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LAVRAS – MG
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INFLUÊNCIA DA ADMINISTRAÇÃO DE VITAMINA A MATERNA OU NEONATAL SOBRE O DESENVOLVIMENTO DOS TECIDOS MUSCULAR E ADIPOSO EM BOVINOS DE CORTE

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Zootecnia, área de concentração em Produção e Nutrição de Ruminantes, para a obtenção do título de Doutor.

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LAVRAS – MG
2025

Ao meu pai José Maria, honrado em ter seu nome, pelo exemplo de perseverança e honestidade.
À minha mãe Nelci pelo apoio absoluto, exemplo de luta e amor mais verdadeiro.
À minha irmã Suelen pelo cuidado, atenção e carinho incondicionais.
Dedico

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“Aprender para fazer, fazer para aprender”

RESUMO

Este estudo teve como objetivo avaliar os efeitos da injeção de vitamina A (VA) no desenvolvimento de tecidos muscular e adiposo, no desempenho de crescimento durante a fase de cria, e na transcriptômica do músculo esquelético em bovinos Angus × Nelore. Para isso, 40 vacas prenhes foram utilizadas em dois experimentos simultâneos, em delineamento de blocos completos casualizados. As vacas foram pesadas, blocadas por ordem de parto e inseminadas com sêmen de um único touro. Após a sexagem fetal, as vacas prenhes foram separadas de acordo com o sexo fetal e alocadas no Experimento 1 ou Experimento 2. No 250º dia de gestação, as vacas de cada experimento foram designadas a um dos três tratamentos: controle sem injeção de vitamina A nas vacas ou bezerros (CON: sem VA), uma única injeção de VA em vacas prenhes no 250º dia de gestação (VAV; 2.000.000 UI de VA), ou duas injeções de VA em bezerros recém-nascidos (VAB: 200.000 UI por dose), uma ao nascer e outra aos 60 dias de idade. Os bezerros foram pesados aos 60 dias de idade, e biópsias do músculo *longissimus thoracis* (LT) foram coletadas aos 60 dias e no desmame. No Experimento 1, os bezerros dos grupos VAB e VAV apresentaram maior peso ao desmame ($P = 0,02$) e ganho médio diário (GMD; $P = 0,02$) em comparação ao grupo CON. Os bezerros tratados com VA (VAB + VAV) também apresentaram maior profundidade da garupa ($P = 0,03$) e tendência a menor índice de musculatura ($P = 0,07$) em relação aos do grupo CON. A análise transcriptômica revelou 139 genes diferencialmente expressos (DEGs; FDR < 0,10) no contraste VAB+VAV vs CON. No contraste VAB vs VAV, 189 DEGs (FDR < 0,10) foram identificados. Bezerros machos do grupo VAV apresentaram maior expressão ($P < 0,01$) do *RARA*, *PAX7* ($P < 0,02$), e tendência a maior expressão de *ZFP423* ($P = 0,08$). Eles também tiveram maior expressão de *MYF5*, *DLK1*, *CEBPA*, e do *IGF1R* ($P < 0,05$) em comparação aos grupos VAB e CON. Além disso, no Experimento 2, as fêmeas do grupo CON tenderam a apresentar maior peso ao desmame ($P = 0,08$) em relação às fêmeas dos grupos VAB e VAV. O grupo CON também mostrou maior área de olho de lombo ($P = 0,05$), enquanto as fêmeas tratadas com VA apresentaram tendência a menor relação do músculo longissimus ($P = 0,07$). As fêmeas do grupo VAV tenderam a maior expressão de *MYH1* ($P = 0,06$) em comparação ao grupo CON. Os grupos tratados com VA exibiram maior expressão de *MYH1* ($P = 0,03$) e tendência a maior expressão do *PPARD* ($P = 0,08$) em relação às fêmeas do grupo CON. No contraste VAB vs VAV, as fêmeas do grupo VAV expressaram mais o *RARB* ($P = 0,05$) e apresentaram tendência a maior expressão de *RARA* e de *MTOR* ($P < 0,08$). Portanto, a injeção de VA em vacas prenhes ou em bezerros recém-nascidos melhora o desempenho e modula vias de desenvolvimento importantes, especialmente em bezerros machos.

Palavras-chave: crescimento muscular; desenvolvimento tecidual; transcriptômica; vitamina A.

ABSTRACT

This study aimed to evaluate the effects of Vitamin A (VA) injection on muscular and adipose tissue development, growth performance during cow-calf phase, and skeletal muscle transcriptomics in Angus × Nellore cattle. We hypothesized that VA injection in pregnant cows would be effective as VA injection in newborn calves to stimuli angiogenesis and adipogenesis. In this regard, 40 pregnant cows were utilized in two simultaneous trials in a randomized complete block design. Cows were weighed, blocked by parity and same-sire inseminated in two fixed-time artificial insemination (FTAI). After fetal sexing assessment, pregnant cows were sorted according to fetal sex and allotted in Trial 1 (cows pregnant with male, n = 20) or Trial 2 (cows pregnant with female, n = 20). Following, at 250d of gestation, cows from each trial were assigned into one of the three treatments: control with no VA injection in cows or calves (CON: no vitamin A), single VA injection in pregnant cows at 250d of gestation (VAcow; 2,000,000 IU of VA), two vitamin A injections in newborn calves (VAcalf: 200,000 IU of VA each dose), one at birth and one at 60d of age. Calves were weighed at 60d of age, and biopsied in the longissimus thoracis (LT) muscle at 60 d of age and at weaning to assess gene expression. From FTAI to weaning, cow-calf pairs were raised as a common herd under the same conditions. In Trial 1, calves from the VAcalf and VAcow groups exhibited higher weaning BW ($P = 0.02$) and average daily gain (ADG; $P = 0.02$) compared to the CON group. VA-treated (VAcalf + VAcow) calves also had greater rump depth ($P = 0.03$) and tended to have a lower muscularity index ($P = 0.07$) than CON calves. Transcriptomic analysis revealed 139 differentially expressed genes (DEGs; FDR < 0.10) when contrasting VAcalf + VAcow vs. CON. Comparing VAcalf vs. VAcow, 189 DEGs (FDR < 0.10) were identified. VAcow male calves displayed higher expression ($P < 0.01$) of *RARA*, *PAX7* ($P < 0.02$), and tended to express more *ZFP423* ($P = 0.08$). They also had greater expressions of *MYF5*, *DLK1*, *CEBPA*, and *IGF1R* ($P < 0.05$) compared to VAcalf and CON groups. In addition, in Trial 2, CON female calves tended to have higher weaning BW ($P = 0.08$) than VAcalf and VAcow female calves. The CON group also showed a greater longissimus muscle area ($P = 0.05$), while VA-treated female calves tended to have a lower longissimus muscle ratio ($P = 0.07$). VAcow female calves tended to express more *MYH1* ($P = 0.06$) than CON. VA-treated groups exhibited higher expression of *MYH1* ($P = 0.03$) and a trend for higher *PPARD* expression ($P = 0.08$) compared to CON female calves. Contrasting VAcalf vs VAcow, VAcow female calves expressed more *RARB* ($P = 0.05$) and tended to express more *RARA* and *MTOR* ($P < 0.08$). In summary, VA injection in pregnant cows or newborn calves enhance growth performance and modulate key developmental pathways especially in male calves.

Keywords: early life development; muscle growth; transcriptomics; vitamin A.

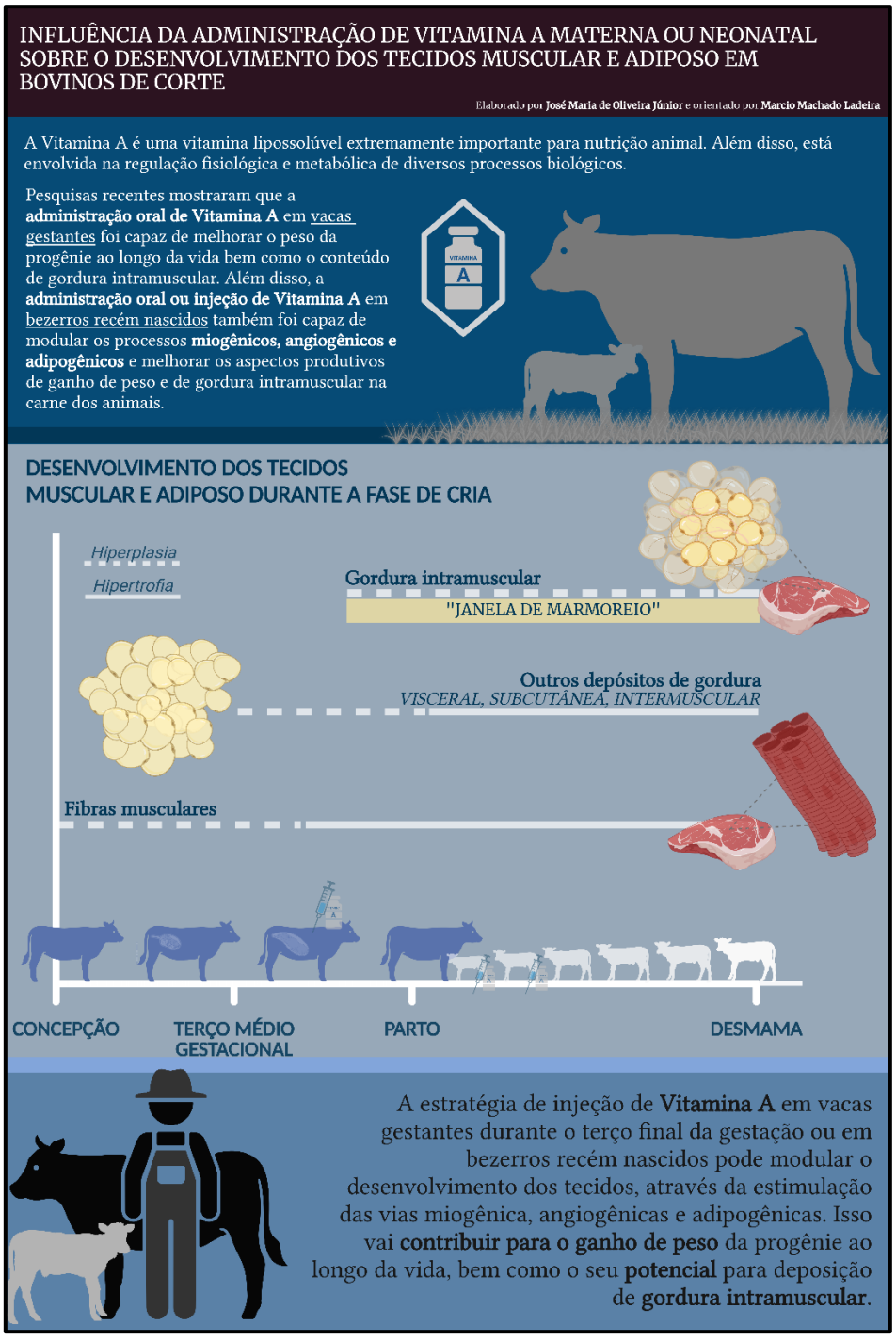
INDICADORES DE IMPACTO

Os resultados desta pesquisa oferecem potenciais impactos significativos em diversas dimensões, especialmente nos âmbitos social, econômico, tecnológico e de saúde animal, com benefícios diretos e em potencial para a pecuária de corte no Brasil e outros países. O estudo avaliou os efeitos de injeções de vitamina A em vacas prenhes e bezerros recém-nascidos sobre o desenvolvimento muscular e adiposo, bem como o desempenho de crescimento, utilizando abordagens avançadas de transcriptômica. Os resultados demonstraram que a suplementação com vitamina A pode otimizar o ganho de peso e o desenvolvimento de tecidos em bezerros, impactando positivamente a produtividade e a eficiência econômica da pecuária. Esses achados têm relevância tecnológica e de produção (área temática 7 da Política Nacional de Extensão), pois geram conhecimento aplicado que pode ser implementado por pecuaristas e indústrias, melhorando a competitividade e sustentabilidade do setor. O trabalho também se alinha aos Objetivos de Desenvolvimento Sustentável (ODS) da ONU, especialmente os ODS 2 (Fome Zero e Agricultura Sustentável), 3 (Saúde e Bem-Estar), e 12 (Consumo e Produção Responsáveis), ao promover práticas que otimizam a produção de alimentos de alta qualidade nutricional com menor impacto ambiental. No âmbito social, os dados gerados podem beneficiar comunidades rurais e pequenos produtores, promovendo capacitação técnica e melhoria na qualidade de vida. O estudo envolveu docentes, técnicos e estudantes da UFLA, promovendo integração acadêmica e contribuindo para a formação de recursos humanos altamente qualificados. Apesar de ter caráter prioritariamente científico, o trabalho apresenta potencial extensionista ao possibilitar a disseminação dos resultados junto a produtores, por meio de parcerias futuras com associações e cooperativas do setor agropecuário. O território impactado abrange principalmente as regiões produtoras de gado de corte no Brasil, com possibilidade de expansão global para mercados interessados em tecnologias voltadas à produção sustentável. Em resumo, a pesquisa contribui para o avanço científico e tecnológico no campo da nutrição e manejo de bovinos, promovendo impactos em saúde animal, produtividade e sustentabilidade ambiental.

IMPACT INDICATORS

The results of this research offer significant potential impacts across social, economic, technological, and animal health domains, with direct and potential benefits for beef cattle production in Brazil and other countries. The study evaluated the effects of vitamin A injections in pregnant cows and newborn calves on muscle and adipose tissue development, as well as growth performance, using advanced transcriptomic approaches. Findings demonstrated that vitamin A supplementation can optimize weight gain and tissue development in calves, positively impacting productivity and economic efficiency in cattle farming. These outcomes hold relevance in technology and production (theme 7 of the National Extension Policy), as they generate applied knowledge that can be implemented by farmers and industries to enhance competitiveness and sustainability in the sector. The study also aligns with the United Nations Sustainable Development Goals (SDGs), particularly SDG 2 (Zero Hunger and Sustainable Agriculture), SDG 3 (Good Health and Well-Being), and SDG 12 (Responsible Consumption and Production), by promoting practices that optimize the production of high-quality nutritional food with reduced environmental impact. On a social level, the data generated can benefit rural communities and small-scale producers, fostering technical training and improving quality of life. The study involved faculty, technicians, and students from UFLA, promoting academic integration and contributing to the training of highly qualified professionals. Although primarily scientific, the research holds extension potential by enabling the dissemination of results to producers through future partnerships with associations and cooperatives in the agricultural sector. The impacted territory primarily includes Brazil's cattle farming regions, with global expansion potential to markets interested in technologies aimed at sustainable production. In summary, the research advances scientific and technological development in cattle nutrition and management, fostering impacts on animal health, productivity, and environmental sustainability.

RESUMO INTERPRETATIVO E RESUMO GRÁFICO



O estudo avaliou os efeitos de injeções de vitamina A (VA) em vacas prenhes e bezerros recém-nascidos sobre o desempenho produtivo na fase de cria e o transcriptoma do músculo esquelético em bovinos. A suplementação com VA, modulou vias moleculares importantes para o crescimento e o desenvolvimento dos tecidos, especialmente em machos, promovendo avanços no manejo nutricional e produtivo de bovinos.

Tese de Doutorado em Zootecnia na UFLA, defendida em 29/11/2024.

SUMÁRIO

Chapter 1

Literature review

1 Introduction

Vitamin A (VA) is a fat-soluble substance that plays important roles in several biological processes. Its bioactive functions in mammalian physiology and metabolism have been studied for several decades, especially in key pathways that can affect animal production, modulate developmental stages, and health. It is role in intramuscular fat (IMF) deposition and muscle growth has gained attention, particularly when administered during late gestation or early postnatal life in cattle (Dean et al., 2024; Harris et al., 2018; Jo et al., 2020; Maciel et al., 2022; Peng et al., 2020; Wang et al., 2018). The manner in which VA acts increasing IMF was newly confirmed by Yu et al. (2022). According to the authors, VA upregulates the Vascular endothelial growth factor (*VEGF*) and stimulates vascular capillary development intramuscularly, increasing intramuscular adipose progenitors and contributing to adipocyte formation. The active form of VA, retinoic acid (RA), plays a specific role in this transcription modulation. In order to modulate gene expression, RA first forms a complex with Retinoid X Receptor (*RXR*) and then binds to Retinoic Acid Receptor (*RAR*), forming a heterodimer that is able to change transcription patterns in target genes (Chawla et al., 2001; Rochette-Egly and Germain, 2009).

Several studies have explored VA administration in cattle. Harris et al. (2018) demonstrated that injecting VA twice (at birth and one at 1 month of age; with 150,000 or 300,000 IU) resulted in a quadratic increase in IMF at weaning and slaughter. Aiming to simplify management and labor, Maciel et al. (2022) evaluated a single VA injection at birth (300,000 IU) and observed that VA injection increased IMF deposition.

Oral supplementation during pregnancy has shown promise. Jo et al. (2020) reported that supplementing pregnant cows at 78,000 IU/d from 225d of gestation to delivery increased birth weight in the offspring and an upregulated genes related to muscle and preadipocyte development.

Furthermore, in a follow up study, Peng et al. (2020) observed that supplementing newborn calves (45,000 IU/d) from day 5 to 2mo of age increased calves body weight and upregulated muscle and preadipocyte pathway genes. Similarly, Dean et al. (2024) found that higher dietary VA level (12.2 KIU/kg) to pregnant cows (from day 180 of gestation to parturition) improved IMF content, upregulated expression of key genes and proteins.

Beyond IMF, VA supplementation appears to enhance growth performance parameters. Some of the studies mentioned above that were conducted focusing on IMF also evaluated animal growth performance. Harris et al. (2018), observed higher average daily gain (ADG) and consequently heavier body weight at weaning (210d) and at 308d (before entry in feedlot). This led to further investigation into VA's impact on muscle development and fiber type composition. In this regard, Wang et al. (2018) reported VA upregulated myogenic transcription factors, increased protein content, density of satellite cells, animal muscle size, and oxidative muscle fibers. These findings align with studies by Jo et al. (2020), that observed increased calves birth weight and upregulation in the expression of myogenic factors, and Peng et al. (2020), that reported also an upregulation in myogenic factors expression and higher BW at 45 and 60 d of age in treated animals.

Adipogenesis of intramuscular adipose tissue peaks in late pregnancy and remains to around 250 d of age, period referred as marbling window (Du et al., 2015). On the other hand, myogenesis predominantly occurs during prenatal phase, where primary myofibers are early formed in the first trimester of pregnancy, while secondary myofibers develop during second trimester (Du et al., 2010). Final muscle fiber maturation and contractile and metabolic differentiation also occurs in late pregnancy (Picard et al., 2002; Du et al., 2010). In this sense, the timing of VA administration in cattle might synchronously encounter key developmental process of skeletal muscle or intramuscular adipocytes and enhance important aspects for meat production.

In this sense, we hypothesized that VA injection in pregnant cows would be effective as VA injection in newborn calves to stimuli angiogenesis and adipogenesis. Thus, the objectives of the present study were to evaluate the effects of VA injection on muscular and adipose tissue development, growth performance during cow-calf phase, and skeletal muscle transcriptomics in Angus × Nellore cattle.

2 Muscle and adipose tissues development in cattle

Tissues development in cattle can be divided into 2 phases: prenatal and postnatal phases. Prenatal phase involves embryonic and fetal development, that will partially define growth potential in adult stage. However, both phases can impact tissues growth and development, modulating hyperplasia and/or hypertrophy processes depending on tissue type.

Skeletal muscle tissue development is a complex process and is characterized by the formation of muscle fibers, adipocytes, and fibroblasts trough myogenesis, adipogenesis, and fibrogenesis processes respectively (Du et al., 2013). Myogenesis event occurs predominantly in prenatal phase, and in cattle no postnatally significant increment in muscle fibers number is observed (Picard et al., 2002; Du et al., 2010; Du et al., 2013). On the other hand, the development of adipose tissue occurs in both prenatal and postnatal phases, depending on the type of adipose tissue being formed: abdominal/visceral, intermuscular, subcutaneous, or intramuscular fat (Du et al., 2013; Du et al., 2015).

