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Prepartal dietary cobalt source alters holstein calf semitendinosus muscle abundance of mTOR and insulin signaling proteins and intermediates of one-carbon metabolism

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Requirement for Cobalt and folic acid (FOA) in late-pregnant dairy cows is unknown, but dietary supply of one or both could impact activity of one-carbon metabolism. Holstein cows were fed the same basal diet supplemented with Cobalt glucoheptonate (CoPro, $n = 16$) or a slow-release Cobalt polysaccharide (CoPectin, $n = 14$) for the last 30 days prepartum to assess impacts on calf growth and skeletal muscle metabolism. Cobalt treatments delivered 1 ppm Cobalt/kg DM and both diets supplied 50 mg ruminally-available FOA/day. Calves were weighed at birth and growth performance recorded weekly through 9-weeks of age. Prior to weaning (day 42), calves ($n = 7$ and 8 for CoPro and CoPectin group, respectively) were subjected to biopsy of semitendinosus muscle for Western blotting and targeted metabolomics using LC-MS-MS. Although birth measures of development did not differ ($P > 0.05$), calves born from CoPectin cows had greater hip width (HW) at weeks 8–9 (Diet $\times$ Time, $P = 0.03$). Overall, withers height (WH) tended (84.6 vs. 82.4 ± 0.9 cm; $P = 0.10$) to be greater in CoPectin than CoPro calves. Metabolomic profiling revealed greater concentrations of betaine ($5.11 \pm 0.36 \times 10^6$ vs. $4.12 \pm 0.36 \times 10^6$ AUC; $P = 0.04$) and S-adenosylmethionine ($3.87 \pm 0.42 \times 10^6$ vs. $2.61 \pm 0.42 \times 10^6$ AUC; $P = 0.02$), with tendencies for greater cystathionine ($1.02 \pm 0.10 \times 10^6$ vs. $0.71 \pm 0.10 \times 10^6$ AUC; $P = 0.06$) and choline ($8.04 \pm 1.15 \times 10^6$ vs. $5.83 \pm 1.15 \times 10^6$ AUC; $P = 0.10$) in CoPectin compared with CoPro calves. Protein abundance (relative to GAPDH) of INSR (1.34 ± 0.07 vs. 1.12 ± 0.05 ; $P = 0.05$), p-AKT (1.22 ± 0.08 vs. 1.01 ± 0.06 ; $P = 0.05$), and p-AKT : AKT ratio (1.37 ± 0.09 vs. 1.00 ± 0.07 ; $P = 0.001$) were greater, whereas total 4EBP1 (0.81 ± 0.06 vs. 1.03 ± 0.05 ; $P = 0.03$) and MRF4 (0.75 ± 0.05 vs. 0.96 ± 0.07 ; $P = 0.04$) were lower in CoPectin calves. These

results suggest that the slow-release cobalt source (CoPectin) enhanced maternal cobalt utilization and fetal one-carbon metabolism, leading to greater activation of the insulin–AKT–mTOR pathway in calf skeletal muscle. Further research could help determine the degree to which slow-release Cobalt alters ruminal synthesis of vitamin B₁₂ and its impact on the physiology of the neonatal calf.

KEYWORDS

nutritional programming, maternal cobalt supplementation, muscle metabolism, metabolomics, dairy calf

1 Introduction

Fetal programming represents the process through which offspring performance is shaped by a wide range of maternal and environmental factors including nutritional deficits or excesses, disease states, environmental conditions (e.g., heat stress), and exposure to toxicant and/or drugs, all of which are relevant in dairy cattle (Cattaneo et al., 2022; Lopreiato et al., 2023b; Ouellet et al., 2020). In this context, the nutritional management of cows during late-gestation plays a particularly important role in determining postnatal growth, metabolism, and productivity (Barcelos et al., 2022; Christofaro Fernandes et al., 2023; Polizel et al., 2021; Schalch Junior et al., 2022). Several studies have pointed out that nutritional status of the dam during late-pregnancy influences fetal life (Alharthi et al., 2019; Hulbert and Moisés, 2016; Jacometo et al., 2018; Lopes et al., 2021). Several metabolic functions such as growth, development, reproduction, and immunity require trace elements. Besides the strategy to feed trace minerals in order to enhance their status in cattle (Spears and Weiss, 2008), some data indicated that feeding trace minerals complexed with organic compounds such as amino acids especially in late-gestation could represent an alternative to further optimize postnatal offspring performance (Marques et al., 2016). For example, there is evidence that maternal diets induce transcriptomic changes in a stage-dependent manner in the liver of pigs (Oster et al., 2016) and dairy calves (Palombo et al., 2021), pointing to molecular routes that contribute to the adaptation of the organism.

Trace minerals are essential for fetal development (Hostetler et al., 2003), since the fetus depends completely on the dam for proper supply of these elements (Marques et al., 2016). Inadequate supply from the dam might impair fetal development and postnatal performance. Among micronutrients, the minerals Zn, Cu, Mn, and Co are required for proper development of the fetal nervous, reproductive, and immune systems (Hostetler et al., 2003; Pepper and Black, 2011). Cobalt (Co) and folic acid (FOA) play complementary roles in vitamin B₁₂ metabolism. Cobalt is an essential component for ruminal synthesis of vitamin B₁₂ by microorganisms, as it constitutes the central atom of the cobalamin molecule. Folic acid, on the other hand, is a key factor

in the one-carbon metabolism pathway, where vitamin B₁₂ functions as a cofactor. Thus, adequate folic acid availability enhances the metabolic utilization of vitamin B₁₂ synthesized in the rumen (Duplessis et al., 2022; Girard and Duplessis, 2023; Tiffany et al., 2006, 2003).

