scientific®, Oregon, USA) following the manufacturer's recommendations. The RNA samples were sent to the ESALQ Genomics Center (Piracicaba, São Paulo, Brazil) for library construction and RNA-sequencing. The RNA quantity and quality were determined by Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). Sequencing was performed on an Illumina HiSeq2500 instrument (Illumina, Inc., San Diego, CA, USA), generating 100 bp paired-reads in a unique flow cell.

The quality of raw reads was evaluated with FastQC (Andrews 2010). Sequence reads for each sample were mapped to *Bos taurus* ARS-*UCD1.2* reference genome, and the number of counts was obtained using STAR (Dobin *et al.* 2013). A total of 232 696 053 paired-ends reads were generated, with an average of 23 269 605 reads/sample. Reads were mapped to a total of 27 607 genes (85.39% of the 32 350 genes annotated in the *Bos taurus* reference genome ARS-*UCD1.2*). Genes with less than two counts per sample and more than six samples with zero counts were eliminated to avoid low counts across multiple samples, resulting in a final set of 15 394 genes. The Trimmed Mean of M-values (TMM) method was used to calculate normalizing factors in R software (R Core Team, 2019) using the *TMM* package (Robinson & Oshlack 2010).

Statistical Analyses for the differentially expressed genes (DEG) identification

The gene expression data were analyzed with a negative binomial model using a log-link function (Di *et al.* 2011) to identify differentially expressed genes (DEG) between maternal dietary treatments, according to the following model:

$$Y_{ijk} = \mu + T_i + R_j + P_k + L_j$$

where, Y_{ijk} is the raw number of read counts of the gene being analyzed; μ is the intercept, T_i is the fixed-effect of the i^{th} Maternal Dietary Treatment; R_j is the covariate of RIN score of the j^{th} individual; P_k is the cow parity level (primiparous or multiparous), and L_j is the TMM-normalized library size, used as an offset. Analyses were performed in SAS 9.4 with the GLIMMIX procedure.

False-discovery rate was used to adjust the *P*-values (*q*-values) of model terms due to multiple testing. Significant DEG were identified at *q*-value ≤ 0.05. *q*-values were obtained in R, using the function *p.adjust* of the *stats* package. Prior to subsequent multivariate and functional analyses, the gene expression data were pre-adjusted for the fixed effect of RIN score to allow for proper comparison between maternal dietary treatments (Tu *et al.* 1997). In other words, the fitted gene expression data was adjusted to the average RIN score.

Functional Annotation Analysis

The enrichment of Gene Ontology (GO) terms associated with DEG was analyzed using PANTHER Enrichment Analysis (Mi *et al.* 2017). A DEG list were created based on the significance (*q*-value < 0.05) of diet effect in the model. The final set of genes identified in the samples (15 394 genes) was used as the background list. Biological Processes was considered significant at *P*-value < 0.05.

Weighed Gene Co-Expression Network Analysis (WGCNA)

Gene co-expression networks were constructed to elucidate the complex genetic interactions as a result of maternal nutrition. Data of all genes were used in this analysis. RNA network construction and module detection were done using the one-step network construction with a soft-threshold power of $\beta = 9$ ($R^2 > 0.73$), which represents the smallest value of power such that approximate scale free topology is reached. The topological overlap distance calculated from the adjacency matrix was then clustered with the average linkage hierarchical clustering. The default minimum cluster merge height of 0.25 was retained. The modules eigengenes (MEs), which are the major component in the principal component analysis and are considered the expression pattern within a given module, were correlated with treatments to identify the relevant modules. Analyses were performed using the *WGCNA* package (Langfelder & Horvath 2008) in R. The three significant correlated gene modules (*P*-value ≤ 0.05) were selected as modules of interest. The three hub genes with greater association within their respective modules (i.e., higher Module Membership) were selected for subsequent functional analysis. Finally, to clarify the mechanism underlying the impact of modules, genes of the module with the higher correlation with the diets were uploaded PANTHER for enrichment of Gene Ontology (GO) enrichment analysis, using the final set of genes identified in the samples (15 394 genes) as the background list.

Results

Supplemented cows presented average daily gain of 0.308 kg/day from 100 days of gestation up to parturition, while CON cows lost -0.010 kg/day ($P \leq 0.01$). When only maternal tissues average daily gain was considered, SUP cows lost in average 0.008 kg/day while CON cows lost

–0.232 kg/day ($P \leq 0.01$). The progeny from SUP cows gained more weight per day until weaning compared to CON progeny (0.875 vs 0.818 kg/day, respectively, $P = 0.05$). These data indicates that the treatment application was effective to simulate different maternal nutritional conditions during gestation impacting long-term postnatal progeny growth.

DEG identification

A total of 310 DEG (q-value < 0.05) were identified in the muscle samples for the effect of diet. There were 187 and 123 genes down-regulated and up-regulated in the SUP diet, respectively. The volcano plot for the effect of diet is shown in Fig. 1.

Major DEG

The 10 most significant down- and up-regulated DEG in SUP are summarized in Table 2. The most significant down- and up-regulated genes in the SUP were *CEACAM19* and ENSBTAG00000055143, with $\log_2$ fold changes ($\log_2$FC) [95% confidence interval] of -2.70 [-3.92, -1.48] (q-value < 0.001) and 5.45 [2.84, 8.05] (q-value < 0.001), respectively. The genes with most extremes $\log_2$FC were *AK9*, with -3.02 [-5.20, -0.84] (q-value = 0.004) and the same unassigned gene (ENSBTAG00000055143) in the previous sentence.

