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Animal Health and Well Being

Disrupted one-carbon metabolism in heifers negatively affects their health and physiology

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Abstract

The objective of this study was to determine the dose-dependent response of one-carbon metabolite (**OCM**: methionine, choline, folate, and vitamin B_{12}) supplementation on heifer dry matter intake on fixed gain, organ mass, hematology, cytokine concentration, pancreatic and jejunal enzyme activity, and muscle hydrogen peroxide production. Angus heifers ($n = 30$; body weight [**BW**] = 392.6 ± 12.6 kg) were individually fed and assigned to one of five treatments: **0XNEG**: total mixed ration (**TMR**) and saline injections at days 0 and 7 of the estrous cycle, **0XPOS**: TMR, rumen-protected methionine (**MET**) fed at 0.08% of the diet dry matter, rumen-protected choline (**CHOL**) fed at 60 g/d, and saline injections at days 0 and 7, **0.5X**: TMR, MET, CHOL, 5-mg B_{12}, and 80-mg folate injections at days 0 and 7, **1X**: TMR, MET CHOL, 10-mg vitamin B_{12}, and 160-mg folate at days 0 and 7, and **2X**: TMR, MET, CHOL, 20-mg vitamin B_{12}, and 320-mg folate at days 0 and 7. All heifers were estrus synchronized but not bred, and blood samples were collected on days 0, 7, and at slaughter (day 14) during which tissues were collected. By design, heifer ADG did not differ ($P = 0.96$). Spleen weight and uterine weight were affected cubically ($P = 0.03$) decreasing from 0XPOS to 0.5X. Ovarian weight decreased linearly ($P < 0.01$) with increasing folate and B_{12} injection. Hemoglobin and hematocrit percentage were decreased ($P < 0.01$) in the 0.5X treatment compared with all other treatments. Plasma glucose, histotroph protein, and pancreatic α-amylase were decreased ($P \leq 0.04$) in the 0.5X treatment. Heifers on the 2X treatment had greater pancreatic α-amylase compared with 0XNEG and 0.5X treatment. Interleukin-6 in plasma tended ($P = 0.08$) to be greater in the 0XPOS heifers compared with all other treatments. Lastly, 0XPOS-treated heifers had reduced ($P \leq 0.07$) hydrogen peroxide production in muscle compared with 0XNEG heifers. These data imply that while certain doses of OCM do not improve whole animal physiology, OCM supplementation doses that disrupt one-carbon metabolism, such as that of the 0.5X treatment, can induce a negative systemic response that results in negative effects in both the dam and the conceptus during early gestation. Therefore, it is necessary to simultaneously establish an optimal OCM dose that increases circulating concentrations for use by the dam and the conceptus, while avoiding potential negative side effects of a disruptive OCM, to evaluate the long-term impacts of OCM supplementation of offspring programming.

Lay Summary

The feeding of one-carbon metabolites (including methionine and B vitamins) has been shown to improve fetal growth and milk production in species such as mice, sheep, and dairy cattle. Extending this to beef cattle around the time of breeding is a growing area of research. Our group previously determined that one-carbon metabolite supplementation to beef heifers altered the abundance of circulating methionine-folate cycle intermediates in a dose-dependent manner. Therefore, we aimed to determine a whole-body response to one-carbon metabolite supplementation in heifers by measuring the effects on specific physiological systems as well as a total systemic response. We determined that treatments that negatively altered the methionine-folate cycle yielded a fundamental negative whole-body response to supplementation.

Key words: estrous cycle, heifers, one-carbon metabolism

Abbreviations: 0XNEG, total mixed ration and sham im injections of saline at days 0 and 7 of the estrous cycle; 0XPOS, total mixed ration, rumen-protected methionine fed at 0.08% of the diet DM, rumen-protected choline; fed at 60 g/d, and sham im injections of saline at days 0 and 7 of the estrous cycle; 0.5X, total mixed ration, rumen-protected methionine, rumen-protected choline, and im injection of 5-mg vitamin B_{12}, and 80-mg folate at days 0 and 7 of the estrous cycle; 1X, total mixed ration, rumen-protected methionine, rumen-protected choline, and im injection of 10-mg vitamin B_{12}, and 160-mg folate at days 0 and 7 of the estrous cycle; 2X, total mixed ration, rumen-protected methionine, rumen-protected choline, and im injection of 20-mg vitamin B_{12}, and 320-mg folate at days 0 and 7 of the estrous cycle; AA, amino acid; CHOL, rumen-protected choline; CL, corpus luteum; DM, dry matter; DMI, dry matter intake; GnRH, gonadotropin releasing hormone; IFN-γ, interferon-gamma; IL-1α, interleukin-1-alpha; IL-1β, interleukin-1-beta; IL-6, interleukin-6; IL-8, interleukin-8; IL-10, interleukin-10; IL-17A, interleukin-17A; IL-36RA, interleukin-36 receptor agonist; IP-10, IFN-γ-induced protein; MCP-1, monocyte chemoattractant protein-1; MCH, mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MET, rumen-protected methionine; MIP1α, macrophage inflammatory protein-1-alpha; MIP1β, macrophage inflammatory protein-1-beta; OCM, one-carbon metabolites; PUN, plasma urea nitrogen; RBC,

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red blood cell; RDW-CV, red cell distribution width percent; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; TMR, total mixed ration; TNF-α, tumor necrosis factor-alpha; VEGF-A, vascular endothelial growth factor A; WBC, white blood cell

Introduction

The roles of biochemicals involved in one-carbon metabolism, which include choline, B vitamins (vitamin B_{12}, vitamin B_6, riboflavin, and folate [vitamin B_9]), minerals (cobalt [component of vitamin B_{12}], sulfur [component of methionine]), and amino acids (methionine, serine, and glycine) that provide a one-carbon moiety for pathways and physiological functions (i.e., DNA methylation, nucleotide synthesis, etc.) have been a topic of increasing research and focus, particularly in dairy cattle (Clare et al., 2019; Khan et al. 2020a). Recently, Crouse et al., (2023) determined that the one-carbon metabolite (**OCM**; methionine, choline, folate, and vitamin B_{12}) dose that was sufficient to maintain an increased OCM concentration was 0.08% of the diet dry matter (**DM**) of rumen-protected methionine (**MET**), 60 g/d of rumen-protected choline (**CHOL**), 320 mg of folate injected once weekly, and 20 mg of vitamin B_{12} injected once weekly. In the same report, they determined that feeding the same quantity of methionine and choline but injecting only 80 mg of folate and 5 mg of B_{12} decreased heifer methylation potential, as indicated by a decrease in S-adenosyl methionine:S-adenosyl homocysteine (**SAM:SAH** ratio) in jugular venous serum.

In humans, decreased methylation potential is present in multiple disease states including cancer, liver diseases (cirrhosis, hepatitis, carcinoma, and liver failure), neurological diseases (Parkinson's depression), kidney disease, type 2 diabetes, high blood pressure, heart disease, and inflammation (Hao et al., 2016). Additionally, Hao et al. (2016) determined that methylation potential could be used as an index of health and/or stages of disease state. Even in a nondiseased state, increased SAH resulting in decreased methylation potential can result in DNA, RNA, and protein hypomethylation, changes in gene expression, and altered cellular differentiation and chromatin conformation, ultimately leading to changes in cellular and organismal phenotype (Jill et al., 2002).

The effects of OCM supplementation, particularly during the periparturient period, have been well-documented in dairy cattle (McFadden et al., 2020; Girard and Duplessis, 2023). MET supplemented at 0.08% of the diet DM increased dry matter intake (**DMI**), circulating insulin in the cows, and calf body weight at birth (Batistel et al., 2017). Furthermore, methionine supplementation increased placentome mRNA transcript abundance of multiple glucose and amino acid (**AA**) transporters as well as increased abundance of multiple mTOR regulatory proteins (Batistel et al., 2017). MET and choline reduced oxidative stress, enhance immune function, and therefore, improved health and metabolic function (Osorio et al., 2013, 2014; Shahsavari et al., 2016; Zhou et al., 2016; Batistel et al., 2018; Coleman et al., 2020). Folate supplementation increased circulating concentrations of IFN-γ and interleukin-17 and stimulate B-cell-mediated immunity in transition dairy cows (Ouattara et al., 2016; Khan et al., 2020a, b). Folate and vitamin B_{12} also improved dairy cow metabolism during lactation (Girard and Duplessis, 2023). When fed or injected in combination, folate and vitamin B_{12} increased milk production, milk component yield, and plasma glucose concentrations, while decreasing hepatic lipids (Graulet et al., 2007; Preynat et al., 2009; Wang et al., 2019). Furthermore, there were no changes in DMI or body weight with folate and vitamin B_{12} injection, demonstrating improvements in metabolism by reducing mobilization of body reserve for energy to meet the demands of lactation (Graulet et al., 2007; Preynat et al., 2009; Wang et al., 2019).

In beef cattle, however, data concerning the immediate effects of OCM supplementation to the heifer/cow and offspring performance are limited. Multiple studies in which both cows and heifers were fed methionine hydroxy analogs during the periparturient period show a lack of effect on milk yield and growth performance of cows and calves, respectively (Huber et al., 1984; Waterman et al., 2007; Clements et al., 2017; Collins et al., 2019; Moriel et al., 2020; Redifer et al., 2023). Supplementation of methionine hydroxy analogs, however, did increase milk fat and milk solids (Lawson et al., 2019; Redifer et al., 2023). Cows supplemented with methionine during the periconceptual period had calves with greater gain:feed, greater total GI tract neutral detergent and acid detergent fiber digestibility, and decreased plasma glucose concentration during a 42-day metabolism study relative to calves from control-fed cows (Silva et al., 2021). Feeding MET to heifers upon arrival in a feedlot resulted in decreased haptoglobin concentrations compared with their control counterparts suggesting that methionine may help alleviate oxidative stress and inflammatory responses in receiving cattle (Grant et al., 2022).