It was established several years ago that young ruminant diets deficient in Co content might result in a lower vitamin B₁₂ (cobalamin) biosynthesis in the rumen (Stangl et al., 1999). This in turn leads to lower post-ruminal supply and average daily gain (ADG), decreased plasma and liver vitamin B₁₂, elevated plasma methylmalonic acid (MMA), and homocysteine (Stangl et al., 1999). At the tissue level, the decrease in ruminally-derived vitamin B₁₂ can decrease methionine synthase and methylmalonyl-CoA mutase activities (Kennedy et al., 1990). Until the ruminal environment develops a microbiota population, at least for vitamin B₁₂, newborn calves rely on placental and colostral transfer to cover its requirements. Duplessis and Girard (2019) demonstrated that supplementing cows with biotin, folic acid, and vitamin B₁₂ in late-gestation effectively boosts the concentrations of these vitamins in both colostrum and the newborn calf's plasma. Thus, there is growing recognition that dietary supply of micronutrients including Co and the methyl donors such as methionine, choline, and/or FOA, all ensure benefits to both dams and offspring in terms of production and health (Coleman et al., 2020, 2021; Lopes et al., 2021; Lopreiato et al., 2023a; Zenobi et al., 2018).

Numerous studies have linked maternal micronutrient supplementation with positive responses in energy metabolism, inflammation, antioxidant status, and birth weight of the offspring (Alharthi et al., 2019; Duplessis and Girard, 2019; Jacometo et al., 2016). Micronutrient supplementation, including Zn, Se, and Co has also led to improvements in reproductive performance, lamb production, and health indices (Aliarabi et al., 2019; Hatfield et al., 1995; Rock et al., 2001). Furthermore, maternal supplementation of Zn, Se, and Co via slow-release ruminal bolus during late-pregnancy enhanced mineral status in ewes and their offspring until weaning, resulting in higher lamb body weight at weaning (Aliarabi et al., 2019).

In a previously studied cohort of calves reported by Lopes et al. (2021), which underwent the same experimental conditions as those

presented in the current study, we observed that maternal supplementation of FOA, Co sources varying in ruminal release (e.g., Co glucoheptonate, Co pectin), and rumen-protected methionine influenced specific plasma biomarkers of inflammation, and molecular responses associated with inflammatory mechanisms during the neonatal period. For example, there is evidence that maternal diets can induce transcriptomic and metabolic reprogramming in offspring tissues, pointing to molecular routes that contribute to postnatal adaptation. In ruminants, maternal plane of nutrition or trace mineral supply during late-gestation altered hepatic and muscle transcriptome profiles (Polizel et al., 2025) and modified metabolomic pathways associated with energy metabolism, amino acid turnover, and one-carbon metabolism (Elolimy et al., 2019; Shang et al., 2024). These findings highlight the complexity of nutrient-mediated fetal programming and support the hypothesis that trace mineral bioavailability during gestation can influence the metabolic phenotype of newborn calves.

We reported evidence for maternal effects of organic trace mineral supplementation on calf circulating immune cell microRNA profiles (Jacometo et al., 2015), but we are unaware of similar studies focused on offspring growth and muscle metabolism. Although several studies have evaluated prepartum cobalt supplementation in ruminants, most have focused on maternal serum or ruminal parameters (e.g., vitamin B₁₂ concentration or ruminal fermentation), whereas the potential programming effects on offspring tissues have received far less attention. In particular, the molecular mechanisms linking maternal trace mineral nutrition to the development of neonatal muscle remain poorly characterized. Furthermore, potential differences in the bioavailability and physiological effects among cobalt chemical forms are still unclear. Slow-release or organic cobalt sources may provide a more stable cobalt supply to rumen microbes, sustaining vitamin B₁₂ synthesis and methyl-donor production in the dam, which could in turn influence fetal one-carbon metabolism and muscle development.

We hypothesized that, compared with an inorganic cobalt source, the slow-release cobalt pectin complex (CoPectin) would provide a more consistent cobalt release and higher vitamin B₁₂ availability, thereby enhancing methyl-donor intermediates (e.g., betaine and S-adenosylmethionine) and activating the insulin-AKT-mTOR signaling pathway in the skeletal muscle of newborn calves. Thus, the aim of the current study was to use a subset of calves from the study of Lopes et al. (2021) to investigate the influence of feeding Co sources to Holstein cows in late-pregnancy on the abundance of proteins in the mTOR and insulin signaling pathway as well as intermediates of one-carbon metabolism in skeletal muscle of neonatal calves.

2 Materials and methods

2.1 Animal ethics

Calves used in the present study were a subset from the experiment reported recently by Lopes et al. (2021) which was

approved by the Institutional Animal Care and Use Committee (protocol no. 17168) of the University of Illinois, Urbana-Champaign. A total of 72 multiparous Holstein cows were used in the original study.

2.2 Experimental design and management

In the original study, a total of 72 multiparous Holstein cows were utilized. Details of the experimental design, diet composition, and nutrient profiles were reported by Lopreiato et al. (2023a). Briefly, from -30 days from calving until delivery (close-up period), cows were housed in a sand-bedded freestall barn with an individual Calan gate feeding system (American Calan, Northwood, NH, USA). They were fed a total mixed ration (TMR) daily at 0600 h (1.37 Mcal/kg of dry matter, 14.6% crude protein; Table 1). Weekly samples of the TMR were collected and frozen at -20 °C before making a composite that was analyzed for DM (method 942.05; AOAC, 2006), crude protein (method 990.03; AOAC, 2006), acid detergent fiber (method 973.18; AOAC, 1997), neutral detergent fiber (Van Soest et al., 1991), starch (Bach Knudsen, 1997; YSI 2950D-1 Biochemistry Analyzer, YSI Inc., Yellow Springs, OH), crude fat (method Am 5-04; AOCS, 2013), Ca, P, Mg, K, Na, Cl, and S (CEM-Dairy One digestion method; analysis in Thermo iCAP Pro XP Inductively Coupled Plasma Radial Spectrometer, Waltham, MA, USA) using wet chemistry methods at a commercial laboratory (Dairy One Cooperative Inc., Ithaca, NY, USA).