From the 10 most down-regulated genes in SUP, we identified genes involved in nucleobase-containing compound metabolic process (*AK9*) and regulation of transcription by RNA polymerase II (*SIM2*). In contrast, among the up-regulated genes were found genes related to insulin receptor signaling pathway (*LOC107131843*); negative regulation of apoptotic process (*ANGPTL4* and *KRT18*); extrinsic apoptotic signaling pathway (*KRT8*)*;* negative regulation of

lipoprotein lipase activity (*ANGPTL4*); sarcomere organization (*KRT19*) and potassium ion transmembrane transport (*KCNH3*).

DEGs Enrichment Analysis

The results of the Gene Ontology (GO) terms enrichment analysis for diet are presented in Table 3. Results showed genes involved in multiple general complex biological processes, such as Microtubule polymerization or depolymerization (GO:0031109, GO:0046785); transmembrane potassium ions transport (GO:0071805, GO:0006813); positive regulation of cellular component biogenesis (GO:0044089); positive regulation of developmental process (GO:0051094); positive regulation of multicellular organismal process (GO:0051240); positive regulation of cellular component organization (GO:0051130); ribose phosphate biosynthetic process (GO:0046390) and cellular response to DNA damage stimulus and DNA repair (GO:0006974, GO:0006281).

WGCNA

A total of 27 modules were identified (Fig. 2). Two modules (*Salmon* and *Cyan*; *P*-value ≤ 0.056) were positively associated with the effect of the SUP maternal treatment, while one module (*Orange*; *P*-value = 0.002) was negatively associated with it. In other words, the expression of the genes in the *Salmon* and *Cyan* modules, and in the *Orange* module, were greater and lower, respectively, in the SUP compared to the CONT group. Therefore, the genes in the *Salmon* and *Cyan* modules have positive Gene Significance (GS) values, whereas those in the *Orange* module have negative GS values.

The functional analysis of the 9 selected genes with the effect of diet in the WGCNA analysis are presented in Table 4. The hub genes of the key module negatively associated with diet

(*Orange* module; GS < 0) were *ANGPTL4, KCNH3* and *ASCL2.* The hub genes of the key modules positively correlated with diet (*Salmon* and *Cyan* modules; GS > 0) were *B3GALT6, PERP, ANKRD6, KCNN3, ABTB2* and *KLHL10.*

Within these 9 hub genes there were genes involved in negative regulation of apoptotic process and negative regulation of lipoprotein lipase activity (*ANGPTL4*); negative regulation of canonical Wnt signaling pathway and positive regulation of JNK cascade (*ANKRD6*); cellular response to toxic substance (*ABTB2*); homeostasis of number of cells within a tissue (*KLHL10*); potassium ion transmembrane transport (*KCNN3* and *KCNH3*); desmosome organization (*PERP*); positive regulation of transcription by RNA polymerase II and cell development (*ASCL2*) and protein glycosylation (*B3GALT6*).

Functional Enrichment Analysis of the Gene Module of Interest

The biological functions of the module (*Orange* module) with higher correlation with diet are presented in Table 5. This analysis resulted in overrepresented biological process related to monovalent inorganic cation transport (GO:0015672); positive regulation of transcription by RNA polymerase II (GO:0045944); cellular process (GO:0009987); metabolic process (GO:0008152) and macromolecule metabolic process (GO:0043170).

Discussion

Major DEG

From the 310 DEG identified in this study, we further investigated the functions of the 10 genes showing largest log_2FC within each treatment. Many of these genes are involved in major complex biological processes, such as insulin signaling pathway, lipid metabolism, and apoptosis.

Insulin is an essential hormone for maintaining glucose homeostasis and cell growth and differentiation. It increases peripheral glucose uptake, especially in muscle and adipose tissues (Pessin & Saltiel 2000; Qaid & Abdelrahman 2016). During periods of nutrient abundance, insulin-like signaling stimulates the Akt3-mTOR pathway in skeletal muscle, which increases amino acid uptake and promotes protein synthesis (Glass 2003; Long *et al.* 2011), leading to muscle hypertrophy. In addition to these effects in the muscle tissue, insulin also stimulates lipogenesis in the adipocytes and reduces lipolysis. Aragão *et al.*, 2014 showed that rats born from protein restricted dams presented decreased plasma concentrations of triacylglycerols (TAG), indicating that early protein restriction induces a long-lasting metabolic state favoring lipid catabolism and insulin function. Of these 20 DEG, one gene has been shown to participate in the insulin pathway. Animals in the SUP group had overexpression of the insulin receptor substrate 1-like gene (*LOC107131843*) compared to animals in the CON group. Hence, greater expression of this gene may indicate that animals in the SUP group may have greater glucose uptake, in addition to greater protein synthesis and muscle cells hypertrophy than those in the CON group.

