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Simeng Yi,
China Agricultural University, China

*CORRESPONDENCE
Marcio S. Duarte
mduarte@uoguelph.ca;
marcio.duarte@ufv.br

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Effects of maternal supplementation of guanidinoacetic acid on skeletal muscle growth and metabolism in beef offspring

Luiza V. Kladt[1], Thaís C. Costa[1], Luiz C. O. Sousa[1], Marta M. Santos[1], Jenifer K. C. Varizi[1], Livia S. Rodrigues[1], Luiz J. M. Motta[1], Walmir Silva[2], Pedro V. R. Paulino[3], Tales L. Resende[4], Claudia B. Sampaio[1], Mateus P. Gionbelli[5] and Marcio S. Duarte[1,2]*

[1]Department of Animal Science, Federal University of Viçosa, Viçosa, Minas Gerais, Brazil, [2]Department of Animal Bioscience, University of Guelph, Guelph, ON, Canada, [3]Cargill Animal Nutrition, Campinas, São Paulo, Brazil, [4]Evonik Brasil Ltda, São Paulo, Brazil, [5]Department of Animal Science, Federal University of Lavras, Lavras, Minas Gerais, Brazil

Guanidinoacetic acid (GAA) is a precursor of creatine and is an arginine-sparing compound that may improve energy metabolism and muscle growth. Its potential in beef cow–calf systems, however, is still poorly understood. This study evaluated the effects of supplementing pregnant cows with GAA during late gestation on muscle development and adipogenesis in beef calves. A total of 24 pregnant Brahman cows carrying male or female fetuses received either a control diet or a diet supplemented with 0.2% GAA from day 180 to day 270 of gestation. Cows were weighed at the beginning and at the end of the trial to assess body weight (BW), and daily feed intake was recorded. Blood was collected on day 227 of gestation for plasma amino acid profiling, and the carcass traits were assessed via ultrasound. At 45 days of age, muscle biopsies were collected for mRNA expression and protein abundance. All statistical analyses were performed in SAS Studio using a mixed model including the fixed effects of treatment and offspring sex. In cows, GAA supplementation did not affect the BW, average daily gain, or feed intake ($p > 0.05$), but increased the plasma arginine, citrulline, and ornithine levels ($p \leq 0.02$) and the final ribeye area ($p = 0.01$). The calves from GAA-supplemented cows exhibited increased p-Akt/Akt ($p = 0.03$) and p-mTOR/mTOR ($p < 0.01$) ratios, with treatment × sex interactions ($p = 0.02$). The *MYOD1* mRNA expression was upregulated ($p = 0.01$), whereas *MYOG* remained unchanged ($p = 0.14$). The PAX7 protein tended to be higher ($p = 0.07$) and PAX3 reduced ($p = 0.01$) in GAA calves. No differences

were detected for the adipogenic markers. These findings suggest that maternal GAA supplementation can stimulate muscle development in beef calves without altering intramuscular adipogenesis, indicating a potential strategy to enhance muscle growth programming in cow–calf production systems.

KEYWORDS

arginine, creatine, fetal programming, myogenesis, beef cattle

1 Introduction

Pregnancy is a nutritionally demanding process, requiring substantial amounts of ATP to support conceptus development through biosynthesis, nutrient transport, metabolism, and tissue remodeling (Aldoretta and Hay, 1995; Johnson et al., 2023; Seo et al., 2021). In beef cattle production, maternal nutrition directly influences fetal skeletal muscle development and ultimately affects the growth efficiency and long-term productivity of the offspring (Costa et al., 2021; Santos et al., 2022; Barcelos et al., 2022). Maternal supplementation with protein and energy may result in different outcomes on postnatal performance by modulating the energy metabolism and muscle growth (Shokrollahi et al., 2024; Kladt et al., 2025; Shokrollahi et al., 2025) and by increasing the intramuscular fat deposition (Marquez et al., 2017; Nascimento et al., 2024; Sanglard et al., 2023; Carvalho et al., 2022). Moreover, nutrition strategies or feed additives that enhance placental vascularization may increase fetal nutrient availability, promoting a more efficient fetal development and an improved offspring performance after birth (Reynolds et al., 2023).

Guanidinoacetic acid (GAA) is derived from amino acids and functions as a direct precursor of creatine, which is an essential molecule in the energy synthesis of the cell (Ostojic, 2015). In metabolically active tissues such as the skeletal muscle, the creatine (Cr)–creatine kinase (CK)–phosphocreatine (PCr) system plays a crucial role in buffering and shuttling ATP. The uterus, placenta, and fetus can synthesize creatine and contribute to its compartmentalization in fetal fluids, which serve as nutrient reservoirs for fetal development (Sah et al., 2025). Studies have shown the dynamic expression of the Cr–CK–PCr system at the maternal–placental interface, with increased creatine biosynthesis and ATP transport activity during critical phases such as implantation (Seo et al., 2021; Guingand et al., 2024). Although creatine supplementation in late-gestation sows did not alter the Cr–CK–PCr system, the detection of its key components in the endometrium and fetal muscle highlights the importance of this system in meeting the elevated energy demands during late gestation and in supporting fetal development (Lopez et al., 2025).

Due to its thermal instability and high production cost, creatine has not been widely adopted in animal nutrition as a feed additive (Baker, 2009). GAA, on the other hand, has been widely studied not only as a creatine precursor but also as an efficient arginine-sparing molecule (Liu et al., 2015; Sousa et al., 2024; Yan et al., 2021). Compared with creatine, it presents higher bioavailability due to the presence of multiple transporters, effectively elevates hepatic and muscular creatine, shows good thermal stability and lower cost (Khajali et al., 2020; Giraldi et al., 2024), and has been proven safe in monogastric species, with no concerns for consumer safety under approved conditions of use (EFSA, 2022). Arginine is an essential amino acid particularly relevant in fetal development due to its involvement in multiple metabolic processes, such as cellular signaling and protein synthesis (Wu and Morris, 1998; Morris, 2007).