2.1 Skeletal muscle development

The formation of muscle tissue is a multi-stage process defined as myogenesis, that occurs primarily during the prenatal phase in cattle and originates from the mesodermal cells. This process is initiated from the paraxial mesoderm, which is progressively segmented into paired somites on either side of the notochord, a phenomenon known as somitogenesis (Tzahor, 2009; Bonnet et al., 2010; Tani et al., 2020). These somites subsequently differentiate into two structures: the sclerotome, that contributes to the vertebral cartilage, and the dermomyotome, from which the majority of skeletal muscle arises (Zhao, 2023; Bonnet, 2010). Cells commitment for myogenic lineage in the embryo initiates with the signaling originating from the surrounding tissues, notochord and neural tube (Chargé and Rudnicki, 2004).

The earliest phase of myogenesis, or primary myogenesis; also referred as embryonic myogenesis, involves the differentiation of myogenic progenitors from the dermomyotome, which expresses a range of signals such as Wingless and Int (*WNT*) and Catenin beta (*CTNNB1)*, that act as a system regulating the expression of several proteins and genes, such as Paired box 3 (*PAX3*)

and 7 (*PAX7*), and the Glioma-associated oncogene homolog 1 (*GLI*), initiating the Myogenic regulatory factors (MRFs). Moreover, *CTNNB1* plays a dual role in promoting cell proliferation and differentiation, ensuring that myogenic progenitors are maintained and directed toward muscle-specific lineage (Suzuki et al., 2015). The MRFs, especially Myogenic factor 5 (*MYF5*) and Myogenic differentiation 1 (*MYOD1*), begin differentiating myogenic progenitors into mononucleated myoblasts (Stern et al., 1995; Buckingham, 2001). These initial myoblasts fuse to form primary myofibers expressing slow myosin heavy chains (*MYHC*) type 1 (*MYH7*) in most muscles (Picard et al., 1994). In cattle, primary myogenesis occurs within the first trimester of gestation, with myogenic cells appearing around 60 days of fetal life (Picard et al., 2002). Additionally, the number of primary myofibers is largely controlled by genetic factors (Deveaux et al., 2001).

Following primary myogenesis, fetal myogenesis, or secondary myogenesis, initiates. In this phase, fetal myoblasts develop into secondary myofibers, primarily expressing fast *MYHC* isoforms type IIx (*MYH1*) (Bonnet et al., 2010). This phase is crucial as it builds upon the structure established by primary myofibers (Zhao et al., 2023; Bonnet, 2010). Signaling molecules such as fibroblast growth factor (FGF) aid in maturing myogenic precursors by promoting epithelial-to-mesenchymal transition within the dermomyotome. Conversely, transforming growth factor-beta (*TGFB*) and bone morphogenetic protein (BMP) serve as inhibitory signals to regulate fetal myoblast differentiation (Plikus et al., 2021). Furthermore, myostatin (*MSTN*), a member of the *TGFB* family, acts as a negative regulator in myogenesis, limiting muscle growth by inhibiting myoblast proliferation (Ríos et al., 2002). Myostatin's inhibitory role is essential for proper muscle patterning and size, ensuring balance between muscle growth and tissue homeostasis (Ríos et al., 2002). During muscle development, a portion of precursors differentiate into fetal myoblasts, downregulating *PAX3* and upregulating *PAX7*, remaining undifferentiated until fetal stage (Hutcheson et al., 2009). The shift of $Pax3^{+}/Pax7^{-}$ embryonic myoblasts to $Pax7^{+}$ fetal myoblasts is mediated by transcription factors like Nuclear factor I X (*NFIX*), which orchestrates the activation of fetal-specific genes essential for muscle development (Messina et al., 2010). A subset of these $PAX7^{+}$ precursors remain undifferentiated after birth, becoming tissue-resident stem cells or satellite cells. These cells are located adjacent to myofibers and play a significant role in muscle growth and regeneration in postnatal stages (Relaix et al., 2005). In cattle, secondary myogenesis occurs during the second trimester of gestation, with myogenic cells appearing around 90 days of

fetal life (Picard et al., 2002). Unlike primary myofibers that are controlled by animal genetics, secondary myofibers seems to be more controlled by epigenetics factors caused by maternal nutrition and intrauterine environment (Picard et al., 2002).

As myogenesis progresses, other MRFs such as Myogenin (*MYOG*) and Myogenic factor 6 (*MYF6*) become essential for the terminal differentiation and maturation of muscle fibers. Myogenin specifically acts in the transition of myoblasts into mature myotubes, promoting their fusion to form multinucleated structures that contribute to fiber formation (Hasty et al., 1993). Also, *MYF6* is predominantly expressed during late fetal and postnatal stages, helps maintain muscle fiber integrity and plays a vital role in muscle fiber maintenance in postnatal phase (Kassar-Duchossoy et al., 2004). Collectively, *MYOG* and *MYF6* expressions ensure the completion of muscle fiber development, essential for skeletal muscle maturation that occurs during late gestation in cattle (Du et al., 2010).

Finally, in the postnatal period, muscle growth predominantly occurs through hypertrophy rather than hyperplasia. This growth is facilitated by satellite cells of mature muscle fibers, which proliferate and fuse with existing fibers (Stickland, 1978; Kuang et al., 2007). Although most satellite cells are committed to the myogenic lineage, some retain multipotency, giving them the potential to differentiate into other cell types, such as adipocytes or fibroblasts (Kuang et al., 2008; Yablonka-Reuveni et al., 2008).

2.2 Adipose tissue development

Adipogenesis is a multi-stage process of the formation of adipocytes from precursor stem cells. Basically, adipogenesis is established by mesenchymal progenitor cells (MSC) commitment into fibro-adipogenic lineages and then adipogenic differentiation. In cattle, the development of adipose tissues considering their location is similar but occurs asynchronously with visceral adipose tissue forming first, followed by intermuscular, subcutaneous, and finally intramuscular fat, which is essential for marbling in beef (Pethick et al., 2004). The formation of these tissues begins around mid-gestation and continues until approximately 250 days after birth (Du et al., 2015). The period between last trimester of gestation and weaning is often referred to as the "marbling window," during which manipulation of intramuscular adipogenesis can be most

effective due to increased recruitment of progenitor cells to the adipogenic lineage (Du et al., 2017).

Since MSCs are pluripotent, they can differentiate into various lineages, including myogenic, fibrogenic, adipogenic, or osteogenic cells (Ghaben and Scherer, 2019). The commitment of MSCs to the adipogenic lineage often overlaps with secondary myogenesis during fetal development, resulting in a temporal competition between myogenesis and fibro-adipogenesis for the allocation of MSCs (Du et al., 2017). Significantly, the *WNT*/*CTNNB1* signaling pathway plays a central role in adipogenesis or fibro-adipogenic progenitors' recruitment. The *WNT*/*CTNNB1* signaling modulates the expression of *MYF5*, a myogenic factor, directing MSCs either toward the myogenic or osteogenic pathway. When *WNT*/*CTNNB1* is inhibited, MSCs are diverted to the fibro-adipogenic lineage, producing fibro-adipogenic progenitors (FAPs) or preadipocytes (Du et al., 2010).

In the late gestation period, as the potential for myogenic differentiation diminishes, muscle fiber hypertrophy becomes more predominant. This shift allows for the inhibition of *WNT*/*CTNNB1* signaling, which can increase the recruitment of $MYF5^{-}$ MSCs for the fibro-adipogenic lineage (Du et al., 2010). Additionally, the transcription factor Zinc-finger protein (*ZFP423*), acts as an early marker of adipogenesis and plays a crucial role in recruiting progenitor cells to preadipocytes during the determination phase of adipogenesis (Ghaben and Scherer, 2019).

After MSCs commit to the preadipocyte lineage, these cells undergo proliferation. Early proliferation signals stimulate the expression of members of the CCAAT-enhancer-binding protein family (*CEBPA*, *CEBPB*, *CEBPG*), which subsequently activate the expression of Peroxisome proliferator activated receptor gamma (*PPARG*), a key transcription factor in adipogenesis. The interplay between *PPARG* and *CEBPA* in preadipocytes establishes a self-regulatory loop that promotes further adipocyte proliferation. Also, *ZFP423* supports *PPARG* expression, which then induces *CEBPA*, increasing *PPARG* levels and facilitating robust adipocyte proliferation (Duarte et al., 2013). When both *PPARG* and *CEBPA* are expressed, they drive the activation of cell cycle arrest genes in adipocytes, halting proliferation and enabling terminal differentiation (Ladeira et al., 2016).

For terminal differentiation, adipocytes require the coordinated action of *PPARG* and *CEBPA* to initiate lipid synthesis and other adipocyte-specific functions. However, active *WNT*/*CTNNB1* signaling inhibits differentiation by repressing *PPARG* and *CEBPA*. Thus, to

promote adipogenesis, the *WNT*/*CTNNB1* pathway must be inhibited (Moseti et al., 2016). Additionally, bone morphogenetic proteins (BMPs) play a pro-adipogenic role, particularly BMP4, which aids in the differentiation of MSCs into adipocytes by inhibiting *WNT*/*CTNNB1* signaling and thereby allowing *PPARG* expression (Gupta et al., 2010; Hausman et al., 2014).

The transcription factor Sterol regulatory element-binding protein 1c (*SREBF1*), further drives the final stages of adipocyte differentiation by activating the transcription of lipogenic genes, such as Fatty acid binding protein 4 (*FABP4*) (Tang and Lane, 2012). In this sense, *FABP4* expression is prominent in mature adipocytes and is involved in fatty acid absorption and transport, making it a marker for adipocyte number in muscle tissue (Del Pino et al., 2017). Moreover, during this phase, adipocytes acquire insulin sensitivity, a trait associated with increased insulin receptor numbers and glucose transporters like Solute Carrier Family 2 Member 4 (*SLC2A4*) (Ladeira et al., 2016).

This complex sequence of events underlying adipogenesis in cattle is essential not only for the animal's energy storage but also influences marbling, a desirable trait in beef production. The abundance of intramuscular fat (IMF, i.e., marbling) content is determined by number and size of intramuscular adipocytes, which is established by hyperplasia and hypertrophy processes respectively (Du et al., 2015), and in this case, hyperplasia can be referred to as adipogenesis. Adipose tissue hypertrophy peaks around the time that the animal reaches puberty and maturity, where lean tissue deposition is reduced in proportion to the total weight gain. Adipogenesis of IMF occurs from late gestation to nearly 250d of age. Based on this, it is critical to establish the most efficient stage to utilize strategies and manipulate IMF adipogenesis as stimulus could affect the expected phenotype potential. So, the marbling window would be the most desirable period to stimulate adipogenesis.

3 Vitamin A strategy in developmental studies

Vitamin A (VA) is a fat-soluble vitamin and plays important roles in several biological processes. Its bioactive functions in mammalian physiology and metabolism have been studied, especially in pathways that can affect animal production or modulate key developmental stages. Recent studies highlight VA's impact on IMF deposition and muscle growth, particularly when administered during late gestation or early postnatal life in cattle (Harris et al., 2018; Wang et al., 2018; Jo et al., 2020; Peng et al., 2020; Maciel et al., 202; Dean et al., 2024). The manner which VA acts was newly confirmed by Yu et al. (2022). According to the authors, VA upregulates the vascular endothelial growth factor (*VEGF*) and stimulates vascular capillary development intramuscularly, increasing intramuscular adipose progenitors and contributing to adipocyte formation. In other words, this vascularization effect of VA fosters IMF accumulation, which is essential for meat quality in beef cattle. The active form of VA, retinoic acid (RA), plays a specific role in this transcription modulation. In order to modulate gene expression, RA first forms a complex with Retinoid X Receptor (*RXR*) and then binds to Retinoic Acid Receptor (*RAR*), forming a heterodimer that is able to change transcription patterns in target genes (Chawla et al., 2001; Rochette-Egly and Germain, 2009).

The developmental timeline of adipose tissues is crucial for targeting VA administration. As previously discussed, adipogenesis of IMF occurs from late gestation to approximately 250 days postnatal, a period termed as "marbling window". Administering VA during this window can be effective for enhancing adipogenesis and potentially maximizing IMF deposition. Summarizing, to stimulate IMF adipogenesis VA could be administered from late pregnancy (250d of gestation) to weaning (nearly 250 of age), however further research is needed to investigate the effects of VA administration in periods later than 60d of age in the offspring.

Several studies have explored VA administration in cattle. One of the initial studies injecting VA in newborn calves was conducted by Dr. Min Du's research group. Harris et al. (2018) tested two VA dosages (150,000 or 300,000 IU) injected twice, one at birth and one at 1 month of age, in Angus steers calves and they observed a quadratically increase in IMF at weaning and slaughter. Aiming to simplify management and labor, our research group led by Dr. Marcio Ladeira designed a streamlined protocol with a single VA injection at birth (300,000 IU) in Montana × Nellore calves, showing that VA injection increased IMF deposition (Maciel et al., 2022).

In addition to injectable VA, oral supplementation during pregnancy has shown promise. A research group from Korea published two papers investigating the effects of oral VA supplementation during late-gestation and in neonatal stage in Korean native calves (Hanwoo cattle). In the first study, Jo et al. (2020) supplementing pregnant cows at 78,000 IU/d from 225d of gestation to delivery reported higher birth weight in the offspring from supplemented dams, and an upregulation in genes related to muscle and preadipocyte development. Furthermore, the follow up study supplemented the newborn calves (45,000 IU/d) from day 5 to 2mo of age, when they were weaned. Peng et al. (2020) observed VA supplementation in neonatal stage increased calves body weight (until 60d of age), and upregulated gene expression of muscle and preadipocyte development pathway genes without activating mature adipocytes development. Dean et al., 2024 further explored this by offering a higher dietary VA level (12.2 KIU/kg) to pregnant beef cows from day 180 of gestation to parturition. Skeletal muscle of newborns from VA-supplemented dams showed higher expression of Retinoic acid receptor alpha (*RARA*), increased protein abundance of Delta Like Non-Canonical Notch Ligand 1 **(***DLK1*) and PPARG, and greater IMF content across postnatal life stages, without affecting subcutaneous fat, carcass weight, or dressing percentage. These findings suggest that gestational VA supplementation can promote intramuscular adipogenesis in offspring without impacting other carcass traits. The results from these studies regarding oral VA supplementation in pregnancy and neonatal stages and its effects on calves confirm VA per se has an impact on animal physiology and metabolism.

Apart from influencing IMF, VA appears to enhance growth performance parameters. Some of the studies mentioned above that were conducted focusing on IMF also evaluated animal growth performance. In Harris et al. (2018) experiment testing two VA dosages (150,000 or 300,000 IU) injected twice (one at birth and one at 1mo of age) in Angus steers calves, was observed higher average daily gain (ADG) and consequently heavier body weight at weaning (210d) and at 308d (before entry in feedlot) but no differences were found in final body weight (at 438d) when comparing animals receiving 300,000 IU VA twice with non-treated animals. The authors suggested that VA plays a role in muscle development or animal growth. This led to further investigation into VA's impact on muscle development and fiber type composition. Wang et al. (2018) reported VA upregulated myogenic transcription factors (*PAX3*, *PAX7*, *MYF5*, *MYOD1* and *MYOG*), increased MYOG protein content, density of satellite cells, animal muscle size, and oxidative muscle fibers. Authors justified the higher number of oxidative muscle fibers due to the

greater expression of the transcription factor *PPARG*, which is related to the formation of oxidative muscle fibers. The research group from Korea also evaluated animal performance and muscle growth and development in their studies with Hanwoo cattle using oral VA supplementation in pregnant cows and newborn calves. Jo et al. (2020) supplementing VA in pregnant cows at 78,000 IU/d from 225d of gestation to delivery observed VA increased calves birth weight and upregulated the expression of *MYOD1*, *MYF6* and *MYOG*. Further, Peng et al. (2020) supplementing VA to the respective newborn calves (45,000 IU/d) from day 5 to 2 months of age, when they were weaned, reported upregulation in *MYF5*, *MYF6* and *MYOD* expression and higher BW at 45 and 60 d of age in VA animals, but no differences were observed in ADG. Those results indicate that VA stimulates fetal and postnatal muscle development.

4 Exploring knowledge gaps and strategic considerations for future research

Currently, there are concerns regarding effectiveness of VA injection in pregnant cows compared to VA injection in newborn calves and its best dosage recommendations (Ladeira and Oliveira Junior, 2022). If maternal VA injections can similarly enhance IMF deposition in offspring, this could provide producers with a practical tool to target marbling traits in early development, potentially reducing the need for postnatal interventions. In this sense, would be effective to evaluate the best dosage to be injected in the dam, placental VA transferring, VA transferring via colostrum, and residual effects of VA injection in late pregnancy on reproductive traits of the subsequent reproductive protocols.

Additionally, the ideal VA dosage and timing for neonatal calves to maximize IMF depositions remains uncertain. Understanding how long VA administration remains effective in promoting IMF deposition in young calves could optimize supplementation strategies. Evidence suggests that VA effects during lipogenesis events, when the adipocyte is mature, acts in the opposite way than its effects when adipogenesis is occurring, so IMF content could be altered depending on when VA would be administered. In this sense, VA in calves during suckling phase would increase IMF potential through upregulation in angiogenesis and adipogenesis (Yu et al., 2022) but during finishing phase would downregulate lipogenesis and upregulate oxidation, reducing marbling content (Gorocica-Buenfil et al., 2007; Pickworth et al., 2012).

Literature data show that the active form of VA, retinoic acid (RA), activates *PPARA* in mature adipocytes, inducing lipid oxidation (Berry & Noy, 2023). When RA is transported by Fatty acid binding protein 5 (*FABP5*) it will bind RA with the retinoid X receptor (*RXR*), forming a heterodimer with *PPAR* and promoting *PPARA* expression that will stimulate the expression of lipid oxidative genes. The effect of VA restriction during finishing phase in marbling increasement was reported only in breeds and crossbreeds with high potential to marble (Gorocica-Buenfil et al., 2007; Pickworth et al., 2012; Knutson et al., 2020). It is important to highlight VA restriction during finishing phase was effective to increase IMF content either in early weaned or traditionally weaned cattle (Pickworth et al., 2012). These reports with VA restriction or adopting no supplemental VA in finishing diets were regarding Angus and Angus crossbred cattle. So, there is still a lack of information concerning VA restriction during the finishing phase in *Bos indicus*-influenced cattle, such as Nellore breed and their respective crossbreeds.

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Chapter 2

5 Article

Manuscript formatted according to the Journal of Animal Science (JAS) guidelines

Influence of maternal or neonatal Vitamin A administration on muscle and adipose tissue development in beef cattle

6 Lay summary

This study explored how Vitamin A (VA) injections influence growth and development in Angus × Nellore cattle during the early stages of life. Researchers tested whether injecting VA in pregnant cows or newborn calves could improve muscle and fat development and overall growth. Pregnant cows were divided into three groups: no Vitamin A injections; injection in cows during late pregnancy at 250 d of gestation; or two injections in calves shortly after birth and at 60 d of age. Male calves receiving VA showed improved growth, including higher weaning weights and average daily gain. Additionally, their muscle samples revealed increased activity in genes linked to muscle growth and fat regulation. Female calves showed mixed results; those without VA injections had larger muscle areas, while those treated with VA had greater activity in specific muscle-related genes. The findings highlight that VA injections can enhance growth and regulate important pathways for muscle and fat development, particularly in male calves. These results provide valuable insights for farmers and researchers looking to improve cattle growth and productivity through nutritional strategies during pregnancy or early life.