The objective of this study was to determine the effects of increasing doses of folate and vitamin B_{12} with a consistent delivery amount of methionine and choline to heifers during an estrous cycle on measures of physiological health and function. We hypothesized that OCM supplementation improves health and physiology of beef heifers in a dose-dependent manner.

Materials and Methods

This experiment was approved by the United States Meat Animal Research Center Institutional Animal Care and Use Committee (EO # 128.1) in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching.

Animals, housing, and treatments

A full description of animals, housing, and treatments can be found in Crouse et al. (2023). Briefly, Angus heifers (n = 30, ~15 mo of age; initial BW = 392.6 ± 12.6 kg) were trained to consume feed from individual feeders (American Calan, Northwood, NH) and were fed a total mixed ration (**TMR**) consisting of 75% grass/alfalfa hay, 21% corn silage, and 4% of a mineral pellet on a DM basis. Due to individual feeding, heifer was the experimental unit. For the treatments containing rumen-protected supplements, the TMR was dried weekly for DM analysis to ensure methionine was delivered at 0.08% of the DM. Additionally, both methionine and choline were mixed in with the TMR for each individual animal to guarantee accurate and consistent supplement delivery. Heifer weight and blood samples were collected prior to feeding on days 0, 2, 5, 7, 9, and 12 and feed intake was adjusted after each weight to target 0.45 kg/d average daily gain. Orts were collected weekly for measurement of DMI.

All heifers underwent synchronization of estrus as described by Crouse et al. (2023) and were randomly assigned to one of five treatments (n = 6/treatment) that were initiated on day 0 (day of second Gonadotropin Releasing Hormone and referred to as day 0 of the estrous cycle) of the study. These treatments were **0XNEG**: TMR and injections of saline at days 0 and 7 of the estrous cycle, **0XPOS**: TMR, MET (Smartamine M, Adisseo, Alpharetta, GA) fed at 0.08% of the diet DM, CHOL (ReaShure, Balchem Inc., New Hampton, NY) fed at 60 g/d, and injections of saline at days 0 and 7 of the estrous cycle, **0.5X**: TMR, MET, CHOL, injections of 5-mg vitamin B_{12}, and 80-mg folate at days 0 and 7 of the estrous cycle, **1X**: TMR, MET CHOL, injections of 10-mg vitamin B_{12}, and 160-mg folate at days 0 and 7 of the estrous cycle, **2X**: TMR, MET, CHOL, injections of 20-mg vitamin B_{12}, and 320-mg folate at days 0 and 7 of the estrous cycle. Injections of saline (0.9% NaCl; 10 mL), vitamin B_{12} (5,000 µg/mL; Neogen Vet, Lansing, MI) and/or folic acid (5 mg/mL; Fresenius Kabi, Bad Homburg, Germany) were administered intramuscularly with no more than 10 mL of solution administered in a single injection site. As previously described in Crouse et al. (2023), treatment doses of folate and vitamin B_{12} (referred to as vitamin) were based on published literature in Holstein cows where the current 1X vitamin dose was equal to that of Preynat et al. (2009). MET and CHOL were fed according to manufacturer recommendations at the same inclusion quantity across all supplemented treatments.

Sample collection and analysis

Blood samples were collected before treatment initiation on days 0 and 7, and before slaughter on day 14 via jugular venipuncture using 10-mL EDTA vacutainer tubes (Becton Dickinson HealthCare, Franklin Lakes, NJ). Before centrifugation, whole blood was used for hematology as described below. After hematology analysis, the blood was centrifuged at 1,500 × g for 20 min, plasma decanted, and stored at −80 °C. On day 14 after synchronization of estrus, all heifers were slaughtered at a federally inspected abattoir at the USDA, ARS, US Meat Animal Research Center. After exsanguination, the uterus was collected, weighed, and transported to the laboratory for additional measurements. The width of each uterine horn was measured with calipers approximately 1 inch from the uterine bifurcation and both horn measurements were summed for a total horn diameter. The uterus was then clamped cranially to the cervix, flushed with 20 mL of phosphate-buffered saline into the contralateral horn and recovered histotroph collected from the ipsilateral horn. Ovaries were weighed and all visible surface follicles were counted. The corpus luteum (**CL**) was removed from the ovary and weighed. A representative cross section of the ovary contralateral to the CL was fixed and embedded in paraffin and ovarian histology was conducted using previously published methods to determine the number of primordial, primary, and secondary follicles per section (Cushman et al., 1999, 2001, 2007). After evisceration, the liver and spleen were collected and weighed, and a sample of muscle from the semitendinosus was collected for mitochondrial analysis as described below. Allometric data was collected for liver, spleen, uterus, and ovary weights and were recorded as grams per kilogram of live weight on day of slaughter. Due to the method of evisceration in the abattoir, samples of the pancreas were collected for enzyme activity analysis, but pancreatic weight was not measured because it was not excised from the body whole. A 1-m segment of the jejunum was isolated from the small intestine, cut laterally, and scraped with a glass slide to isolate the mucosa. Pancreas and jejunal mucosa samples were flash-frozen in liquid nitrogen and stored at −80 °C for enzymatic activity analysis as described below.

Hematology analysis

Whole blood samples were transported to the laboratory for hematology analysis. Samples were placed on gentle rotation on a tube rocker at room temperature until analysis. Samples were tested for 14 different white blood cell (**WBC**) and red blood cell (**RBC**) hematology parameters using a Heska Element HT5 Veterinary Analyzer (Loveland, CO). Hematology parameters included total white blood cells (10^3/µL), neutrophils (10^3/µL), lymphocytes (10^3/µL), monocytes (10^3/µL), eosinophils (10^3/µL), basophils (10^3/µL), red blood cells (10^6/µL), hemoglobin (g/dL), percent hematocrit, mean corpuscular volume (fL), mean corpuscular hemoglobin (pg), mean corpuscular hemoglobin concentration (g/dL), red cell distribution width percent (**RDW-CV**), platelets (10^3/µL), and mean platelet volume (fL).

Plasma and histotroph cytokine/chemokine multiplex

Plasma and histotroph samples were used for the simultaneous quantification of cytokines and chemokines using the MILLIPLEX Bovine Cytokine/Chemokine Magnetic Bead Panel 1- Immunology Multiplex Assay (Millipore Sigma, Burlington, MA). This assay simultaneously quantifies 15 cytokines and chemokines: interferon-gamma (**IFN-γ**), interleukin-1-alpha (**IL-1α**), interleukin-1-beta (**IL-1β**), interleukin-6 (**IL-6**), interleukin-8 (**IL-8**), interleukin-10 (**IL-10**), interleukin-17A (**IL-17A**), macrophage inflammatory protein-1-alpha (**MIP-1α**), interleukin-36 receptor agonist (**IL-36RA**), IFN-γ-induced protein-10 (**IP-10**), monocyte chemoattractant protein-1 (**MCP-1**), macrophage inflammatory protein-1 (**MIP-1β**), tumor necrosis factor-alpha (**TNF-α**), and vascular endothelial growth factor A (**VEGF-A**). The cytokine/chemokine analysis was only performed on plasma samples from days 0 and 14 of the study. Concentrations of all cytokines are reported as pg/mL (intraplate coefficient of variation [CV] across all analytes: 21.45%).

Plasma and histotroph metabolites

Urea nitrogen (**PUN**) concentration was analyzed in plasma samples from days 0, 7, and 14 according to previously published methods (intraplate CV = 6.31%; interplate CV = 12.07%; Fawcett and Scott, 1960; Chaney and Marbach, 1962). Plasma and histotroph glucose concentrations were determined using the Infinity Glucose Hexokanse Liquid Stable Reagent (Thermo Fisher Scientific, Waltham, MA). For glucose analysis, 5 µL of plasma or histotroph was added with 250 µL of reagent in a 96-well flat bottom microplate (intraplate CV = 4.03%; interplate CV = 3.97%). Histotroph total protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific; intraplate CV = 7.71%; interplate CV = 9.42%). Urea nitrogen, glucose, and protein were microplate assays and used the BioTek Synergy H1 microplate reader (Agilent Technologies, Inc, Santa Clara, CA).

Pancreatic and jejunal protein and enzyme activity analysis

All procedures are described in detail by Trotta et al. (2023). Briefly, pancreas and jejunal mucosal samples were homogenized (Kinematica Polytron PT 3100; Brinkmann Instruments Inc.) and total protein was measured with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Pancreatic α-amylase activity was determined using the procedure outlined by Wallenfels et al. (1978) using the Amylase Reagent Set (Teco Diagnostics; Anaheim, CA) with p-nitrophenyl-D-maltoheptaoside as the substrate. One unit (U) of enzyme activity equals 1 µmol of p-nitrophenol produced per minute. Jejunal enzyme activity assays followed the methods of Dahlqvist (1964) for maltase and isomaltase and the methods of Kidder et al. (1972) for glucoamylase. Procedures were modified for use with cattle tissues as recommended by Kreikemeier et al. (1990) and Siddons (1968). Following enzymatic hydrolysis, glucose concentrations were measured as previously described. About 1 U of enzyme activity equals 1 µmol of glucose produced per minute for glucoamylase and 2 µmol of glucose produced per minute for maltase and isomaltase. Tissue blanks were quantified for endogenous glucose concentrations which were subtracted from the total amount of product produced per minute. Because cattle do not have measurable sucrase activity (Trotta et al., 2022), α-glucosidase activity is equal to the sum of maltase, isomaltase, and glucoamylase activity.