Although the fetal origins of muscle development and energy metabolism are increasingly recognized, the role of maternal GAA supplementation in these processes remains only partially understood. Sousa et al. (2024), in a companion study conducted within the same experimental framework, demonstrated that maternal GAA supplementation can enhance the placental blood flow through nitric oxide (NO) pathways and highlighted its biochemical capacity as an arginine-sparing compound. While these findings provide important mechanistic insights, they were focused on maternal and placental adaptations and did not address downstream effects on fetal muscle tissue. Therefore, the present study uniquely investigates how maternal GAA supplementation during late gestation influences early skeletal muscle development and intramuscular adipogenesis in beef calves. This study expands on these previous findings by directly evaluating postnatal skeletal muscle and adipogenic pathways, providing novel insights into how maternal GAA supplementation during late gestation may influence offspring growth and metabolic programming.

2 Materials and methods

2.1 Animals, treatments, and experimental design

All animal care and handling procedures were previously approved by the Animal Care and Use Committee of the Department of Animal Science at the Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil (protocol 04/2022).

The methodology used in this study has been previously described by Sousa et al. (2024). A total of 24 pregnant Brahman

cows (532 ± 15.1 kg) carrying male (n = 12) or female (n = 12) fetuses were used. Briefly, cows were subjected to a fixed-time artificial insemination protocol with four attempts using semen from the same sire. Therefore, four groups of cows were established based on the days of gestation. After pregnancy confirmation, the cows were managed as a single herd on Marandu palisade grass (*Urochloa brizantha*). On day 170 of gestation, the cows were housed individually for a 10-day adaptation period and fed a basal diet *ad libitum* (Sousa et al., 2024). From day 180 to day 270, the cows received either a control (CON) diet or a diet supplemented with 0.2% GAA (dry matter basis) (GuanAMINO®; Evonik Operations GmbH, Hanau-Wolfgang, Germany). GAA (GuanAMINO®; Evonik Operations GmbH, Hanau-Wolfgang, Germany) was incorporated into the mineral mixture (Probeef Reprodução Seca; Cargill Nutrição Animal, Itapira, SP, Brazil). On day 270, the cows were moved to pasture for calving. Postpartum, the cow–calf pairs remained as a single herd until weaning (210 days). From day 90 onward, calves had access to creep-feeding (5.0 g/kg body weight, BW). The composition of the supplement provided through the creep-feeding system is shown in Table 1.

2.2 Cows' performance and metabolic evaluation

The cows were weighed at the beginning and at the end of the experiment, following a 16-h fasting period, to determine the initial body weight (IBW) and the final body weight (FBW) and to calculate the average daily gain (ADG) during the experimental period.

The voluntary feed intake of each cow was individually recorded daily throughout the experimental period. Concentrate samples were collected separately for each production batch, while forage and orts samples were collected daily and stored at −20°C for subsequent nutrient composition analysis.

On day 227 of gestation, blood samples were taken via jugular venipuncture into vacuum tubes (Vacuplast® Collect Time, Cotia, SP, Brazil) containing sodium heparin for analysis of the plasma amino acid profile and immediately frozen (−20°C) for subsequent analysis.

TABLE 1 Nutrient composition of the supplement (Probeef® Bambini Creep) provided to the calves through the creep-feeding system.

Item	Level
Dry matter (DM), g/kg as fed	826.2
Organic matter, g/kg (DM basis)	704.7
Crude protein, g/kg (DM basis)	237.9
NDFap[a], g/kg (DM basis)	307.0
Ether extract, g/kg (DM basis)	9.3

Guaranteed mineral levels per kilogram of the supplement: 33 g of calcium (max), 18 g of calcium (min), 3.1 mg of cobalt (min), 60 mg of cooper (min), 1 mg of chromium (min), 3 g of sulfur (min), 2,000 mg of fluoride (max), 6 g of phosphorus (min), 2.8 mg of iodine (min), 3,000 mg of magnesium (min), 112 mg of manganese (min), 0.5 mg of selenium (min), 10 g of sodium (min), and 181 mg of zinc (min).
[a]Neutral detergent fiber corrected for ash and protein.

Plasma amino acids, such as arginine, citrulline, and ornithine, were quantified by high-performance liquid chromatography (Xevo™ TQD; Waters Corporation, Milford, MA, USA).

Carcass measurements of the cows were evaluated at the beginning and at the end of the experiment via ultrasound (Aloka SSD500II; Mitaka, Tokyo, Japan) using a 3.5-MHz linear probe. The initial and final ribeye areas (IREA and FREA, respectively) were measured at the *Longissimus lumborum* (12th–13th ribs). Images were analyzed using BioSoft Toolbox® II for Beef (Biotronics, Ames, IA, USA).

2.3 Calf skeletal muscle sampling

At 45 days of age, *Longissimus lumborum* muscle samples (located between the 12th and 13th ribs) were biopsied from the calves. Briefly, the area was cleaned with 70% ethanol, and then the incision was made 10 min after local anesthesia (2% lidocaine). The muscle sample was washed with sterile saline solution (0.9% NaCl). The muscle samples were immediately snap-frozen and powdered on liquid nitrogen using a pre-cold mortar and pestle. The samples were stored at −80°C until mRNA and protein expression analyses.