Apoptosis is responsible for protein turnover in the muscle, which may lead to muscle hypertrophy. Three of the major DEG play roles in apoptosis. Keratin, type II cytoskeletal 8 (*KRT8)*; keratin, type I cytoskeletal 18 (*KRT18)* and keratin, type I cytoskeletal 19 *(KRT19),* that encode the proteins (CK8), cytokeratin 18 (CK18) and cytokeratin 19 (CK19), respectively, are co-expressed and serve as structural molecules that maintain cytoplasmic structure and resist external stresses (Dong *et al.* 2015). CK18 is an integral component of the intermediate filament network of skeletal muscle that contributes significantly to the network's function in force transmission and resisting injury (Muriel *et al.* 2020) and is also important for cellular processes such as apoptosis, mitosis, cell cycle progression, and cell signaling (Weng *et al.* 2012). These

genes are involved in the negative regulation of apoptosis and were up-regulated in the SUP group compared to the CON group. Therefore, the greater expression of these genes in SUP animals indicates that animals born from cows receiving protein supplementation may have lower apoptosis activity than those born from cows not receiving this supplement. Hence, it is expected that SUP animals have greater hypertrophy and muscle development than CON animals.

Angiopoietin like 4 (*ANGPTL4*) was up-regulated in SUP group. It regulates lipid metabolism by inhibiting lipoprotein lipase (LPL) activity and increasing adipose tissue hormone-sensitive lipase (HSL, also known as LIPE) expression, resulting in lipolysis of white adipose tissue (WAT) and increased levels of plasma triacylglycerols (TAG) and fatty acids. (Robciuc *et al.* 2011; Zhu *et al.* 2012; La Paglia *et al.* 2017). *ANGPTL4* is produced in response to long chain fatty acids via Peroxisome proliferator-activated receptor-delta (PPAR–δ). It is conceivable that promotion of white adipose tissue lipolysis via PPAR–δ–mediated *ANGPTL4* production represents a mechanism that prevents too strong decrements of plasma non-esterified fatty acid (NEFA) levels and, in this way, ensures ongoing fuel supply of skeletal muscle. Such mechanism would favor the efficient use of stored lipids, as opposed to glucose, during periods of increased energy demand (Staiger *et al.* 2009).

Compared to those in the SUP group, animals in the CON group had greater expression of *ENSBTAG00000032057*, a gene that encodes a protein involved in the ATP synthesis coupled proton transport. This protein is a mitochondrial membrane ATP synthase that produces ATP from ADP in the presence of a proton gradient across the membrane generated by electron transport complexes of the respiratory chain (Bateman 2019). A higher mitochondrial coupling efficiency triggers additional energy saving by channeling the energy of oxidized substrates to ATP synthesis and cellular energy-demanding processes. During food deprivation, animals must develop

physiological responses to maximize energy conservation and survival. At the subcellular level, energy conservation is mainly achieved by a reduction in mitochondrial activity and an upregulation of oxidative phosphorylation efficiency (Roussel *et al.* 2018). The thrifty phenotype hypothesis says that metabolic adaptations by the undernourished fetus may lead to adverse consequences later in life under conditions of both adequate nutrition and overnutrition (Park *et al.* 2004). Our results suggest that animals born from cows without supplementation (i.e., from the CON group) during gestation may have efficient mitochondrial metabolism, as an adaptative response to a nutrient restricted environment.

DEGs Enrichment Analysis

In the enrichment of Gene Ontology (GO) terms associated with DEGs were found biological process related with ions transport, such as potassium ion transmembrane transport (GO:0071805). Na, K-ATPase (NKA) is a transmembrane pump that transports three Na from the cell and two K into the cell against their concentration gradients and is intimately linked with energy metabolism in skeletal muscle (Pirkmajer & Chibalin 2016). Besides that, in muscle mitochondria, the potassium channels (mitoK) and uncoupling proteins (UCPs) dissipate the energy formed by the electron transport chain. These two types of energy-dissipating systems can potentially influence the efficiency of oxidative phosphorylation and therefore the cellular energy homeostasis of the tissue (Jarmuszkiewicz & Szewczyk 2019). However, under oxidative stress conditions, the enhanced level of reactive oxygen species (ROS) inhibits ATP synthesis, activating mitoK channels and UCPs, which produce mild uncoupling, resulting in the attenuation of further mitochondrial ROS formation. This feedback-induced decrease in mitochondrial ROS synthesis protects mitochondria against harmful ROS levels (Laskowski *et al.* 2016). Therefore, despite

being a form of energy dissipation, mitoK channels activation is also important for cellular protection against oxidative stress.

Another overrepresented GO terms were the microtubule polymerization or depolymerization (GO:0031109, GO:0046785). Microtubules are the major components of cytoskeletal systems that are responsible for the regulation of the mitochondrial distribution in the cell. It has been proposed that ROS cause microtubules depolymerization, which in turn induces mitochondrial biogenesis (Lee *et al.* 2005). Besides that, positive regulation of cellular component biogenesis (GO:0044089) also was a biological process overrepresented, reinforcing the hypothesis mitochondria proliferation. These results indicate that cow's supplementation in mid-gestation may imply adaptation mechanisms in muscle energy metabolism at the mitochondrial level. Which also was demonstrated in DEG analysis, with the differential expression of *ENSBTAG00000032057* gene, involved in the mitochondrial ATP synthesis.

WGCNA

From the genes present in the modules identified in the analysis of weighted gene co-expression network analysis, we selected the 9 hub genes most associated with the modules to investigate the biological functions.

Kelch family members are involved in several cellular and molecular processes such as cell migration, cytoskeletal arrangement, regulation of cell morphology, protein degradation, gene expression and, in skeletal muscle, many Kelch proteins are known to regulate the proliferation and differentiation of muscle cells (Gupta & Beggs 2014). The Kelch like family member 10 *(KLHL10)* was found in the module positively related to diet. It is associated with the biological process of homeostasis of number of cells within a tissue, which includes regulation of apoptosis,

cell proliferation and cell differentiation involved in tissue homeostasis as direct descendants GO terms (Huntley *et al.* 2015).