This larger study involved two experimental sources of Co with different rates of ruminal release of the mineral fed in combination with the same experimental ruminally-available folic acid (FOA) source, and one of the Co sources fed with FOA plus rumen-protected methionine (RPM; Smartamine M from Adisseo, Alpharetta, GA). The level of FOA supplemented from -30 to 30 days in milk (DIM) was 50 mg, and the Co treatments provided 1 mg of Co/kg of dry matter (CoPro or CoPectin from Zinpro Corp.). The cobalt inclusion level exceeded the NRC (2001) requirement to ensure adequate cobalt supply during the late-gestation period when feed intake typically declines and microbial vitamin B₁₂ synthesis may be limited. All supplements were administered by top-dressing onto the TMR. For the data in the present study, 16 calves were used in the CoPro group and 14 in the CoPectin group. Some of these were also included in the study by Lopes et al. (2021) dealing with mRNA abundance of immune markers in whole blood. The management of the calves was described by Lopes et al. (2021). Briefly, a digital scale was used to weigh calves at birth and 3.8 L first-milking fresh colostrum from their respective dams provided. Subsequently, calves were housed in individual outdoor hutches bedded with straw. Milk replacer (Advance Excelerate, Milk Specialties; 28.5% crude protein, 15% fat) was fed at 0600 and 1800 h daily until 35 d of age. Then, calves were only fed once daily until weaning at 42 d of age. In detail, calves received 4.54 L of milk replacer (0.59 kg of milk replacer in 3.95 L of water) from 1 to 10 d of age, 5.90 L (0.77 kg of milk replacer in 5.13 L of water) from 11 to 20 d of age, 7.26 L (0.94 kg of milk replacer in 6.32 L of water) from 21 to 35 d of age, and 3.63 L

TABLE 1 Ingredient and chemical composition of the total mixed ration fed from -30 days from parturition until parturition.

Ingredient	% of DM
Corn silage	37.47
Ground shelled corn	11.60
Wheat straw	24.00
Canola meal	11.67
Soybean meal	6.30
Soychlor ^{® 1}	3.37
Corn gluten feed	2.80
Mineral–vitamin mix ²	1.19
ProvAAL2 AADvantage ^{® 3}	0.47
Biotin ⁴	0.10
AjiPro-L ^{® 5}	0.06
Availa ^{® Dairy 6}	0.05
Rumensin ^{® 7}	0.19
Calcium sulfate	0.53
Magnesium oxide	0.10
Salt	0.10
Chemical composition	
Dry matter (%)	42.88
Crude protein	14.60
Neutral detergent fiber	39.15
Acid detergent fiber	31.08
Starch	17.17
Crude fat	2.53
NE _L (Mcal/kg of DM ⁸)	1.37
Rumen degradable protein ⁸	8.92
Rumen undegradable protein ⁸	5.68
Ca	0.66
P	0.33
Na	0.12
Cl	0.78
Mg	0.45
K	1.36
S	0.33

¹Landus (Des Moines, IA, USA). On DM basis: 20.06% of crude protein, 1.5% of fat, 0.82% of starch, 10.53% of chloride, 4.54% of calcium, 2.84% of magnesium, 0.76% of potassium, 0.53% of phosphorus, 0.37% of sulfur. DCAD of -2980 mEq/kf DM.

²Non-medicated mineral/vitamin mix. On DM basis: 1.10 ppm of Co, 18.30 of ppm Cu, 1.28 ppm of I, 92.60 ppm of Mn, 0.33 ppm of Se, 87.05 ppm of Zn, 104,509 IU of vitamin A, 31,788 IU of vitamin D, and 1,034 IU of vitamin E.

³Perdue AgriBusiness (Salisbury, MD, USA). On DM basis: 82.7% of crude protein, 4.8% of lysine, 0.6% of methionine, 31.2% of essential amino acids.

⁴ADM Animal Nutrition (Quincy, IL, USA).

⁵Ajinomoto Health & Nutrition North America, Inc. (Itasca, IL, USA). On DM basis: 50% of L-lysine monohydrochloride, 49% of fat, 1% of soy lecithin

⁶Zinpro Corp. (Eden Prairie, MN, USA). On DM basis: 26.4% of crude protein, 23% of ash, 6.67% of zinc, 3.34% of manganese

⁷Elanco Animal Health (Greenfield, IN, USA).

⁸Estimated from NRC (2021).

(0.47 kg of milk replacer in 3.16 L of water) in a single feeding from 36 to 42 d of age. During the whole period, calves had ad-libitum access to textured starter (Ampli-Calf Starter 20, 19.9% crude protein and 13.5% neutral detergent fiber, Purina Animal Nutrition). None of these calves experience an episode of diarrhea or other clinical diseases.

2.3 Growth measurements

Growth measurements (n = 16 in CoPro and 14 in CoPectin) were conducted weekly during the first 9 weeks of age by using a scale, wherein two individuals measured body weight, body length, hip height, wither height, and hip width for each calf. All calves included in the present study remained clinically healthy throughout the experiment. Procedures were exactly as described in our previous publications (Jacometo et al., 2015; Alharthi et al., 2018).

2.4 Muscle biopsy

Semitendinosus biopsies were harvested from a total of 5 heifers and 3 bulls in the CoPro group and 4 heifers and 3 bulls in the CoPectin group at 42 d of age before weaning. Briefly, an area of approximately 10 × 10 cm over the tuber ischia was closely clipped and scrubbed thoroughly with betadine and 70% ethanol. Local anesthesia was achieved with 2% lidocaine (5 mL s.c.; NexGen Animal Health, Weatherford, TX, USA). A 2-cm incision was made using a sterile scalpel blade. Approximately 200 mg of tissue was harvested using a biopsy needle (Bard MAGNUM; 12 gauge × 16 cm; C. R. Bard, Inc., Murray Hill, NJ) from the right hind leg. Once the biopsies were completed, a sterile gauze was used to apply pressure in order to stop any external bleeding and the blood on the surrounding area was cleansed with sterile saline. Afterward, the incision was closed with 3 surgical staples and an antimicrobial ointment was then applied to the incision site. Calves were monitored for 7 days post biopsy for swelling or infection of the incision site, and any staples that remained removed followed by application of iodine ointment.