2.4 Total RNA extraction and mRNA expression analyses

Total RNA was extracted from 0.1 g of tissue using TRIzol® (Invitrogen™, Thermo Fisher Scientific®, Hillsboro, OR, USA) following the manufacturer's recommendations. The total RNA was quantified with a NanoDrop spectrophotometer (Thermo Fisher Scientific®, Waltham, MA, USA), ensuring an optimal 260:280 ratio between 1.8 and 2.0, and the integrity was assessed in a 1% agarose gel. The RNA samples were reverse-transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The primers (Table 2) for the amplification of the target and endogenous genes were designed using PrimerQuest Software (PrimerQuest–design qPCR assays | IDT; idtdna.com) with sequences obtained from GenBank (GenBank Overview; nih.gov). Real-time quantitative PCR was performed in the thermal cycler QuantStudio 3 (Applied Biosystems, Foster City, CA, USA) using the SYBR Green detection method (Applied Biosystems, Foster City, CA, USA) and the SYBR™ Green PCR Master Mix (Invitrogen™, Thermo Fisher Scientific®, Hillsboro, OR, USA). The results of gene expression were calculated according to the methods described by Steibel et al. (2009).

2.5 Protein extraction and Western blotting analyses

Total protein was extracted from 0.1 g of tissue in 1 ml of lysis buffer [10 mM of Tris–HCl (pH 7.6), 150 mM of NaCl, 1% of Triton X-100, 0.5% sodium deoxycholate, 1% sodium dodecyl sulfate (SDS), and 1% of protease inhibitor cocktail mammalian cells and

TABLE 2 List of primers for mRNA expression by RT-qPCR.

Gene symbol	NCBI accession no.	Primer
Target genes		
MYOD1	NM_001040478.2	F:TTCCGACGGCATGATGGACTAG R: AAGTGCGGTCGTAGCAGTCCC
MYOG	NM_001111325.1	F:TACAGACGCCCACAATCTGCAC R: AGCGACATCCTCCATGTGATG
PPARγ	NM_181024.2	F: TGGAGACCGCCCAGGTTTGC R: AGTTGGGAGGACTCGGGGGTG
ZFP423	NM_001101893.1	F: TCCGTGACAGCATCAGGAGG R: CACGCTGTTCCTGTCTTCCA
Endogenous gene		
18s	NM_001304989.2	F: CCAGTAAGTGCGGGTCATAA R: CCATCCAATCGGTAGTAGCG

F, forward; *R*, reverse.

tissues] (Sigma-Aldrich®, St. Louis, MO, USA), and the lysate was sonicated. The total protein content was estimated using the Bradford protein assay (Bio-Rad, Hercules, CA, USA), aliquoted, and stored at −80°C. The proteins were separated using a 10% SDS-PAGE gel loaded with 40 µg of protein per sample, transferred into a 0.45-µm nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) using a semi-dry Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, CA, USA), and blocked for 1 h at room temperature with 3% bovine serum albumin (BSA) (Sigma Aldrich®, St. Louis, MO, USA) in 1× Tris-buffered saline (TBS1×; 50 mM Tris–HCl, pH 7.5, 150 mM NaCl) (Sigma Aldrich®, St. Louis, MO, USA). Subsequently, the membranes were incubated for 12 h at 4°C with primary antibodies (Table 3) diluted in blocking solution. After 12 h of incubation, the membranes were washed three times for 5 min with Tris-buffered saline and 0.1% Tween® (TBST) and incubated with a secondary antibody (Table 3) diluted in blocking solution for 1 h at room temperature. The membranes were then washed with TBST three times for 7 min, revealed with the ECL Plus Western Blotting Detection System (GE HealthCare, Buckinghamshire, UK), and the images generated with the ChemiDoc XRS+ System (Bio-Rad, Hercules, CA, USA) and evaluated using Image Lab Software (Bio-Rad, Hercules, CA, USA). For internal control, two reference samples from the same tissue and experiment were loaded on each gel. The internal control that had a greater band intensity (expressed in optical densitometry units) was used to normalize the remaining samples as described by Cruzen et al. (2014).

2.6 Statistical analysis

The protein abundance data were analyzed using a mixed model that included the fixed effects of maternal treatment (CON or GAA) and sex and the random effect of gestation group. The variables were assessed for homoscedasticity of the error variances between treatments using Levene's test. Prior to the final analysis, the residuals were evaluated for normality. For each analysis, data points were removed one at a time until absolute Studentized residuals were lower than 3 and had non-significant ($p > 0.01$) Shapiro–Wilk's test for normality (only one data point was excluded in the analysis of PPARγ protein abundance). Expected means were generated from the final models and separated using Tukey's test when significant ($p < 0.05$).

Prior to the analysis of the RT-PCR data, the CT values were adjusted (adjCT) for their respective primer efficiency and back-transformed to the $\log_2$ scale. Afterward, the adjCT data were analyzed according to the linear mixed model below, following the strategy proposed by Steibel et al. (2009), where the data on the target and endogenous control genes are analyzed simultaneously. Treatment and sex effects were assessed through orthogonal contrasts, as in Steibel et al. (2009). Expected adjCT were computed as $-\Delta$adjCT, such that the differences between the levels of treatment and sex were estimated as $-\Delta\Delta$adjCT and then used to compute the gene ratio expression (GRE) as:

$$GRE = 2^{-\Delta\Delta CT}$$

Estimates of GREs were separated using contrasts when significant ($p < 0.05$).

When pertinent, the IBW and IREA were used as covariates. If the effect of these variables was found to be non-significant, the model was reparameterized by excluding them. All analyses were performed in SAS Studio 3.81 (Enterprise Edition, SAS Institute Inc., Cary, NC, USA).

3 Results

3.1 Maternal performance and metabolism

The complete results on the performance of cows were thoroughly discussed by Sousa et al. (2024). In brief, the IBW ($p = 0.78$) and the FBW ($p = 0.69$) did not differ between treatments, nor did the ADG (in kilograms per day) during the experimental period ($p = 0.79$) (Table 4).

