





# One-carbon metabolite supplementation to heifers for the first 14 d of the estrous cycle alters the plasma and hepatic one-carbon metabolite pool and methionine-folate cycle enzyme transcript abundance in a dose-dependent manner

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## Abstract

The objective of this study was to determine the dose of folate and vitamin B<sub>12</sub> in beef heifers fed rumen protected methionine and choline required to maintain increased B<sub>12</sub> levels and intermediates of the methionine-folate cycle in circulation. Angus heifers (n = 30; 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 day 0 and 7 of the estrous cycle, **0XPOS**: TMR, rumen protected methionine (MET) fed at 0.08% of the diet DM, rumen protected choline (CHOL) fed at 60 g/d, and saline injections at day 0 and 7, **0.5X**: TMR, MET, CHOL, 5 mg B<sub>12</sub>, and 80 mg folate at day 0 and 7, **1X**: TMR, MET, CHOL, 10 mg vitamin B<sub>12</sub>, and 160 mg folate at day 0 and 7, and **2X**: TMR, MET, CHOL, 20 mg B<sub>12</sub>, and 320 mg folate at day 0 and 7. All heifers were estrus synchronized but not bred, and blood was collected on day 0, 2, 5, 7, 9, 12, and 14 of a synchronized estrous cycle. Heifers were slaughtered on day 14 of the estrous cycle for liver collection. Serum B<sub>12</sub> concentrations were greater in the 0.5X, 1X, and 2X, compared with 0XNEG and 0XPOS on all days after treatment initiation ( $P < 0.0001$ ). Serum folate concentrations were greater for the 2X treatment at day 5, 7, and 9 of the cycle compared with all other treatments ( $P \leq 0.05$ ). There were no differences ( $P \geq 0.19$ ) in hepatic methionine-cycle or choline analyte concentrations by treatment. Concentrations of hepatic folate cycle intermediates were always greater ( $P \leq 0.04$ ) in the 2X treatment compared with the 0XNEG and 0XPOS heifers. Serum methionine was greater ( $P = 0.04$ ) in the 0.5X and 2X heifers compared with 0XNEG, and S-adenosylhomocysteine (SAH) tended ( $P = 0.06$ ) to be greater in the 0.5X heifers and the S-adenosylmethionine (SAM):SAH ratio was decreased ( $P = 0.05$ ) in the 0.5X treatment compared with the 0XNEG, 0XPOS, and 2X heifers. The hepatic transcript abundance of *MAT2A* and *MAT2B* were decreased ( $P \leq 0.02$ ) in the 0.5X heifers compared with the 0XNEG, 0XPOS, and 2X heifers. These data support that beef heifers fed rumen protected methionine and choline require 20 mg B<sub>12</sub> and 320 mg folate once weekly to maintain increased concentrations of B<sub>12</sub> and folate in serum. Furthermore, these data demonstrate that not all supplementation levels are equal in providing positive responses, and that some levels, such as the 0.5X, may result in a stoichiometric imbalance in the one-carbon metabolism pathway that results in a decreased SAM:SAH ratio.

## Lay Summary

The strategic inclusion of one-carbon metabolites, which include vitamins and minerals that are found in human prenatal vitamins, to beef cattle feeding and management protocols during the periconceptual period (the time around breeding) is a novel concept. Therefore, this study aimed to identify the feeding and injection doses of one-carbon metabolites in beef heifers to maintain increased circulating concentrations of one-carbon metabolites for use as a model from which other studies could base their treatments on. We determined that daily feeding of methionine and choline at 0.08% of dry matter and 60 g/d, respectively, and administration of vitamin B<sub>12</sub> and folate at 20 mg and 320 mg once per week, respectively resulted in sustained elevated concentrations of one-carbon metabolites.