2.6 Western blotting

The Western blotting protocol employed in our studies utilized a "two-step probing" approach, without stripping the blot. Briefly, Semitendinosus muscle samples (50 mg) were homogenized in 1 mL of protein extraction reagent (catalog no. 78510; Thermo Fisher Scientific) supplemented with 1 µL of HaltTM protease and phosphatase inhibitor cocktail (100x, catalog no. 78442; Thermo Fisher Scientific). Total protein concentration was quantified using a Nano-Drop ND-1000 (Thermo Fisher Scientific). Protein samples (40 µg) were denatured by heating at 37 °C for 5 min. Denatured samples were loaded onto 4–20% Mini-PROTEAN[®] TGXTM Precast Protein Gels (Bio-Rad Laboratories) and electrophoresed

at 180 V for 10 min followed by 120 V for 50 to 60 min. A polyvinylidene fluoride membrane (catalog number 1620261; Bio-Rad) was activated with methanol for 1 min. Proteins were transferred onto polyvinylidene fluoride membranes (catalog number 1620261; Bio-Rad) using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). Membranes were activated with methanol and blocked for 90 min at room temperature in 1x Tris-buffered saline (1xTBST) with 5% nonfat milk. Membranes were then incubated overnight at 4 °C with primary antibodies targeting specific proteins, including mTOR, phosphorylated (p)-mTOR, eIF2alpha, 4EBP1, p-4EBP1, S6K1, p-S6K1, TSC2, ERK, INSR, PI3K, AKT, p-AKT, MRF4, SLC2A4, PPARGC1alpha, SLC1A3, SLC38A1, SLC1A5, and BCKDK, along with the internal control anti-GAPDH (catalog no. 2118S; Cell Signaling Technology; catalogue numbers and dilution ratios are included in [Supplementary Table 1](#)). Although we did not perform validations for any of these antibodies, per the manufacturers, each are polyclonal and some are reported to crossreact with bovine samples (AKT, p-AKT, GAPDH, ERK1/2). Furthermore, we have used these antibodies in several previous publications dealing with different types of bovine tissues ([Coleman et al., 2022a, b](#)). After washing 5 times for 2 min with 1x TBST, membranes were incubated with secondary antibody (HRP conjugated anti-rabbit IgG, catalog no. 7074S; Cell Signaling Technology) for 1 h at room temperature. Following another wash step, membranes were exposed to ECL reagent (catalog no. 170-5060; Bio-Rad) for 3 min in the dark, and images were captured using the ChemiDOC MP Imaging System (Bio-Rad). Band intensities were quantified using Image Lab software (version 3.0, Bio-Rad). Specific target protein band densities were normalized to GAPDH density values, which served as an internal control with stable expression across tissues and variation in intensity of 10% or less.

2.8 Targeted metabolomics

Metabolites were extracted from 50–80 mg of tissue using a solution of 4 mL/g of cold methanol and 0.85 mL/g of cold water, following the method outlined in our previous publication ([Vailati-Riboni et al., 2020](#)). Following centrifugation at 4 °C for 10 min at 12,000 × g, the resulting supernatant was collected and divided into two aliquots. The first aliquot was utilized to determine the protein concentration using the Bradford assay (no. 500–0205, Bio-Rad). The second aliquot was submitted to the Metabolomics unit at the Roy J. Carver Biotechnology Center (University of Illinois, Urbana) for targeted analyses [liquid chromatography (LC)–MS–MS] employing commercial standards (Sigma-Aldrich, St. Louis, MO, USA) previously measured by our group in bovine liver. The instrumentation, run settings, and data acquisition details remained consistent with those reported previously ([Vailati-Riboni et al., 2020](#)). Before statistical analysis, the peak area under the curve (AUC) was normalized by the protein concentration of each sample. Notably, the following metabolites were undetectable: N-methyl-glycine, 5-methyltetrahydrofolic acid, succinyl-CoA,

acetyl-CoA, vitamin B₁₂, NADPH, NADH, trimethyl-lysine, homocysteine, vitamin B₆, cysteinesulfinic acid, nicotinic acid, ATP, GTP, fructose-1,6 bisphosphate, and folic acid.

2.9 Statistical analysis

The statistical analysis was conducted using SAS 9.4 (SAS Institute Inc., Cary, NC, United States). Repeated measures analysis of body measurements and starter intake was performed using the MIXED procedure according to the following general model:

$$y_{ijk} = \mu + B_i + M_j + T_k + TM_{jk} + \varepsilon_{ijk}$$

Where Y_{ijk} the dependent, continuous variable; μ the overall mean; B_i is the random effect of calf within maternal diet (CoPro or CoPectin); the fixed effects in the model include maternal diet (M_j , j = CoPro or CoPectin), Time (T_k), and interactions. ε_{ijk} is the residual error. The AR (1) covariance structure was used. Cow DMI prior to parturition was analyzed using a similar model including diet (CoPro or CoPectin), time, and their interaction. Metabolomics and protein abundance data of skeletal muscle were analyzed using the MIXED procedure according to the following model:

$$y_{ij} = \mu + B_i + M_j + \varepsilon_{ij}$$

Where Y_{ij} the dependent, continuous variable; μ the overall mean; B_i is the random effect of calf within maternal diet (CoPro or CoPectin); the fixed effect in the model included maternal diet (M_j , j = CoPro or CoPectin). ε_{ijk} is the residual error. The normality and homoscedasticity of model residuals were assessed using the PROC UNIVARIATE procedure in SAS, with the NORMAL option and associated diagnostic plots. All means were compared using the PDIF statement of SAS, and Tukey-Kramer's *post hoc* adjustment was applied. It was determined that all data were normally distributed and did not require log transformation. Significance was determined at $P \leq 0.05$ whereas tendencies were declared at $P \leq 0.10$.