In addition, no differences were observed in the voluntary intake (in grams per kilogram of BW) of dry matter ($p = 0.65$), organic matter ($p = 0.65$), crude protein ($p = 0.64$), or neutral detergent fiber ($p = 0.37$) (Table 4).

The plasma concentrations of arginine ($p = 0.01$), citrulline ($p = 0.02$), and ornithine ($p = 0.01$) were greater in GAA cows compared with the CON (Figure 1).

Regarding the carcass ultrasound measurements (in millimeters), no differences were observed in the IREA ($p = 0.20$) between groups. However, the cows from the GAA group showed greater FREA ($p = 0.01$) compared with the CON group (Table 4).

3.2 Protein abundance and mRNA expression of the muscle development markers in offspring

Calves born to GAA-supplemented cows exhibited a greater p-Akt/Akt ratio compared with those in the CON group ($p = 0.03$). A

TABLE 3 List of antibodies used in the Western blotting analysis.

Antibody	Host	Dilution	Manufacturer	Catalog no.
Primary antibodies				
Akt	Rabbit monoclonal IgG	1:1,000	Cell Signaling	9272S
p-Akt	Rabbit monoclonal IgG	1:1,000	Cell Signaling	9271S
DLK1	Rabbit polyclonal IgG	1:500	Boster Biological Technology®	A00513-4
mTOR	Rabbit monoclonal IgG	1:1,000	Cell Signaling	2972S
p-mTOR	Rabbit monoclonal IgG	1:1,000	Cell Signaling	2971S
PAX3	Rabbit polyclonal IgG	1:500	Boster Biological Technology®	A00285-1
PAX7	Mouse monoclonal IgG	1:1,000	NeoBiotechnologies	5081-MSM1-P0
PDGFRα	Rabbit polyclonal IgG	1:2,000	Thermo Fisher Scientific®	500–2694
PPARγ	Rabbit polyclonal IgG	1:500	Boster Biological Technology®	PA1320
Secondary antibodies				
HRP	Goat anti-rabbit IgG	1:5,000	Thermo Fisher Scientific®	A16096
HRP	Goat anti-rabbit IgG	1:5,000	Boster Biological Technology®	BA1054
HRP	Goat anti-mouse IgG	1:5,000	Boster Biological Technology®	BA1050

treatment × sex interaction was observed (p = 0.02), where the GAA-supplemented male calves had a higher p-Akt/Akt ratio than the CON males, while no differences were detected between the female calves from both groups (Figure 2).

Similarly, the phospho-mechanistic target of rapamycin (p-mTOR)/mechanistic target of rapamycin (mTOR) ratio was higher in calves from the GAA-supplemented cows than in those from the CON group (p = 0.004). A significant treatment × sex interaction was found (p = 0.02), with the GAA-supplemented female calves showing a greater p-mTOR/mTOR ratio than the CON females, whereas no differences were observed among males (Figure 3).

The protein abundance of PAX3 was greater in CON calves compared with GAA calves (p = 0.01). A treatment × sex interaction was observed (p = 0.02), where CON males exhibited higher PAX3 abundance than GAA males, but no differences were observed among females. A tendency for greater PAX7 abundance was found in GAA calves compared with CON calves (p = 0.07), with no treatment × sex interaction detected (Figure 4).

Regarding mRNA expression, the expression of *MYOD1* was significantly higher in GAA calves than in CON calves (p = 0.01), whereas the expression of *MYOG* did not differ between groups (p = 0.14) (Figure 5).

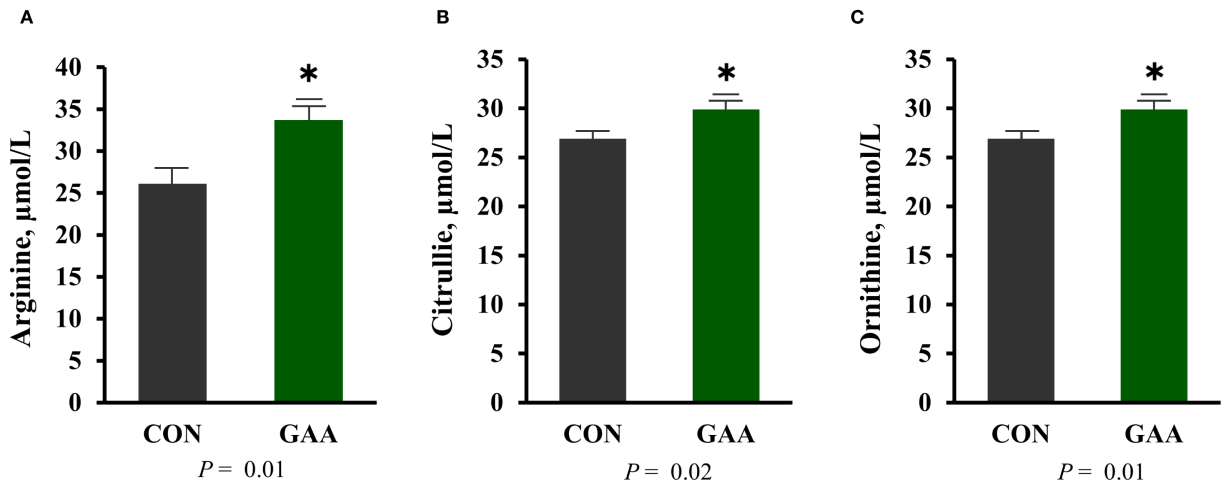

FIGURE 1
(**A**–**C**) Least squares mean ± SEM of the maternal plasma concentration (in micromoles per liter) of arginine **(A)**, citrulline **(B)**, and ornithine **(C)**. Differences (*) were considered when $p \leq 0.05$.