Mitochondrial hydrogen peroxide analysis

Mitochondria were isolated via differential centrifugation, with modifications (Chappel and Perry, 1954; Makinen and Lee, 1968. Approximately 1.0 g of muscle was diced in four volumes of homogenization buffer (140 mM KCl, 20 mM HEPES, 5 mM MgCl6•H2O, 2mM EGTA, 1 mB ATP, 10 mg/mL fatty acid-free BSA, pH 7.4). One hundred µL subtilisin A protease (1.5 AU/mL; Sigma) per g of muscle was added and the sample was shaken at 4 °C for 9 min. The protease incubation mixture was washed 6-fold in homogenization buffer and filtered through a 100-µm sieve (BD Bioscience). The tissue was then homogenized in 12 mL of homogenization buffer per g muscle in a Dounce homogenizer (Wheaton). The slurry was centrifuged at 500 × *g* at 4 °C and the pellet containing cellular debris was discarded. The supernatant was then centrifuged at 9,600 × *g* at 4 °C for 10 min. The supernatant was discarded, and the pellet was resuspended and washed twice in wash buffer (140 mM KCl, 20 mM HEPES, 5 mM MgCl6•H2O, 1 mM EGTA, 10 mg/mL fatty acid-free BSA, pH 7.4), centrifuging at 4,300 × *g* at 4 °C between washes. The sample was centrifuged again at 4,300 × *g* at 4 °C and the pellet was resuspended in 200-µL isolation medium (140 mM KCl, 20 mM HEPES, 5 mM MgCl6•H2O, 1 mM EGTA). The protein concentration of the extracts was determined using the bicinchoninic acid reagent (Smith et al., 1985).

Hydrogen peroxide (H_2O_2) production from the mitochondria was used to determine reactive oxygen species production via 2,7 dichlorofluorescin diacetate (DCFH), as previously described by Iqbal et al. (2001), with modifications. All reagents were made fresh each day. A H_2O_2 standard curve was used to calculate production values. Fluorescence of DCFH was measured on a BioTek Synergy H4 microplate reader (BioTek, USA) at 38 °C at an excitation/emission wavelength of 480/530 nm. Samples and standards were run in triplicate on black polystyrene 96-well plates. Twenty units of superoxide dismutase and 51-µM DCFH were added in 45-µL isolation buffer to each well. In addition, either 8-µm glutamate or succinate was added as an energy substrate for electron transport complexes I and II, respectively. Individual electron transport complexes were inhibited by 10-µM rotenone, 8-µM 4,4,4-trifluoro-1-[2-thienyl]-1,3-butanedione, 13-µM antimycin A, or a combination of the three. Either H_2O_2 standards or 100 µg of mitochondrial protein per well was added and blank wells containing no mitochondria were used to calculate background fluorescence. Readings were taken every 5 min from 0 to 40 min.

Due to time constraints to accurately collect and analyze peroxides from muscle mitochondria, no heifers from the 2X treatment and only five of the six heifers from the remaining treatments were used.

Statistical analysis

All statistical analysis of hematology and metabolite data was conducted using the MIXED procedure of SAS 9.4 (SAS Institute, Cary, NC) with repeated measures using AR(1) as the covariate based on the lowest AIC/BIC, with day, treatment, and the interaction in the model as fixed effects and animal nested within treatment as the random effect. Statistical significance was met at $P \leq 0.05$, and tendencies $P > 0.05$ but less than $P \leq 0.10$. Least squares means were computed using the LSMEANS statement and mean separation was conducted with the PDIFF statement in SAS.

Weights, DMI, organ measurements, pancreatic and jejunal protein concentration, enzyme activity analysis, and H_2O_2 analysis were analyzed using PROC GLM and means were separated using LSMEANS. Polynomial contrast coefficients were generated using PROC IML for unequal spacing between treatments. To test the effects of MET and CHOL (Negative vs. Positive), a contrast was performed for 0XNEG vs. 0XPOS. To test the effect of folate and vitamin B_{12} supplementation in combination with MET and CHOL, vs methionine and choline without additional folate and vitamin B_{12}, (0XPOS vs. Vitamin) a contrast statement was performed for 0XPOS vs. 0.5X, 1X, and 2X. Finally, linear, quadratic, and cubic orthogonal contrast statements were generated to evaluate concentrations of folate and vitamin B_{12} supplementation from 0XPOS, 0.5X, 1X, and 2X. For mitochondrial hydrogen peroxide analysis, Met + Choline vs. Vitamin and linear and quadratic orthogonal contrasts were conducted due to not analyzing the 2X treatment. Probability values were considered significant when $P \leq 0.05$, and tendencies were noted when $0.05 > P \leq 0.10$.

Results

Heifer DMI and gain

Heifer DMI and gain data are presented in Table 1. By design, there were no differences in initial weight, final weight, or ADG across treatments ($P \geq 0.89$). Daily DMI and average daily DMI adjusted per kg of heifer body weight were affected cubically ($P = 0.04$) such that intake increased from the 0XPOS to the 0.5X treatment then decreased to the at the 1X and 2X intake.

Hematology analysis

All hematology data are presented in Table 2. Reference ranges as well as minimum and maximum recorded values

Table 1. Body weight, ADG, and Intake following a timed estrus synchronization protocol in heifers as influenced by nutritional treatment and injection strategy

	Treatment[1]						*P* value[2]					
Measurement[3]	0XNEG	0XPOS	0.5X	1X	2X	SEM[4]	Trt	0XNEG vs. 0XPOS	0XPOS vs. vitamin	Lin	Quad	Cub
Initial weight, kg	397	390	288	399	384	11.5	0.89	0.90	0.74	0.84	0.47	0.98
Final weight, kg	404	397	404	406	392	12.0	0.90	0.90	0.90	0.79	0.79	0.70
ADG	0.51	0.49	0.49	0.52	0.48	0.04	0.96	0.96	0.84	0.76	0.58	0.88
DMI/d, kg	10.4	8.8	11.5	9.14	10.1	0.79	0.16	0.38	0.17	0.75	0.50	0.04
DMI, g/kg BW	25.1	21.6	28.0	22.7	25.3	1.86	0.15	0.17	0.12	0.53	0.54	0.04

[1]0XNEG: basal TMR. 0XPOS: basal TMR plus MET fed at 0.08% of the diet DM plus 60 g/d CHOL. 0.5X: 0XPOS ration plus 80-mg injectable folic acid plus 5-mg injectable vitamin B_{12}. 1X: 0XPOS ration plus 160-mg injectable folic acid plus 10-mg injectable B_{12}. 2X: 0XPOS ration plus 320-mg injectable folic acid plus 20-mg injectable vitamin B_{12}.
[2]Probability values for the main effect of nutritional treatment/injection (trt). The following are analyzed as contrasts: 0XNEG vs. 0XPOS and 0XPOS vs. Vitamin (0.5X, 1X, and 2X treatments). Linear (Lin), quadratic (Quad), and cubic (Cub) polynomial contrasts were conducted for 0XPOS, 0.5X, 1X, and 2X with PROC IML used to determine coefficients for unequal spacing.
[3]ADG: average daily gain, DMI: dry matter intake as a unnormalized measurement and scaled per kilogram of body weight.
[4]Average standard error of the mean for the main effect of nutritional treatment/injection.

are presented in Supplementary Table 1. There were no differences for any hematology parameter for the day × treatment interaction ($P \geq 0.62$). There were no differences in the concentration of WBC, neutrophils, monocytes, or basophils ($P \geq 0.25$). The number of lymphocytes was greater ($P = 0.02$) in 2X compared with 0XPOS- and 1X-treated heifers, with 0XNEG- and 0.5X-treated heifers being intermediate and not different to both. Eosinophils were greater ($P = 0.03$) in 0XNEG- and 0XPOS- compared with 1X-treated heifers with 0.5X- and 2X-treated heifers being intermediate and equal all other treatments. There were no differences ($P \geq 0.29$) in the number of WBC, lymphocytes, monocytes, eosinophils, or basophils across the estrous cycle; however, the number of neutrophils decreased with advancing day, being lesser ($P = 0.01$) on days 7 and 14 compared with day 0 of the estrous cycle. The number of lymphocytes increased linearly ($P = 0.01$) from 0XPOS- to 2X-treated heifers, the number of eosinophils was greater ($P = 0.01$) in those heifers on the 0XPOS treatment compared with heifers receiving vitamin-injection (0.5, 1X, and 2X).

The RBC count was greater ($P < 0.01$) in 0XNEG, 0XPOS, and 1X compared with 0.5X- and 2X-treated heifers. The hemoglobin count and hematocrit were greater ($P < 0.01$) in 0XNEG, 0XPOS, and 1X compared to 2X, which was greater than 0.5X-treated heifers. The RBC and hemoglobin counts, as well as the hematocrit were all affected similarly across day of the estrous cycle, being greater ($P \leq 0.02$) on day 0 compared with days 7 and 14 of the cycle. The MCV was greatest ($P < 0.01$) in the 2X-treated heifers, the 0XPOS and 0.5X were intermediate and equal to both the 0XNEG and 1X. The MCH was greater ($P < 0.01$) in the 2X heifers compared to the 0XNEG heifers, which were greater than or equal to the 0XPOS-, 0.5X-, and 1X-treated heifers. The MCHC was greater ($P < 0.01$) in 0.5X heifers compared with 1X and 2X heifers with 0XNEG and 0XPOS heifers being intermediate and equal to both the 0.5X and 2X treatments. The RDW-CV percentage was greater ($P < 0.01$) in the 0XNEG- and 0.5X-treated heifers compared to all other treatments. The RBC count was affected cubically ($P = 0.01$) with increasing vitamin dose, decreasing from 0XPOS to 0.5X, increasing at the 1X dose, and subsequently decreasing at the 2X dose to be equal to the 0.5X dose. The MCV and RDW-CV were greater ($P \leq 0.01$) in 0XNEG compared with 0XPOS-treated heifers, and MCH and platelets tended ($P \leq 0.10$) to be greater in 0XNEG compared with 0XPOS-treated heifers. The number of RBC, hemoglobin, and platelets as well as the hematocrit were greater ($P \leq 0.05$) in 0XPOS-treated heifers compared with those receiving B_{12} and folate injections. The number of RBC, hemoglobin, the MCH, MCHC, as well as the hematocrit and RDW-CV were affected cubically ($P \leq 0.01$) with increasing vitamin dose.