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

**Abbreviations:** 0XNEG, total mixed ration and sham injections of saline at day 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 injections of saline at day 0 and 7 of the estrous cycle; 0.5X, total mixed ration, rumen protected methionine, rumen protected choline, 5 mg vitamin B<sub>12</sub>, and 80 mg folate at day 0 and 7 of the estrous cycle; 1X, total mixed ration, rumen protected methionine, rumen protected choline, 10 mg vitamin B<sub>12</sub>, and 160 mg folate at day 0 and 7 of the estrous cycle; 2X, total mixed ration, rumen protected methionine, rumen protected choline, 20 mg vitamin B<sub>12</sub>, and 320 mg folate at day 0 and 7 of the estrous cycle; ACTB, beta actin; AUC, area under the curve; CHOL, rumen protected choline; DHF, dihydrofolic acid; DNMT1, DNA methyltransferase 1; DNMT3A, DNA methyltransferase 3A; FBP, folate binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GNMT, glycine N-methyltransferase; GnRH, gonadotropin releasing hormone; HIF, hog intrinsic factor; HPRT1, hypoxanthine phosphoribosyltransferase; MAT1A, methionine adenosyltransferase 1A; MAT2A, methionine adenosyltransferase 2A; MAT2B, methionine adenosyltransferase 2B; MET, rumen protected methionine; MTHFR, methyltetrahydrofolate reductase; MTR, methionine synthase; MTRR, methionine synthase reductase; OCM, one-carbon metabolites; PRMT1, protein methyltransferase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; THF, tetrahydrofolic Acid; TMR, total mixed ration

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## Introduction

One-carbon metabolites (OCM) include choline, B-vitamins (vitamin B<sub>12</sub>, vitamin B<sub>6</sub>, riboflavin [vitamin B<sub>2</sub>], and folate [vitamin B<sub>9</sub>]), minerals (cobalt [component of vitamin B<sub>12</sub>], sulfur [component of methionine], and amino acids (methionine, serine, and glycine; [Clare et al., 2019](#)). At its core, one-carbon metabolism centers around the methionine and folate cycles which are responsible for the transfer of methyl groups to DNA, RNA, and proteins, but branch into pathways for the synthesis of nucleotides, polyamines, amino acids, creatine, and phospholipids ([Mason, 2003](#); [Clare et al., 2019](#)). Because of the interconnected nature of the cycles involved in one-carbon metabolism, it is inherently understood that the perturbation of a metabolite(s) in one part of the pathway results in compensatory changes to metabolite synthesis and availability in other portions of the pathway ([Zeisel, 2011](#)).

Epigenetic modifications including DNA methylation rely on the availability of methyl donors such as those generated via OCM ([Van den Veyver, 2002](#)). Furthermore, during early gestation, the bovine conceptus undergoes intensive epigenetic reprogramming with global demethylation taking place shortly after fertilization, followed by a wave of DNA methylation beginning at the blastocyst stage ([Morgan et al., 2005](#); [Dobbs et al., 2013](#); [Jiang et al., 2018](#); [Duan et al., 2019](#); [Ivanova et al., 2020](#)). Multiple studies have determined that the presence or absence of OCM during the periconceptual period of gestation results in immediate effects on the embryo with lasting phenotypic effects on the offspring in sheep ([Sinclair et al., 2007](#)) and cattle ([Peñagaricano et al., 2013](#); [Acosta et al., 2016](#); [Estrada-Cortés et al., 2020, 2021](#)) such as altered fat metabolism and accretion and insulin resistance in male offspring of methyl deficient ewes as well as increased birth and 205-d adjusted weaning weights in brahman calves supplemented as embryos.

In beef cattle, the knowledge base surrounding the role of OCM supplementation during the periconceptual period is severely limited. In fact, the committee that drafted the 2016 version of the Nutrient Requirements of Beef Cattle ([National Academies of Sciences and Medicine, 2016](#)) indicated that our current understanding of the roles of one-carbon metabolites in beef cattle is “insufficient”, and therefore, “additional research in one-carbon metabolism, folic acid, and other factors affecting the metabolic one-carbon pool would likely provide needed insight into developmental and epigenetic events during the fetal and perinatal periods of growth.”