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TABLE 4 Least squares mean ± SEM of the maternal performance and carcass ultrasound measurements.

Item	Experimental treatment		*p*-value
	CON	GAA	
Maternal performance			
IBW (kg)	528 ± 22.6	537 ± 22.5	0.78
FBW (kg)	599 ± 7.90	603 ± 7.90	0.69
ADG (kg/day)	0.67 ± 0.08	0.69 ± 0.08	0.79
Intake (g/kg of body weight)			
Dry matter	14.3 ± 1.05	14.7 ± 1.04	0.65
Organic matter	13.4 ± 1.01	13.6 ± 0.99	0.65
Crude protein	1.77 ± 0.14	1.81 ± 0.14	0.64
Neutral detergent fiber	6.85 ± 0.35	7.12 ± 0.35	0.37
Carcass ultrasound			
IREA (mm)	65.4 ± 2.75	61.2 ± 2.78	0.20
FREA (mm)	58.1 ± 1.75	62.9 ± 1.70	0.01

CON, control; *GAA*, guanidinoacetic acid; *IBW*, initial body weight; *FBW*, final body weight; *ADG*, average daily gain; *IREA*, initial ribeye area; *FREA*, final ribeye area.

3.3 Protein abundance and mRNA expression of the fibroadipogenic and adipogenic markers in the skeletal muscle of calves

No differences were observed between the GAA and CON groups in the protein abundance of DLK1 (p = 0.10), PPARγ (p = 0.10), or PDGFRα (p = 0.29) (Table 5).

Similarly, the mRNA expression of *PPARγ* (p = 0.95) and *ZFP423* (p = 0.39) did not differ between groups (Table 5).

4 Discussion

Given the energy demands on gestation and the central role of creatine in cellular energy buffering, strategies that modulate creatine metabolism, such as GAA supplementation, have been extensively studied in different species and production phases (Yan et al., 2021; Esser et al., 2018). In our study, cows that received GAA supplementation during late gestation exhibited greater ribeye area compared with the non-supplemented cows, despite no differences in the ADG or dry matter intake between treatments. This finding suggests that GAA supplementation may have attenuated the extent of maternal tissue mobilization typically observed during late gestation, possibly by enhancing the efficiency of energy metabolism and reducing the metabolic burden on maternal reserves. Despite the observed changes, it must be noted that, ruminal degradation may have limited the systemic availability of dietary GAA, as only 47%–49% is estimated to escape the rumen intact (Speer et al., 2020). This constraint has been highlighted in studies employing post-ruminal GAA infusion, which demonstrated that bypassing ruminal degradation significantly enhances the creatine supply in cattle (Ardalan et al., 2020). These findings underscore the importance of the delivery method in determining the physiological response to GAA supplementation. It is noteworthy that, in the present study, the serum and urinary creatine concentrations remained unchanged (Sousa et al., 2024). Previous research has shown that newly synthesized creatine is rapidly taken up by high-demand tissues such as the muscle and the brain, with the plasma creatine levels typically returning to baseline within 1–3 h post-synthesis or supplementation (Wyss and

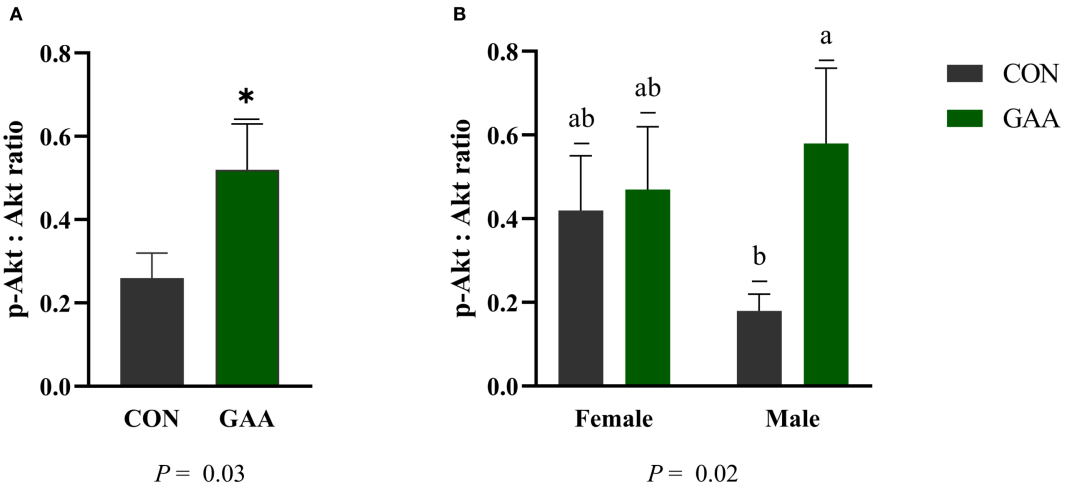

FIGURE 2
(A) Least squares mean ± SEM of the protein abundance of the phosphoprotein kinase B (p-Akt)/protein kinase B (Akt) ratio in the skeletal muscle of calves born to beef cows supplemented with guanidinoacetic acid **(GAA)** (n = 12) or the control (CON) (n = 12) at late gestation. Differences (*) were considered when $p \leq 0.05$. **(B)** Least squares mean ± SEM of the protein abundance of the p-Akt/Akt ratio in the skeletal muscle of calves born to beef cows supplemented with GAA or CON at late gestation, showing the interaction between treatment and sex. Differences **(A, B)** were considered when $p \leq 0.05$.