7 Abstract

This study aimed to evaluate the effects of Vitamin A (VA) injection on muscular and adipose tissue development, growth performance during cow-calf phase, and skeletal muscle transcriptomics in Angus × Nellore cattle. We hypothesized that VA injection in pregnant cows would be effective as VA injection in newborn calves to stimuli angiogenesis and adipogenesis. In this regard, 40 pregnant cows were utilized in two simultaneous trials in a randomized complete block design. Cows were weighed, blocked by parity and same-sire inseminated in two fixed-time artificial insemination (FTAI). After fetal sexing assessment, pregnant cows were sorted according to fetal sex and allotted in Trial 1 (cows pregnant with male, n = 20) or Trial 2 (cows pregnant with female, n = 20). Following, at 250d of gestation, cows from each trial were assigned into one of the three treatments: control with no vitamin A injection in cows or calves (CON: no vitamin A), single vitamin A injection in pregnant cows at 250d of gestation (VAcow; 2,000,000 IU of vitamin A), two vitamin A injections in newborn calves (VAcalf: 200,000 IU of vitamin A each dose), one at birth and one at 60d of age. Calves were weighed at 60d of age, and biopsied in the longissimus thoracis (LT) muscle at 60 d of age and at weaning to assess gene expression. From FTAI to weaning, cow-calf pairs were raised as a common herd under the same conditions. In Trial 1, calves from the VAcalf and VAcow groups exhibited higher weaning BW ($P = 0.02$) and average daily gain (ADG; $P = 0.02$) compared to the CON group. VA-treated (VAcalf + VAcow) calves also had greater rump depth ($P = 0.03$) and tended to have a lower muscularity index ($P = 0.07$) than CON calves. Transcriptomic analysis revealed 139 differentially expressed genes (DEGs; FDR < 0.10) when contrasting VAcalf + VAcow vs. CON. Comparing VAcalf vs. VAcow, 189 DEGs (FDR < 0.10) were identified. VAcow male calves displayed higher expression of *Retinoic acid receptor alpha* (*RARA*), *Paired box 7* ($P < 0.02$), and tended to express more *Zinc finger protein 423* ($P = 0.08$). They also had greater expression of *Myogenic factor 5*, *Delta like non-canonical notch ligand 1*, *CCAAT Enhancer binding protein alpha*, and *Insulin like growth factor 1 receptor* ($P < 0.05$) compared to VAcalf and CON groups. In addition, in Trial 2, CON female calves tended to have higher weaning BW ($P = 0.08$) than VAcalf and VAcow female calves. The CON group also showed a greater longissimus muscle area ($P = 0.05$), while VA-treated female calves tended to have a lower longissimus muscle ratio ($P = 0.07$). VAcow female calves tended to express more Myosin heavy chain 1 (*MYH1*; $P = 0.06$) than CON. VA-treated groups exhibited higher expression

of *MYH1* (P = 0.03) and a trend for higher *Peroxisome proliferator activated receptor delta* expression (P = 0.08) compared to CON female calves. Contrasting VAcalf vs VAcow, VAcow female calves expressed more *Retinoic acid receptor beta* (P = 0.05) and tended to express more *RARA* and *Mechanistic target of rapamycin kinase* (P < 0.08). In summary, VA injection in pregnant cows or newborn calves enhance growth performance and modulate key developmental pathways especially in male calves.

Keywords: early life development, muscle growth, transcriptomics, vitamin A

8 Introduction

Vitamin A (VA) is a fat-soluble substance that plays important roles in several biological processes. Its bioactive functions in mammalian physiology and metabolism have been studied for several decades, especially in key pathways that can affect animal production, modulate developmental stages, and health. It is role in intramuscular fat (IMF) deposition and muscle growth has gained attention, particularly when administered during late gestation or early postnatal life in cattle (Dean et al., 2024; Harris et al., 2018; Jo et al., 2020; Maciel et al., 2022; Peng et al., 2020, 2021; Wang et al., 2018). The manner in which VA acts increasing IMF was newly confirmed by Yu et al. (2022). According to the authors, VA upregulates the Vascular endothelial growth factor (*VEGF*) and stimulates vascular capillary development intramuscularly, increasing intramuscular adipose progenitors and contributing to adipocyte formation. The active form of VA, retinoic acid (RA), plays a specific role in this transcription modulation. In order to modulate gene expression, RA first forms a complex with Retinoid X Receptor (*RXR*) and then binds to Retinoic Acid Receptor (*RAR*), forming a heterodimer that is able to change transcription patterns in target genes (Chawla et al., 2001; Rochette-Egly and Germain, 2009).

Several studies have explored VA administration in cattle. Harris et al. (2018) demonstrated that injecting VA twice (at birth and one at 1 month of age; with 150,000 or 300,000 IU) resulted in a quadratic increase in IMF at weaning and slaughter. Aiming to simplify management and labor, Maciel et al. (2022) evaluated a single VA injection at birth (300,000 IU) and observed that VA injection increased IMF deposition.

Oral supplementation during pregnancy has shown promise. Jo et al. (2020) reported that supplementing pregnant cows at 78,000 IU/d from 225d of gestation to delivery increased birth weight in the offspring and an upregulated genes related to muscle and preadipocyte development. Furthermore, in a follow up study, Peng et al. (2020) observed that supplementing newborn calves (45,000 IU/d) from day 5 to 2mo of age increased calves body weight and upregulated muscle and preadipocyte pathway genes. Similarly, Dean et al., 2024 found that higher dietary VA level (12.2 KIU/kg) to pregnant cows (from day 180 of gestation to parturition) improved IMF content, upregulated expression of key genes and proteins.

Beyond IMF, VA supplementation appears to enhance growth performance parameters. Some of the studies mentioned above that were conducted focusing on IMF also evaluated animal

growth performance. Harris et al. (2018), observed higher average daily gain (ADG) and consequently heavier body weight at weaning (210d) and at 308d (before entry in feedlot). This led to further investigation into VA's impact on muscle development and fiber type composition. In this regard, Wang et al. (2018) reported VA upregulated myogenic transcription factors, increased protein content, density of satellite cells, animal muscle size, and oxidative muscle fibers. These findings align with studies by Jo et al. (2020), that observed increased calves birth weight and upregulation in the expression of myogenic factors, and Peng et al. (2020), that reported also an upregulation in myogenic factors expression and higher BW at 45 and 60 d of age in treated animals.

Adipogenesis of intramuscular adipose tissue peaks in late pregnancy and remains to around 250 d of age, period referred as marbling window (Du et al., 2015). On the other hand, myogenesis predominantly occurs during prenatal phase, where primary myofibers are early formed in the first trimester of pregnancy, while secondary myofibers develop during second trimester (Du et al., 2010). Final muscle fiber maturation and contractile and metabolic differentiation also occurs in late pregnancy (Picard et al., 2002; Du et al., 2010). In this sense, the timing of VA administration in cattle might synchronously encounter key developmental process of skeletal muscle or intramuscular adipocytes and enhance important aspects for meat production.

In this sense, we hypothesized that VA injection in pregnant cows would be effective as VA injection in newborn calves to stimuli angiogenesis and adipogenesis. Thus, the objectives of the present study were to evaluate the effects of VA injection on muscular and adipose tissue development, growth performance during cow-calf phase, and skeletal muscle transcriptomics in Angus × Nellore cattle.

9 Materials and methods

All experimental procedures and protocols in this study were approved by the Ethics Committee on Animal Use of the Universidade Federal de Lavras (UFLA; protocol number 014/21). The trial was carried out at a commercial farm located in Extrema, Minas Gerais, Brazil, and at UFLA (Lavras, Minas Gerais, Brazil). The analyses were performed in the Molecular Biology Laboratory of the Animal Science Department at UFLA (Lavras, Minas Gerais, Brazil) and in the Genetics and Biotechnology Laboratory (LaGenBio) at the Universidade Federal de São Carlos (UFSCar, São Carlos, São Paulo, Brazil).

9.1 Experimental design, treatments and animals

Initially, a total of 52 pregnant Nellore cows with an average initial body weight (BW) of 438 ± 48.9 kg and initial body condition score (BCS) of 5.1 ± 0.97 were used in two simultaneous trials in a randomized complete block design. Cows were weighed, blocked by parity and same-sire inseminated in two fixed-time artificial insemination (FTAI). Pregnancy diagnostic was checked at 21 d post insemination and fetal sexing was assessed around 60 d post insemination. Pregnant cows were then sorted according to fetal sex (male or female) and assigned to either Trial 1 (cows carrying male fetuses, n = 22) or Trial 2 (cows carrying female fetuses, n = 24). The decision to run separate trials for fetal sex was based on the following explanation: due to the high cost of RNA sequencing analyses, this transcriptomic data was obtained only in male calves and in one time point (60 d of age); 2- the effect of vitamin A injection in the intramuscular content found in the males (Figure 1); 3- the present study did not aim to compare angiogenesis or adipogenesis in male *versus* female calves.

Following, at 250d of gestation, cows from each trial were assigned into one of the three treatments: control (CON), with no VA injection in cows or calves; a single VA injection in pregnant cows at 250d of gestation (VAcow) with 2,000,000 IU of VA; or two VA injections in newborn calves (VAcalf), each with 200,000 IU of VA, administered at birth and again at 60d of age. The form of VA used in the injections was retinyl palmitate/vitamin A palmitate (Monovin A, Bravet, RJ, Brazil). The dose of VA injection in newborn calves was chosen based on previous

research by our research group (Maciel et al., 2022b) and by Dr. Min Du's research group (Harris et al., 2018b). In addition, given the lack of available data regarding VA injection in cows, the dosage of VA injection in pregnant cows of VAcow group was based on the dosage used in VAcalf treatment in proportion to the metabolic body weight ($BW^{0.75}$).

At birth, calves were individually identified, had their navels treated with iodine solution (10% concentration), and calves from VAcalf received first VA injection (200,000 IU of vitamin A). At 60 d of age, all calves were weighed and calves from VAcalf group received the second VA injection (200,000 IU). On this same day (average of 60 d of age), skeletal muscle was biopsied in the longissimus thoracis (LT) muscle the 12th rib. The lumbar area was shaved and a local anesthetic (lidocaine hydrochloride HCl, 20 mg/mL, 6 mL) was injected subcutaneously. The site was disinfected with Betadine, and a 1-cm incision was made with a scalpel. A sterile Bergström muscle biopsy cannula (Eskilds Tuna, Sweden), 5 mm in diameter, was used to collect 1 g of muscle tissue, which was immediately placed in liquid nitrogen for transportation and later stored at -80°C. After the procedure, the incision was rinsed with sterile saline and water, sealed with veterinary tissue-glue, and sprayed with an antibiotic. The calves were monitored for 72 h post-biopsy and treated with antibiotics (20 mg of oxytetracycline/kg BW) if signals of infection were observed. From 90d of age to weaning, calves were fed *ad libitum* supplementation (total digestible nutrients (TDN) = 78.8%; crude protein (CP) = 22.0%) through creep-feeding technique. From FTAI until weaning, cow-calf pairs from all treatments and trials were raised as a single herd in a common paddock with *Urochloa decumbens,* in a stocking rate of ~1 animal unit/ha.

Both FTAI groups were simultaneously weaned to assist farm management. In this sense, calves from FTAI group 1 were weaned, averaging 311 d of age, whereas calves from FTAI group 2 averaging 267 d of age. At weaning, all calves were weighed, and carcass ultrasonography was performed in calves at the 12th rib and rump to access muscle growth development parameters. Ultrasonography images were collected using Aloka 500-V (Corometrics Medical Systems, Wallingford, CT) with a 3.5-MHz, 17.2-cm linear array transducer and processed using ImageJ® software (National Institutes of Health, Bethesda, Maryland, USA). Also at weaning, calves were biopsied again in the LT muscle, following the same procedure previously described.

Undesirably, unforeseen predator attacks, snake accidents, and conflict accidents caused loss and damage to a few experimental units, which we decided to remove from the final data

analysis. In summary, from the initially 52 cow-calf pair it ended up with 40 cows (28 primiparous and 12 multiparous) and 40 calves.

9.2 Gene expression analysis

9.2.1 RNA-sequencing (RNA-seq)

Based on phenotypic data of intramuscular fat content after the feedlot (Figure 1), and to reduce analysis expenses, the transcriptome profile was analyzed in samples (CON n = 5; VAcalf n = 6; VAcow n = 3) collected at 60 d of age in Trial 1 (male calves) using the RNA-seq technique. Following manufactures' instructions, total RNA was extracted from ≈80 mg of LT muscle using RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany) and treated to eliminate genomic DNA with gDNA Removal Kit (Jena Bioscience, Jena, Germany). Extracted RNA was quantified using Qubit™ RNA High Sensitivity (Invitrogen, Carlsbad, CA, USA) in the Qubit 4.0 Fluorometer (ThermoFisher Scientific, Waltham, MA, USA). Quality control of RNA was assessed with RNA 6000 Pico Kit (Agilent, Santa Clara, CA, USA) in the 2100 Bioanalyzer Instrument (Agilent, Santa Clara, CA, USA), and all samples presented RNA Integrity Number (RIN) ≥ 7.2. The selection of mRNA was prepared from 500 ng of total RNA through QIAseq Stranded mRNA Enrichment Kit (Qiagen, Valencia, CA, USA). The libraries preparation was made following the steps as suggested by kit (QiaSeq Stranded RNA Lib Enzyme), which consisted in RNA fragmentation, first-strand synthesis, second-strand synthesis, end repair, A-addition, and strand-specific ligation. The index sequence used was QIAseq UDI Y-adapter kit (Qiagen, Valencia, CA - USA). Library size was quantified using Qubit™ dsDNA Assay Kit High Sensitivity (Invitrogen, Carlsbad, CA, USA) in the Qubit 4.0 Fluorometer (ThermoFisher Scientific, Waltham, MA, USA). To assess library quality control, it was used High Sensitivity DNA Kit (Agilent, Santa Clara, CA, USA) in the 2100 Bioanalyzer Instrument (Agilent, Santa Clara, CA, USA). Following, libraries were grouped, and their concentrations were adjusted for 4 nM. Sequencing step was performed in the NextSeq 500/550 (Illumina, San Diego, CA, USA) using High Output Kit v2.5 (75 cycles; Illumina, San Diego, CA, USA) and considering reads size of 1×75 pb.

Bioinformatics analyses were performed using the RStudio software (version 2024.09.0+375). The sequences were checked in Trim Galore (v. 0.6.10) considering a length of 70 bp and a Phred quality score of 33. The reference genome used for alignment was ARS-UCD-2.0, and annotated gene read counts were obtained using the featureCounts function of Rsubread package (Liao et al., 2019). Differentially expressed genes (DEGs) were identified and analyzed using pre-defined contrasts (CON vs. VAcalf + VAcow; VAcalf vs. VAcow) in the DESeq2 package (Love et al., 2014). Read count filtering was applied based on the following criteria: genes with zero counts across all samples were excluded and classified as non-expressed, genes with fewer than 5 counts per sample and 20 counts considering all samples were excluded as very lowly expressed, and genes not detected in at least 50% of the samples within each treatment group were removed and classified as rarely expressed (Appendix A). DEGs were identified based on adjusted *P*-values (FDR < 0.10) and log2 fold-change values exceeding |0.50|. Additionally, gene-gene associations and transcription factors involved in co-expression networks were explored using the CeTF package (Biagi Junior et al., 2021). A network analysis was used to explore co-expressed genes and identify transcription factors (TFs). Functional enrichment analysis of DEGs and transcription factors TFs was performed with the ClusterProfiler package in R, focusing on Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes Genomes (KEGG) pathways.

9.2.2 Real-time quantitative polymerase chain reaction (RT-qPCR)

Gene expression of samples collected at weaning from Trial 1 (male calves) and Trial 2 (female calves) was analyzed using the RT-qPCR technique. For this, total RNA was extracted from ≈170 mg of muscle tissue with the SV Total RNA Isolation System (Promega, Madison, WI, USA) adapting the manufacturer's recommendations using 350 µL of the lysate and including 100 µL of QIAzol (Qiagen, Valencia, CA, USA) before 3 min at 70°C incubation step. Extracted RNA was quantified with a nano spectrophotometer (DeNovix DS-11, Wilmington, DE, USA) and to picture RNA integrity, extracted RNA was subjected to electrophoresis in agarose gel that was visualized using E-Gel Imager Camera Hood (Life Technologies, Neve Yamin, Israel). The synthesis of the complementary deoxyribonucleic acid (cDNA) was done using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA).

Details of the primers used in the reactions are described in Table 1. Primers' design was based on sequences available in NCBI platform (National Center for Biotechnology Information) and the specific primers sequences were selected using the web interface program Primer3PLus and analyzed on Oligo Analizer 3.1 and Premier Biosoft softwares. Primers were synthesized by Invitrogen (Carlsbad, CA, USA).

The SYBR Green detection system (Applied Biosystems, USA) was used in the Eppendorf Realplex2 thermocycler (Eppendorf, Hamburg, Germany). All RT–qPCR assay was based on the cDNA from 40 biological replicates and 3 technical replicates for each. Relative expression levels are expressed relative to the average of Actin beta (*ACTB*) and Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and were calculated using the 2 Delta Delta Ct method (Livak and Schmittgen, 2001).

9.3 Statistical analysis

Performance and RT-qPCR data was analyzed using the MIXED procedure of SAS 9.4 software (SAS Inst. Inc., Cary, NC, USA). To assess data normality, the Shapiro-Wilk test was performed, and data were transformed using the RANK procedure when did not present a normal distribution. Trials were analyzed as randomized complete block design. Cows' parity (primiparous and multiparous) and FTAI events (group 1 and group 2) were considered as blocks and were balanced among treatments. Cows' initial BW was used as covariate adjustment. Cow-calf pair was considered as experimental unit. Cow parity, FTAI group, and treatment were considered as fixed effects, while cow-calf pair was considered as random effect. Orthogonal contrasts (CON vs VAcalf + VAcow and VAcalf vs VAcow) were used to partition treatment effects into pre-planned comparisons, allowing focused hypothesis testing. Statistical differences were considered when $P \leq 0.05$ and trends were discussed when $0.05 < P \leq 0.10$.

10 Results

10.1 Trial 1 - Male calves

10.1.1 Offspring performance

No significant differences were observed in cow initial body weight (BW) and calf weight at 60 days ($P > 0.50$) across treatments (Table 2). When contrasting VA-treated groups with CON, calves from VAcalf and VAcow groups had higher BW at weaning ($P = 0.02$), and greater ($P = 0.02$) ADG from 60d of age until weaning. On the other hand, when contrasting VAcalf vs VAcow group no significant differences were observed ($P > 0.26$).

10.1.2 Body ultrasound in the offspring

No significant treatment differences were observed ($P > 0.13$) in the measurements, except for rump depth (Table 3). Contrasting VA-treated groups with CON, calves from VAcalf and VAcow groups presented higher ($P = 0\ .03$) rump depth and tended to have lower ($P = 0\ .07$) muscularity index compared to CON calves. However, when contrasting VAcalf vs VAcow group no significant differences were observed ($P > 0.16$).

10.1.3 Gene expression at 60d of age (RNA-seq)

Sequencing obtained 84.87% of uniquely mapped reads and 16.95 million reads. With all genes expressed, the principal component analysis (PCA) showed a contribution of 19.7% of the variability in Principal Component 1 and 13.3% in Principal Component 2 (Figure 2).