Previous work in ruminants investigating the roles of OCM on embryonic development and postnatal performance have been limited to the supplementation or depletion of one or two OCM. Our laboratory recently reported that the supplementation of methionine, choline, vitamin B<sub>12</sub>, and folate to bovine embryonic fibroblasts cultured in divergent energy supplies (media containing low or high glucose) improved growth rate and mitochondrial respiration in cells treated with increasing concentrations of OCM ([Crouse et al., 2022](#)). Therefore, we hypothesized that increasing OCM supplementation to heifers during the first 14 d of the estrous cycle would increase circulating OCM concentrations and improve methylation potential in a dose dependent manner. The objective of the present study aimed to translate our in vitro model to an in vivo model and determine the optimal supplementation rate of the same four OCM (methionine, choline, vitamin B<sub>12</sub>, and folate) to heifers during the first 14 d of the estrous cycle

to maintain increased circulating concentrations of OCM for use as a model in early pregnancy studies.

## 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

Angus heifers ( $n = 30$ , ~ 15 mo of age; average initial BW =  $392.6 \pm 12.6$  kg) were trained to consume feed from individual feeders (American Calan, Northwood, NH) for at least one month prior to the start of the study. Heifers were stratified across five pens with six heifers per pen. Each treatment was represented in each pen with one treatment being represented twice in each pen. Heifers were fed a total mixed ration (TMR) consisting of 75% grass/alfalfa hay, 21% corn silage, and 4% mineral pellet on a dry matter basis which met or exceeded the recommended metabolizable energy and metabolizable protein requirements for heifers fed to target 0.45 kg/d gain (actual gain =  $0.44 \pm 0.11$  kg) as established in NASEM ([National Academies of Sciences, Engineering, and Medicine, 2016](#)).

All heifers were administered 100 µg Gonadotropin Releasing Hormone (GnRH; Cystorelin, Boehringer Ingelheim, Ingelheim am Ehin, Germany), followed in 7 d by 25 mg Prostaglandin (Lutalyse, Zoetis, Parsippany-Troy Hills, NJ), followed in 48 h by 100 µg GnRH, with the final injection of GnRH considered day 0 of the study. None of the heifers were bred as part of the synchronization protocol. Heifers were randomly assigned to one of five treatments ( $n = 6$ /treatment) which were initiated after day 0 (referred to as day 0 of the estrous cycle) of the study and are described in [Table 1](#). These treatments were: 0XNEG: Total mixed ration (TMR) and injections of saline at day 0 and 7 of the estrous cycle, 0XPOS: TMR, rumen protected methionine (MET; Smartamine M, Adisseo, Alpharetta, GA) fed at 0.08% of the diet DM, rumen protected choline (CHOL; ReaShure, Balchem Inc., New Hampton, NY) fed at 60 g/d, and injections of saline at day 0 and 7 of the estrous cycle, 0.5X: TMR, MET, CHOL, 5 mg vitamin B<sub>12</sub>, and 80 mg folate at day 0 and 7 of the estrous cycle, 1X: TMR, MET, CHOL, 10 mg vitamin B<sub>12</sub>, and 160 mg folate at day 0 and 7 of the estrous cycle, 2X: TMR, MET, CHOL, 20 mg vitamin B<sub>12</sub>, and 320 mg folate at day 0 and 7 of the estrous cycle. Injections of saline (0.9% NaCl; 10 mL), vitamin B<sub>12</sub> (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 provided in a single injection site. Vitamin B<sub>12</sub> and folate injection levels were based on previously published literature in Holsteins ([Preynat et al., 2009b](#)) where the injection dosage of vitamin B<sub>12</sub> and folate, equivalent to the 1X treatment, increased milk yield as well as folate and B<sub>12</sub> concentrations in milk. Methionine and CHOL were fed at the same inclusion level across all supplemented treatments as per manufacturer's recommendations.