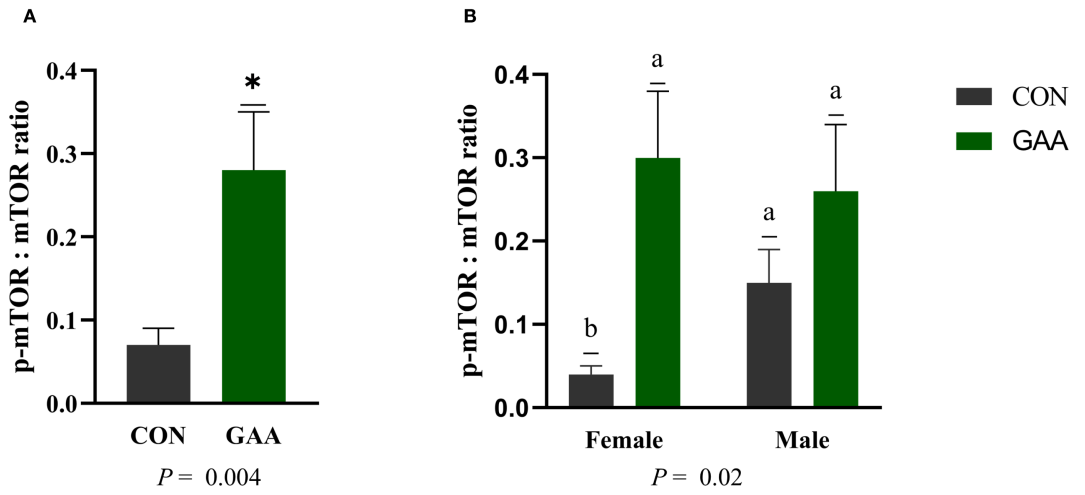

FIGURE 3
(A) Least squares mean ± SEM of the protein abundance of the phospho-mechanistic target of rapamycin kinase (p-mTOR)/mechanistic target of rapamycin kinase (mTOR) ratio in the skeletal muscle of calves born to beef cows supplemented with guanidinoacetic acid (GAA) (n = 12) or the control (CON) (n = 12) at late gestation. Differences (*) were considered when $p \leq 0.05$. **(B)** Least squares mean ± SEM of the protein abundance of the p-mTOR/mTOR ratio in the skeletal muscle of calves born to beef cows supplemented with GAA or CON at late gestation, showing the interaction between treatment and sex. Differences **(A, B)** were considered when $p \leq 0.05$.

Kaddurah-Daouk, 2000; Brosnan and Brosnan, 2007). In addition, the kidneys reabsorb a significant portion of filtered creatine, limiting urinary excretion unless the tissue stores are saturated (Asiriwardhana et al., 2024). Given that the cows in this study were at late gestation, a stage marked by substantially elevated energy and nutrient demands due to the exponential growth of the fetuses, it is plausible that any increase in the systemic creatine supply was rapidly utilized by maternal and fetal tissues to support the intensified metabolic activity. This heightened demand may help explain the absence of detectable changes in the blood and urinary creatine concentrations following GAA supplementation. Although creatine was likely rapidly utilized to meet the heightened metabolic demands of late gestation, the concurrent increases in plasma arginine, ornithine, and citrulline suggest a metabolic adaptation favoring efficient resource allocation. These elevations indicate a reduced need for arginine as a substrate for endogenous GAA synthesis, as supported by the observed downregulation of hepatic AGAT activity (Sousa et al., 2024). Moreover, the greater plasma

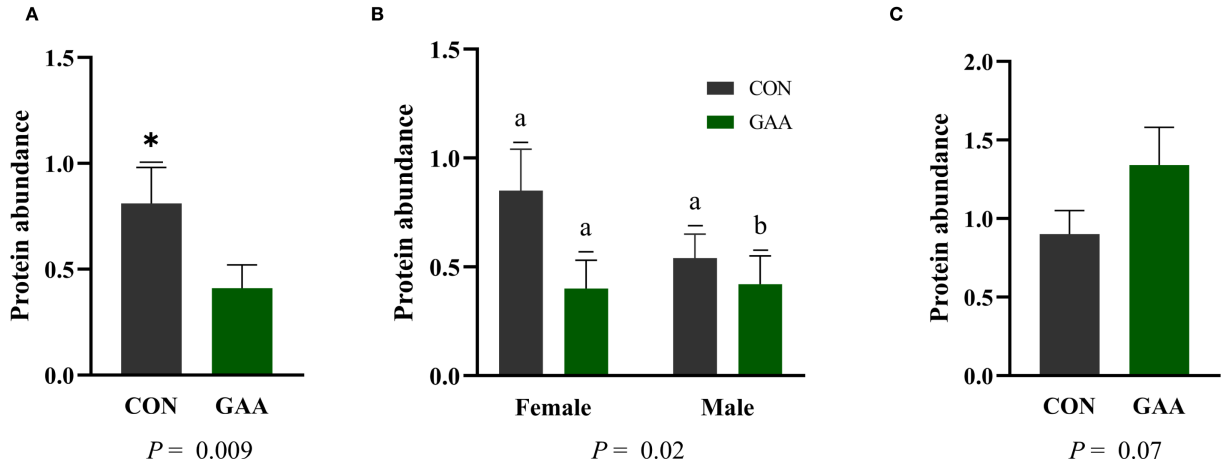

FIGURE 4
(A) Least squares mean ± SEM of the protein abundance of paired box 3 (PAX3) in the skeletal muscle of calves born to beef cows supplemented guanidinoacetic acid (GAA) (n = 12) or the control (CON) (n = 12) at late gestation. Differences (*) were considered when $p \leq 0.05$. **(B)** Least squares mean ± SEM of the protein abundance of PAX3 in the skeletal muscle of calves born to beef cows supplemented with guanidinoacetic acid (GAA) (n = 12) or control (CON) (n = 12) at late gestation, showing the interaction between treatment and sex. Differences **(A, B)** were considered when $p \leq 0.05$. **(C)** Least squares mean ± SEM of the protein abundance of paired box 7 (PAX7) in the skeletal muscle of calves born to beef cows supplemented with GAA (n = 12) or CON (n = 12) at late gestation. Differences were considered when $p \leq 0.05$.