The Ankyrin repeat domain 6 gene *(ANKRD6)* was found in the module positively related to diet. It was initially named Diversin (Schwarz-Romond *et al.* 2002) and encodes a protein which is involved in the Wnt signaling pathway. The *ANKRD6* gene inhibits the canonical Wnt signaling pathway and promotes the planar-cell-polarity (PCP) pathway, also called Wnt/Jun N-terminal kinase (JNK) pathway (Schwarz-Romond *et al.* 2002; Bateman 2019). Wnt signaling plays an essential role during embryonic muscle development and in the maintenance of skeletal muscle homeostasis in the adult. In adult skeletal muscle, canonical Wnt signaling regulates the differentiation of muscle stem cells (satellite cells) (Brack *et al.* 2008; von Maltzahn *et al.* 2012), whereas non canonical signals mediate the self-renewal of satellite stem cells and the growth of muscle fibers (Brack *et al.* 2008; von Maltzahn *et al.* 2012). Furthermore, canonical Wnt signaling promotes slow myofiber phenotype in the postnatal skeletal muscle (Kuroda *et al.* 2013) and its inhibition induces the commitment of mesenchymal stem cells into preadipocyte differentiation (Karczewska-Kupczewska *et al.* 2016).

Additionally, the Achaete-scute homolog 2 gene *(ASCL2)* gene, found in the module negatively related to diet, is a member of the basic helix-loop-helix (BHLH) family of transcription factors (Safran *et al.* 2010) that maintains somatic stem cell population within a tissue (Huntley *et al.* 2015). Wang *et al*. (2017) reported that *Ascl2* inhibits myogenic differentiation by targeting myogenic regulatory factors (MRFs), such as MyoD, which is known to be essential for satellite cells differentiation (Megeney *et al.* 1996; Yin *et al.* 2013). These findings support the hypothesis that progeny born from supplemented dams have more myogenesis, recruitment of satellite cells and adipocyte differentiation.

Conclusion

Maternal protein supplementation during mid-gestation increased the expression of genes related to insulin uptake and negative regulation of apoptosis, indicating greater protein synthesis and muscle cells hypertrophy. Animals born from cows without supplementation presented evidence of efficient mitochondrial metabolism, which may be an adaptative response to a nutrient restricted environment and agrees to enriched biological process, which were related to mitochondria biogenesis and metabolism. Furthermore, the analysis of co-expression genes network also showed possible changes in mechanisms involved in myogenesis, recruitment of satellite cells and adipocyte differentiation.

References

Andrews S. (2010) FastQC A Quality Control tool for High Throughput Sequence Data.

Aragão R.D.S., Guzmán-Quevedo O., Pérez-García G., Manhães-De-Castro R. & Bolaños-Jiménez F. (2014) Maternal protein restriction impairs the transcriptional metabolic flexibility of skeletal muscle in adult rat offspring. *British Journal of Nutrition* **112**, 328–337.

Bateman A. (2019) UniProt: A worldwide hub of protein knowledge. *Nucleic Acids Research* **47**, D506–D515.

Brack A.S., Conboy I.M., Conboy M.J., Shen J. & Rando T.A. (2008) A Temporal Switch from Notch to Wnt Signaling in Muscle Stem Cells Is Necessary for Normal Adult Myogenesis. *Cell Stem Cell* **2**, 50–59.

Costa T.C., Du M., Nascimento K.B., Galvão M.C., Meneses, J.A.M., Schultz E.B., Gionbelli M.P. & Duarte M.S. (2021) Skeletal Muscle Development in Postnatal Beef Cattle Resulting from Maternal Protein Restriction during Mid-Gestation. *Animals* **11**.

Di Y., Schafer D.W., Cumbie J.S. & Chang J.H. (2011) The NBP negative binomial model for assessing differential gene expression from RNA-Seq. *Statistical Applications in Genetics and Molecular Biology* **10**.

Dobin A., Davis C.A., Schlesinger F., Drenkow J., Zaleski C., Jha S., Batut P., Chaisson M. & Gingeras T.R. (2013) STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics* **29**, 15–21.

Dong Q.M., Ling C. & Zhao L. (2015) Immunofluorescence analysis of cytokeratin 8/18 staining is a sensitive assay for the detection of cell apoptosis. *Oncology Letters* **9**, 1227–1230.

Du M., Tong J., Zhao J., Underwood K.R., Zhu M., Ford S.P. & Nathanielsz P.W. (2010) Fetal programming of skeletal muscle development in ruminant animals. *Journal of animal science* **88**.

Ford S.P., Hess B.W., Schwope M.M., Nijland M.J., Gilbert J.S., Vonnahme K.A., Means W.J., Han H. & Nathanielsz P.W. (2007) Maternal undernutrition during early to mid-gestation in the ewe results in altered growth, adiposity, and glucose tolerance in male offspring. *Journal of Animal Science* **85**, 1285–1294.

Gicquel C., El-Osta A. & Le Bouc Y. (2008) Epigenetic regulation and fetal programming. *Best Practice and Research in Clinical Endocrinology and Metabolism* **22**, 1–16.