Contrasting CON vs VAcalf + VAcow, PCA showed a contribution of 39.7% of the variability in Principal Component 1 and 11.5% in Principal Component 2 (Figure 3). Transcriptomics showed 13,988 expressed genes and 139 DEGs (FDR < 0.10; Appendix B). In this case, 71 were upregulated for VA-treated groups (VAcalf + VAcow) and 68 were

downregulated in comparison with CON. Also, *FZD10* was exclusively identified to VA-treated groups (VAcalf + VAcow) compared to CON. Moreover, 14 key TFs were identified, which *ESRRA*, *HOXC9*, *ETS1*, *NFIX*, *ZFP64*, *E2F4*, and *ZIC1* were upregulated in VA-treated groups (VAcalf + VAcow) in comparison with CON, while *ELK1*, *ETV3*, *TSC22D3*, *ZZZ3, SMAD5, CDC5L,* and *NFYC* were downregulated in VA-treated groups (VAcalf + VAcow) compared to CON (Appendix C). Functional enrichment analysis highlighted these terms as overrepresented (Figure 4): regulation of canonical Wnt signaling pathway (GO:0060828, $P < 0.01$), response to BMP (GO:0071772, $P < 0.01$), cellular response to BMP stimulus (GO:0071773, $P < 0.01$), pattern specification process (GO:0007389, $P < 0.01$), regulation of Wnt signaling pathway (GO:0030111, $P = 0.01$), tube development (GO:0035295, $P = 0.01$), growth factor activity (GO:0008083, $P = 0.02$), canonical Wnt signaling pathway (GO:0060070, $P = 0.03$), anatomical structure formation involved in morphogenesis (GO:0048646, $P = 0.05$), humoral immune response (GO:0006959, $P = 0.05$), and regulation of cellular component biogenesis (GO:0044087, $P = 0.09$) in Gene Ontology database (Appendix D). Additionally, lipid and atherosclerosis (bta05417, $P < 0.01$), FoxO signaling pathway (bta04068, $P < 0.01$), MAPK signaling pathway (bta04010, $P = 0.04$), TGF-beta signaling pathway (bta04350, $P < 0.01$), HIF-1 signaling pathway (bta04066, $P < 0.01$), sphingolipid signaling pathway (bta04071, $P < 0.01$), signaling pathways regulating pluripotency of stem cells (bta04550, $P = 0.01$), PPAR signaling pathway (bta03320, $P = 0.01$), platelet activation (bta04611, $P = 0.04$), and AMPK signaling pathway (bta04152, $P = 0.04$) were enriched spotlight pathways in KEGG (Appendix E). Moreover, smear plot was utilized to visualize the direct impact of TFs on gene expression. Here some targeted genes are presented: TF *SREBF1* targeted genes as *AGP2*, *ZC3H12C*, *YPEL5*, *PPP1R27*, *CRYAB*, *RPLP1*, *NFIX*, *HJV*, *ACO2*, *MYOM2*, *LOC101905499*, *ENTREP1*, *MSS51*, *MLF1*, and *LOC113633884* in VA groups (VAcalf + VAcow) and *EEF1A2*, *ADCY2*, *JSRP1*, and *LOC101906743* genes in CON (Figure 5); TF *PPARD* targeted *HMOX2*, *AMD1*, *XIRP1*, *PDK4*, *HSPA1A*, *H19*, *MYH2*, *CA3*, *FOXO6*, *CRY2*, *FOS*, *WFIKKN2*, *FABP4*, and *ADIPOQ* genes in VA groups (VAcalf + VAcow) and *LOC132346930* gene in CON (Figure 6); TF *PPARA* targeted *HBB*, *WFIKKN2*, *LPL*, *CA3*, *LOC112442408*, *SDHA*, *EEF1A2*, *PHETA1*, and *BTNL9* genes in VA groups (VAcalf + VAcow) and *GSTM3*, *CRY2*, *PPFIBP2*, *PTDSS2*, *PFKM*, *LMO7*, *CRYAB*, *FLNC*, *KLHL40*, *HIPK2*, *RNF115*, *ESCO1*, *HABP4*, and *GNPTAB* genes in CON (Figure 7); TF *MYF6* targeted *ESCO1*, *ADAM19*, *CCNG1*, *RPLP1*, *LOC616200*, *HSPB6*, *OBSCN*, *TNNC2*, *MYOZ2*, *MYH7*, and *CKM*

genes in VA groups (VAcalf + VAcow) and *CRY2* and *IP6K3* genes in CON (Figure 8); TF *CREBZF* targeted *ADIPOQ*, *FOS*, *MCAM*, *ALDH1A1*, *NDUFB8*, *SMIM19*, *GOT2*, *GPI*, *MYBPC2*, *SCD4*, *EIF4G2*, *TRIM63*, *FLNC*, *PDK4*, *KLHL40*, *XIRP1*, and *PPP1R15A* genes in VA groups (VAcalf + VAcow) and *ABAT*, *CRY2*, *VAPB*, *LMOD2*, and *ATP1B4* genes in CON (Figure 9); TF *CEBPG* targeted *LOC101904174*, *MYL2*, *PADI2*, *CAPN3*, *VCL*, *RCAN1*, *RRAD*, *MEF2C*, *SMAD1*, and *ZC3H12C* genes in VA groups (VAcalf + VAcow) and *CTNNBIP1*, *CLU*, *MYH3*, *ALDH1A1*, *SPARC*, *USP25*, *SDHA*, *MBNL1*, *H19*, and *PDE7A* genes in CON (Figure 10).

On the other hand, when contrasting VAcalf vs VAcow, PCA showed a contribution of 55.3% of the variability in Principal Component 1 and 16.4% in Principal Component 2 (Figure 11). Also, transcriptomic profiling showed 14,221 expressed genes including 189 DEGs (FDR < 0.10; Appendix F) were identified, where 73 were upregulated 116 were downregulated for VAcow compared to VAcalf. Also, *LOC132345999*, *LOC112442229*, *LOC132343087*, and *LOC104975489* were exclusively identified to VAcalf, whereas *HTR1F*, *LOC104971346*, *NUGGC*, *SNTG2*, *ODAD2*, *LOC132342506*, *TMEM132B*, *TRPV3*, *LOC112443200*, and *LOC112443867* were identified as exclusive genes in VAcow. Additionally, the key TFs *BCL6* and *GABPA* were identified and were upregulated in VAcalf group compared to VAcow (Appendix G). Functional enrichment analysis highlighted these terms as overrepresented (Figure 12): protein tyrosine/threonine phosphatase activity (GO:0008330, $P < 0.01$), MAP kinase tyrosine/serine/threonine phosphatase activity (GO:0017017, $P < 0.01$), circadian regulation of gene expression (GO:0032922, $P < 0.01$), MAP kinase phosphatase activity (GO:0033549, $P < 0.01$), transcription factor binding (GO:0008134, $P < 0.01$), cellular response to organonitrogen compound (GO:0071417, $P < 0.01$), negative regulation of intracellular signal transduction (GO:1902532, $P < 0.01$), cellular response to peptide hormone stimulus (GO:0071375, $P < 0.01$), cellular response to peptide (GO:1901653, $P < 0.01$), negative regulation of MAPK cascade (GO:0043409, $P < 0.01$), RNA polymerase II-specific DNA-binding transcription factor binding (GO:0061629, $P < 0.01$), response to peptide hormone (GO:0043434, $P < 0.01$), response to peptide (GO:1901652, $P < 0.01$), DNA-binding transcription factor binding (GO:0140297, $P < 0.01$), cellular response to insulin stimulus (GO:0032869, $P < 0.01$), regulation of MAPK cascade (GO:0043408, $P < 0.01$), MAPK cascade (GO:0000165, $P = 0.01$), cellular response to hormone stimulus (GO:0032870, $P = 0.01$), cellular response to endogenous stimulus (GO:0071495, $P = 0.01$), muscle structure development (GO:0061061, $P = 0.01$), myofibril (GO:0030016, $P = 0.01$),

contractile fiber (GO:0043292, $P = 0.01$), regulation of developmental process (GO:0050793, $P = 0.02$), regulation of cell cycle (GO:0051726, $P = 0.02$), positive regulation of DNA-templated transcription (GO:0045893, $P = 0.02$), positive regulation of RNA biosynthetic process (GO:1902680, $P = 0.02$), positive regulation of RNA metabolic process (GO:0051254, $P = 0.06$), positive regulation of transcription by RNA polymerase II (GO:0045944, $P = 0.07$), regulation of cell differentiation (GO:0045595, $P = 0.08$), tissue development (GO:0009888, $P = 0.09$), and microtubule (GO:0005874, $P = 0.09$) in Gene Ontology database (Appendix H). Furthermore, cytoskeleton in muscle cells (bta04820, $P < 0.01$), ABC transporters (bta02010, $P = 0.04$), FoxO signaling pathway (bta04068, $P = 0.02$), motor proteins (bta04814, $P = 0.02$), and cAMP signaling pathway (bta04024, $P = 0.07$) were enriched spotlight pathways in KEGG (Appendix I). Additionally, here are some targeted genes by the TF observed in the smear plot: TF *ADK* targeted genes as *TTC9*, *GAB2*, *MYH14*, *DUSP26*, *SPEG*, *EGLN1*, *CFL2*, *CASTOR2*, *MAP4*, *ANK1*, *SYNM*, *HSPB8*, *CNBP*, *SYPL2*, *MYL1*, *CCDC88C*, *NDUFS11*, *ATP5MC3*, *NMRK2*, *CARNMT1*, *ACAT1*, *EGLN3*, *MYL9*, *FABP3*, *ETNPPL*, *FRZB*, *ID1*, *IGDCC4*, *DHDH*, *METTL21E*, *ST8SIA5*, and *NQO1* in VAcalf (Figure 13); TF *PPARA* targeted genes as *FKBP4*, *CMYA5*, *SYNM*, *LOC112442545*, *PARVB*, *SDHA*, *PHKA1*, *ADSS1*, *ASB12*, *PGP*, *MYL9*, *TMT1A*, *CARNS1*, *DHDH*, *LOC100851323*, *DHRS4* in VAcalf (Figure 14); TF *CEBPG* targeted genes as *ZMYM4*, *TRD7*, *P4HA2*, *LRP4*, *C11H2orf68*, *EFNB2*, *MYOD1*, *DUSP29*, *INTS8*, *ABRA*, *DNAJB4*, *GYG1*, *SQSTM1*, *NNT*, *PLN*, *AK1*, *PYGM*, *CAST*, *DEK*, *ALDH2*, *XIRP2*, *C28H10orf71*, *PADI2*, *ACACB*, *ARID5B*, *FOXP1*, *LOC112447844* in VAcalf (Figure 15).

10.1.4 Gene expression at weaning (RT-qPCR)

No treatment differences were observed ($P > 0.17$) in *RARB*, *RARG*, *RXRB*, *RXRG*, *FABP4*, *MTOR*, *PPARG*, *MYOD1*, *PDGFRA*, *MYH1*, *MYH7*, *MYH2*, *MYOG*, *MYF6*, *GHR*, *WNT*, and *PPARD* expressions at weaning (Table 4). When contrasting CON with VA-treated groups (VAcalf + VAcow), *RARA* and *RXRA* expressions were greater ($P \leq 0.04$) and *ZNF423* tended to be greater ($P = 0.06$) in VA-treated groups than in CON male calves. Additionally, when contrasting VAcalf with VAcow group, VAcow male calves presented greater ($P \leq 0.02$) expressions of *RARA*, *MYF5*, *PAX7*, *CEBPA*, *IGF1R* and *DLK1*, and a tendency for *ZNF423* and *VEGFA* to be greater ($P = 0.07$) compared to VAcalf male calves.

10.2 Trial 2 - Female calves

10.2.1 Offspring performance

No significant treatment differences were found for cow initial BW, calf weight at 60 days, calf ADG, or calf BW at weaning ($P > 0.19$, Table 5). However, when contrasting VA groups with CON, CON calves tended to have higher ($P = 0.08$) BW at weaning compared to calves from VAcalf and VAcow groups. Also, when contrasting VAcalf vs VAcow group no significant differences were observed ($P > 0.12$).

10.2.2 Body ultrasound in the offspring

No significant treatment differences were observed ($P > 0.14$) in backfat thickness, muscularity index, longissimus muscle width, longissimus muscle depth, rump fat thickness, rump depth, and overall fat thickness (Table 6). VA-treated groups (VAcalf + VAcow) compared to the CON, had lower ($P = 0.05$) longissimus muscle area, and longissimus muscle ratio tended to be lower ($P = 0.07$) in VA-treated groups (VAcalf + VAcow) than in CON female calves. Also, when contrasting VAcalf with VAcow group, longissimus muscle ratio tended to be greater ($P = 0.09$) in VAcow than in VAcalf female calves.

10.2.3 Gene expression at weaning (RT-qPCR)

No treatment differences were observed ($P > 0.11$) in *ZNF423*, *RARG*, *RXRA*, *RXRB*, *RXRG*, *MYF5*, *FABP4*, *PPARG*, *PAX7*, *CEBPA*, *VEGFA*, *IGF1R*, *MYOD1*, *PDGFRA*, *MYH7*, *MYH2*, *MYOG*, *MYF6*, *GHR*, *WNT*, and *DLK1* expressions at weaning (Table 7). However, comparing VA-treated groups (VAcalf + VAcow) with CON, *MYH1* were greater ($P = 0.03$) and *PPARD* tended to be greater ($P = 0.08$) in VA-treated (VAcalf + VAcow) groups than in CON female calves. Additionally, when contrasting VAcalf with VAcow group, VAcow female calves

presented greater ($P = 0.05$) expression of *RARB* and tended to have greater ($P < 0.08$) expression of *RARA* and *MTOR* compared to VAcalf female calves.

11 Discussion

In our study, the objectives were to evaluate the effects of VA injection on muscular and adipose tissue development, growth performance during cow-calf phase, and skeletal muscle transcriptomics in Angus × Nellore cattle. It was hypothesized that VA injection in pregnant cows would be effective as VA injection in newborn calves to stimuli angiogenesis and adipogenesis. This hypothesis was constructed based on previous results, where VA injection and administration during late pregnancy or early calf live stimulated angiogenesis and adipogenesis, triggering modulation of IMF content (Harris et al., 2018; Wang et al., 2018; Jo et al., 2020; Peng et al., 2020; Maciel et al., 2022; Dean et al., 2024). However, data on the effects of VA injection specifically during late pregnancy remain scarce. Thus, our first investigation focused on the earliest skeletal muscle transcriptomics, using a large-scale approach with RNA-seq technique to uncover possible key pathways modulation by VA.

Our findings provide critical insights into the impact of VA injection on early developmental processes, including growth performance, body composition, and skeletal muscle transcriptomics. The observed increase in male calves ADG and BW at weaning in VA-treated groups compared to CON supports the premise that VA administration might enhance calf performance by modulating key physiological processes as myogenesis, corroborating with previous data published by Wang et al. (2018) and Peng et al. (2020). On the other hand, some other research did not find any improvement in growth performance when injecting or administering VA in cattle (Jo et al., 2020b; Maciel et al., 2022b; Dean et al., 2024). In this sense, it is important to emphasize that the studies that observed increase in ADG and BW were those where VA was administered orally for approximately 60 d in calves' early life or injected in two doses also in early life. So, it appears that there is a timing-dependent relationship between VA and its effects on growth, where would be necessary a booster dose or an extended administration to influence growth. The enriched pathways, including regulation of canonical Wnt signaling pathway (GO:0060828), response to BMP (GO:0071772), cellular response to BMP stimulus (GO:0071773), pattern specification process (GO:0007389), regulation of Wnt signaling pathway (GO:0030111), growth factor activity (GO:0008083), canonical Wnt signaling pathway (GO:0060070), and FoxO signaling pathway (bta04068), together with targeted genes by the TFs in VA-treated calves, such as *SREBF1* (*YPEL5* and *NFIX*), *PPARD* (*MYH2*), *MYF6* (*MYOZ2* and

MYH7), *CREBZF* (*MYBPC2* and *TRIM63*), and *CEBPG* (*MYL2*), support the changes in ADG and BW phenotypes, showing the influence of VA on myogenic pathway and muscle tissue metabolism. Notably, the identification of *FZD10* exclusively in VA-treated groups highlights its potential involvement in *Wnt* signaling and myogenesis. In summary, VA's effects on growth performance were previous discussed and agrees with our findings regarding key myogenic factors upregulation (Harris et al., 2018; Peng et al., 2020). Still, VA may have influenced growth performance by modulation of animal immune response, supported by the enriched pathway of humoral immune response (GO:0006959), and the downregulation of genes related to cellular stress and immune response (*SMAD5*, *NFYC*) suggesting a potentially protective role of vitamin A during early development. Conversely, based on the lower calf BW at weaning in VA-treated female calves in comparison with CON female calves, it seems that the effect of VA on growth performance is sex-dependent but a clear reason for this remains unclear and require a deep focused investigation in specific epigenetics effects such as DNA methylation, and possibly VA impact in reproductive tract early development.

Transcriptomics data also revealed significant modulation of pathways related to growth, angiogenesis, and adipogenesis in VA-treated groups. Key TFs such as *ESRRA*, *SMAD5*, and *ETS1*, which were more frequent in VAcalf + VAcow groups, suggest the role of VA in activating signaling pathways like Wnt and BMP, also highlighting the potential impact on mitochondrial biogenesis and energy metabolism supported by enriched pathways such as AMPK signaling pathway (bta04152) and FoxO signaling pathway (bta04068). On the other hand, the enriched pathways, including Wnt signaling pathway (GO:0060828), response to BMP (GO:0071772), cellular response to BMP stimulus (GO:0071773), regulation of Wnt signaling pathway (GO:0030111), and canonical Wnt signaling pathway (GO:0060070) emphasize VA's regulatory role in muscle and adipose tissue development. These pathways are known to orchestrate the balance between myogenic and adipogenic lineage commitment, which is crucial for IMF deposition and overall muscle quality. In addition, VA-treated groups also presented enriched pathways, including TGF-beta signaling pathway (bta04350), signaling pathways regulating pluripotency of stem cells (bta04550), PPAR signaling pathway (bta03320), and AMPK signaling pathway (bta04152). Besides enriched pathways, it was identified TFs targeting specific genes in VA-treated groups, as *CREBZF* (*ADIPOQ* and *FOS*), *PPARD* (*ADIPOQ*, *FOS*, and *FABP4*). These findings, especially AMPK pathway plus *FOS* and *FABP4* targeting by TFs, and *TGFB* and *CEBPA*

DEGs upregulation in VA-treated groups gather an implication of a possible modulation of angiogenic and adipogenic pathways, that would contribute to marbling potential later in life. On the other hand, when contrasting VAcow vs VAcalf groups in the gene expression at weaning, we observed an upregulation in VAcow male calves in key genes such as *ZNF423*, *CEBPA*, *VEGFA*, *DLK1,* plus the dramatically upregulation in *RARA*. This slight shift in molecular signaling raises a hypothesis of a possible better potential of injecting VA in pregnant cows in comparison with two injections in newborn calves to modulate angiogenesis and adipogenesis.

The distinct transcriptomic profiles between VAcalf and VAcow groups underscore the influence of the timing of VA administration. While VAcalf male calves showed exclusive expression of genes like *LOC132345999* and *HTR1F*, suggesting a direct effect on early calf development, VAcow male calves exhibited upregulation of genes associated with maternal programming effects. The identification of pathways such as the MAPK signaling cascade (GO:0000165) in VAcow highlights VA's role in modulating stress and growth responses at the cellular level. Moreover, TFs such as *BCL6* and *GABPA*, which were more frequent in VAcalf, suggest a potential mechanism through which VA influences specific gene networks during postnatal development. These findings raise the idea that maternal VA supplementation may have long-lasting effects on offspring, potentially through epigenetic modifications or altered transcriptional activity. In this regard, it is important to highlight that transcriptomics profiling performed in this study show modulation of the first VA injection in VAcalf group since their second injection was at 60d of age. The timing of second injection in this group, later than observed in previous studies (Maciel et al., 2022; Harris et al., 2018), can have triggered in re-modulation of key genes and no differences were observed in the gene expression of some expected genes at weaning.

The trend for a lower muscularity index in VA-treated male calves can be clarified due to the higher BW at the time of the evaluation (weaning), since the muscularity index represents LMA in proportion to 100 kg of BW. In addition, the increased rump depth observed in VA-treated male calves compared to CON male calves can be explained by the earlier development in the muscles close to body extremities compared to other muscles. However, the explanation for the lower LMA and longissimus muscle ratio in VA-treated female cale compared to CON female calves remains unclear and need to be further investigated.