### Sample collection and analysis

Serum and plasma samples were collected on day 0, 2, 5, 7, 9, 12, and 14 of the estrous cycle via jugular venipuncture using

**Table 1.** Treatment structure

	0XNEG	0XPOS	0.5X	1X	2X
Feed <sup>1</sup>	TMR	TMR + 0.08% MET + 60 g/d CHOL	TMR + 0.08% MET + 60 g/d CHOL	TMR + 0.08% MET + 60 g/d CHOL	TMR + 0.08% MET + 60 g/d CHOL
Injection <sup>2</sup>	0.9% Saline	0.9% Saline	5mg B <sub>12</sub> + 80 mg FA	10 mg B <sub>12</sub> + 160 mg FA	20 mg B <sub>12</sub> + 320 mg FA

<sup>1</sup>TMR, 75% grass/alfalfa hay, 21% corn silage, and 4% mineral pellet on a dry matter basis fed to gain 0.45 kg/d. MET, Rumen Protected Methionine fed at 0.08% inclusion on a Dry Matter Basis. CHOL, Rumen Protected Choline.

<sup>2</sup>B<sub>12</sub>, Vitamin B<sub>12</sub> (Cyanocobalamin) injected after blood collection on day 0 and 7 of the synchronized estrous cycle. FA, Folic Acid (pteroylglutamic acid) injected after blood collection on day 0 and 7 of the synchronized estrous cycle.

10-mL serum and EDTA vacutainer tubes (Becton Dickinson HealthCare, Franklin Lakes, NJ). On injection days (day 0 and 7) and the day of slaughter (day 14), serum and plasma were collected prior to injection or transport to the abattoir, respectively. The serum was allowed to set for 20 min at room temperature and both serum and plasma were centrifuged at 1,500 × g for 20 min, separated from blood constituents, and were stored at -80 °C. Heifers were slaughtered at the U.S. Meat Animal Research Center abattoir on day 14 of the synchronized estrous cycle. Livers were collected at slaughter and 500 mg of sample collected from the left hepatic lobe and snap frozen in liquid nitrogen and stored at -80 °C.

### Methionine-folate cycle intermediates

Serum vitamin B<sub>12</sub> and folate analysis was conducted at IDEXX Laboratories (Westbrook, ME). For vitamin B<sub>12</sub>, the IMMULITE 2000 (Siemens Medical Solutions, Malvern, PA) performs a one-cycle sample treatment with dithiothreitol and a sodium hydroxide/potassium cyanide solution in a reaction tube containing no bead. After a 30-min incubation, the treated sample was transferred to a second reaction tube containing a vitamin B<sub>12</sub>-coated polystyrene bead and hog intrinsic factor (HIF). During this subsequent 30-min incubation, the vitamin B<sub>12</sub> released from the endogenous binding proteins during sample treatment competes with immobilized vitamin B<sub>12</sub> for binding with HIF. Alkaline phosphatase-labeled anti-hog intrinsic factor was then introduced, which binds to any HIF that was immobilized on the B<sub>12</sub>-coated bead in the final 30-min incubation. Unbound enzyme conjugate was removed by centrifugal wash. Substrate was added and the procedure continued as described for typical immunoassays. For folate, the IMMULITE 2000 performs a 2-cycle, on-board sample treatment of serum. The sample, along with ligand-labelled folic acid was first treated with dithiothreitol in a reaction tube containing no bead, and then sodium hydroxide/potassium cyanide in a second treatment cycle. The treated sample was transferred to a second reaction tube containing a murine anti-folate binding protein antibody-coated polystyrene bead and folate binding protein (FBP). During a 30-min incubation, folic acid released from binding proteins in the sample competes with ligand-labeled folic acid for binding with FBP. The bead was washed, and alkaline phosphatase labeled anti-ligand was added. During the final 30-min incubation, the alkaline phosphatase labeled anti-ligand binds to the ligand-labeled folate that was bound to the bead during the first incubation. The unbound enzyme conjugate was removed by centrifugal wash. Substrate was added and the procedure continues as describe for typical immunoassays.

Hepatic folic acid intermediates were measured as previously described by [Nandania et al. \(2018\)](#) with 80 mg of

initial tissue for homogenization. Folate cycle intermediates: folic acid, dihydrofolic acid (DHF), tetrahydrofolate (THF), 5,10-methylene-THF, and 5-methyl-THF were purchased from Schircks Laboratories (Switzerland). Quality control standards (QC standards containing all five metabolite standards of interest) were analyzed between samples within runs (intra run CV = 9.14%; across run CV = 14.27%).