3 Results

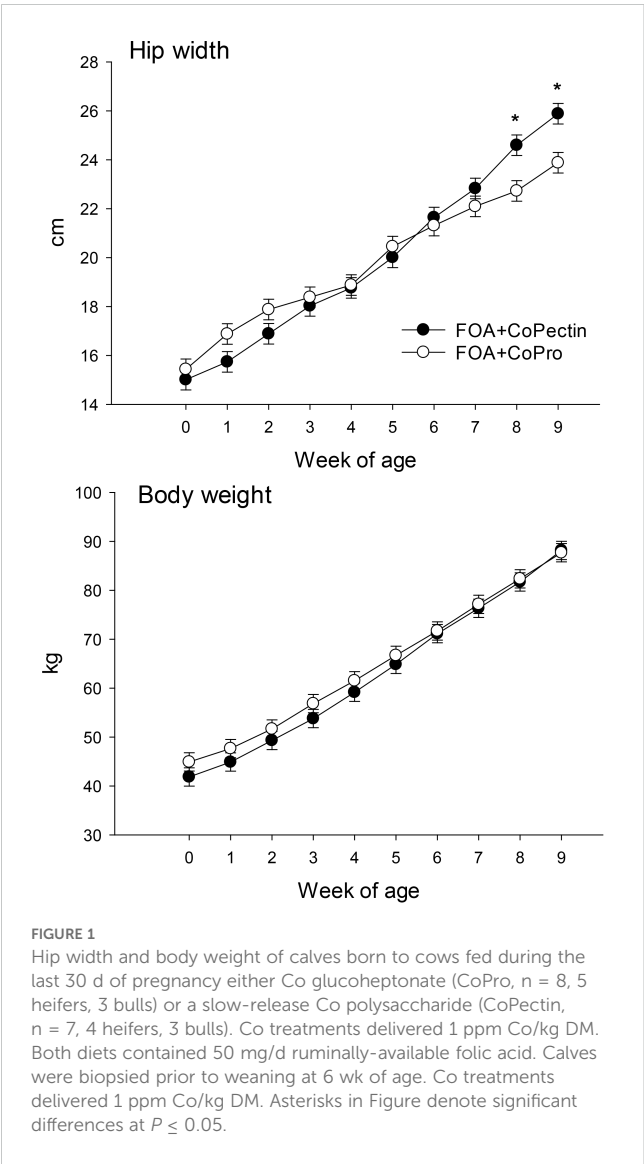
Feed intake by the pregnant cows during the close-up period did not differ due to source of Co ($P = 0.98$; [Table 2](#)). In terms of postnatal growth, only hip width had an interaction (Diet × Time, $P = 0.01$; [Figure 1](#)) over the 9 wk of age because of a greater response in CoPectin calves at 8 and 9 wk of age. All other growth measurements were not affected by the maternal treatment ($P > 0.05$; [Table 2](#), [Figure 2](#)).

Calves fed to cows supplemented with CoPectin also had alterations of certain metabolites within the one-carbon metabolism pathway ([Table 3](#)). Compared with CoPro calves, there was a greater concentration of betaine ($P = 0.04$), S-5'-adenosyl-methionine ($P = 0.02$), and a tendency for greater concentration of cystathionine ($P = 0.06$) and choline ($P = 0.10$) in CoPectin calves.

TABLE 2 Cow dry matter intake (DMI) during the last 30 days prepartum (close-up period) and growth performance of calves during the first 9-weeks of age.

Item	Maternal Co treatment		SEM	P value		
	CoPro	CoPectin		Diet	Time	Diet×Time
Cow DMI (kg/d)	13.0	13.2	0.58	0.98	<0.001	0.24
Starter intake, kg/d	0.92	0.95	0.12	0.83	<0.01	0.99
Body weight, kg	64.8	63.1	1.6	0.43	<0.01	0.49
Body length, cm	126	123	1.5	0.16	<0.01	0.93
Hip height, cm	85.4	87.1	0.9	0.22	<0.01	0.42
Wither height, cm	82.4	84.6	0.9	0.10	<0.01	0.20
Hip width, cm	19.8	19.9	0.3	0.95	<0.01	0.01

Cows were fed during the last 30 d of pregnancy either Co glucoheptonate (CoPro, n = 8, 5 heifers, 3 bulls) or a slow-release Co polysaccharide (CoPectin, n = 7, 4 heifers, 3 bulls). Co treatments delivered 1 ppm Co/kg DM. Both diets contained 50 mg/d ruminally-available folic acid. ^{a-b}Within a row, means with different superscript letters differ, Diet×Time $P \leq 0.05$.



Results of the Western blotting analysis (Table 4) revealed that, compared with calves born to cows fed CoPro, calves born to cows fed CoPectin had greater abundance of INSR ($P = 0.05$), p-AKT ($P = 0.05$), and p-AKT/AKT ratio ($P = 0.001$), which is used as a proxy of AKT activity. In contrast, these calves had lower abundance of total 4EBP1 ($P = 0.03$) and MRF4 ($P = 0.04$).

4 Discussion

The present study demonstrates that the chemical form of cobalt supplemented to the dam during late gestation can modulate the postnatal muscle molecular response of calves. Because cobalt is a structural component of vitamin B₁₂, its bioavailability directly influences one-carbon metabolism and the generation of methyl donors such as S-adenosylmethionine (SAM). The higher concentrations of SAM and betaine observed in calves from CoPectin-fed cows suggest an enhanced methylation potential that may favor activation of the insulin–AKT–mTOR signalling pathway.