Plasma and histotroph cytokine/chemokine analysis

The concentration of IL-4 was below detectable limit in most samples for both plasma and histotroph and was therefore omitted from the results and discussion. All of the other plasma cytokine/chemokine analysis data are presented in Table 3. There were no differences for the concentration of any cytokine in plasma due to the day × treatment interaction ($P \geq 0.22$). The concentration of IL-6 in plasma tended ($P = 0.08$) to be greater in 0XPOS-treated heifers compared with all other treatments and was greater ($P = 0.03$)

Table 2. Hematology analysis of whole blood at days 0, 7, and 14 following a timed estrus synchronization protocol in heifers as influenced by nutritional treatment and injection strategy

		Treatment[1]							*P* value[2]							
Path. Meas.[3]	Day[4]	0XNEG	0XPOS	0.5X	1X	2X	Day avg.[5]	SEM[6]	Trt	Day	Trt × Day	0XNEG vs. 0XPOS	0XPOS vs. vitamin	Lin	Quad	Cub
WBC	0	9.64	9.18	9.81	9.21	10.04	9.58	0.82	0.48	0.93	0.98	0.11	0.38	0.21	0.55	0.21
	7	10.36	8.82	9.30	9.13	10.05	9.53									
	14	9.25	9.53	9.65	8.76	9.85	9.41									
	Trt Avg.[7]	9.75	9.18	9.59	9.03	9.98										
Lym	0	5.81	5.22	5.58	5.40	6.75	5.75	0.65	0.04	0.35	0.84	0.15	0.12	0.01	0.16	0.07
	7	6.73	5.31	6.41	5.84	6.62	6.18									
	14	5.93	6.24	6.18	5.38	7.02	6.15									
	Trt Avg.	6.16^{ab}	5.59^{b}	6.06^{ab}	5.54^{b}	6.80^{a}										
Neu	0	3.25	3.36	3.78	3.34	2.76	3.30	0.32	0.37	0.02	0.62	0.53	0.91	0.18	0.51	0.99
	7	3.04	2.86	2.49	2.91	2.88	2.84									
	14	2.80	2.65	2.96	3.02	2.23	2.73									
	Trt Avg.	3.03	2.96	3.08	3.09	2.62										
Mon	0	0.39	0.32	0.39	0.35	0.36	0.36	0.05	0.91	0.89	0.92	0.99	0.76	0.79	0.86	0.48
	7	0.33	0.37	0.33	0.34	0.37	0.35									
	14	0.30	0.34	0.40	0.32	0.36	0.34									
	Trt Avg.	0.34	0.34	0.37	0.34	0.36										
Eos	0	0.13	0.13	0.11	0.09	0.10	0.11	0.02	0.08	0.63	0.99	0.91	0.01	0.09	0.03	0.83
	7	0.12	0.13	0.08	0.09	0.11	0.11									
	14	0.16	0.14	0.12	0.09	0.11	0.12									
	Trt Avg.	0.14	0.14	0.11	0.09	0.10										
Bas	0	0.07	0.06	0.04	0.05	0.05	0.05	0.04	0.85	0.38	0.73	0.83	0.58	0.99	0.33	0.79
	7	0.04	0.05	0.05	0.04	0.07	0.05									
	14	0.05	0.05	0.05	0.04	0.03	0.04									
	Trt Avg.	0.05	0.05	0.05	0.04	0.05										
RBC	0	8.32	8.64	8.10	8.88	7.85	8.36^{x}	0.33	<0.01	0.05	0.98	0.24	0.01	0.01	0.20	0.01
	7	8.07	8.44	7.51	8.55	7.60	8.04^{xy}									
	14	8.24	8.35	7.62	8.30	7.42	7.99^{y}									
	Trt Avg.	8.21^{a}	8.48^{a}	7.73^{b}	8.59^{a}	7.63^{b}										
HGB	0	13.93	13.97	13.05	14.04	13.40	13.68	0.30	<0.01	<0.01	0.75	0.98	<0.01	0.18	0.24	<0.01
	7	13.47	13.74	12.27	13.48	13.07	13.21									
	14	13.77	13.71	12.61	13.17	12.74	13.20									
	Trt Avg.	13.7^{a}	13.8^{a}	12.6^{c}	13.6^{a}	13.1^{b}										
HCT (%)	0	42.9	43.1	40.3	43.8	41.8	42.4^{x}	1.05	<0.01	<0.01	0.84	0.97	0.02	0.55	0.43	<0.01
	7	41.4	42.2	37.4	42.0	40.5	40.7^{y}									
	14	42.6	42.1	38.3	41.1	39.4	40.7^{y}									
	Trt Avg.	42.3^{a}	42.47^{a}	38.7^{c}	42.3^{a}	40.6^{b}										

Table 2. Continued

		Treatment[1]							*P* value[2]							
Path. Meas.[3]	Day[4]	0XNEG	0XPOS	0.5X	1X	2X	Day avg.[5]	SEM[6]	Trt	Day	Trt × Day	0XNEG vs. 0XPOS	0XPOS vs. vitamin	Lin	Quad	Cub
MCV	0	51.5	50.3	49.7	49.6	54.0	51.0	1.78	<0.01	0.93	0.99	0.01	0.35	<0.01	<0.01	0.20
	7	51.4	50.3	49.8	49.3	54.1	51.0									
	14	51.8	50.7	50.1	49.7	54.0	51.3									
	Trt Avg.	51.6^{b}	50.4^{bc}	50.1^{bc}	49.4^{c}	53.8^{a}										
MCH	0	16.75	16.22	16.25	15.85	17.28	16.47	0.53	<0.01	0.80	0.99	0.08	0.39	<0.01	<0.01	0.01
	7	16.69	16.37	16.45	15.76	17.37	16.53									
	14	16.75	16.48	16.55	15.88	17.38	16.61									
	Trt Avg.	16.73^{b}	16.36^{b}	16.41^{b}	15.83^{c}	17.34^{a}										
MCHC	0	32.5	32.3	32.5	32.0	32.1	32.3	0.24	<0.01	0.42	0.99	0.21	0.27	0.02	0.35	<0.01
	7	32.5	32.6	32.8	32.1	32.3	32.5									
	14	32.3	32.6	32.8	32.1	32.3	32.4									
	Trt Avg.	32.5^{ab}	32.5^{ab}	32.7^{a}	32.1^{c}	32.3^{b}										
RDW-CV	0	23.3	21.7	23.7	21.3	21.6	22.3	0.75	<0.01	0.82	0.99	<0.01	0.44	0.04	0.30	<0.01
	7	23.0	21.7	23.4	21.0	21.3	22.1									
	14	22.9	21.7	23.3	21.5	21.1	22.1									
	Trt Avg.	23.1^{a}	21.7^{b}	23.5^{a}	21.2^{b}	21.4^{b}										
PLT	0	375.0	473.6	393.0	455.7	474.6	434.4	57.6	0.52	0.72	0.83	0.10	0.88	0.54	0.63	0.44
	7	446.4	508.3	451.3	422.0	476.7	460.9									
	14	404.1	417.6	456.9	532.9	480.9	458.5									
	Trt Avg.	408.7	466.9	432.2	469.8	477.3										
MPV	0	6.24	6.25	5.96	6.10	6.09	6.13	0.23	0.20	0.83	0.99	0.99	0.05	0.18	0.29	0.24
	7	6.28	6.12	5.88	6.04	5.98	6.06									
	14	6.37	6.44	5.93	6.06	5.94	6.05									
	Trt Avg.	6.28	6.26	5.98	6.06	6.00										

[1]0XNEG: basal TMR. 0XPOS: basal TMR plus MET fed at 0.08% of the diet DM plus 60 g/d CHOL. 0.5X: 0XPOS ration plus 80-mg injectable folic acid plus 5-mg injectable vitamin B_{12}. 1X: 0XPOS ration plus 160-mg injectable folic acid plus 10-mg injectable B_{12}. 2X: 0XPOS ration plus 320-mg injectable folic acid plus 20-mg injectable vitamin B_{12}.
[2]Probability values for the main effect of nutritional treatment/injection (trt) and the main effect of day following a timed estrus synchronization protocol. The following are analyzed as contrasts: 0XNEG vs. 0XPOS and 0XPOS vs. Vitamin (0.5X, 1X, and 2X treatments). Linear (Lin), quadratic (Quad), and cubic (Cub) polynomial contrasts were conducted for 0XPOS, 0.5X, 1X, and 2X with PROC IML used to determine coefficients for unequal spacing.
[3]WBC: white blood cells (10^3/μL), Neu: Neutrophils (10^3/μL), Lym, Lymphocytes (10^3/μL), Mon: Monocytes (10^3/μL), Eos: eosinophils (10^3/μL), Bas: basophils (10^3/μL), RBC: red blood cells (10^6/μL), HBG: hemoglobin (g/dL), HCT: percent hematocrit, MCV: mean corpuscular volume (fL), MCH: mean corpuscular hemoglobin (pg), MCHC: mean corpuscular hemoglobin concentration (g/dL), RDW-CV(%): red cell distribution width percent, PLT: platelets (10^3/μL), MPV: mean platelet volume (fL).
[4]Day 0 = whole blood sample collected 48 hours after injection with prostaglandin.
[5]Average measurement across treatment within day of the synchronized estrous cycle.
[6]Average standard error of the mean for the interaction of treatment and day following timed estrus synchronization.
[7]Average measurement across day within treatment.
[a,b,c]Means without common superscripts differ by treatment.
[x,y]Means without common superscripts differ by day.