Gionbelli T.R.S., Veloso C.M., Rotta P.P. et al. (2017) Foetal development of skeletal muscle in bovines as a function of maternal nutrition, foetal sex and gestational age. *Journal of Animal Physiology and Animal Nutrition* **102**, 545–556.

Glass D.J. (2003) Signalling pathways that mediate skeletal muscle hypertrophy and atrophy. *Nature Cell Biology* **5**, 87–90.

Gupta V.A. & Beggs A.H. (2014) Kelch proteins: Emerging roles in skeletal muscle development and diseases. *Skeletal Muscle* **4**, 1–12.

Huntley R.P., Sawford T., Mutowo-Meullenet P., Shypitsyna A., Bonilla C., Martin M.J. & O'Donovan C. (2015) The GOA database: Gene Ontology annotation updates for 2015. *Nucleic Acids Research* **43**, D1057–D1063.

Jarmuszkiewicz W. & Szewczyk A. (2019) Energy-dissipating hub in muscle mitochondria: Potassium channels and uncoupling proteins. *Archives of Biochemistry and Biophysics* **664**, 102–109.

Jennings T.D., Gonda M.G., Underwood K.R., Wertz-Lutz A.E. & Blair A.D. (2016) The influence of maternal nutrition on expression of genes responsible for adipogenesis and myogenesis in the bovine fetus. *Animal* **10**, 1697–1705.

Karczewska-Kupczewska M., Stefanowicz M., Matulewicz N., Nikołajuk A. & Straczkowski M. (2016) Wnt signaling genes in adipose tissue and skeletal muscle of humans with different degrees of insulin sensitivity. *Journal of Clinical Endocrinology and Metabolism* **101**, 3079–3087.

Kuroda K., Kuang S., Taketo M.M. & Rudnicki M.A. (2013) Canonical Wnt signaling induces BMP-4 to specify slow myofibrogenesis of fetal myoblasts. *Skeletal Muscle* **3**, 5.

Langfelder P. & Horvath S. (2008) WGCNA: An R package for weighted correlation network analysis. *BMC Bioinformatics* **9**.

Larson D.M., Martin J.L., Adams D.C. & Funston R.N. (2009) Winter grazing system and supplementation during late gestation influence performance of beef cows and steer progeny. *Journal of Animal Science* **87**, 1147–1155.

Laskowski M., Augustynek B., Kulawiak B., Koprowski P., Bednarczyk P., Jarmuszkiewicz W.

& Szewczyk A. (2016) What do we not know about mitochondrial potassium channels? *Biochimica et Biophysica Acta - Bioenergetics* **1857**, 1247–1257.

Lee C.F., Liu C.Y., Hsieh R.H. & Wei Y.H. (2005) Oxidative Stress–Induced Depolymerization of Microtubules and Alteration of Mitochondrial Mass in Human Cells. In: Annals of New York Academy of Science. p. 246–254.

Lillycrop K.A., Slater-Jefferies J.L., Hanson M.A., Godfrey K.M., Jackson A.A. & Burdge G.C. (2007) Induction of altered epigenetic regulation of the hepatic glucocorticoid receptor in the offspring of rats fed a protein-restricted diet during pregnancy suggests that reduced DNA methyltransferase-1 expression is involved in impaired DNA methylation and . *British Journal of Nutrition* **97**, 1064–1073.

Long Y.C., Cheng Z., Copps K.D. & White M.F. (2011) Insulin Receptor Substrates Irs1 and Irs2 Coordinate Skeletal Muscle Growth and Metabolism via the Akt and AMPK Pathways. *Molecular and Cellular Biology* **31**, 430–441.

von Maltzahn J., Chang N.C., Bentzinger C.F. & Rudnicki M.A. (2012) Wnt Signaling in Myogenesis. *Trends in Cell Biology* **22**, 602–609.

Megeney L.A., Kablar B., Garrett K., Anderson J.E. & Rudnicki M.A. (1996) MyoD is required for myogenic stem cell function in adult skeletal muscle. *Genes and Development* **10**, 1173–1183.

Mi H., Huang X., Muruganujan A., Tang H., Mills C., Kang D. & Thomas P.D. (2017) PANTHER version 11: Expanded annotation data from Gene Ontology and Reactome pathways, and data analysis tool enhancements. *Nucleic Acids Research* **45**, D183–D189.

Muriel J.M., O'Neill A., Kerr J.P., Kleinhans-Welte E., Lovering R.M. & Bloch R.J. (2020) Keratin 18 is an integral part of the intermediate filament network in murine skeletal muscle.

American Journal of Physiology - Cell Physiology **318**, C215–C224.

La Paglia L., Listì A., Caruso S., Amodeo V., Passiglia F., Bazan V. & Fanale D. (2017) Potential Role of ANGPTL4 in the Cross Talk between Metabolism and Cancer through PPAR Signaling Pathway. *PPAR Research* **2017**.

Paradis F., Wood K.M., Swanson K.C., Miller S.P., McBride B.W. & Fitzsimmons C. (2017) Maternal nutrient restriction in mid-to-late gestation influences fetal mRNA expression in muscle tissues in beef cattle. *BMC Genomics* **18**, 1–14.

Park H.K., Jin C.J., Cho Y.M., Park D.J., Shin C.S., Park K.S., Kim S.Y., Cho B.Y. & Lee H.K. (2004) Changes of mitochondrial DNA content in the male offspring of protein-malnourished rats. *Annals of the New York Academy of Sciences* **1011**, 205–216.