Wither height represents the best parameter for evaluating skeletal growth of heifers/calves because this measure is not affected by body condition and is correlated with body weight (Heinrichs et al., 1992). In one of our previous studies, a greater wither height in dairy calves born to cows fed an amino acid-complexed mixture of Zn, Mn, and Cu, and Co glucoheptonate compared with sulfate sources was suggestive of enhanced bone growth (Jacometo et al., 2015). Among the potential mechanisms that could affect bone development *in utero*, processes such as endochondrial ossification and cartilage formation require trace minerals (Chen et al., 2012) and recent data suggested a direct role of Co on angiogenesis and bone formation in rats (Zheng et al., 2024). Although no differences in dam DMI were achieved during the last period of pregnancy, similar to our previous study (Jacometo et al., 2015), the differences in the bioavailability of Co from CoPro versus CoPectin could have had an impact on its supply to the developing calf.

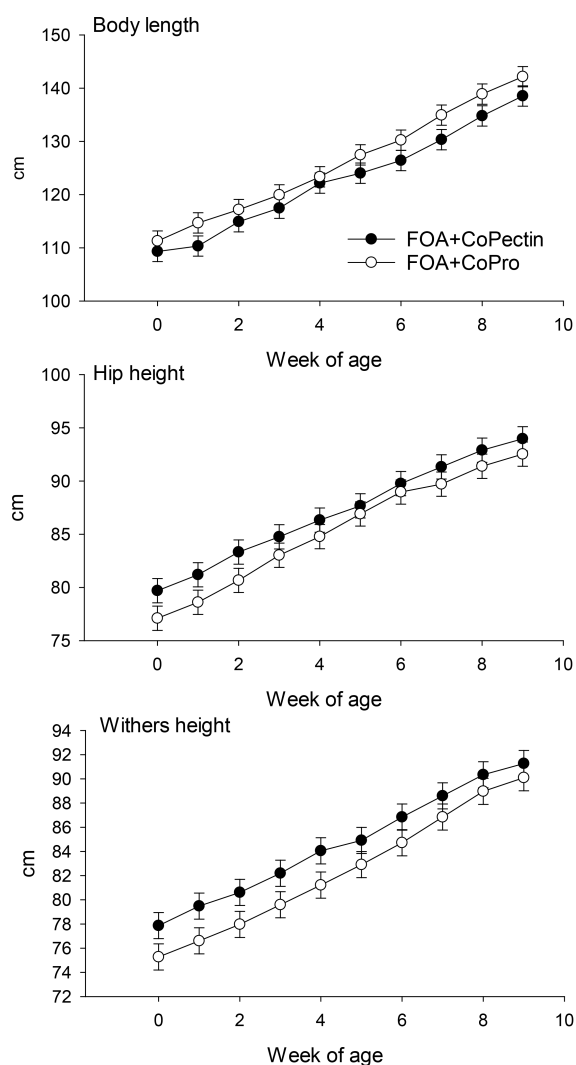


FIGURE 2
Body length, hip height, and withers height Hip width and body weight of calves born to cows fed during the last 30 d of pregnancy either Co glucoheptonate (CoPro, $n = 8$, 5 heifers, 3 bulls) or a slow-release Co polysaccharide (CoPectin, $n = 7$, 4 heifers, 3 bulls). Co treatments delivered 1 ppm Co/kg DM. Both diets contained 50 mg/d ruminally-available folic acid. Calves were biopsied prior to weaning at 6 wk of age. Co treatments delivered 1 ppm Co/kg DM. Asterisks in Figure denote significant differences at $P \leq 0.05$.

Both CoPro and CoPectin are rumen-available sources of Co (Zinpro Corp.), but the speed at which Co releases differs between sources because pectin is more resistant to microbial action than glucoheptonate. In fact, pectin itself can induce changes in the ruminal microbiota (Liu et al., 2015; Poulsen et al., 2012), which in turn can alter the assimilation of Co for the synthesis of vitamin B₁₂ (McDowell, 2000). Dietary Co content positively influences ruminal vitamin B₁₂ synthesis (Nutrient Requirements of Dairy Cattle, 2021). Thus, although either source results in a gradual release of Co in the rumen, feeding CoPectin would be expected to enhance Co availability to bacteria and potentially the animal.

From a molecular and physiological standpoint, the muscle protein abundance data also help understand the effect of maternal

supplementation of Co sources on postnatal calf growth. In tissues such as the liver, it is well-established in non-ruminants and ruminants (Coleman et al., 2021) that the activity of methionine synthase (MTR; a key component of the folic and Met cycles) requires vitamin B₁₂. This enzyme catalyzes the transfer of a methyl group from 5-methyltetrahydrofolate (5-MTH) to homocysteine to generate methionine, and vitamin B₁₂ (cobalamin) acts as a cofactor in this reaction (MTR is a vitamin B₁₂-dependent enzyme) (Krupenko, 2020). We recently demonstrated that the Holstein bovine fetal skeletal muscle at the last third of gestation expresses MTR and other enzymes with key roles in one-carbon metabolism (Aboragah et al., 2023). Thus, Co supplementation over currently recommended dosages [doubled from 0.11 to 0.20 mg/kg of dry matter in the latest Nutrient Requirements of Dairy Cattle, 8th edition (2021)] and during the last period of pregnancy (the most critical period where voluntary DMI is reduced) could further improve the production of methyl groups via the activity of methionine adenosyl transferase 1A (MAT1A) within the Met cycle. This idea is supported by recent data we generated demonstrating that MAT1A protein is highly-abundant in the bovine fetal (Aboragah et al., 2023) and adult cow skeletal muscle (Thanh et al., 2023).

The greater abundance of p-AKT (and in turn the greater p-AKT/AKT ratio) and INSR in CoPectin calves underscored the likely existence of improved skeletal muscle insulin sensitivity, a response that would be advantageous for postnatal calf growth (Hammon et al., 2012). To fully meet its requirements for glucose immediately after birth, the neonatal calf must activate glycogenolysis and especially gluconeogenesis to maintain normal glycemia (Baldwin et al., 2007). Hence, at equal amount of lactose provided by milk or milk replacer, the faster the calf acquires the ability to generate energy endogenously the better it can deal with the extrauterine demands of the body. As calves grow and receive increasing amounts of solid feed, the development of a ruminal microbial population ensures that propionate becomes the predominant gluconeogenic substrate (Donkin and Armentano, 1995).