Table 3. Cytokine and chemokine (pg/mL) analysis of plasma at days 0 and 14 following a timed estrus synchronization protocol in heifers as influenced by nutritional treatment and injection strategy

		Treatment[1]							*P* value[2]							
Cyt[3]	Day[4]	0XNEG	0XPOS	0.5X	1X	2X	Day avg.[5]	SEM[6]	Trt	Day	Trt × Day	0XNEG vs. 0XPOS	0XPOS vs. Vitamin	Lin	Quad	Cub
IFN-γ	0	2.13	3.17	2.91	1.78	3.04	2.60	0.78	0.26	0.85	0.72	0.46	0.93	0.19	0.30	0.57
	14	2.64	2.88	2.08	1.15	3.84	2.52									
	Trt Avg.[7]	2.39	3.03	2.49	1.46	3.44										
IL-1α	0	25.02	18.60	9.48	17.46	12.93	16.69	7.50	0.56	0.13	0.22	0.43	0.14	0.24	0.24	0.79
	14	25.28	13.41	6.49	7.91	16.19	13.86									
	Trt Avg.	25.14	16.01	7.98	12.68	14.56										
IL-1β	0	16.01	9.90	22.46	11.81	6.89	13.41	7.30	0.72	0.41	0.42	0.35	0.40	0.50	0.90	0.35
	14	20.55	9.45	10.85	8.25	6.88	11.20									
	Trt Avg.	18.28	9.67	16.65	10.03	6.88										
IL-6	0	11.86	19.18	6.30	9.08	6.89	10.66	2.80	0.08	0.34	0.33	0.12	0.03	0.12	0.80	0.91
	14	9.70	12.87	8.51	6.93	8.53	9.31									
	Trt Avg.	10.78	16.03	7.40	8.00	7.71										
IL-8	0	283	391	237	261	235	281	70.9	0.45	0.53	0.62	0.16	0.69	0.42	0.52	0.11
	14	257	408	273	230	325	298									
	Trt Avg.	270	400	254	245	280										
IL-10	0	228	161	122	161	146	163	80.1	0.57	0.50	0.22	0.75	0.48	0.22	0.70	0.27
	14	206	337	82	93	219	187									
	Trt Avg.	216	249	102	127	182										
IL-17A	0	2.49	1.00	1.35	1.50	0.22	1.51	0.50	0.72	0.36	0.44	0.18	0.23	0.77	0.24	0.47
	14	1.12	1.11	1.15	0.44	1.39	1.24									
	Trt Avg.	1.80	1.06	1.25	1.47	1.30										
MIP-1α	0	566	588	394	615	548	524	160	0.75	0.64	0.31	0.48	0.95	0.54	0.93	0.23
	14	552	831	469	399	527	555									
	Trt Avg.	559	709	431	507	492										
IL-36RA	0	124.1	150.1	95.0	83.1	118.2	114.1	30.2	0.33	0.79	0.73	0.52	0.52	0.09	0.82	0.22
	14	137.6	167.7	88.8	64.9	124.2	116.6									
	Trt Avg.	130.9	158.9	91.9	74.0	121.2										
IP-10	0	468	341	408	387	380	397	125.8	0.56	0.46	0.34	0.50	0.30	0.33	0.65	0.81
	14	503	428	335	282	703	450									
	Trt Avg.	485	384	371	334	542										
MCP-1	0	230	312	263	128	206	250	70.7	0.23	0.96	0.24	0.09	0.67	0.27	0.40	0.06
	14	239	451	167	1547	228	248									
	Trt Avg.	234	381	215	196	217										

Table 3. Continued

		Treatment[1]							*P* value[2]							
Cyt[3]	Day[4]	0XNEG	0XPOS	0.5X	1X	2X	Day avg.[5]	SEM[6]	Trt	Day	Trt × Day	0XNEG vs. 0XPOS	0XPOS vs. Vitamin	Lin	Quad	Cub
MIP-1β	0	156	275	283	349	272	267	58.9	0.32	0.63	0.91	0.16	0.05	0.06	0.47	0.48
	14	172	268	254	294	283	254									
	Trt Avg.	164	272	269	321	278										
TNF-α	0	2091	1389	807	2051	936	1454	950	0.65	0.86	0.32	0.53	0.89	0.59	0.72	0.19
	14	1258	3422	703	898	1417	1540									
	Trt Avg.	1674	2406	755	1474	1177										
VEGF-A	0	46.1	65.4	53.3	44.9	42.8	50.5	16.5	0.27	0.85	0.41	0.09	0.57	0.33	0.34	0.07
	14	40.5	94.7	31.6	29.4	48.3	48.9									
	Trt Avg.	43.3	80.0	42.5	37.1	45.6										

[1]0XNEG: basal TMR. 0XPOS: basal TMR plus MET fed at 0.08% of the diet DM plus 60g/d CHOL. 0.5X: 0XPOS ration plus 80-mg injectable folic acid plus 5-mg injectable vitamin B_{12}. 1X: 0XPOS ration plus 160-mg injectable folic acid plus 10-mg injectable B_{12}. 2X: 0XPOS ration plus 320-mg injectable folic acid plus 20-mg injectable vitamin B_{12}.
[2]Probability values for the main effect of nutritional treatment/injection (trt) and the main effect of day following a timed estrus synchronization protocol. The following are analyzed as contrasts: 0XNEG vs. 0XPOS and 0XPOS vs. Vitamin (0.5X, 1X, and 2X treatments). Linear (Lin), quadratic (Quad), and cubic (Cub) polynomial contrasts were conducted for 0XPOS, 0.5X, 1X, and 2X with PROC IML used to determine coefficients for unequal spacing.
[3]IFN-γ: interferon-gamma, IL-1α: interleukin-1-alpha, IL-1β: interleukin-1-beta, IL-6: interleukin-6, IL-8: interleukin-8, IL-10: interleukin-10, IL-17A: interleukin-17A, MIP1α: macrophage inflammatory protein-1-alpha, IL-36RA: interleukin-36 receptor agonist, IP-10: IFN-γ-induced protein-10, MCP-1: monocyte chemoattractant protein-1, MIP-1β: marcophage inflammatory protein-1, TNF-α: tumor necrosis factor-alpha, VEGF-A: vascular endothelial growth factor A.
[4]Day 0 = whole blood sample collected 48 hours after injection with prostaglandin.
[5]Average measurement across treatment within day of the synchronized estrous cycle.
[6]Average standard error of the mean for the interaction of treatment and day following timed estrus synchronization.
[7]Average measurement across day within treatment.
[a,b,c]Means without common superscripts differ by treatment.
[x,y]Means without common superscripts differ by day.

in 0XPOS-treated heifers compared with 0.5X-, 1X-, and 2X-treated heifers. No other analyte was affected by treatment nor day of the estrous cycle ($P \geq 0.23$). Both MCP-1 and VEGF-A tended ($P = 0.09$) to be greater in 0XPOS- compared with 0XNEG-treated heifers. The concentration of IL-36RA tended ($P = 0.09$) to decrease linearly with increasing vitamin dose. The concentration of MIP-1β was greater ($P = 0.05$) in heifers receiving vitamin B_{12} and folate injections compared with 0XPOS and tended ($P = 0.06$) to decrease linearly with increasing vitamin dose. Lastly, VEGF-A tended ($P = 0.07$) to be altered cubically being greater in 0XPOS and decreasing and remaining constant from 0.5X- to 2X-treated heifers. There were no differences by treatment or contrasts for any cytokine analytes in histotroph (Table 4; $P \geq 0.11$).

Plasma and histotroph metabolites

All plasma and histotroph metabolite data are presented in Table 5. There was no difference due to the interaction of treatment and day, or the main effects of treatment or day for the concentration of PUN ($P \geq 0.47$). The PUN concentration tended ($P = 0.08$) to increase in heifers with increasing vitamin dose. Plasma glucose was not affected by the day × treatment interaction or the main effect of day ($P \geq 0.71$). Plasma glucose concentration differed by treatment ($P < 0.01$) and did so cubically ($P = 0.02$) such that the concentration of glucose in 0.5X heifers was less than that of all other treatments. While there were no overall treatment differences in the concentration of glucose in histotroph ($P = 0.31$), the orthogonal contrast evaluating inclusion of MET and CHOL revealed a tendency ($P = 0.06$) for glucose to be greater in 0XPOS- compared with 0XNEG-treated heifers. Similar to plasma glucose, the concentration of glucose in histotroph was affected cubically ($P = 0.04$) such that it was decreased from 0XPOS to 0.5X and returned to be equal to that of the 0XPOS-treated heifers by the 1X and 2X treatments. Histotroph protein concentration was affected by treatment ($P = 0.04$) such that 0XPOS and 1X treatments were greater than the 0XNEG and 0.5X treatments with 2X being intermediate and equal to all other treatments. Furthermore, total histotroph protein was greater ($P = 0.02$) in 0XPOS-treated heifers compared with 0XNEG and tended ($P = 0.06$) to be greater in 0XPOS-treated heifers compared with those receiving vitamin injection. Similar to the glucose pattern across treatment, histotroph protein was altered cubically with increasing vitamin dosage, being decreased ($P = 0.01$) from 0XPOS to 0.5X then returned to the equivalent concentration of the 0XPOS at the 1X and 2X vitamin dosages.

Visceral organ and reproductive tract measurements

All organ weight data are presented in Table 6. There were no differences in liver weight or liver weight when scaled to heifer body weight ($P \geq 0.50$). Unnormalized and scaled spleen weights were not affected by a treatment difference ($P \geq 0.26$); however, both were affected cubically ($P = 0.03$) with increasing vitamin dose such that the weight decreased from 0XPOS to 0.5X, increased from the 0.5X to the 1X, and remained equivalent from the 1X to the 2X doses. Uterine weight was greater ($P = 0.05$) in the 0XNEG and 0XPOS treatments compared with the 0.5X-treated heifers, with the 1X and 2X being intermediate and not different from all other treatments. Similarly, when uterine weight was scaled to heifer body weight, it tended ($P = 0.07$) to be lesser in the 0.5X treatment compared with all other treatments. Both unnormalized and scaled uterine weight were greater ($P \leq 0.01$) in the 0XPOS-treated heifers compared with those receiving vitamin injections. Both unnormalized and scaled ovarian weight tended ($P = 0.08$) to be reduced in the 1X- and 2X-treated heifers compared with the 0XPOS-treated heifers. Both unnormalized and scaled ovarian weights were greater ($P \leq 0.03$) in 0XPOS compared with vitamin injected heifers. Furthermore, there was a linear decrease ($P \leq 0.01$) in both unnormalized and scaled ovarian weights with increasing vitamin supplementation. Unnormalized nor scaled CL weights, uterine horn diameter, or primordial, primary, secondary, or surface follicle counts differed among treatments ($P \geq 0.15$). Although there was no overall treatment difference in the largest follicle diameter ($P = 0.17$), follicle diameter tended ($P = 0.10$) to be affected quadratically, decreasing from 0XPOS to 0.5X and 1X, and subsequently increasing at the 2X treatment.