Peñagaricano F., Wang X., Rosa G.J.M., Radunz A.E. & Khatib H. (2014) Maternal nutrition induces gene expression changes in fetal muscle and adipose tissues in sheep. *BMC Genomics* **15**, 1–13.

Pessin J.E. & Saltiel A.R. (2000) Signaling pathways in insulin action : molecular targets of insulin resistance Find the latest version : Signaling pathways in insulin action : molecular targets. *Journal of Clinical Investigation* **106**, 165–169.

Pirkmajer S. & Chibalin A. V. (2016) Na,K-ATPase regulation in skeletal muscle. *American Journal of Physiology - Endocrinology and Metabolism* **311**, E1–E31.

Qaid M.M. & Abdelrahman M.M. (2016) Role of insulin and other related hormones in energy metabolism - A review. *Cogent Food & Agriculture* **2**, 1–18.

R Core Team (2019) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Robciuc M.R., Naukkarinen J., Ortega-Alonso A. et al. (2011) Serum angiopoietin-like 4 protein

levels and expression in adipose tissue are inversely correlated with obesity in monozygotic twins. *Journal of Lipid Research* **52**, 1575–1582.

Robinson M.D. & Oshlack A. (2010) A scaling normalization method for differential expression analysis of RNA-seq data. *Genome Biology* **11**, R25.

Rodrigues L.M., Schoonmaker J.P.S., Resende F.D., Siqueira G.R., Machado Neto, O.R., Gionbelli M.P. & Gionbelli, T.R.S. Ladeira M.M. (2020) Effects of protein supplementation on Nellore cows ' reproductive performance , growth , myogenesis , lipogenesis and intestine development of the progeny. *Animal Production Science.*

Roussel D., Boël M. & Romestaing C. (2018) Fasting enhances mitochondrial efficiency in duckling skeletal muscle by acting on the substrate oxidation system. *Journal of Experimental Biology* **221**.

Safran M., Dalah I., Alexander J. et al. (2010) GeneCards Version 3: the human gene integrator. *Database : the journal of biological databases and curation* **2010**, 1–16.

Sanglard L.P., Nascimento M., Moriel P., Sommer J., Ashwell M., Poore M.H., Duarte M.S. & Serão N.V.L. (2018) Impact of energy restriction during late gestation on the muscle and blood transcriptome of beef calves after preconditioning. *BMC Genomics* **19**, 1–18.

Schwarz-Romond T., Asbrand C., Bakkers J., Kühl M., Schaeffer H.J., Huelsken J., Behrens J., Hammerschmidt M. & Birchmeier W. (2002) The ankyrin repeat protein diversin recruits casein kinase Iε to the β-catenin degradation complex and acts in both canonical Wnt and Wnt/JNK signaling. *Genes and Development* **16**, 2073–2084.

Staiger H., Haas C., Machann J. et al. (2009) Muscle-derived angiopoietin-like protein 4 is induced by fatty acids via peroxisome proliferator-activated receptor (PPAR)-δ and is of metabolic relevance in humans. *Diabetes* **58**, 579–589.

Symonds M.E., Pearce S., Bispham J., Gardner D.S. & Stephenson T. (2004) Timing of nutrient restriction and programming of fetal adipose tissue development. *Proceedings of the Nutrition Society* **63**, 397–403.

Tu A.X.M., Kowalski J., Randall J., Shear M.K., Monk T.H., Frank E. & Kupfer D.J. (1997) Generalized Covariance-Adjusted Discriminants : Perspective and Application. *International Biometric Society* **53**, 900–909.

Wang C., Wang M., Arrington J., Shan T., Yue F., Nie Y. & Tao W.A. (2017) Ascl2 inhibits myogenesis by antagonizing the transcriptional activity of myogenic regulatory factors. *Stem Cells and Regeneration* **144**, 235–247.

Weng Y.R., Cui Y. & Fang J.Y. (2012) Biological functions of cytokeratin 18 in cancer. *Molecular Cancer Research* **10**, 485–493.

Wu G., Bazer F.W., Cudd T.A. & Cynthia J. Meininger T.E.S. (2004) Maternal Nutrition and Fetal Development. *The Journal of Nutrition* **134**, 2169–2172.

Yin H., Price F. & Rudnicki M.A. (2013) Satellite cells and the muscle stem cell niche. *Physiological Reviews* 23–67.

Zhu P., Goh Y.Y., Chin H.F.A., Kersten S. & Tan N.S. (2012) Angiopoietin-like 4: A decade of research. *Bioscience Reports* **32**, 211–219.

Tables and Figures

Table 1 Nutrient content of the diet offered to the cows during gestation

		Basal Diet		
Chemical composition	Supplement	Early gestation	Mid gestation	Late gestation
DM[1] (g/kg DM)	881.10	284.90	418.26	330.30
OM[2] (g/kg DM)	957.80	916.70	951.05	941.40
CP[3] (g/kg DM)	360.71	154.33	53.31	72.20
NDFap[4] (g/kg DM)	213.03	568.00	651.48	549.20
NFC[5] (g/kg DM)	342.10	112.57	242.13	290.70
EE[6] (g/kg DM)	41.20	25.50	24.12	29.20

[1] Dry matter.
[2] Organic matter.
[3] Crude protein.
[4] Neutral detergent fiber.
[5] Non-fiber carbohydrate.
[6] Ether extract.