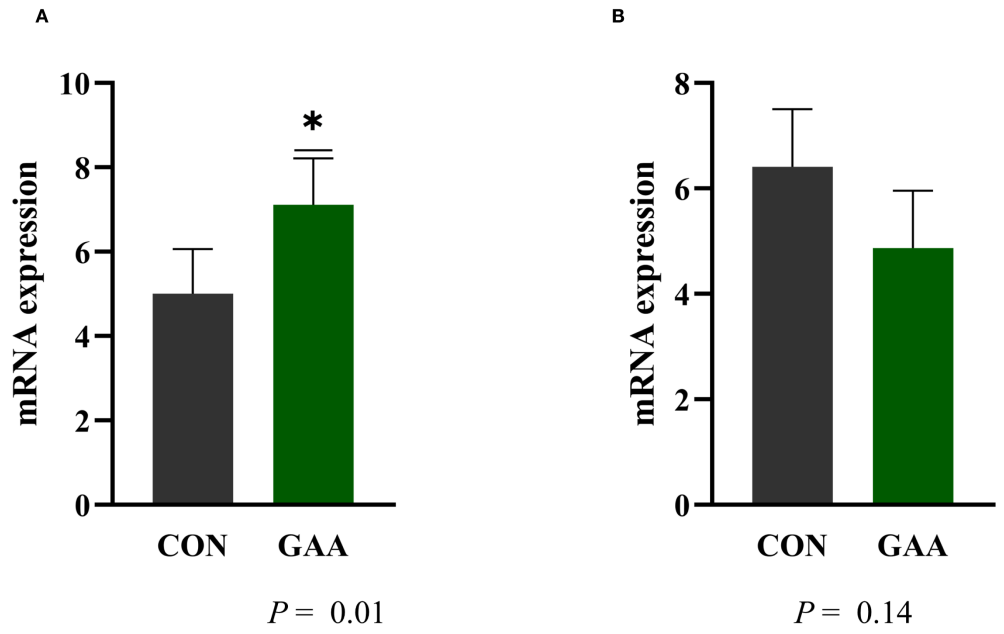

FIGURE 5
Least squares mean ± SEM of the mRNA expression of myogenic differentiation 1 (*MYOD1*) **(A)** and myogenin (*MYOG*) **(B)** in the skeletal muscle of calves born to beef cows supplemented with guanidinoacetic acid (GAA) (n = 12) or the control (CON) (n = 12) at late gestation. Differences (*) were considered when $p \leq 0.05$.

ornithine and citrulline concentrations may have enhanced arginine regeneration through the urea cycle, thereby supporting higher systemic arginine availability.

Citrulline also serves as a precursor for *de novo* arginine synthesis and participates in the arginine–NO pathway, which directly supports NO production. The elevated plasma arginine and citrulline concentrations observed in the GAA-supplemented cows may have contributed to the increased NO synthesis, as confirmed by the higher serum NO in GAA cows (Sousa et al., 2024). As NO is a crucial vasodilator that enhances placental blood flow (Morris, 2007; Elmetwally et al., 2022), its increase may have supported the greater placental vascularization observed in GAA cows (Sousa et al., 2024). This increased placental blood flow may enhance the nutrient delivery to the fetus, with glucose being the primary substrate transferred (Baumann et al., 2002).

TABLE 5 Least squares mean ± SEM of the protein abundance and mRNA expression of the fibroadipogenic and adipogenic markers in the skeletal muscle of the offspring per treatment.

Item	Maternal treatments		*p*-value
	CON	GAA	
Protein abundance			
DLK1	0.50 ± 0.23	0.97 ± 0.48	0.10
PDGFRα	0.38 ± 0.09	0.54 ± 0.11	0.29
PPARγ	0.77 ± 0.10	1.02 ± 0.10	0.10
mRNA expression			
PPARγ	13.07 ± 1.06	13.14 ± 1.06	0.95
ZFP423	3.99 ± 1.14	3.07 ± 1.13	0.39

CON, control; *GAA*, guanidinoacetic acid.

In previous studies, arginine supplementation not only increased the plasma insulin concentrations but also activated the IGF-1/insulin/Akt/mTOR signaling pathway, which is the main pathway involved in protein synthesis and muscle growth (Dou et al., 2023; Yao et al., 2008). This mechanistic link provides a potential explanation for the greater activation of Akt and mTOR observed in calves born to GAA-supplemented cows. Two primary mechanisms may underline this effect: firstly, the increased nutrient availability, potentially driven by the higher creatine concentrations derived from GAA, which could elevate the levels of IGF-1, a key upstream regulator of Akt (Liu et al., 2021; Li et al., 2022); secondly, the greater availability of arginine itself, which can directly activate mTOR in a NO-dependent manner (Wang et al., 2018a). Nevertheless, the lack of direct measurements of IGF-1 or insulin, as well as the downstream markers of energy metabolism such as mitochondrial function and ATP content, represents a limitation of the present study.