Pancreatic and jejunal enzyme activity

All pancreatic and jejunal protein and enzyme analysis data are presented in Table 7. Total pancreatic protein was not affected by treatment ($P = 0.22$); however, it was affected cubically ($P = 0.04$) such that the protein concentration was decreased from 0XPOS- to 0.5X-treated heifers and increased at the 1X and 2X doses to be equal to that of the 0XPOS-treated heifers. There were no effects of treatment ($P \geq 0.36$) in jejunal total protein concentration.

Pancreatic α-amylase activity per gram of pancreas and per gram of pancreatic protein followed the same trends being greater ($P = 0.01$) in 2X-treated heifers compared with 0XNEG and 0.5X-treated heifers. Furthermore, both activity per gram of pancreas and per gram of pancreatic protein were affected cubically with increasing vitamin dose ($P \leq 0.02$) decreasing from 0XPOS to 0.5X, returning to the equivalent activity of 0XPOS by the 1X treatment and continuing to increase to the 2X-treated heifers. The α-glucosidase, maltase, and glucoamylase activity per gram of jejunum or per gram of jejunal protein were not affected by treatment ($P \geq 0.16$). Isomaltase activity was not affected by overall treatment ($P \geq 0.13$); however, activity per gram of jejunum and per gram of jejunal protein decreased linearly ($P = 0.04$) with increasing vitamin injection dose.

Muscle hydrogen peroxide accumulation rate

All H_2O_2 accumulation rate data are presented in Table 8. Hydrogen peroxide accumulation rate in heifer muscle when using glutamate as a substrate and inhibiting Complex I and II of the electron transport chain were similar in that the 0XNEG-treated heifers had a greater H_2O_2 accumulation rate than the 0XPOS- and 0.5X-treated heifers. When both complexes one and three were inhibited, 0XNEG-treated heifers tended ($P = 0.07$) to accumulate H_2O_2 at a greater rate than 0XPOS- and 0.5X-treated heifers. When using glutamate as a substrate, the H_2O_2 accumulation rate was decreased in the 0XPOS- compared to the 0XNEG-treated heifers when either none ($P = 0.07$) of the complexes were inhibited, or when complexes one ($P = 0.01$), two ($P = 0.01$), three ($P = 0.07$), or one and three were inhibited together ($P = 0.01$. Furthermore, there was a linear increase in H_2O_2 accumulation with increasing folate and B_{12} injection dose. When using succinate as the substrate, H_2O_2 accumulation was not affected ($P \geq 0.13$) by treatment in any complex; however, H_2O_2 accumulation was

Table 4. Cytokine and chemokine (pg/mL) analysis of histotroph at day 14 following a timed estrus synchronization protocol in heifers as influenced by nutritional treatment and injection strategy

	Treatment[1]							*P* value[2]				
Cyt[3]	0XNEG	0XPOS	0.5X	1X	2X	Trt SEM[4]	Trt	0XNEG vs. 0XPOS	0XPOS vs. Vitamin	Lin	Quad	Cub
IFN-γ	0.33	0.19	0.41	0.21	0.18	0.14	0.57	0.44	0.68	0.68	0.63	0.20
IL-1α	2.17	3.37	2.12	3.73	2.17	0.99	0.72	0.38	0.38	0.38	0.94	0.36
IL-1β	16.5	19.9	40.5	50.9	38.7	17.6	0.57	0.89	0.29	0.11	0.87	0.68
IL-6	4.25	7.17	12.98	14.85	1.22	5.57	0.67	0.71	0.26	0.16	0.68	0.75
IL-8	69.6	79.8	109.0	110.4	97.6	20.7	0.56	0.73	0.22	0.14	0.56	0.60
IL-10	9.44	13.96	17.22	16.87	10.82	4.73	0.70	0.51	0.24	0.28	0.47	0.95
IL-17A	1.86	1.64	1.81	2.24	1.66	0.79	0.98	0.84	0.97	0.68	0.75	0.90
MIP-1α	201.0	267.4	264.1	245.1	219.0	31.0	0.50	0.14	0.12	0.48	0.46	0.55
IL-36RA	13.6	11.9	16.9	14.0	16.9	5.0	0.93	0.80	0.91	0.84	0.78	0.52
IP-10	513.8	394.8	570.3	424.2	682.6	221.1	0.88	0.69	0.83	0.87	0.88	0.58
MCP-1	108.0	101.6	177.7	138.7	179.9	39.1	0.48	0.91	0.47	0.44	0.44	0.30
MIP-1β	9.6	19.8	18.6	16.5	11.1	5.3	0.58	0.19	0.17	0.52	0.25	0.55
TNF-α	133.2	109.2	163.9	155.2	159.6	37.9	0.83	0.66	0.83	0.51	0.93	0.38
VEGF-A	113.1	100.6	116.8	111.9	104.5	50.0	0.53	0.95	0.99	0.99	0.99	0.94

[1]0XNEG: basal TMR. 0XPOS: basal TMR plus MET fed at 0.08% of the diet DM plus 60g/d CHOL. 0.5X: 0XPOS ration plus 80-mg injectable folic acid plus 5-mg injectable vitamin B_{12}. 1X: 0XPOS ration plus 160-mg injectable folic acid plus 10-mg injectable B_{12}. 2X: 0XPOS ration plus 320-mg injectable folic acid plus 20-mg injectable vitamin B_{12}.
[2]Probability values for the main effect of nutritional treatment/injection (trt). The following are analyzed as contrasts: 0XNEG vs. 0XPOS and 0XPOS vs. Vitamin (0.5X, 1X, and 2X treatments). Linear (Lin), quadratic (Quad), and cubic (Cub) polynomial contrasts were conducted for 0XPOS, 0.5X, 1X, and 2X with PROC IML used to determine coefficients for unequal spacing.
[3]IFN-γ: interferon-gamma, IL-1α: interleukin-1-alpha, IL-1β: interleukin-1-beta, IL-6: interleukin-6, IL-8: interleukin-8, IL-10: interleukin-10, IL-17A: interleukin-17A, MIP1α: macrophage inflammatory protein-1-alpha, IL-36RA: interleukin-36 receptor agonist, IP-10: IFN-γ-induced protein-10, MCP-1: monocyte chemoattractant protein-1, MIP-1β: marcophage inflammatory protein-1, TNF-α: tumor necrosis factor-alpha, VEGF-A: vascular endothelial growth factor A.
[4]Average standard error of the mean for the main effect of nutritional treatment/injection.

Table 5. Urea nitrogen (mg/dL) and glucose (mM) concentration in plasma at days 0, 7, and 14 as well as glucose and protein concentration (µg) in histotroph at day 14 following a timed estrus synchronization protocol in heifers as influenced by nutritional treatment and injection strategy

		Treatment[1]							*P* value[2]							
Meas.	Day[3]	0XNEG	0XPOS	0.5X	1X	2X	Day Avg.[4]	SEM[5]	Trt	Day	Trt × Day	0XNEG vs. 0XPOS	0XPOS vs. Vitamin	Lin	Quad	Cub
Plasma																
Urea N.	0	6.03	5.62	6.48	7.08	7.16	6.47	0.63	0.47	0.93	0.35	0.59	0.17	0.08	0.70	0.23
	7	5.57	6.39	6.76	6.06	7.00	6.36									
	14	7.29	6.36	6.58	5.63	6.62	6.50									
	Trt Avg.[6]	6.30	6.12	6.61	6.26	6.93										
Glucose	0	4.59	4.93	3.95	5.13	5.23	4.77	0.41	<0.01	0.71	0.92	0.97	0.17	0.51	0.01	0.02
	7	4.72	4.58	3.85	4.77	4.75	4.53									
	14	5.36	5.08	3.58	4.53	4.68	4.65									
	Trt Avg.	4.90[a]	4.88[a]	3.79[b]	4.80[a]	4.88[a]										
Histo.																
Glucose		2.77	3.88	3.03	4.03	3.79	—	1.15	0.31	—	—	0.06	0.24	0.10	0.61	0.04
Protein		94[b]	179[a]	92[b]	173[a]	147[ab]	—	24.5	0.04	—	—	0.02	0.06	0.11	0.82	0.01

[1]0XNEG: basal TMR. 0XPOS: basal TMR plus MET fed at 0.08% of the diet DM plus 60 g/d CHOL. 0.5X: 0XPOS ration plus 80-mg injectable folic acid plus 5-mg injectable vitamin B_{12}. 1X: 0XPOS ration plus 160-mg injectable folic acid plus 10-mg injectable B_{12}. 2X: 0XPOS ration plus 320-mg injectable folic acid plus 20-mg injectable vitamin B_{12}.
[2]Probability values for the main effect of nutritional treatment/injection (trt) and the main effect of day following a timed estrus synchronization protocol. The following are analyzed as contrasts: 0XNEG vs. 0XPOS and 0XPOS vs. Vitamin (0.5X, 1X, and 2X treatments). Linear (Lin), quadratic (Quad), and cubic (Cub) polynomial contrasts were conducted for 0XPOS, 0.5X, 1X, and 2X with PROC IML used to determine coefficients for unequal spacing.
[3]Day 0 = whole blood sample collected 48 hours after injection with prostaglandin.
[4]Average measurement across treatment within day of the synchronized estrous cycle.
[5]Average standard error of the mean for the interaction of treatment and day following timed estrus synchronization.
[6]Average measurement across day within treatment.
[a,b]Means without common superscripts differ by treatment.