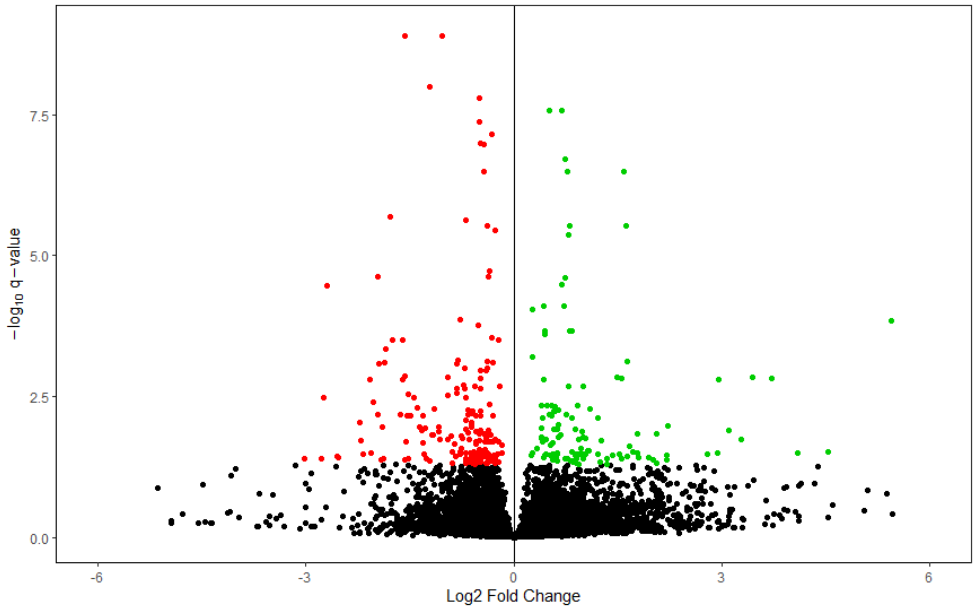


Figure 1 Volcano plot for the effect of diet in the muscle tissue. The Y-axis shows the –log10 *q*-values, whereas the X-axis represents the $\log_2$ fold change (FC), with $\log_2$FC equal 0 representing equal expression between the groups. Negative and positive $\log_2$FC values represent the up-regulation of DEG in control and supplemented groups, respectively. Genes with significant (*q*-value < 0.05) effect of diet are highlighted in red or green, representing up-regulation in the control and supplemented groups, respectively.

Table 2 The 10 most significant down- and up-regulated differentially expressed genes[1] in muscle tissue for the effect of Diet[2]

Gene Symbol	Biological Process	Log2FC [CI]	*q*-value
AK9	Nucleobase-containing compound metabolic process	-3.02 [-5.20, -0.84]	3.98E-02
ENSBTAG00000043480	N/A	-2.78 [-4.79, -0.78]	3.93E-02
LOC510904	Amino acid transport; L-arginine import across plasma membrane; L-ornithine transmembrane transport	-2.75 [-4.32, -1.18]	3.37E-03
CEACAM19	N/A	-2.70 [-3.92, -1.48]	3.48E-05
ENSBTAG00000031354	N/A	-2.56 [-4.38, -0.73]	3.72E-02
RRH	N/A	-2.53 [-4.35, -0.72]	3.87E-02
ENSBTAG00000032057	ATP synthesis coupled proton transport	-2.22 [-3.59, -0.85]	9.24E-03
SIM2	Regulation of transcription by RNA polymerase II	-2.21 [-3.66, -0.75]	1.95E-02
ENSBTAG00000051469	N/A	-2.18 [-3.71, -0.65]	3.41E-02
LOC112449293	Steroid metabolic process	-2.07 [-3.20, -0.94]	1.62E-03
LOC107131843	Insulin receptor signaling pathway	2.78 [0.84, 4.73]	3.27E-02
ENSBTAG00000055063	N/A	2.94 [0.90, 4.97]	3.15E-02
CLSTN3	Homophilic cell adhesion via plasma membrane adhesion molecules	2.96 [1.35, 4.56]	1.60E-03
ANGPTL4	Negative regulation of apoptotic process; negative regulation of lipoprotein lipase activity	3.10 [1.14, 5.06]	1.25E-02
ANKEF1	N/A	3.28 [1.13, 5.43]	1.84E-02
KRT8	Extrinsic apoptotic signaling pathway	3.44 [1.59, 5.30]	1.46E-03
KRT18	Negative regulation of apoptotic process	3.72 [1.71, 5.74]	1.49E-03
KRT19	Sarcomere organization	4.08 [1.23, 6.93]	3.24E-02
KCNH3	Potassium ion transmembrane transport	4.53 [1.40, 7.65]	3.06E-02
ENSBTAG00000055143	Electron transport chain	5.45 [2.84, 8.05]	1.41E-04

[1]*q*-value < 0.05.
[2]Down- and up-regulated in the supplemented group compared with the control are represented by values negative and positive values, respectively.
$Log_2FC = Log_2$ fold change.
CI = 95% Confidence Interval.

Table 3. Enrichment analysis showing the overrepresented[1] Biological Process of differentially expressed genes[2] for diet effect in muscle tissue.