Hepatic methionine cycle intermediates were measured by the Duke University Proteomics and Metabolomics Shared Resource (Duke University, Durham, NC). For liver tissue, 500 microliters of a 50% methanol solution was added to 50 mg of liver samples and homogenized with a Percellys 24 bead blaster (Bertin instruments, Montigny-le-Bretonneux, France) at 4 °C for 3 cycles of 10 s each at 10,000 rpm with a 20-s pause between each burst. All sample extracts were centrifuged at 20,000 rcf for 5 min at 4 °C. Fifty microliters of supernatant and standard solutions were transferred into the well of a 1 mL 96-well NUNC plate (Nalge Nunc Internation Corporation, Roskilde, Denmark) and mixed with 20 microliters of 1 µg/mL L-methionine methyl-D3 (Cambridge Isotope Laboratories, Inc, Tewksbury, MA) and S-adenosylhomocystein-d4 (Cayman Chemical, Ann Arbor, MI), then subjected to complete dryness at room temperature under nitrogen. One hundred microliters of methanol solution was added into the dried wells and incubated at room temperature while shaking for 20 min at 500 rpm, and then 10 min for 2,000 rpm. Finally, the plate was centrifuged at 500 rcf for 5 min before instrument injection. Samples were analyzed with a 6500+ QTRAP LC-MS/MS system (Sciex, Framingham, MA). Software Analyst 1.7.1 was used for data acquisition and analysis. The Sciex ExionLC UPLC system includes a degasser, an AD autosampler, an AD column oven, a controller, and an AD pump. The LC separation was performed on an Agilent SB-Aq RRHD column (2.1 × 50 mm, 1.8 µm) with mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile). The flow rate was 0.4 mL/min. The linear gradient was as follows: 0–1 min, 100% A; 2–3.5 min, 0% A; 3.6–4 min, 50% A; 4.1–5.5 min, 100% A. The autosampler was set at 10 °C and the column was kept at 40 °C. The injection volume was 1 µL. Mass spectra were acquired under positive electrospray ionization with the ion spray voltage of 4,500 V. The source temperature was 450 °C. The curtain gas, ion source gas 1, and ion source gas 2 were at 32, 60, and 60 psi, respectively. Multiple reaction monitoring was used for quantitation: methionine (MET; m/z 150.0 → m/z 56.0), S-adenosylhomocysteine (SAH; m/z 385.1 → m/z 136.0), S-adenosylmethionine (SAM; m/z 399.0 → m/z 97.1), DL-homocysteine (m/z 136.0 → m/z 56.0), and internal standards L-methionine methyl-d3 (m/z 153.0 → m/z 107.0), S-adenosylhomocysteine-d4 (m/z 389.1 → m/z 136.0). The average assay CV for all 4 analytes in hepatic tissue was 2.29%.

Plasma methionine cycle intermediates were analyzed on samples from day 14 of the estrous cycle by the Duke University Proteomics and Metabolomics Shared Resource. Fifty microliters of plasma was mixed with 200 microliters of methanol and 20 microliters 1  $\mu\text{g}/\text{mL}$  L-methionine-d3 and S-adenosylhomocysteine-d4 followed by vigorous vortexing. Samples were then kept at  $-20\text{ }^{\circ}\text{C}$  for 20 min, centrifuged at 15,000 rcf for 5 min at  $4\text{ }^{\circ}\text{C}$ , and the supernatant was transferred into the well of a 1 mL 96-well NUNC plate and subjected to complete dryness at room temperature by nitrogen. One hundred microliters of 50% methanol solution was added into the dried wells and incubated at room temperature while shaking for 20 min at 500 rpm, and then for 5 min at 2,000 rpm. Finally, the plate was centrifuged at 500 rcf for 5 min before instrument injection. After injection, mass spectrometry methods follow the same protocols as outlined for hepatic tissue. The average assay coefficient of variation for all 4 analytes in plasma was 1.78%.