Table 6. Organ weight and ovarian follicle counts following a timed estrus synchronization protocol in heifers as influenced by nutritional treatment and injection strategy

Measurement	Treatment[1]					SEM[3]	*P* value[2]					
	0XNEG	0XPOS	0.5X	1X	2X		Trt	0XNEG vs. 0XPOS	0XPOS vs. Vitamin	Lin	Quad	Cub
Liver, g	5,526	5,300	5,108	5,231	5,086	218	0.68	0.50	0.54	0.60	0.93	0.57
Liver, g/kg BW	14.04	13.46	12.97	13.29	12.92	0.55	0.62	0.53	0.57	0.63	0.98	0.61
Spleen, g	628	638	526	626	601	38.2	0.29	0.89	0.20	0.99	0.42	0.03
Spleen, g/kg BW	1.60	1.62	1.34	1.59	1.53	0.10	0.26	0.90	0.22	0.99	0.45	0.03
Uterus, g	298^{a}	328^{a}	248^{b}	278^{ab}	273^{ab}	17.8	0.05	0.33	<0.01	0.12	0.03	0.03
Uterus, g/kg BW	0.76^{x}	0.72^{x}	0.61^{y}	0.69^{x}	0.70^{x}	0.05	0.07	0.49	0.01	0.29	0.03	0.04
Ovary, g	14.1^{xy}	16.6^{x}	15.6^{x}	13.0^{y}	12.5^{y}	1.11	0.08	0.21	0.03	<0.01	0.34	0.41
Ovary, g/kg BW	0.036^{xy}	0.041^{x}	0.038^{x}	0.032^{y}	0.032^{y}	0.003	0.08	0.27	0.02	0.01	0.20	0.43
CL, g	3.85	4.64	5.85	5.20	4.67	0.85	0.56	0.63	0.48	0.74	0.32	0.41
CL, g/kg	0.010	1.011	0.014	0.013	0.012	0.002	0.66	0.71	0.43	0.89	0.38	0.37
Horn Diameter[4], mm	42.3	40.7	37.5	40.9	37.7	2.42	0.56	0.64	0.46	0.53	0.90	0.24
Primordial Follicles	84.9	88.7	95.9	80.7	96.5	14.2	0.92	0.80	0.89	0.82	0.69	0.47
Primary Follicles	20.2	16.1	18.5	19.3	21.6	3.0	0.77	0.44	0.23	0.15	0.81	0.85
Secondary Follicles	1.73	1.93	2.00	1.43	1.46	0.38	0.76	0.55	0.53	0.32	0.78	0.48
Surface Follicles	41.8	40.8	38.5	33.7	29.2	7.1	0.69	0.92	0.39	0.22	0.92	0.87
Follicle Diameter[5], mm	12.66	12.79	11.12	11.48	12.86	0.62	0.17	0.88	0.24	0.45	0.10	0.17

[1]0XNEG: basal TMR. 0XPOS: basal TMR plus MET fed at 0.08% of the diet DM plus 60 g/d CHOL. 0.5X: 0XPOS ration plus 80-mg injectable folic acid plus 5-mg injectable vitamin B_{12}. 1X: 0XPOS ration plus 160-mg injectable folic acid plus 10-mg injectable B_{12}. 2X: 0XPOS ration plus 320-mg injectable folic acid plus 20-mg injectable vitamin B_{12}.
[2]Probability values for the main effect of nutritional treatment/injection (trt). The following are analyzed as contrasts: 0XNEG vs. 0XPOS and 0XPOS vs. Vitamin (0.5X, 1X, and 2X treatments). Linear (Lin), quadratic (Quad), and cubic (Cub) polynomial contrasts were conducted for 0XPOS, 0.5X, 1X, and 2X with PROC IML used to determine coefficients for unequal spacing.
[3]Average standard error of the mean for the main effect of nutritional treatment/injection.
[4]Summed diameter of both uterine horns.
[5]Diameter of the largest follicle.
[a,b]Means without common superscripts differ by treatment ($P \leq 0.05$).
[x,y]Means without common superscripts tend to differ by treatment ($0.05 > P \leq 0.10$).

Table 7. Pancreatic and jejunal protein and enzyme activity following a timed estrus synchronization protocol in heifers as influenced by nutritional treatment and injection strategy

Measurement	Treatment[1]					SEM[3]	*P* value[2]					
	0XNEG	0XPOS	0.5X	1X	2X		Trt	0XNEG vs. 0XPOS	0XPOS vs. Vitamin	Lin	Quad	Cub
Protein												
mg/g pancreas	115	121	98.5	120	117	7.21	0.22	0.57	0.92	0.74	0.43	0.04
mg/g jejunum	81.0	79.2	75.9	76.8	74.8	4.13	0.82	0.77	0.36	0.52	0.84	0.72
α-Amylase activity												
U/g pancreas	263^{bc}	359^{ab}	189^{c}	352^{ab}	399^{a}	43.2	0.01	0.13	0.21	0.11	0.10	0.01
U/g protein	2284^{bc}	2961^{ab}	1880^{c}	2953^{ab}	3421^{a}	304	0.01	0.13	0.14	0.05	0.09	0.02
α-Glucosidase activity[4]												
U/g jejunum	0.259	0.289	0.264	0.154	0.181	0.0548	0.36	0.70	0.55	0.17	0.40	0.44
U/g protein	3.20	3.79	3.47	1.95	2.39	0.698	0.34	0.56	0.70	0.16	0.36	0.40
Maltase activity												
U/g jejunum	0.100	0.101	0.0819	0.0460	0.0712	0.0274	0.60	0.99	0.41	0.36	0.33	0.64
U/g protein	1.22	1.33	1.05	0.557	0.928	0.342	0.56	0.82	0.52	0.43	0.27	0.61
Isomaltase activity												
U/g jejunum	0.0639	0.0674	0.0680	0.0265	0.0217	0.0170	0.14	0.88	0.35	0.04	0.66	0.28
U/g protein	0.810	0.898	0.916	0.357	0.321	0.223	0.13	0.78	0.43	0.04	0.60	0.23
Glucoamylase activity												
U/g jejunum	0.0951	0.121	0.115	0.0816	0.0883	0.0172	0.43	0.29	0.54	0.17	0.45	0.45
U/g protein	1.17	1.56	1.50	1.07	1.17	0.219	0.43	0.22	0.55	0.20	0.47	0.44

[1]0XNEG: basal total mixed ration. 0XPOS: basal total mixed ration plus MET fed at 0.08% of the diet DM plus 60 g/d CHOL. 0.5X: 0XPOS ration plus 80-mg injectable folic acid plus 5-mg injectable vitamin B_{12}. 1X: 0XPOS ration plus 160 mg injectable folic acid plus 10-mg injectable B_{12}. 2X: 0XPOS ration plus 320-mg injectable folic acid plus 20-mg injectable vitamin B_{12}.
[2]Probability values for the main effect of nutritional treatment/injection (trt). The following are analyzed as contrasts: 0XNEG vs. 0XPOS and 0XPOS vs. Vitamin (0.5X, 1X, and 2X treatments). Linear (Lin), quadratic (Quad), and cubic (Cub) polynomial contrasts were conducted for 0XPOS, 0.5X, 1X, and 2X with PROC IML used to determine coefficients for unequal spacing.
[3]Average standard error of the mean for the main effect of nutritional treatment/injection.
[4]Sum of maltase, isomaltase, and glucoamylase activity.
[a,b,c]Means without common superscripts differ by treatment.

Table 8. Muscle hydrogen peroxide accumulation rate (μmol/mg protein/min) following a timed estrus synchronization protocol in heifers as influenced by nutritional treatment and injection strategy

		Treatment[1]				*P* value[2]					
Substrate	Inhibited Complex	0XNEG	0XPOS	0.5X	1X	SEM[3]	Trt	0XNEG vs. 0XPOS	0XPOS vs. Vitamin	Lin	Quad
Glutamate	None	77.1	49.1	58.2	69.2	15.1	0.38	0.07	0.37	0.29	0.95
	One	73.7[a]	38.5[c]	43.4[bc]	66.1[ab]	10.8	0.03	0.01	0.17	0.05	0.43
	Two	79.1[a]	45.4[b]	41.7[b]	68.1[ab]	11.7	0.04	0.01	0.47	0.15	0.26
	Three	64.1	41.0	41.5	56.8	11.5	0.17	0.07	0.50	0.23	0.45
	One and Three	92.8	50.4	57.4	82.0	19.6	0.07	0.01	0.25	0.11	0.59
	One and Two	75.7	53.6	48.5	67.6	16.4	0.48	0.31	0.81	0.52	0.52
Succinate	One	77.7	44.3	50.2	65.2	17.7	0.13	0.03	0.31	0.18	0.73
	One and Two	82.2	52.0	60.5	70.5	20.5	0.48	0.12	0.47	0.39	0.97
	One and Three	72.1	48.6	50.2	65.3	16.9	0.40	0.15	0.54	0.34	0.65
	One, Two, and Three	69.6	43.6	46.3	60.5	15.2	0.29	0.11	0.46	0.28	0.66

[1]0XNEG: basal TMR. 0XPOS: basal TMR plus MET fed at 0.08% of the diet DM plus 60 g/d CHOL. 0.5X: 0XPOS ration plus 80-mg injectable folic acid plus 5-mg injectable vitamin B_{12}. 1X: 0XPOS ration plus 160-mg injectable folic acid plus 10-mg injectable B_{12}. 2X: 0XPOS ration plus 320-mg injectable folic acid plus 20-mg injectable vitamin B_{12}.
[2]Probability values for the main effect of nutritional treatment/injection (trt). The following are analyzed as contrasts: 0XNEG vs. 0XPOS and 0XPOS vs. Vitamin (0.5X, 1X, and 2X treatments). Linear (Lin), quadratic (Quad), and cubic (Cub) polynomial contrasts were conducted for 0XPOS, 0.5X, 1X, and 2X with PROC IML used to determine coefficients for unequal spacing.
[3]Average standard error of the mean for the main effect of nutritional treatment/injection.
[a,b]Means without common superscripts differ by treatment.

decreased ($P = 0.03$) in the 0XPOS-treated heifers compared to 0XNEG-treated heifers when complex one was inhibited.