From a practical standpoint, our results suggest implications for optimizing growth in beef cattle. The timing and dosage of VA administration appear critical, as evidenced by the nuanced differences between VAcalf and VAcow groups. Future research should focus on the long-term effects of different timing of VA injection. Additionally, the integration of transcriptomic data with metabolomic and proteomic analyses could provide a more comprehensive understanding of VA's biological effects. Also, exploring epigenetic modifications and their transgenerational impacts may also yield valuable insights into the potential for maternal supplementation to influence offspring performance in subsequent generations.

12 Conclusion

In summary, our findings suggest that VA injection during late pregnancy or early postnatal life enhance growth performance and modulate key developmental pathways in Angus × Nellore male calves. The observed transcriptomic changes in keys TFs and pathways provide a molecular outline for understanding VA's effects on muscle and adipose tissue development, highlighting its potential as a management tool in beef production systems.

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Tables and figures

Table 1. Primers sequences (5' – 3') used in the RT-qPCR analysis

Gene symbol	NCBI Accession Number	Primer sequence
ACTB	NM_173979.3	F: GTCCACCTTCCAGCAGATGT R: CAGTCCGCCTAGAAGCATTT
CEBPA	NM_176784.2	F: GGCAACGACTTTGACTACCC R: GGTCATTGTCACTGGTCAGC
DLK1	NM_174037.2	F: GGATTCTGCGACGATGACAG R: TGTGGTTGTAGCGCAGATTG
FABP4	NM_174314.2	F: GCTGCACTTCTTTCTCACCT R: TGGACAACGTATCCAGCAGA
GAPDH	NM_001034034.2	F: CACAGTCAAGGCAGAGAACG R: ATTCTCAGTGTGGCGGAGAT
GHR	NM_176608.1	F: CCAGCTTTCCTTGTCAGAGC R: GAAGTTAGCTTGGCAGGGTG
IGF1R	NM_001244612.1	F: GACTCCTGTTTTTCTCCGCC R: CAGTTCCGAAGCGATGTTGT
MTOR	XM_002694043.7	F: GCACATGCAGCACTTTGTTC R: GGATTTTGTTGGCTGCGTTG
MYF5	NM_174116.1	F: AGCGTCTACTGTCCTGATGTA R: GTTGGTGATCCGATCCACTATG
MYF6	NM_181811.2	F: TACCCTGCAGCCCTTAGAAG R: TACAAGCCCAAAGCCGAAAG
MYH1	NM_174117.1	F: CCACTTTGTACGCTGCATCA R: GTGGCGTGTTTCTCCTTCTC
MYH2	NM_001166227.1	F: TCGCAACGCAGAAGAGAAAG F: AGCATCAGGACACGATCACT
MYH7	NM_174727.1	F: CAGAAGAACGCTGTGACCAG R: TCTTGTTCTCGCGCTTGAAG

MYOD1	NM_001040478.2	F: CCTGAGCAAAGTCAACGAGG R: GTAAATCGGGTTGGGGTTCG
MYOG	NM_001111325.1	F: CACAGATGCCACCACTTCTG R: TTCAGCACAGAGACCTTGGT
PAX7	XM_027522151.1	F: GACCCTCCAGTTTCCTCATTT R: CCAGTTATGAAACCCTCCTCTG
PDGFRA	XM_024993021.1	F: CGAGATGGGAGTTTCCAAGAG R: GACAGGTTGAGACCGACTTAAT
PPARD	XM_024983411.1	F: CAGCTACACAGGGCTTCTT R: CACTTGTTGCGGTTCTTCTTC
PPARG	NM_181024.2	F: GACATCAAGCCCTTCACCAC G: GGGGACTGATGTGCTTGAAC
RARA	NM_001014942.4	F: TCTGCCTCATCTGTGGAGAC R: CTGGCATTTGCTGGTGATGA
RARB	XM_059882389.1	F: TCTGTGAGAATCCTGGGAGC R: CCAGCAGTGGTTCTTGTAGC
RARG	NM_001130756.1	F: GCAAGTACACCACGAACTCC R: ATGAGCATCCTGGGGAACAT
RXRA	NM_001304343.1	F: CGAGGTCCTCTGTTTGCAAG R: CTGCTTCACTCTGCTGACAC
RXRB	NM_001083640.1	F: CTGTGACCAACATCTGCCAG R: CTCCATGAGGAAGGTGTCGA
RXRG	NM_001075408.1	F: CAGGAAAGCACTACGGTGTG R: GAAACCGAGCGATGGGAAAA
VEGFA	NM_174216.2	F: ACTTGAGTTGGGAGGAGAATG R: GCTGCCGTAAGAGGGATAAA
WNT1	NM_001114191.1	F: AGAGTCTGCAGCTGGTACTC R: CTGTACGTGCAGAAGTTGGG
ZNF423	NM_001101893.1	F: CCGTTCAAGTGCACCTACTG R: GGACGAAGACTGTGAAGCAC

Actin beta (*ACTB*), CCAAT Enhancer binding protein alpha (*CEBPA*), Delta like non-canonical notch ligand 1 (*DLK1*), Fatty acid binding protein 4 (*FABP4*), Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), Growth hormone receptor (*GHR*), Insulin like growth factor 1 receptor (*IGF1R*), Mechanistic target of rapamycin kinase (*MTOR*), Myogenic factor 5 (*MYF5*), Myogenic factor 6 (*MYF6*), Myosin heavy chain 1 (*MYH1*), Myosin heavy chain 2 (*MYH2*), Myosin heavy chain 7 (*MYH7*), Myogenic differentiation 1 (*MYOD1*), Myogenin (*MYOG*), Paired box 7 (*PAX7*), Platelet derived growth factor receptor alpha (*PDGFRA*), Peroxisome proliferator activated receptor delta (*PPARD*), Peroxisome Proliferator Activated Receptor gamma (*PPARG*), Retinoic acid receptor alpha (*RARA*), Retinoic Acid Receptor beta (*RARB*), Retinoic Acid Receptor gamma (*RARG*), Retinoid x receptor alpha (*RXRA*), Retinoid X Receptor beta (*RXRB*), Retinoid X Receptor gamma (*RXRG*), Vascular endothelial growth factor A (*VEGFA*), Wnt family member 1 (*WNT1*), Zinc finger protein 423 (*ZPF423*).

Table 2. Effects of Vitamin A injection in pregnant cows or newborn calves on calf performance during cow-calf phase – Trial 1 (male calves)

Item	Male			SEM‡	*P*-value	
	CON	VAcalf	VAcow		CON vs VAcalf+VAcowΔ	VAcalf vs VAcow†
Cow initial BW, kg	428	427	454	34.8	0.61	0.50
Calf weight 60d, kg	61.4	65.5	60.7	7.19	0.83	0.70
Calf BW at weaning, kg	204	226	243	11.0	0.02	0.40
Calf ADG, kg/d	0.561	0.632	0.716	0.0415	0.02	0.26

‡SEM = standard error of the means

Δ CON vs VAcalf + VAcow = *P*-value for the contrast between CON versus VAcalf and VAcow treatments.

†VAcalf vs VAcow = *P*-value for the contrast between VAcalf versus VAcow treatments

CON = control treatment with no VA injection, either in cows nor calves; VAcalf = VA injection in newborn calves at birth and 60 d of age; VAcow = VA injection in pregnant cows at 250 d of gestation

Table 3. Effects of Vitamin A injection in pregnant cows or newborn calves on calf body measurements at weaning – Trial 1 (male calves)

Item	Male			SEM‡	*P*-value	
	CON	VAcalf	VAcow		CON vs VAcalf+VAcowΔ	VAcalf vs VAcow†
Backfat thickness, mm	0.632	0.672	0.582	0.0812	0.98	0.68
Longissimus muscle area, cm^2	36.4	38.5	35.7	3.69	0.85	0.67
Muscularity index, cm^2/ 100 kg	17.2	16.5	14.4	8.40	0.07	0.16
Longissimus muscle width, cm	11.0	11.9	11.7	0.63	0.27	0.80
Longissimus muscle depth, cm	4.57	4.81	4.99	0.330	0.37	0.75
Longissimus muscle ratio	2.46	2.53	2.30	0.174	0.81	0.47
Rump fat thickness, mm	0.617	0.697	0.558	0.1114	0.61	0.99
Rump depth, cm	5.54	6.71	6.05	0.315	0.03	0.27
Overall fat thickness, mm	0.619	0.682	0.571	0.0917	0.94	0.49

‡SEM = standard error of the means

Δ CON vs VAcalf + VAcow = *P*-value for the contrast between CON versus VAcalf and VAcow treatments.

CON = control treatment with no VA injection, either in cows nor calves; VAcalf = VA injection in newborn calves at birth and 60 d of age; VAcow = VA injection in pregnant cows at 250 d of gestation

Table 4. Effects of Vitamin A injection in pregnant cows or newborn calves on longissimus thoracis muscle gene expression at weaning – Trial 1 (male calves)

Gene	Male			SEM‡	*P*-value	
	CON	VAcalf	VAcow		CON vs VAcalf+VAcow$^{\Delta}$	VAcalf vs VAcow†
ZNF423	1.00	1.11	4.81	1.540	0.06	0.07
RARA	1.00	32.3	245	63.90	<0.01	<0.01
RARB	1.00	1.44	0.60	0.888	0.58	0.19
RARG	1.00	0.76	0.87	0.856	0.86	0.68
RXRA	1.00	2.72	3.78	1.398	0.04	0.63
RXRB	1.00	1.29	0.43	0.827	0.48	0.31
RXRG	1.00	0.76	0.74	0.803	0.17	0.77
MYF5	1.00	0.09	2.24	0.555	0.99	0.02
FABP4	1.00	2.19	0.78	1.015	0.74	0.66
MTOR	1.00	1.06	1.53	0.890	0.88	0.71
PPARG	1.00	1.11	1.30	0.864	0.55	0.21
PAX7	1.00	0.64	3.02	0.929	0.46	<0.01
CEBPA	1.00	0.46	2.17	0.405	0.44	<0.01
VEGFA	1.00	0.06	1.27	0.559	0.98	0.10
IGF1R	1.00	0.20	2.60	0.723	0.67	<0.01
MYOD1	1.00	0.85	0.47	0.501	0.44	0.63
PDGFRA	1.00	0.84	0.64	0.729	0.30	0.72
MYH1	1.00	0.32	0.73	0.538	0.47	0.51
MYH7	1.00	2.49	0.52	1.295	0.61	0.26
MYH2	1.00	0.41	1.14	0.436	0.82	0.48
MYOG	1.00	0.27	0.99	0.355	0.77	0.44
MYF6	1.00	1.41	0.54	0.675	0.77	0.58
GHR	1.00	0.52	0.04	0.627	0.23	0.96
WNT	1.00	0.04	0.88	0.374	0.35	0.16
PPARD	1.00	0.69	0.87	0.708	0.47	0.81

DLK1	1.00	0.28	1.10	0.282	0.23	0.02

‡SEM = standard error of the means

Δ CON vs VAcalf + VAcow = *P*-value for the contrast between CON versus VAcalf and VAcow treatments

†VAcalf vs VAcow = *P*-value for the contrast between VAcalf versus VAcow treatments

CON = control treatment with no VA injection, either in cows nor calves; VAcalf = VA injection in newborn calves at birth and 60 d of age; VAcow = VA injection in pregnant cows at 250 d of gestation

Table 5. Effects of Vitamin A injection in pregnant cows or newborn calves on calf performance during cow-calf phase – Trial 2 (female calves)

Item	Female			SEM‡	*P*-value	
	CON	VAcalf	VAcow		CON vs VAcalf+VAcowΔ	VAcalf vs VAcow†
Cow initial BW, kg	409	437	454	20.2	0.13	0.56
Calf weight 60d, kg	65.6	56.7	52.3	5.92	0.14	0.59
Calf BW at weaning, kg	221	200	196	10.4	0.08	0.81
Calf ADG, kg/d	0.614	0.564	0.568	0.0312	0.21	0.93

‡SEM = standard error of the means

Δ CON vs VAcalf + VAcow = *P*-value for the contrast between CON versus VAcalf and VAcow treatments.

†VAcalf vs VAcow = *P*-value for the contrast between VAcalf versus VAcow treatments

CON = control treatment with no VA injection, either in cows nor calves; VAcalf = VA injection in newborn calves at birth and 60 d of age; VAcow = VA injection in pregnant cows at 250 d of gestation

Table 6. Effects of Vitamin A injection in pregnant cows or newborn calves on calf body measurements at weaning – Trial 2 (female calves)

Item	Female			SEM‡	*P*-value	
	CON	VAcalf	VAcow		CON vs VAcalf+VAcowΔ	VAcalf vs VAcow†
Backfat thickness, mm	0.683	0.674	0.604	0.1025	0.78	0.39
Longissimus muscle area, cm^2	36.9	31.7	30.4	2.21	0.05	0.66
Muscularity index, cm^2/ 100 kg	16.5	16.1	16.2	1.17	0.77	0.93
Longissimus muscle width, mm	11.9	10.6	11.4	0.66	0.30	0.40
Longissimus muscle depth, mm	4.44	4.73	4.35	0.176	0.63	0.13
Longissimus muscle ratio	2.69	2.25	2.61	0.146	0.07	0.09
Rump fat thickness, mm	0.651	0.710	0.833	0.148	0.37	0.74
Rump depth, mm	5.90	5.70	5.45	0.167	0.12	0.32
Overall fat thickness, mm	0.649	0.693	0.709	0.1120	0.48	0.73

‡SEM = standard error of the means

Δ CON vs VAcalf + VAcow = *P*-value for the contrast between CON versus VAcalf and VAcow treatments

†VAcalf vs VAcow = *P*-value for the contrast between VAcalf versus VAcow treatments

CON = control treatment with no VA injection, either in cows nor calves; VAcalf = VA injection in newborn calves at birth and 60 d of age; VAcow = VA injection in pregnant cows at 250 d of gestation

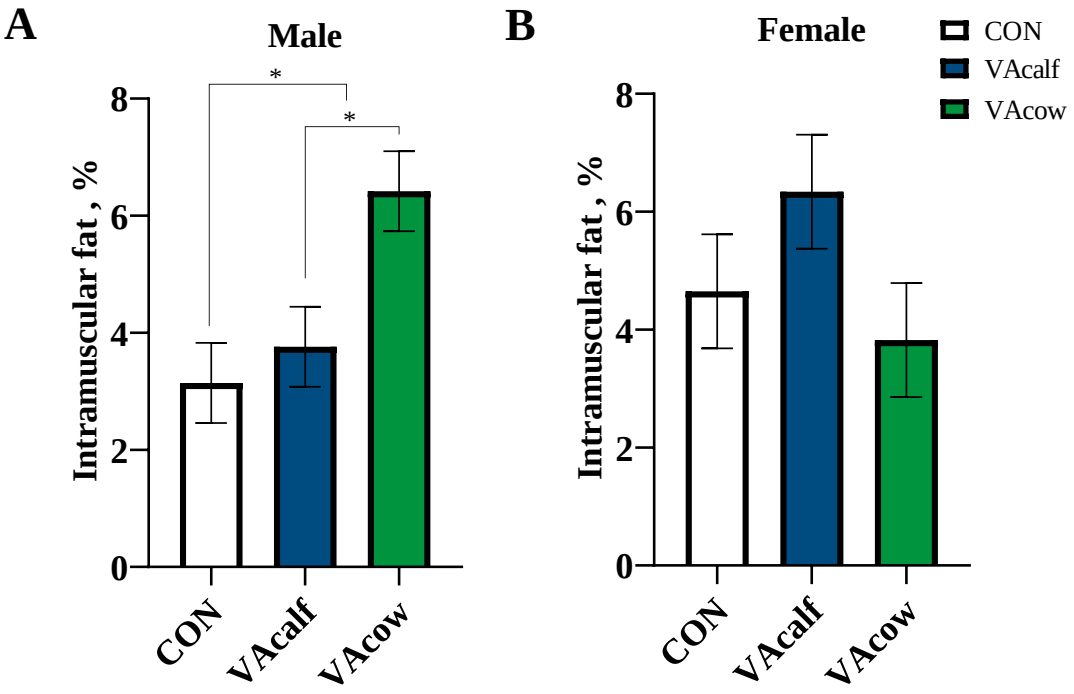


Figure 1. Effects of VA injection in pregnant cows or newborn calves on intramuscular fat content at slaughter after 207 days on feedlot diet. Intramuscular fat content was analyzed in steaks collected at slaughter after 207 d on feeding according to the 2007–04 AOAC method using FoodScan (FOSS, Hillerod, Denmark). **A:** *P*-values for Trial 1 (male calves): 0.027 (contrast between CON versus VAcalf + VAcow), and 0.050 (contrast between VAcalf versus VAcow). Standard error of the means for steers' data: 0.684. **B:** *P*-values for Trial 2 (female calves): 0.923 (contrast between CON versus VAcalf + VAcow), and 0.088 (contrast between VAcalf versus VAcow). Standard error of the means for heifers' data: 0.967. CON = control treatment with no VA injection, either in cows nor calves; VAcalf = VA injection in newborn calves at birth and 60 d of age; VAcow = VA injection in pregnant cows at 250 d of gestation.