At the cellular level, at least in non-ruminants, a greater abundance of p-AKT positively regulates a wide variety of functions including cell proliferation, survival, metabolism, and angiogenesis (Nader, 2005). Activity of p-AKT also feeds into the mTOR signaling pathway which controls the assembly of the eukaryotic translation initiation factor 4F (eIF4E) complex that responds to extracellular signals such as growth factors, nutrients, and cytokines (Powell et al., 2012).

Another potential mechanistic framework that links a greater availability of vitamin B₁₂ with the enhanced insulin signalling [through greater protein abundance of the Insulin Receptor (INSR)] is the intermediate metabolite S-adenosyl methionine (SAM). Based on present results, greater production of the methionine-cycle product SAM could lead to an enhanced activation of AKT-mTOR signalling. In fact, research with non-ruminant models has indicated that products of the Met cycle can activate mTOR. The activation of mTOR via SAM occurs indirectly as a result of SAM binding to the protein SAMTOR, which is an inhibitor of mTOR

TABLE 3 Metabolite concentrations (peak AUC) in semitendinosus muscle from calves born to cows fed during the last 30 d of pregnancy either Co glucoheptonate (CoPro, n = 8, 5 heifers, 3 bulls) or a slow-release Co polysaccharide (CoPectin, n = 7, 4 heifers, 3 bulls).

Metabolite	Maternal Co treatment		SEM	P-value
	CoPro	CoPectin		
One-carbon metabolism				
Methionine	10,917,960	10,545,323	1,189,980	0.81
Glycine	6,907,345	8,530,506	1,608,899	0.42
N,N-DimethylGlycine	208,458	142,410	46,627	0.28
Betaine	4,118,250 ^b	5,107,904 ^a	362,904	0.04
S-5'-adenosyl-homocysteine	1,719,669	1,778,093	60,810	0.47
S-5'-adenosyl-methionine	2,612,862 ^b	3,869,457 ^a	416,798	0.02
Choline	5,826,507 ^x	8,044,102 ^y	1,155,294	0.10
Adenosine	1,663,972	1,962,144	268,202	0.38
Carnitine	9,363,393	8,050,795	575,033	0.12
Butyrobetaine	951,339	1,037,011	70,047	0.33
Transsulfuration				
Serine	2,008,657	2,404,990	227,880	0.20
Cystathionine	711,793 ^y	1,016,655 ^x	96,800	0.06
Cysteine	13,595	14,295	1,990	0.79
Hypotaurine	535,489	527,459	81,603	0.94
Taurine	2,320,171	2,355,007	213,676	0.90
Glutathione	51,163,087	55,392,986	4,461,556	0.48
γ-Glutamylcysteine	166,677	221,815	40,067	0.25
Glycolysis/TCA cycle				
Phosphoenolpyruvate	2,998,064	3,682,106	662,561	0.41
Pyruvate	461,829	419,933	82,237	0.71
Malate	3,704,121	3,716,981	456,758	0.98
Citrate	129,518	139,065	25,225	0.77
α-ketoglutarate	2,798,456	5,201,873	1,487,336	0.13
Glutamate	71,122,366	74,606,603	4,703,329	0.58
Glutamine	385,774	354,591	64,229	0.73
Pentose phosphate pathway				
D-ribose-5-P/D-ribulose-5-P	7,897,146	7,746,429	769,437	0.88
D-fructose-6-P/D-glucose-6-P	20,913,047	19,007,955	2,148,893	0.53
6-phosphogluconic acid	27,778	30,131	4,801	0.71
Nucleotides				
NAD	7,947,667	8,057,495	847,249	0.92
NADP	671,999	654,257	42,161	0.75
FAD	3,889,356	4,362,730	272,705	0.19
UDP	533,304	585,252	45,678	0.40
AMP	28,427	31,781	2,368	0.31
cAMP	215,107	211,602	18,305	0.88

Co treatments delivered 1 ppm Co/kg DM. Both diets contained 50 mg/d ruminally-available folic acid. Calves were biopsied prior to weaning at 6 wk of age. Co treatments delivered 1 ppm Co/kg DM. ^{a, b}Within a row, means with different superscript letters differ, $P \leq 0.05$. ^{x, y}Different superscript letters between means within a row indicate a tendency at $0.10 \geq P \geq 0.05$.

TABLE 4 Abundance of proteins (relative to GAPDH) in semitendinosus muscle from calves born to cows fed during the last 30 d of pregnancy either Co glucoheptonate (CoPro, n = 8, 5 heifers, 3 bulls) or a slow-release Co polysaccharide (CoPectin, n = 7, 4 heifers, 3 bulls).