Discussion

These data are the first to report on the combined effects of supplementing four OCM in coordination with each other on measurements of overall health and performance in beef heifers. The measurements evaluated in this study were taken due to the previously reported effects of methyl donors on intake, hematopoiesis, immune response, energy metabolism, and free radical scavenging in multiple species. We failed to reject our null hypothesis that supplementing increasing doses of OCM would positively impact measures of health and performance in beef heifers; however, we report that a dose that disrupts one-carbon metabolism, as presented in Crouse et al. (2023), that results in decreased methylation potential negatively impacts performance and health parameters of beef heifers. Crouse et al. (2023) previously reported that the 0.5X treatment did not maintain increased circulating OCM concentrations, and, in fact, reduced the methylation potential (SAM:SAH ratio). When evaluating this treatment over the multiple measures conducted in the study, the 0.5X-treated heifers had increased DMI by cubic contrast, but a decrease in RBC, hemoglobin, hematocrit, plasma, and histotroph glucose as well as histotroph protein, spleen weight (g/kg), uterine weight, pancreatic protein concentration, and α-amylase activity. Overall, these data suggest a negative systemic response to aberrant methyl supply in beef heifers during an estrous cycle. Further mechanistic discussion of the decreased energy availability and protein synthesis will be included below.

Multiple reports in dairy cattle demonstrated that supplementation with MET alters DMI in the transition dairy cow (Osorio et al., 2013; Batistel et al., 2017; Cardoso et al., 2021). In beef cattle, Grant et al. (2022), reported that receiving heifers that were limit-fed and received either a control diet or supplemental MET had no difference in average daily gain or DMI due to methionine supplementation. Herein, we report no overall difference in intake amongst treatments when gain was fixed; however, a cubic response in DMI was observed with increasing folate and B_{12} supplementation which was driven primarily by the difference in DMI between 0XPOS and 0.5X.

The role of the spleen includes filtration of erythrocytes and platelets, recycling iron from red blood cells, phagocytic filtration of blood and production of opsonizing antibodies, as well as storage of red blood cells and platelets (Kapila et al., 2023). Folate and vitamin B_{12} are essential for the formation of red blood cells and heme and deficiency in these vitamins results in anemia (Koury and Ponka, 2004). The spleen decreased in mass in the 0.5X treatment and the hematology data showed a decrease in RBC, hemoglobin, hematocrit percentage, and a greater RDW-CV. These differences may suggest altered splenic filtering and storage capacity in the 0.5X treatment. Interestingly, the MCV and MCH were greatest in the 2X treatment with a RBC equal to that of the 0.5X treatment, which suggests the potential for macrocytic anemia, a condition of increased red blood cell size as a result of decreased red blood cell number and is typically associated with folate or vitamin B_{12} deficiency (Moore and Adil, 2023). It should be noted that while we see differences between treatments, all hematology measurements are within reported reference ranges (Supplementary Table 1), although some measurements such as hematocrit, specifically on the 0.5X treatment are low (Herman et al., 2018; Cornell University College of Veterinary Medicine, 2019).

There were few differences in plasma whole blood immune cell concentrations and plasma cytokine concentrations across treatments in the current study. Immune cells are important for protecting the body against disease and pathogens and cytokines are important signaling proteins for modulating immune system activity. Although there were several measurements that collectively suggest a negative systemic response to OCM supply in 0.5X heifers, the lack of change in immune cell concentrations and cytokines indicates that immunoactivation did not occur in 0.5X heifers. Immunoactivation in cattle requires large amounts of glucose and thus, nutrients are repartitioned away from anabolic pathways that result in increased growth (Kvidera et al., 2016, 2017). Thus, the lack of change in immune cell concentrations and cytokines in the current study provides evidence that decreased glucose in response to 0.5X supplementation was not because of immune system activation.

Although there was greater DMI in the 0.5X treatment, glucose concentration in plasma and histotroph was decreased compared with 0XPOS heifers. It has been estimated that approximately 95% of glucose in systemic circulation is derived from gluconeogenesis (Aschenbach et al., 2010), with only a minor proportion contributed from intestinal glucose absorption and/or splanchnic glucose metabolism for cattle consuming low-starch diets (Reynolds et al., 2003; Doepel et al., 2009). Of the potential gluconeogenic precursors (volatile fatty acids, lactate, glycerol, amino acids), propionate is by far the predominant substrate for gluconeogenesis in fed ruminants (Reynolds et al., 2003; Larsen and Kristensen, 2009). Flux of propionate through gluconeogenesis is regulated by the vitamin B_{12}-dependent enzyme, methylmalonyl-coA mutase (Elliot, 1980); however, intramuscular vitamin B_{12} injections have only been reported to increase (Peters and Elliot, 1983; Preynat et al., 2009) or not influence (Duplessis et al., 2017) whole-body glucose rate of appearance from propionate. Serum vitamin B_{12} concentration of 0.5X heifers remained elevated for the duration of the study compared with the control treatment (Crouse et al., 2023), suggesting that another mechanism may be responsible for decreased glucose concentration in plasma and histotroph. Intramuscular injection of 320-mg folic acid was reported to decrease whole-body glucose rate of appearance in dairy cows and those authors suggested folic acid might have changed glucose utilization in peripheral tissues (Duplessis et al., 2017). Increasing SAH and decreasing methylation potential results in decreased mRNA expression of phosphoenolpyruvate carboxykinase 1 (*PCK1*) and glucose-6-phosphatase (*G6PC*) in human hepatocytes (Jackson et al., 2012). Therefore, balance of OCM supply and/or decreased methylation potential of 0.5X heifers may have contributed to decreased plasma and histotroph glucose concentration.

Acinar cells of the exocrine pancreas have the greatest rate of protein synthesis compared with other cell types in mammalian tissues (Pandol et al., 2011). Pancreatic α-amylase activity in ruminants is sensitive to changes in energy intake and energy balance (Trotta et al., 2022), where dietary energy restriction has been shown to decrease pancreatic α-amylase activity in cattle and sheep (Awda et al., 2016; Keomanivong et al., 2016, 2017a, b; Trotta

et al., 2020). Decreased pancreatic protein concentration and α-amylase activity in 0.5X heifers may be related to conservation of energy expenditure by reducing protein activity/synthesis (Wang et al., 2009; Holligan et al., 2013; Wood et al., 2013), as regulated by the mammalian target of rapamycin signaling pathway (Guo et al., 2018a, b, 2019). Additionally, responses of pancreatic protein concentration and α-amylase activity observed in the current experiment were quite similar to the response of SAM:SAH previously reported using the same experimental animals (Crouse et al., 2023). Previous research reported that pancreatic α-amylase activity decreases in response to increases in SAH concentrations and decreases in the SAM:SAH ratio (Capdevila et al., 1997). As supply of folate and vitamin B_{12} increased from 0.5X to 2X in the current study, pancreatic α-amylase activity increased concurrently with increases in methylation potential (SAM:SAH) (Crouse et al., 2023). Therefore, changes in pancreatic protein concentration and α-amylase activity in the current study could potentially be related to changes in whole-body energy utilization, protein turnover, and/or methylation potential.

Although heifers used in the current experiment were not bred, the reproductive tract and histotroph data are interpreted to imply that fertility of the 0.5X heifers would be compromised. Cushman et al. (2013; 2023) reported that both cows and ewes that were open for multiple breeding seasons had reduced uterine size which would be similar to the decrease in uterine size in the 0.5X treatment. Similarly, key components of uterine histotroph such as glucose and protein, which are key regulators of embryonic growth, were decreased (Wang et al., 2016; Moraes et al., 2020). Heifers that conceive earlier in the year have increased uterine glucose and protein concentration compared with heifers that take multiple cycles to establish and maintain a pregnancy (McNeel et al., 2017; Snider et al., 2022). Furthermore, nutritionally compromised pregnancies have decreased abundance of high-capacity glucose transporter SLC2A3 in deep uterine glands and decreased concentration of glucose in fetal fluids compared with those fed to a moderate rate of gain (Crouse et al., 2019, 2021). The concentration differences seen in histotroph may be due to either a decrease in circulating concentration of glucose or simply a decrease in ability to transport nutrients via transporters in the uterus. In either case, with the decrease in glucose, heifers on the 0.5X treatment would have decreased ability to maintain a pregnancy due to decreased nutrients in histotroph for use in embryonic development and thus, an inability to generate an elongated embryo for maternal recognition of pregnancy.

The OCM pathway is linked to peroxide radical scavenging through glutathione production via the transulfuration pathway (Clare et al., 2019). Zhou et al. (2016) did not report a difference in H_2O_2 produced but did report an increase in reduced glutathione with methionine supplementation. While we do not have a measurement of reduced or total glutathione, we report that H_2O_2 was decreased with glutamate and succinate as the substrate when comparing 0XNEG to 0XPOS suggesting improved hydroxy radical scavenging in heifers when supplemented with methionine and choline. Interestingly, there were no differences between the 0.5X and 0XPOS treatments suggesting that the 0.5X treatment was equally effective in hydroxy radical scavenging. This would be counter to the other data showing a negative response to the 0.5X treatment while reduction in H_2O_2 is a positive response to treatment.

In conclusion, these data are interpreted to imply that supplementation with OCM at most concentrations does not positively affect overall heifer health and performance; however, there may be aberrant supplementation doses, such as that of the 0.5X treatment, that can disrupt one-carbon metabolism and thus negatively impact heifer performance which would most likely also negatively impact fertility and embryonic development. Therefore, additional work using doses such as the 2X dose should be conducted in beef cattle throughout pregnancy to determine whether supplementation with OCM improves embryonic and fetal development. Lastly, studies should be conducted with the 0.5X treatment to further understand the mechanisms and negative outcomes associated with reduced methylation potential.

Supplementary Data

Supplementary data are available at *Journal of Animal Science* online.

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Conflict of interest statement

The authors declare no known or perceived conflicts of interest.

Disclosures

Mention of a trade name, proprietary product, or specific agreement does not constitute a guarantee or warranty by the USDA and does not imply approval of the inclusion of other products that may be suitable. USDA is an equal opportunity provider and employer.

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