Table 7. Effects of Vitamin A injection in pregnant cows or newborn calves on longissimus thoracis muscle gene expression at weaning – Trial 2 (female calves)

Gene	Female			SEM‡	*P*-value	
	CON	VAcalf	VAcow		CON vs VAcalf+VAcow$^{\Delta}$	VAcalf vs VAcow†
ZNF423	1.00	2.93	0.82	1.001	0.93	0.15
RARA	1.00	0.09	1.89	0.766	0.99	0.07
RARB	1.00	0.16	0.82	0.436	0.87	0.05
RARG	1.00	0.84	2.49	0.695	0.40	0.16
RXRA	1.00	0.38	0.12	0.565	0.94	0.90
RXRB	1.00	1.28	1.28	0.644	0.90	0.60
RXRG	1.00	1.51	1.03	0.360	0.67	0.33
MYF5	1.00	0.74	1.41	0.335	0.56	0.16
FABP4	1.00	0.82	0.57	0.421	0.67	0.48
MTOR	1.00	0.63	1.21	0.342	0.39	0.06
PPARG	1.00	1.12	0.85	0.402	0.62	0.50
PAX7	1.00	0.70	0.58	0.213	0.61	0.67
CEBPA	1.00	1.07	1.38	0.744	0.93	0.53
VEGFA	1.00	1.36	1.15	0.437	0.39	0.41
IGF1R	1.00	0.54	0.89	0.623	0.88	0.65
MYOD1	1.00	1.34	1.31	0.965	0.75	0.81
PDGFRA	1.00	1.12	1.59	0.279	0.22	0.20
MYH1	1.00	1.95	2.80	0.485	0.03	0.15
MYH7	1.00	0.66	0.86	0.324	0.65	0.77
MYH2	1.00	2.00	2.27	0.784	0.27	0.95
MYOG	1.00	0.89	0.50	0.260	0.46	0.26
MYF6	1.00	2.56	0.51	1.496	0.92	0.22
GHR	1.00	0.91	1.05	0.600	0.41	0.91
WNT	1.00	0.60	0.48	0.345	0.72	0.98
PPARD	1.00	2.00	3.84	0.781	0.08	0.36

DLK1	1.00	0.33	0.92	0.358	0.33	0.33

‡SEM = standard error of the means

Δ CON vs VAcalf + VAcow = *P*-value for the contrast between CON versus VAcalf and VAcow treatments

†VAcalf vs VAcow = *P*-value for the contrast between VAcalf versus VAcow treatments

CON = control treatment with no VA injection, either in cows nor calves; VAcalf = VA injection in newborn calves at birth and 60 d of age; VAcow = VA injection in pregnant cows at 250 d of gestation

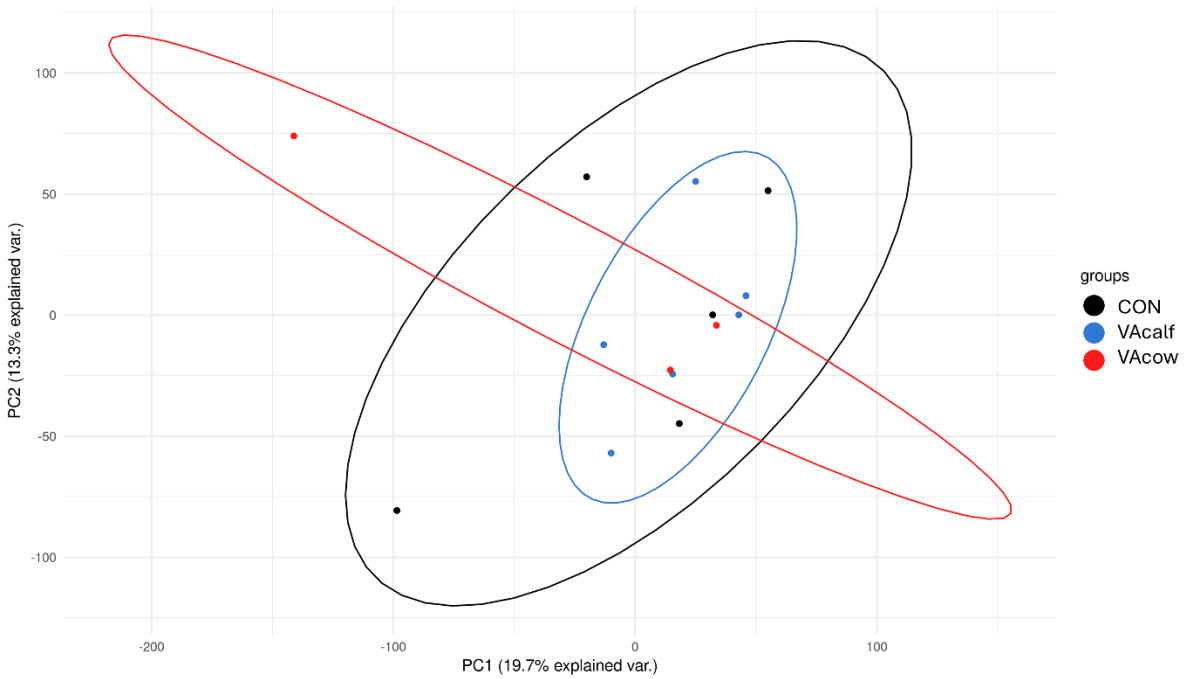


Figure 2. Principal component analysis (PCA) scatter plot. The horizontal axis represents the first principal component (PC1), and the vertical axis represents the second principal component (PC2). CON = control treatment with no VA injection, either in cows nor calves; VAcalf = VA injection in newborn calves at birth and 60 d of age; VAcow = VA injection in pregnant cows at 250 d of gestation.

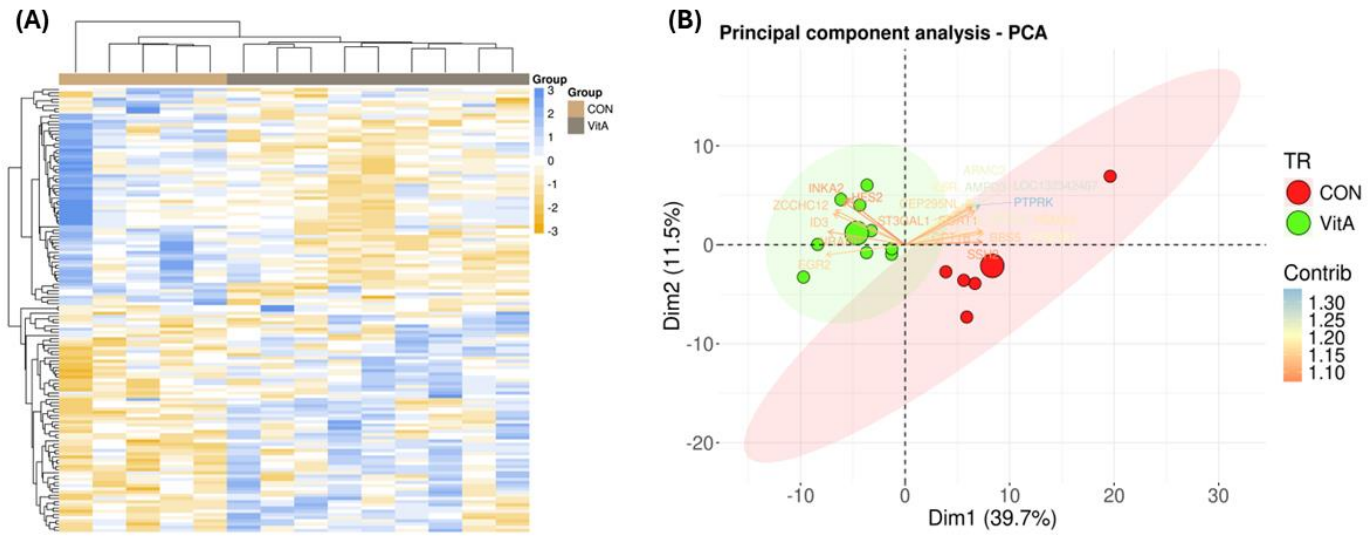


Figure 3. Heatmap graph (A) and Principal Component Analysis (PCA) scatter plot (B) based on differentially expressed genes (DEGs) and transcription factors (TFs) contrasting VA-treated groups (VAcalf + VAcow) vs CON. CON = control treatment with no VA injection, either in cows nor calves; VAcalf = VA injection in newborn calves at birth and 60 d of age; VAcow = VA injection in pregnant cows at 250 d of gestation.

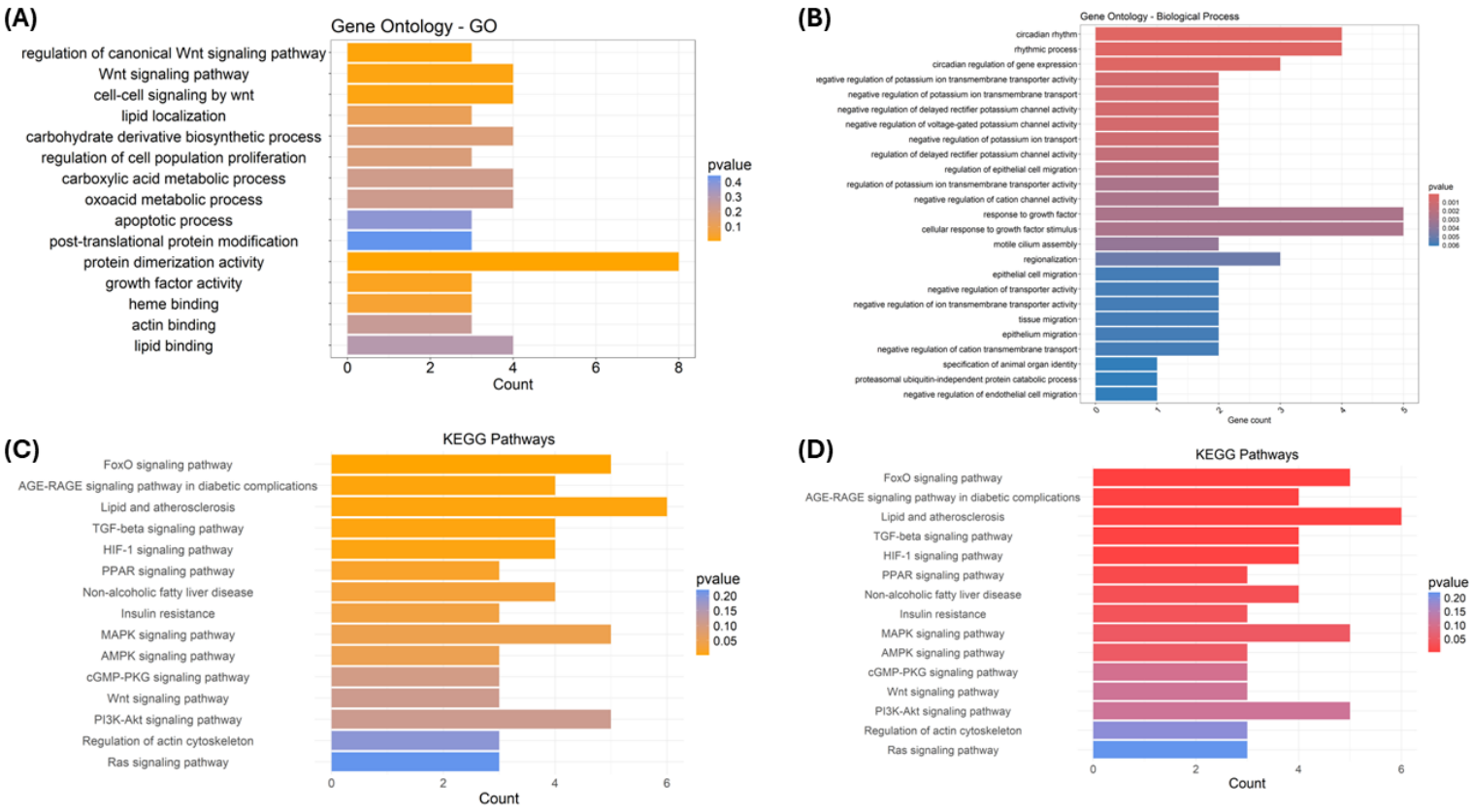


Figure 4. Bar plot graph with the main ontological terms highlighted in gene ontology (GO) (A-B) and with the main pathways highlighted in Kyoto Encyclopedia of Genes and Genomes (KEGG) (C-D) contrasting VA-treated groups (VAcalf + VAcow) vs CON. CON = control treatment with no VA injection, either in cows nor calves; VAcalf = VA injection in newborn calves at birth and 60 d of age; VAcow = VA injection in pregnant cows at 250 d of gestation.

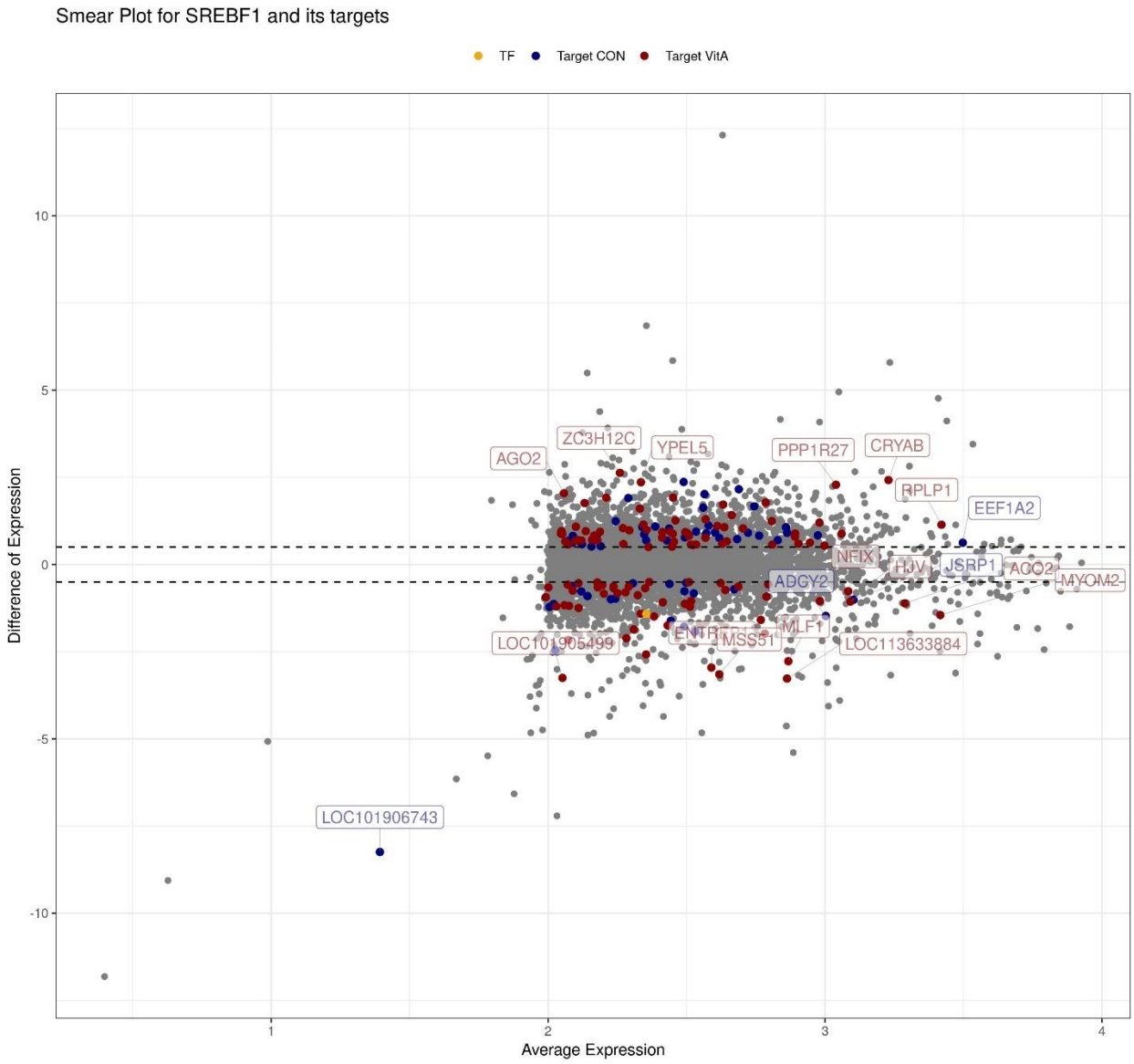


Figure 5. Smearplot illustrating the impact of the transcription factor *SREBF1* on gene expression in CON and VA-treated groups (VAcalf + VAcow) groups. Differentially expressed genes (DEGs) are highlighted in red for VA-treated groups (VAcalf + VAcow) groups and in blue for CON, representing genes directly regulated by *SREBF1*. Non-significant genes are shown in gray. CON = control treatment with no VA injection, either in cows nor calves; VAcalf = VA injection in newborn calves at birth and 60 d of age; VAcow = VA injection in pregnant cows at 250 d of gestation.

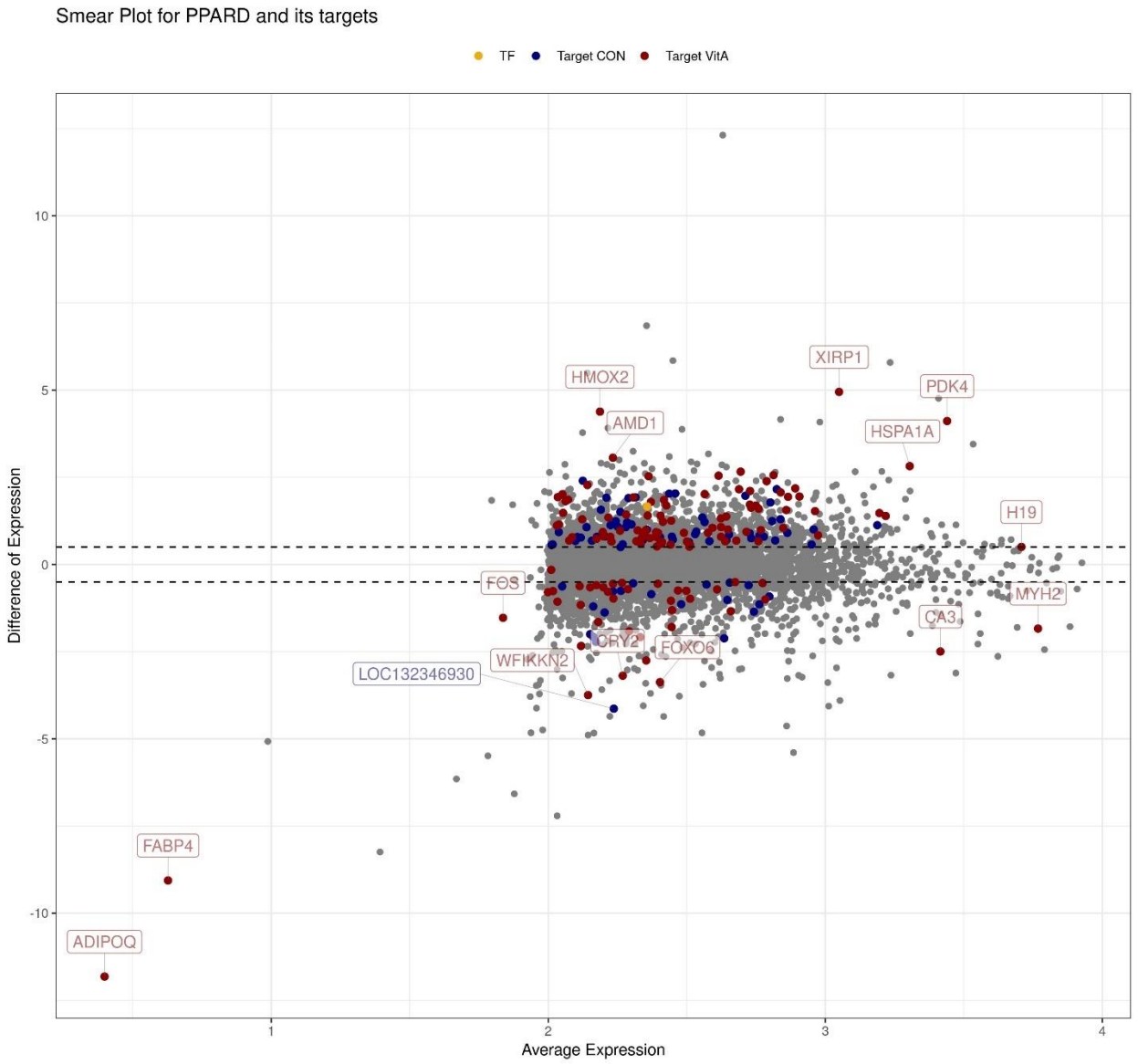


Figure 6. Smearplot illustrating the impact of the transcription factor *PPARD* on gene expression in CON and VA-treated groups (VAcalf + VAcow) groups. Differentially expressed genes (DEGs) are highlighted in red for VA-treated groups (VAcalf + VAcow) groups and in blue for CON, representing genes directly regulated by *PPARD*. Non-significant genes are shown in gray. CON = control treatment with no VA injection, either in cows nor calves; VAcalf = VA injection in newborn calves at birth and 60 d of age; VAcow = VA injection in pregnant cows at 250 d of gestation.