Hepatic choline was measured with the Abcam Total Choline Assay Kit (Abcam, Cambridge, UK). Twenty mg of liver tissue was used and was assayed according to the manufacturer's protocol except for being read by absorbance instead of fluorescence and thus the OD being measured at 576 nm (intra assay CV = 9.90%).

### Methionine-folate cycle enzyme transcript abundance

Ribonucleic acid extraction was performed using the RNeasy Plus Universal Mini Kit (Qiagen, Hilden, Germany) and quantified using the Qubit 3.0 (Invitrogen, Waltham, MA). The RNA was diluted to 2  $\mu\text{g}$  and cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, Waltham, MA). The efficiency of the primer pairs for the 13 genes (*ACTB*, *DNMT1*, *DNMT3A*, *GAPDH*, *GNMT*, *HPRT1*, *MAT1A*, *MAT2A*, *MAT2B*, *MTHFR*, *MTR*, *MTRR*, and *PRMT1*) were tested (Supplementary Table S1). Samples were analyzed in triplicate with a 1:5 serial dilution. Out of the three reference genes selected (*ACTB*, *GAPDH*, and *HPRT1*), *GAPDH* was the most stably expressed across all samples and therefore was the only gene used as a reference gene. Real-time polymerase chain reaction (rt-PCR) was performed using SYBR Green with 1.75 microliters water, 7.5 microliters SYBR Green, 0.375 microliters of the 10  $\mu\text{M}$  forward primer, and 0.375 microliters of the 10  $\mu\text{M}$  reverse primer were included per well. The PCR reaction was performed using the QuantStudio Real-Time PCR system (ThermoFisher Scientific). The program was set to run at  $95\text{ }^{\circ}\text{C}$  for 20–30 s, at  $95\text{ }^{\circ}\text{C}$  for 1–3 s, and finally at  $60\text{ }^{\circ}\text{C}$  for 20–30 s for 35–40 cycles. The primer information can be found in Supplementary Table S1.

The average  $C_t$  value of the triplicates was used for final analysis. First, one animal from the control treatment was designated as the control sample for calculations, and its relative quantification (RQ) was set to 1. Relative quantification for all other genes were calculated as  $\text{RQ} = 2^{-\Delta\Delta C_t}$  where  $\Delta\Delta C_t = (\text{sample gene of interest } C_t - \text{sample reference gene } C_t) - (\text{control gene of interest } C_t - \text{control reference gene } C_t)$ ; Livak and Schmittgen, 2001).

### Statistical analysis

Serum vitamin  $B_{12}$  and folate concentrations were analyzed using the MIXED procedure of SAS 9.4 (SAS Institute, Cary, NC) with repeated measures using Spatial Power as the

covariate, with day, treatment, and the interaction in the model as fixed effects and animal nested within treatment as the random effect. Net area under the curve (AUC) analysis was conducted to account for the variation in starting  $B_{12}$  and folate concentrations across heifers using Proc SQL and macro as previously described by Shiang et al. (2010). Measurements include 1<sup>st</sup> Week = area under the curve for the first week of the estrous cycle (day 0 to 7), 2<sup>nd</sup> Week = area under the curve for day 7 to 14 of the estrous cycle using day 0 as the baseline value, and Total = total area under the curve for the entire sampling period.