Biological Process	# genes	FE	(P-value)
Regulation of synapse organization (GO:0050807)	3	8.76	5.15E-03
Microtubule polymerization (GO:0046785)	3	8.27	6.03E-03
Regulation of synapse structure or activity (GO:0050803)	3	7.09	9.16E-03
Positive regulation of cellular component biogenesis (GO:0044089)	5	4.07	8.37E-03
Microtubule polymerization or depolymerization (GO:0031109)	3	3.92	4.24E-02

Positive regulation of developmental process (GO:0051094)	6	3.59	7.22E-03
Reproductive process (GO:0022414)	6	3.54	7.63E-03
Reproduction (GO:0000003)	6	3.54	7.63E-03
Potassium ion transmembrane transport (GO:0071805)	4	3.31	3.42E-02
Potassium ion transport (GO:0006813)	4	3.31	3.42E-02
Positive regulation of cellular component organization (GO:0051130)	7	2.82	1.32E-02
Positive regulation of multicellular organismal process (GO:0051240)	5	2.64	4.30E-02
Ribose phosphate biosynthetic process (GO:0046390)	5	2.61	4.47E-02
Monovalent inorganic cation transport (GO:0015672)	7	2.41	2.81E-02
DNA repair (GO:0006281)	8	2.39	2.05E-02
Cellular response to DNA damage stimulus (GO:0006974)	9	2.03	3.60E-02

[1]*P*-value < 0.05;
[2]*q*-value < 0.05;
FE = Fold Enrichment.

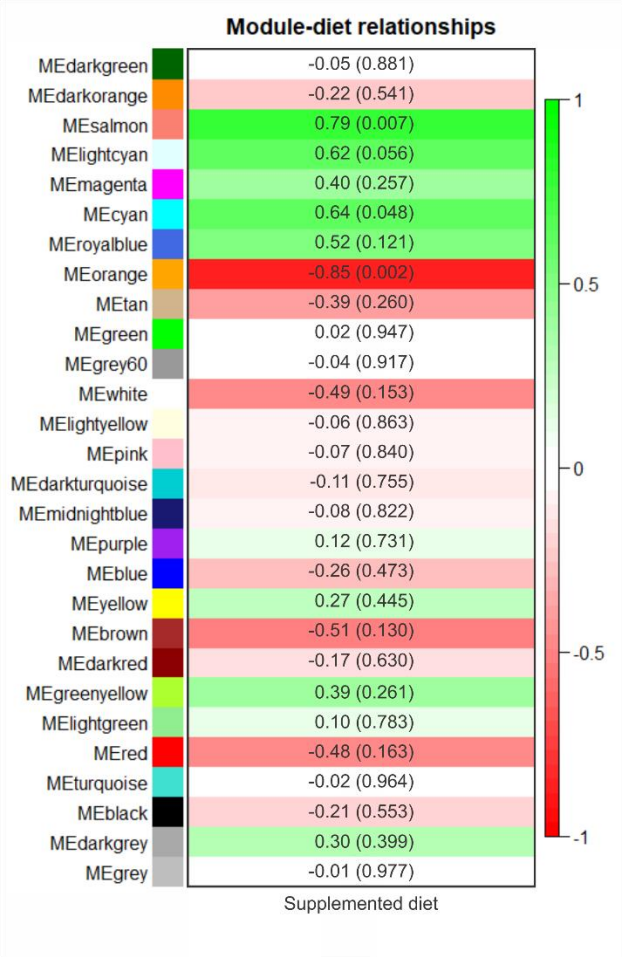


Figure 2 Heatmap of the correlation between module eigengenes (ME) and the diet effect. In parenthesis are the respective *P*-values of the correlation.

Table 4 Genes most associate[1] with the three significant[2] modules for supplemented diet in WGCNA analysis

Gene Name	Gene Symbol	Biological Process	GS[3]
Angiopoietin like 4	*ANGPTL4*	Negative regulation of apoptotic process; Negative regulation of lipoprotein lipase activity	-0.89
Potassium voltage-gated channel subfamily H member 3	*KCNH3*	Potassium ion transmembrane transport	-0.81
Achaete-scute family bHLH transcription factor 2	*ASCL2*	Positive regulation of transcription by RNA polymerase II; cell development	-0.76
Hexosyltransferase	*B3GALT6*	Glycosaminoglycan biosynthetic process; protein glycosylation	0.42
PERP, TP53 apoptosis effector	*PERP*	Desmosome organization	0.54
Ankyrin repeat domain 6	*ANKRD6*	Negative regulation of canonical *Wnt* signaling pathway; positive regulation of *JNK* cascade	0.67
Potassium calcium-activated channel subfamily N member 3	*KCNN3*	Potassium ion transmembrane transport	0.79
Ankyrin repeat and BTB domain containing 2	*ABTB2*	Cellular response to toxic substance	0.81
Kelch like family member 10	*KLHL10*	Homeostasis of number of cells within a tissue	0.82

[1] Higher Module Membership values.
[2] *P*-value < 0.05.
[3] Gene significance.

Table 5 Enrichment analysis showing the overrepresented[1] Biological Process of orange module[2]

Biological Process	# genes	FE	*P*-value
Monovalent inorganic cation transport (GO:0015672)	2	6.09	4.27E-02
Positive regulation of transcription by RNA polymerase II (GO:0045944)	3	4.20	3.44E-02
Cellular process (GO:0009987)	8	0.52	1.03E-02
Metabolic process (GO:0008152)	5	0.50	4.44E-02
Macromolecule metabolic process (GO:0043170)	3	0.37	2.66E-02

[1] *P*-value < 0.05.
[2] Higher correlation with SUP diet.
FE = Fold Enrichment.