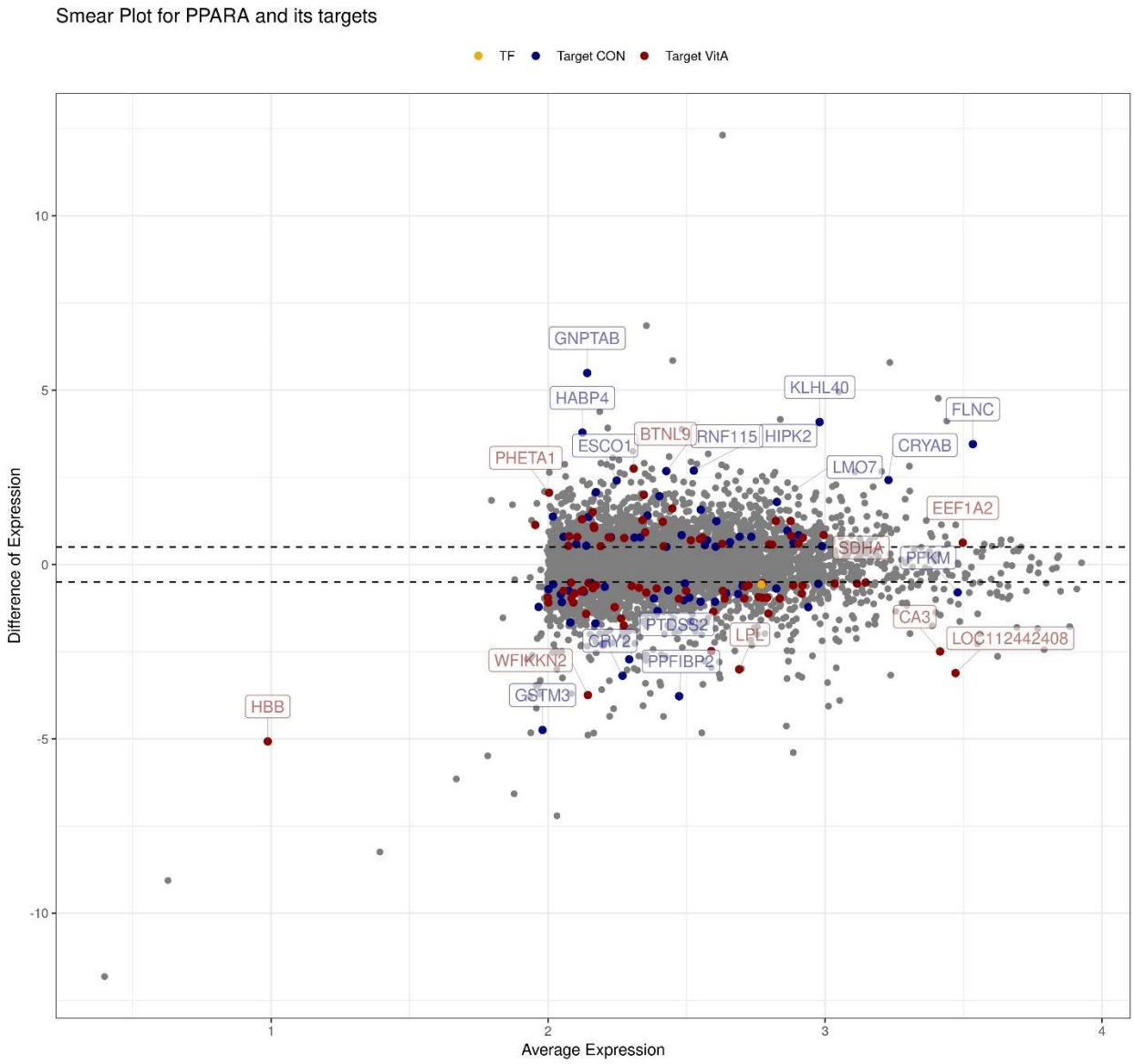


Figure 7. Smearplot illustrating the impact of the transcription factor *PPARA* on gene expression in CON and VA-treated groups (VAcalf + VAcow) groups. Differentially expressed genes (DEGs) are highlighted in red for VA-treated groups (VAcalf + VAcow) groups and in blue for CON, representing genes directly regulated by *PPARA*. Non-significant genes are shown in gray. CON = control treatment with no VA injection, either in cows nor calves; VAcalf = VA injection in newborn calves at birth and 60 d of age; VAcow = VA injection in pregnant cows at 250 d of gestation.

Figure 8. Smearplot illustrating the impact of the transcription factor *MYF6* on gene expression in CON and VA-treated groups (VAcalf + VAcow) groups. Differentially expressed genes (DEGs) are highlighted in red for VA-treated groups (VAcalf + VAcow) groups and in blue for CON, representing genes directly regulated by *MYF6*. Non-significant genes are shown in gray. CON = control treatment with no VA injection, either in cows nor calves; VAcalf = VA injection in newborn calves at birth and 60 d of age; VAcow = VA injection in pregnant cows at 250 d of gestation.

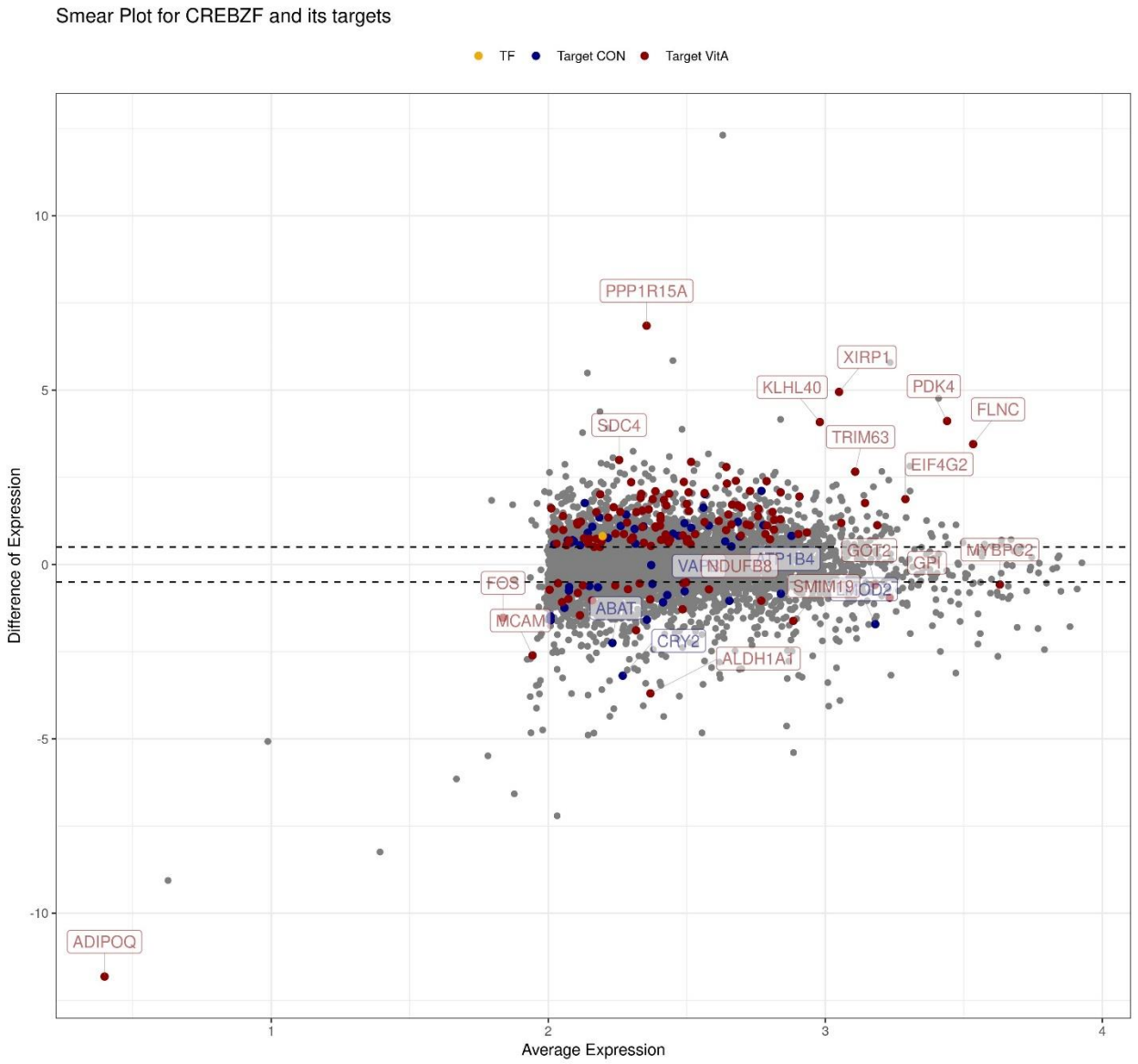


Figure 9. Smearplot illustrating the impact of the transcription factor *CREBZF* on gene expression in CON and VA-treated groups (VAcalf + VAcow) groups. Differentially expressed genes (DEGs) are highlighted in red for VA-treated groups (VAcalf + VAcow) groups and in blue for CON, representing genes directly regulated by *CREBZF*. Non-significant genes are shown in gray. CON = control treatment with no VA injection, either in cows nor calves; VAcalf = VA injection in newborn calves at birth and 60 d of age; VAcow = VA injection in pregnant cows at 250 d of gestation.

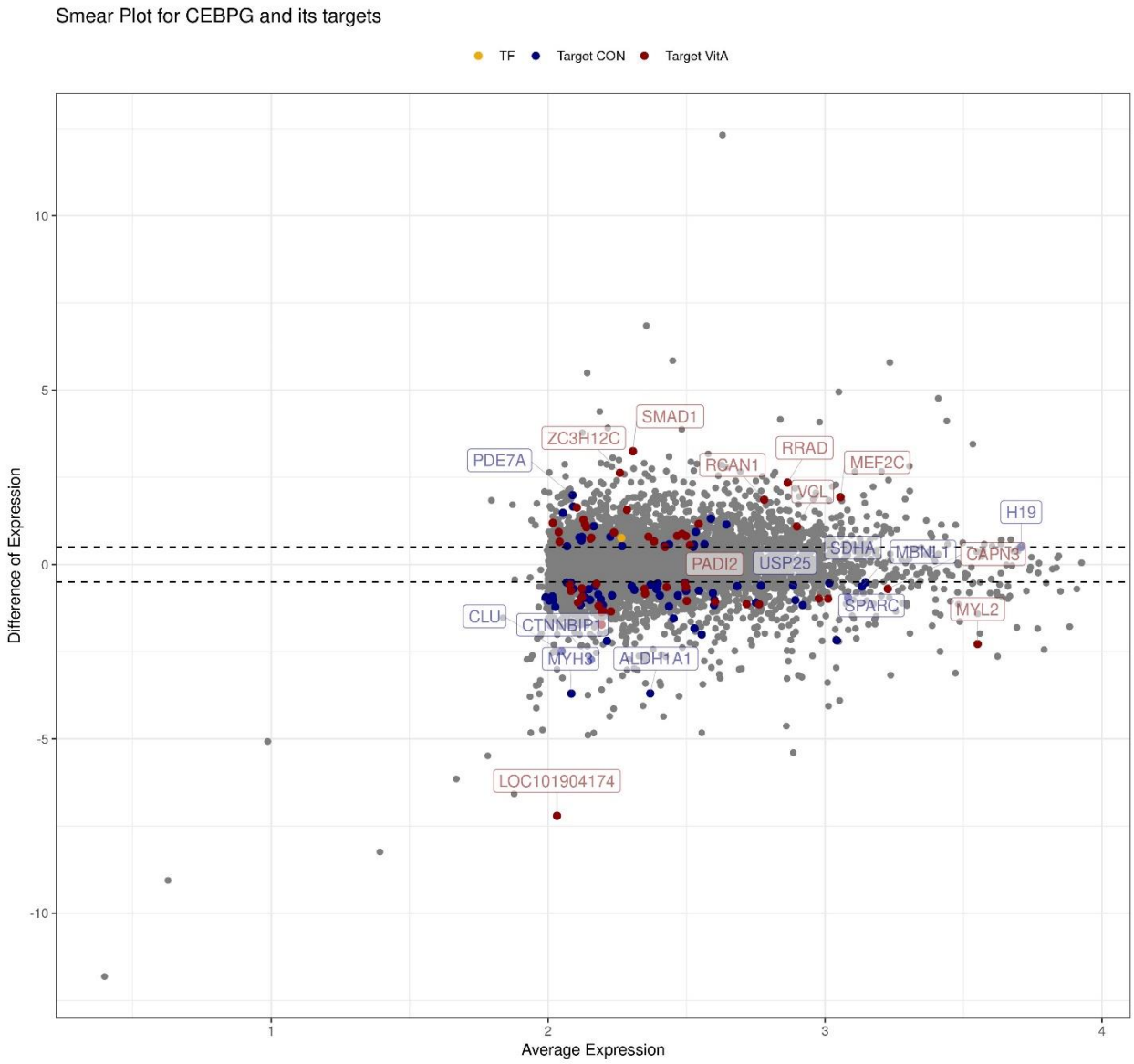


Figure 10. Smearplot illustrating the impact of the transcription factor *CEBPG* on gene expression in CON and VA-treated groups (VAcalf + VAcow) groups. Differentially expressed genes (DEGs) are highlighted in red for VA-treated groups (VAcalf + VAcow) groups and in blue for CON, representing genes directly regulated by *CEBPG*. Non-significant genes are shown in gray. CON = control treatment with no VA injection, either in cows nor calves; VAcalf = VA injection in newborn calves at birth and 60 d of age; VAcow = VA injection in pregnant cows at 250 d of gestation.

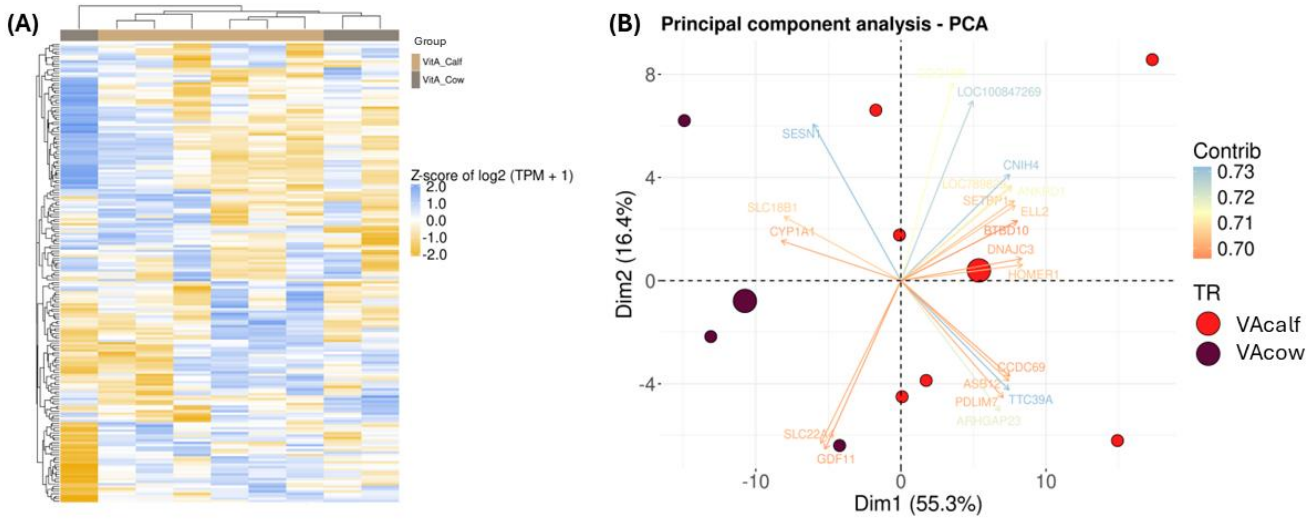


Figure 11. Heatmap graph (A) and Principal Component Analysis (PCA) scatter plot (B) based on differentially expressed genes (DEGs) and transcription factors (TFs) contrasting VAcow vs VAcalf. VAcalf = VA injection in newborn calves at birth and 60 d of age; VAcow = VA injection in pregnant cows at 250 d of gestation. CON = control treatment with no VA injection, either in cows nor calves; VAcalf = VA injection in newborn calves at birth and 60 d of age; VAcow = VA injection in pregnant cows at 250 d of gestation.

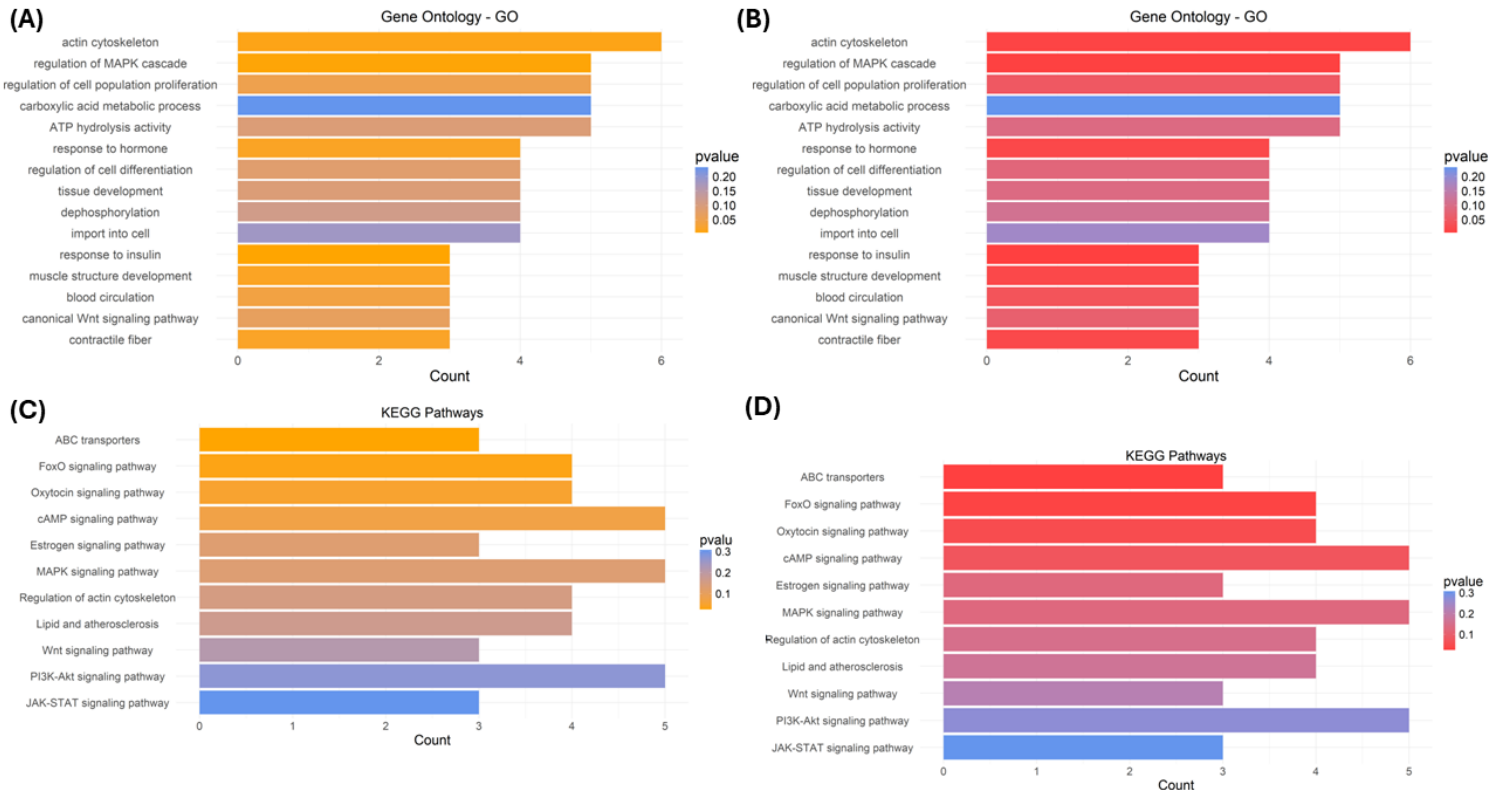


Figure 12. Bar plot graph with the main ontological terms highlighted in gene ontology (GO) (A-B) and with the main pathways highlighted in Kyoto Encyclopedia of Genes and Genomes (KEGG) (C-D) contrasting VAcow vs VAcalf. VAcalf = VA injection in newborn calves at birth and 60 d of age; VAcow = VA injection in pregnant cows at 250 d of gestation. CON = control treatment with no VA injection, either in cows nor calves; VAcalf = VA injection in newborn calves at birth and 60 d of age; VAcow = VA injection in pregnant cows at 250 d of gestation.