Beyond nutrient availability, intrinsic fetal factors such as sex and the developmental stage appear to modulate the responsiveness of fetal tissues to maternal supplementation strategies. Earlier studies have reported sex-related differences in muscle development, with male fetuses displaying greater myogenic gene expression (Gionbelli et al., 2018) and enhanced muscle growth during the same developmental window (Barcelos et al., 2022). More recently, Sah et al. (2025) provided evidence that the creatine biosynthesis

pathway and its regulatory enzymes are modulated in a sex- and stage-specific manner, with female offspring exhibiting higher expression of creatine transporters and biosynthetic enzymes in distinct tissues and gestational stages. These findings reinforce the need to investigate whether maternal GAA supplementation interacts with fetal sex to modulate muscle energy metabolism.

In the present study, sex-specific treatment interactions were also observed, with Akt activation being more pronounced in GAA males and mTOR activation higher in GAA females. However, these findings cannot be fully explained by the current knowledge on sex-related differences in muscle development and creatine metabolism. Evidence from placental and fetal biology supports the existence of sexual dimorphism in mTOR regulation, but the mechanistic basis remains poorly understood. For instance, Akhaphong et al. (2021) reported sex-dependent differences in a genetic model with placental mTOR deletion, although the underlying regulatory pathways were not clarified. Similarly, Sedlmeier et al. (2021) showed sexually dimorphic expression of placental mTOR and amino acid transporters in response to maternal diet, but without a definitive mechanistic explanation. Beetch and Alejandro (2021) also emphasized the need for mechanistic studies to clarify how placental mTOR perturbations drive sexually dimorphic metabolic outcomes. Collectively, these findings indicate that sex-specific regulation of mTOR signaling is evident, but incompletely understood, with epigenetic regulation remaining a plausible contributor. Thus, while our study revealed distinct sex-specific interactions for Akt and mTOR, the mechanistic basis of these observations could not be determined and must be acknowledged as a limitation.

Due to the key role of the Akt/mTOR signaling pathway in regulating satellite cell function and myogenesis (Zhang et al., 2015), we evaluated whether maternal GAA supplementation, in addition to altering the energy metabolism in calf skeletal muscle, would also influence cell commitment toward the myogenic lineage. In myogenesis, PAX3 primarily governs early skeletal muscle formation in the embryo, whereas PAX7 takes precedence in postnatal muscle growth and adult muscle progenitor proliferation to a more differentiated state (Buckingham and Relaix, 2015). Moreover, *MYOD1*, an early myogenic regulatory factor, is crucial for committing progenitor cells to the myogenic lineage and stimulating myoblast proliferation (Jennings et al., 2016; Berkes and Tapscott, 2005). Previous findings have shown that GAA supplementation enhances myogenic differentiation and muscle growth by increasing the expression of *MYOD1*, which facilitates myoblast expansion and early differentiation (Wang et al., 2018b; Yan et al., 2021). A study conducted with broiler embryos supplemented *in ovo* with creatine pyruvate showed increased expression of *MYOD1* and *PAX7* (Zhao et al., 2017). In our study, however, we observed a tendency of elevated PAX7 protein abundance in the GAA group, in addition to a greater expression of *MYOD1*, indicating a potentially expanded satellite cell pool, which is essential for postnatal muscle growth and regeneration. Taken together, increased activation of the Akt/mTOR pathway, particularly with sex-specific patterns, and the upregulation of the satellite (PAX7) and myogenic (*MYOD1*) marker indicate a potential enhancement of the myogenic commitment and expansion phase.

Considering that maternal supplementation during late gestation can improve maternal body condition and the uterine environment, as well as stimulate the fibroadipogenic progenitor cells and promote adipogenic differentiation in the offspring (Du et al., 2013; Duarte et al., 2014; Du et al., 2015, 2023; Du, 2023), and given that GAA functions as a direct precursor of creatine, a key molecule for ATP production, together with our observation of the higher final REA in GAA-supplemented cows, we hypothesized that maternal GAA supplementation during this period could potentially influence the commitment of fibroadipogenic progenitor cells toward mature adipocytes. Nevertheless, no significant differences were observed in the expression of the key fibroadipogenic markers DLK1, PPARγ, PDGFRα, and *ZFP423* between calves born to GAA-supplemented dams and those in the CON group. This lack of detectable effect may reflect the prioritization of the Cr–CK–PCr system in meeting the high energy demands of maternal and fetal tissues during late gestation (Lopez et al., 2025). Consequently, GAA appears to act more efficiently as an arginine-sparing strategy, enhancing the downstream pathways such as Akt/mTOR signaling and NO-mediated metabolism rather than directly modulating the classical adipogenic signaling in fetal skeletal muscle.

5 Conclusions

Maternal GAA supplementation during late gestation in beef cows may reduce the mobilization of maternal reserves, as well as enhance placental function, through the sparing of arginine for NO synthesis and improved vascularization. These systemic changes were accompanied by the activation of Akt/mTOR signaling and the upregulation of key myogenic markers (*MYOD1* and PAX7) in the offspring, indicating evidence of early molecular responses that suggest a potential enhancement in muscle growth programming.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by Animal Care and Use Committee of the Department of Animal Science at the Universidade Federal de Viçosa. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

LK: Data curation, Formal Analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. TC: Investigation,

Methodology, Writing – original draft, Writing – review & editing. LS: Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review & editing. MS: Investigation, Methodology, Writing – original draft, Writing – review & editing. JV: Writing – original draft, Writing – review & editing. LR: Investigation, Methodology, Writing – original draft, Writing – review & editing. LM: Investigation, Methodology, Writing – original draft, Writing – review & editing. WS: Data curation, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. PP: Conceptualization, Investigation, Writing – original draft, Writing – review & editing. TR: Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review & editing. CS: Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. MG: Conceptualization, Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. MD: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

Author TR was employed by the company Evonik Brasil Ltda. Author PP was employed by company Cargill Animal Nutrition.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fanim.2025.1671346/full#supplementary-material

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