Protein ¹	Maternal Co treatment		SEM	P-value
	CoPro	CoPectin		
mTOR pathway (protein synthesis)				
Total mTOR	0.05	0.10	0.041	0.31
Phosphorylated (p) mTOR	0.22	0.30	0.110	0.52
pmTOR:mTOR	3.33	2.08	1.477	0.47
Total eIF2alpha	0.57	0.57	0.062	0.99
Phosphorylated (p) eIF2alpha	0.10	0.11	0.018	0.74
peIF2alpha:eiF2alpha	0.20	0.25	0.038	0.51
Total 4EBP1	0.74 ^a	0.58 ^b	0.063	0.03
Phosphorylated 4EBP1	0.66	0.67	0.158	0.98
p4EBP1:4EBP1	0.95	0.90	0.224	0.88
Total S6K1	0.07	0.06	0.005	0.14
Phosphorylated (p) S6K1	0.06	0.05	0.005	0.36
pS6K1:S6K1	0.86	1.12	0.163	0.31
mTOR regulators				
TSC2	0.14	0.05	0.072	0.25
ERK	0.90	0.82	0.062	0.37
Insulin signalling				
INSR	0.15 ^b	0.35 ^a	0.102	0.05
PI3K	0.35	0.33	0.044	0.72
Total AKT	0.15	0.05	0.092	0.30
Phosphorylated (p) AKT	0.63 ^b	0.83 ^a	0.064	0.05
pAKT : AKT	8.85 ^b	17.7 ^a	7.324	0.001
MRF4	0.25 ^a	0.16 ^b	0.022	0.05
SLC2A4	0.05	0.21	0.154	0.18
Energy metabolism				
PPARGC1alpha	0.27	0.25	0.039	0.72
Amino acid transport/metabolism				
SLC1A3 (acidic AA transport)	0.71	0.52	0.088	0.18
SLC38A1 (glutamine)	0.89	0.79	0.107	0.56
SLC1A5 (Na-dependent)	0.47	0.55	0.092	0.55
BCKDK (branched-chain AA catabolism)	0.25	0.23	0.034	0.72

Co treatments delivered 1 ppm Co/kg DM. Both diets contained 50 mg/d ruminally-available folic acid. Calves were biopsied prior to weaning at 6 wk of age. Co treatments delivered 1 ppm Co/kg DM. ^a, ^bWithin a row, means with different superscript letters differ, $P \leq 0.05$.

The ratio of phosphorylated protein to total protein is a proxy for activity.

complex 1 (MTORC1), and the subsequent inhibition of the association of SAMTOR and GATOR1, allowing MTORC1 to be activated (Gu et al., 2017). Upon activation of mTORC1, this kinase phosphorylates S6K and inhibits it, resulting in an increase of insulin sensitivity and upregulation of *IRS1* and *INSR* (Guo 2016).

The observed differences in muscle metabolites are suggestive of alterations in the Met cycle and the transsulfuration pathway in relation to the availability of Co during the last stages of pregnancy, where cows face physiological challenges that might impinge on their voluntary DMI. Ensuring a slow release of Co in the rumen

would lead to increased availability to ruminal microbes and formation of vitamin B₁₂, which in turn would be available for uptake at the intestinal level at a sustained rate. If such a mechanism holds, then the supply of vitamin B₁₂ to the liver and other extrahepatic tissues of the fetus would increase.

Along with the presence of intermediate metabolites, protein abundance for components of one-carbon metabolism in skeletal muscle allow for greater understanding of the long-term biological impacts on the calf. Thus, together with metabolite data discussed above, the lower protein abundance of MRF4 in muscle of CoPectin calves supports the idea of enhanced growth. At least in non-ruminants, this protein binds DNA and exerts control over transcription of genes associated with muscle cell differentiation and maintenance of postnatal muscle mass (Moretti et al., 2016). Knockout mouse studies demonstrated that inhibition of MRF4 induces muscle protein synthesis via activation of muscle-specific genes (Moretti et al., 2016), thus, along with the greater abundance of the insulin receptor INSR and p-AKT : AKT and the lower total 4EBP1, the semitendinosus muscle in CoPectin calves seemed to have been programmed for more efficient growth.

In the context of the present study, we speculate that besides its direct positive effect on semitendinosus muscle growth, other muscle types including those of the hip region (e.g., iliopsoas and sartorius) and their interactions with surrounding osteoblasts could help explain the tendency for greater wither height and the greater hip width over time. Mechanistically, although not measured in the present study, it is well-known in non-ruminants that skeletal muscle and bone anabolism are tightly coupled during growth such that growth factors produced by muscle could impact growth of the surrounding osteoblasts (Hamrick et al., 2010). Thus, despite the lack of published data with dairy calves, the present data are indicative of a synergistic effect of prenatal nutrition on skeletal muscle development through the pre-weaning stage.

A limitation of the present study is that muscle samples were collected only once, at 42 days of age, which prevents assessment of temporal changes in molecular and metabolic profiles. In addition, only one muscle (semitendinosus) was analysed; other tissues could respond differently to maternal cobalt supplementation. These aspects limit the generalization of the current findings. Future studies should include multiple time points and tissues to better capture the dynamic effects of cobalt source and the persistence of these programming mechanisms.

5 Conclusions

Trace minerals, especially the ones that are important drivers of key metabolic reactions (also known as cofactors), can be essential for adequate growth of the fetus, which depends completely on the dam for supply of these elements. Compared with cobalt glucoheptonate, cobalt pectin supplementation led to higher S-adenosylmethionine and betaine concentrations, along with greater INSR and phosphorylated AKT abundance, suggesting that maternal cobalt source affects the activity of one-carbon metabolism and insulin signaling in calf skeletal muscle. Although growth performance

parameters were largely unaffected, the combined metabolite and protein data indicate that maternal cobalt source may shape early-life muscle metabolic programming. Because muscle tissue was evaluated only once and no functional or anatomical growth measures were performed, these results should be interpreted as molecular-level adaptations rather than physiological outcomes. Future studies should investigate how different cobalt sources modulate vitamin B₁₂ synthesis, one-carbon metabolism, and downstream anabolic signaling across multiple tissues and developmental stages to clarify their long-term implications for calf growth and health.

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 the Institutional Animal Care and Use Committee (protocol no. 17168) of the University of Illinois, Urbana-Champaign. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

VL: Investigation, Writing – original draft. CJ: Data curation, Formal analysis, Methodology, Writing – original draft. AA: Data curation, Formal analysis, Investigation, Writing – review & editing. TI: Investigation, Writing – review & editing. FA: Investigation, Writing – original draft. MS: Resources, Writing – review & editing. JL: Conceptualization, Funding acquisition, Project administration, Resources, Writing – review & editing.

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Conflict of interest

Author MS is employed by the company Zinpro Corporation.

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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.2025.1686755/full#supplementary-material>

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