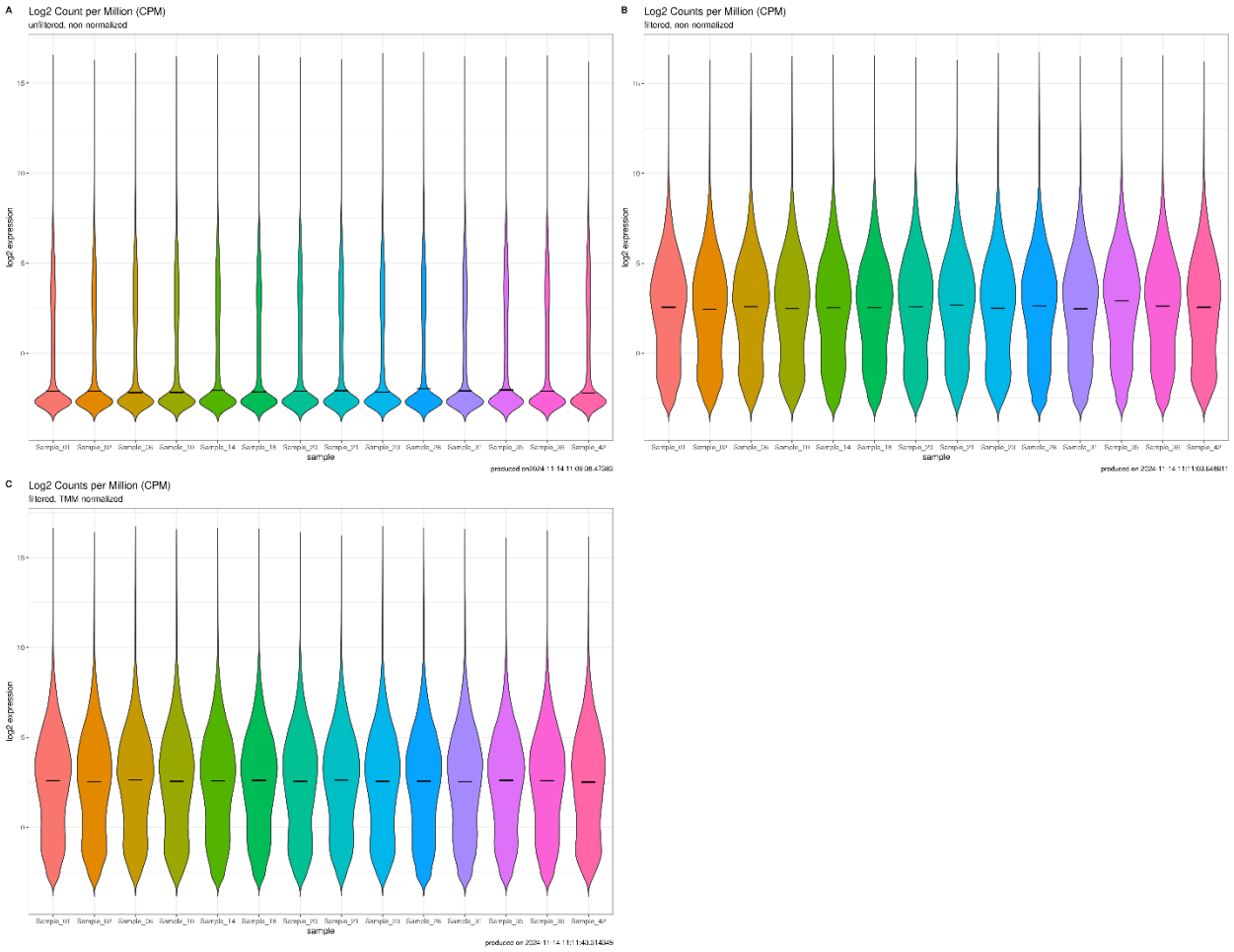
Figure 13. Smearplot illustrating the impact of the transcription factor *ADK* on gene expression in VAcow and VAcalf groups. Differentially expressed genes (DEGs) are highlighted in red for VAcow and in blue for VAcalf, representing genes directly regulated by *ADK*. Non-significant genes are shown in gray. CON = control treatment with no VA injection, either in cows nor calves; VAcalf = VA injection in newborn calves at birth and 60 d of age; VAcow = VA injection in pregnant cows at 250 d of gestation.

Figure 14. Smearplot illustrating the impact of the transcription factor *PPARA* on gene expression in VAcow and VAcalf groups. Differentially expressed genes (DEGs) are highlighted in red for VAcow and in blue for VAcalf, representing genes directly regulated by *PPARA*. Non-significant genes are shown in gray. CON = control treatment with no VA injection, either in cows nor calves; VAcalf = VA injection in newborn calves at birth and 60 d of age; VAcow = VA injection in pregnant cows at 250 d of gestation.

Figure 15. Smearplot illustrating the impact of the transcription factor *CEBPG* on gene expression in VAcow and VAcalf groups. Differentially expressed genes (DEGs) are highlighted in red for VAcow and in blue for VAcalf, representing genes directly regulated by *CEBPG*. Non-significant genes are shown in gray. CON = control treatment with no VA injection, either in cows nor calves; VAcalf = VA injection in newborn calves at birth and 60 d of age; VAcow = VA injection in pregnant cows at 250 d of gestation.

Appendixes

Appendix A – Violin plot of the sample's distribution in log2 expression. (A) Unfiltered and non-normalized data; (B) Filtered and non-normalized data; (C) Filtered and TMM normalized (Trimmed Mean of M-values) data.



Appendix B – List of differentially expressed genes (DEGs) contrasting VAcalf + VAcow vs CON

Gene symbol	log2 FoldChange	FDR
ABHD4	-0.677	<0.001
ADAMTS8	0.965	<0.001
ADAMTS9	0.518	0.007
ADIPOQ	7.285	0.054
AHI1	-0.663	0.051
AMPD3	-0.761	0.004
ANKRD1	-0.570	0.035
ANKRD33B	-0.563	0.003
ANO6	-0.538	0.014
APAF1	-0.600	0.018
ARMC2	-1.829	0.009
BBS5	-0.597	0.084
BHLHE40	-0.562	0.070
BTBD11	0.721	<0.001
BTG2	-0.709	<0.001
C23H6orf132	0.615	0.003
C2CD4B	0.998	0.091
C3H1orf94	-0.815	0.026
CCDC17	0.936	0.064
CEBPA	0.655	0.071
CEMIP2	-0.678	<0.001
CEP295NL	-1.571	0.030
CFB	0.639	0.003
CHP2	-0.566	0.080
CHRNA10	0.906	0.026
CIPC	-0.546	0.086
CPT1B	-0.546	0.081

CXCL9	0.757	<0.001
DKK2	0.555	0.024
EGR2	1.038	0.034
ESCO1	-0.535	0.047
FAM110C	0.539	0.000
FBXO10	-0.658	0.004
FBXO32	-0.945	<0.001
FGF10	-0.899	0.076
FOXO6	0.812	0.005
GNPTAB	-1.291	<0.001
GPR157	-0.537	0.051
GPR4	0.536	0.013
HABP4	-0.819	<0.001
HBB	1.886	0.004
HES2	0.823	<0.001
HIF3A	0.542	0.022
HIPK2	-0.666	0.040
HMOX1	-0.602	0.001
HNMT	-0.514	0.005
HP	-0.910	0.078
HSDL1	0.514	0.013
HSDL2	-0.666	0.025
HSPA6	-0.649	0.083
HSPB8	-0.995	0.051
ID1	0.915	0.005
ID3	0.662	0.028
IL6R	-0.651	0.089
INKA2	0.732	0.023
IRX3	0.510	0.071
IYD	-0.531	0.097
KCNAB1	-0.769	0.017

KCNE3	0.676	0.062
KLF2	0.584	0.064
KLF5	-0.541	<0.001
LDLR	0.514	0.086
LIMA1	0.519	0.020
LOC100336029	-1.034	0.007
LOC101903413	0.564	0.028
LOC101905254	-0.993	0.021
LOC101905779	-0.790	0.078
LOC101907588	1.339	0.043
LOC104968634	1.273	0.014
LOC104971845	-0.831	0.005
LOC112443504	0.509	0.047
LOC112444531	-0.690	0.022
LOC112444653	0.943	0.063
LOC112445964	0.626	0.055
LOC112446457	0.847	0.096
LOC112448430	0.722	0.072
LOC132342467	-1.955	<0.001
LOC132343590	-0.657	0.062
LOC132343659	1.046	0.040
LOC132343745	1.615	0.002
LOC132344937	0.739	0.024
LOC132345192	-0.697	0.054
LOC132345336	0.619	0.059
LPAR6	-0.645	<0.001
MALL	0.734	0.079
MAP1A	-0.886	0.013
MAP3K14	-0.962	0.055
MDK	0.501	0.019
MMRN2	0.548	0.010

MOSPD1	-0.846	0.056
MPZ	0.753	0.021
MRC1	0.671	0.060
NMB	-0.654	0.071
NOL4L	0.642	0.073
NOS3	0.561	0.042
NPPC	1.262	0.002
NPTX1	-0.573	<0.001
NRARP	0.858	0.023
OSBPL7	0.581	0.070
PCBD1	-0.967	0.004
PCMTD2	-0.561	0.002
PDZD2	0.542	0.040
PIGZ	0.533	0.087
PIK3R3	0.587	0.002
PKNOX1	-0.707	0.017
PLCB1	0.574	0.075
PLIN2	-0.844	0.002
PRODH	-0.518	<0.001
PSME4	-0.519	0.010
PTPRK	-1.055	<0.001
PTPRR	-0.798	0.091
QPCT	0.583	0.041
QPRT	0.791	0.064
RAB15	-0.962	0.037
RASA4B	0.581	0.001
RASSF8	-0.633	0.048
RBP4	1.426	0.091
REEP5	-0.524	0.049
RGS16	1.009	0.067
RGS3	0.513	0.047

RNF115	-0.575	0.014
S1PR3	0.572	0.048
SFRP5	0.621	0.002
SLC16A7	1.697	0.031
SLC41A2	0.834	0.089
SLC7A8	-0.523	0.040
SMAD1	-0.580	0.001
SORT1	-0.522	<0.001
SSH2	-0.518	<0.001
ST3GAL1	-1.042	0.087
TAGLN3	1.064	0.041
TGFB3	0.531	0.062
TMEM100	0.612	<0.001
TMEM120B	0.507	<0.001
TMEM86A	-0.803	0.065
UNC80	1.696	0.077
WLS	-0.765	0.059
ZCCHC12	1.369	0.002
ZNF385A	0.666	0.010

Appendix C - List of co-expressed genes and transcription factors (CeTF) contrasting VAcalf + VAcow vs CON

Transcription factor	Average expression	Regulatory impact factors 1	Regulatory impact factors 2	Frequency in CON	Frequency in VA	Frequency difference
ELK1	5.799	-0.677	2.163	132	76	56
ETV3	4.029	-0.309	2.098	109	90	19
TSC22D3	7.435	2.440	1.694	103	115	-12
ESRRA	6.950	2.568	1.658	128	144	-16
HOXC9	5.437	2.063	0.213	59	93	-34
ETS1	5.800	2.673	0.804	94	157	-63
ZZZ3	4.195	2.453	0.661	73	142	-69
NFIX	8.492	2.183	0.585	97	180	-83
SMAD5	4.914	3.205	-1.144	59	210	-151
ZFP64	4.333	2.407	-0.083	83	244	-161
E2F4	4.419	2.504	0.076	83	273	-190
CDC5L	5.171	-0.123	-2.260	52	274	-222
ZIC1	3.941	0.015	-2.172	34	279	-245
NFYC	5.265	1.040	-2.358	43	363	-320

Appendix D – List of overrepresented enriched pathways contrasting VAcalf + VAcow vs CON based on Gene Ontology (GO) database https://docs.google.com/spreadsheets/d/1wjaNxd2M0yYVf8Fi6ZpiiL-k7y-Xf7PB/edit?usp=drive_link&ouid=106720031472680291316&rtpof=true&sd=true

Appendix E – List of overrepresented enriched pathways contrasting VAcalf + VAcow vs CON based on Kyoto Encyclopedia of Genes Genomes (KEGG) database https://docs.google.com/spreadsheets/d/1wjaNxd2M0yYVf8Fi6ZpiiL-k7y-Xf7PB/edit?usp=drive_link&ouid=106720031472680291316&rtpof=true&sd=true

Appendix F – List of differentially expressed genes (DEGs) contrasting VAcow vs VAcalf

Gene symbol	log2 FoldChange	FDR
AASS	-1.175	0.010
ABCA1	0.895	0.001
ACTC1	1.117	0.001
ADAM9	-0.537	0.042
AFF4	-0.635	0.088
ALK	-0.981	0.042
AMPD3	0.648	<0.001
ANKRD1	-1.036	<0.001
ANKRD2	0.665	0.077
ARHGAP23	-1.032	0.015
ARHGAP30	1.804	0.037
ART1	0.653	<0.001
ASB12	-0.530	0.008
ASCC1	-0.817	0.005
ATP2B2	-0.976	0.008
B3GALNT1	-1.166	0.063
B3GALNT2	-1.259	0.027
BAHCC1	0.518	0.002
BCOR	0.946	0.057
BHLHE40	-0.710	<0.001
BMF	0.602	0.098
BTBD10	-0.507	0.028
BTBD11	1.216	0.098
BTG2	-1.064	<0.001
C23H6orf132	-0.563	0.080
C6H4orf54	-0.579	0.048
CA14	-0.533	0.087
CCDC69	-1.192	0.021

CCL5	2.648	0.006
CEP295NL	2.269	0.055
CFB	1.278	0.051
CHD7	-0.779	0.001
CIB2	-1.118	<0.001
CILP	-0.798	<0.001
CMBL	-0.586	0.048
CNIH4	-0.862	0.006
COQ10B	-0.716	<0.001
COX7A1	-0.503	0.052
CRHR1	-1.097	<0.001
CSF3R	2.603	<0.001
CSRNP3	0.738	0.088
CST6	-1.164	0.023
CXCL9	-1.198	0.007
CYP1A1	0.581	<0.001
DACH1	0.927	0.042
DCSTAMP	-3.230	0.054
DDIT4L	0.638	<0.001
DEPTOR	-0.956	0.086
DHDH	-0.934	0.020
DKK1	1.354	0.100
DNAJC3	-1.169	0.001
DSG4	-2.284	0.092
DUSP10	1.018	0.002
DUSP16	-1.132	<0.001
DUSP8	-0.588	0.007
EGFR	-0.508	0.069
ELL2	-1.012	<0.001
ERFE	-1.543	<0.001
FAM13A	-0.836	0.003

FBXO30	0.518	<0.001
FBXO32	0.867	0.012
FOXP1	0.686	0.041
FRAT1	1.115	0.052
FRZB	-0.508	0.034
GADL1	-1.368	0.051
GAS1	0.582	<0.001
GBP1	-0.833	0.001
GDA	1.841	0.093
GDF11	0.683	0.046
GREB1	-0.681	<0.001
GXYLT2	-1.246	0.033
HOMER1	-0.719	0.097
HPCAL1	-0.832	0.020
ID1	-0.684	<0.001
IGHM	1.870	0.031
IKZF2	0.853	0.002
IL4R	0.880	0.075
ING2	-0.817	0.002
IRF2BP2	0.505	<0.001
IRS2	0.781	0.001
ITGB6	-0.621	0.006
JMJD1C	-0.511	0.081
JUND	-0.622	0.001
KCNC4	1.032	<0.001
KCNS3	0.692	0.031
KEH36_t12	-0.578	0.001
KIAA0408	0.740	0.004
KLHL38	0.600	<0.001
KY	-0.786	0.005
LIMD2	0.846	0.092

LITAF	-0.728	0.019
LMCD1	-0.831	0.002
LOC100174924	-1.382	0.014
LOC100847269	-2.135	0.058
LOC100847509	-0.822	0.010
LOC100847574	2.209	0.096
LOC101903038	0.941	0.003
LOC101903765	-4.334	0.010
LOC101905499	-1.204	0.051
LOC101908154	-0.992	0.041
LOC101908406	-0.899	0.009
LOC104973229	1.018	0.071
LOC107131247	5.975	0.006
LOC107132431	1.209	0.038
LOC107132967	-0.555	0.006
LOC112443504	1.240	0.058
LOC112443732	-0.512	0.075
LOC112448103	0.799	0.001
LOC112449303	-0.575	0.003
LOC113633884	-0.881	0.095
LOC132342789	0.773	0.058
LOC132343590	0.878	<0.001
LOC132343745	-0.912	0.005
LOC132344757	4.457	0.043
LOC132344937	-0.585	0.046
LOC132345162	1.372	0.008
LOC132345302	0.862	0.006
LOC132345819	-2.480	0.045
LOC132345824	0.964	<0.001
LOC513055	4.832	0.001
LOC515676	1.188	<0.001

LOC789829	-2.800	0.001
LPIN1	-0.982	0.011
MARCHF3	-1.357	0.005
MAST4	-0.821	0.021
MBNL3	-2.577	0.046
MIEF2	-0.635	0.088
MKI67	0.848	0.097
MLF1	-0.751	0.048
MSTN	-1.984	<0.001
MYL9	-0.549	0.058
MYOG	-1.686	0.011
NES	0.534	0.001
NFIC	0.674	0.089
NFIL3	-0.729	0.027
NHERF2	-0.546	0.009
NOS3	-0.662	0.002
NPPC	-1.772	0.001
NR4A3	-1.166	<0.001
OAT	-0.988	0.015
OSBP2	-1.652	<0.001
PDLIM7	-1.012	0.027
PER2	-0.948	0.092
PIGZ	-1.033	0.093
PMP22	-0.556	<0.001
POU2AF1	3.023	0.017
PPP4R3A	-0.719	<0.001
RAET1G	0.656	0.015
RBM20	-0.992	0.023
RFLNB	0.668	0.033
RGCC	0.506	0.004
RNF207	-1.250	0.045

RPS27L	-0.518	0.057
SERTAD1	-0.893	<0.001
SESN1	0.507	<0.001
SETBP1	-1.101	<0.001
SGCG	-0.757	0.019
SH3RF2	-1.095	0.005
SIAH2	-0.715	0.052
SLC18B1	1.325	0.031
SLC22A4	1.285	0.006
SLC45A4	1.968	0.080
SLC7A2	-0.843	0.048
SLC8A3	-0.648	0.054
SLN	-0.787	<0.001
SPATA20	0.587	<0.001
STON2	-0.610	0.002
SYT4	-1.476	0.001
TBX3	-0.869	0.001
TECRL	-0.763	0.035
TFAP4	0.737	<0.001
TIPARP	-0.622	0.005
TLCD2	-1.109	0.010
TMEM100	-1.147	<0.001
TMEM131L	-1.329	0.018
TMT1A	-1.272	<0.001
TRIM16	-0.510	0.051
TRPM3	1.050	<0.001
TRPV2	-0.713	0.001
TTC39A	-2.237	0.080
TUBA4A	0.599	0.080
TUBA8	0.730	0.096
TUBG1	0.858	0.016

VASH1	0.986	0.016
WDR86	-0.513	0.023
WWP1	-0.524	0.084
ZC3H6	0.816	0.001
ZNF385C	0.523	0.011
ZNF395	0.636	0.038

Appendix G – List of co-expressed genes and transcription factors (CeTF) contrasting VAcow vs VAcalf

Transcription factor	Average expression	Regulatory impact factors 1	Regulatory impact factors 2	Frequency in VAcalf	Frequency in VAcow	Frequency difference
BCL6	6.394	1.401	2.227	162	1	161
GABPA	4.893	-2.067	-1.187	91	1	90

Appendix H – List of overrepresented enriched pathways contrasting VAcow vs VAcalf based on Gene Ontology (GO) database https://docs.google.com/spreadsheets/d/1wjaNxd2M0yYVf8Fi6ZpiiL-k7y-Xf7PB/edit?usp=drive_link&ouid=106720031472680291316&rtpof=true&sd=true

Appendix I – List of overrepresented enriched pathways contrasting VAcow vs VAcalf based on Kyoto Encyclopedia of Genes Genomes (KEGG) database https://docs.google.com/spreadsheets/d/1wjaNxd2M0yYVf8Fi6ZpiiL-k7y-Xf7PB/edit?usp=drive_link&ouid=106720031472680291316&rtpof=true&sd=true