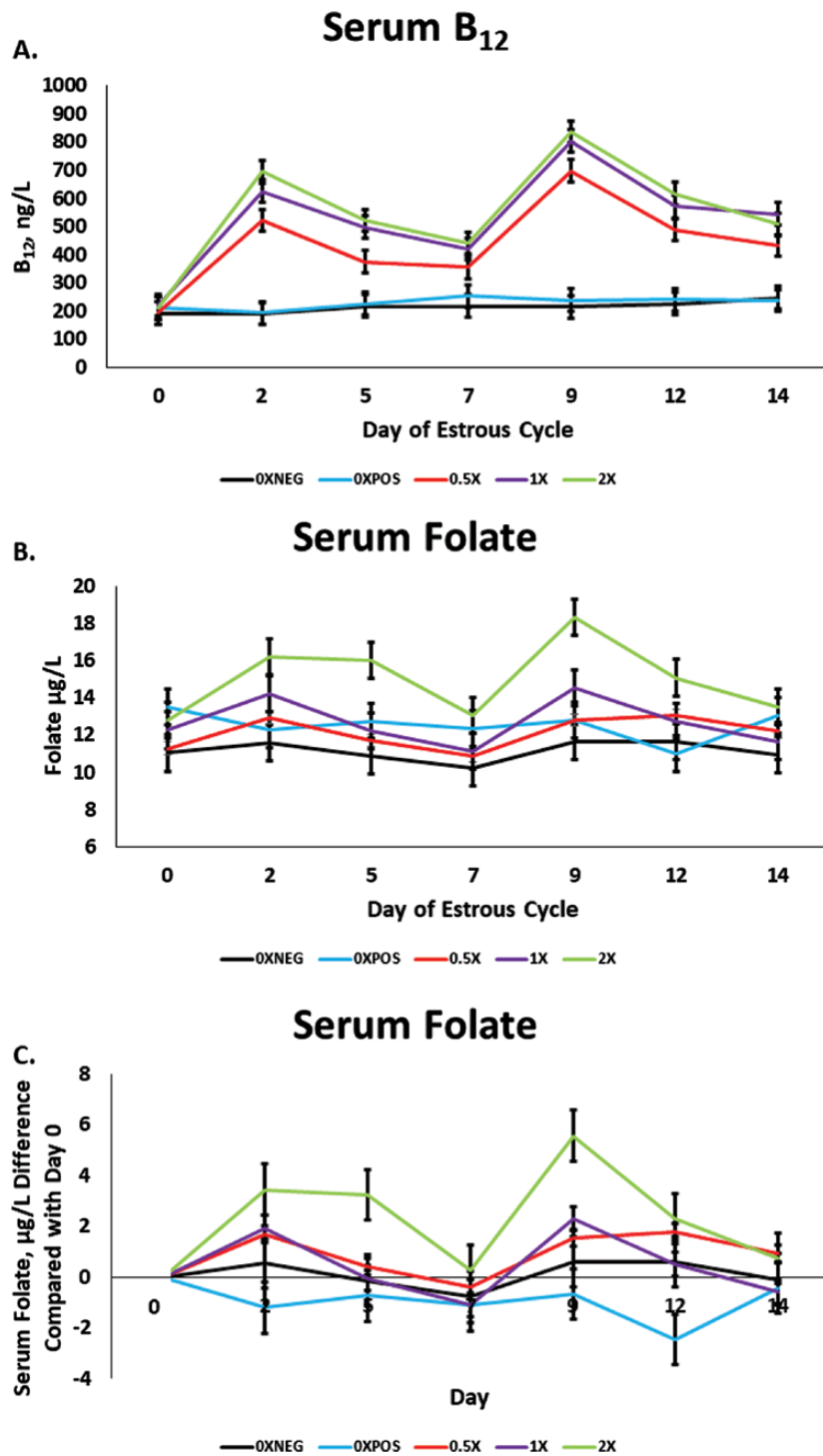
Methionine-folate cycle intermediates in serum and liver as well as the methionine-folate cycle transcript abundance were analyzed using PROC GLM and means were separated using LSMEANS. Polynomial contrast coefficients were generated using PROC IML for unequal spacing between treatments. Contrasts performed were Negative vs. Positive (0XNEG vs. 0XPOS), Met + Choline vs. Folate (0XPOS vs. 0.5X, 1X, and 2X) as well as linear quadratic, and cubic contrasts from 0XPOS, 0.5X, 1X, and 2X for increasing folate and  $B_{12}$  concentrations.

## Results

### Serum vitamin $B_{12}$ and folate

Serum vitamin  $B_{12}$  was influenced by the day  $\times$  treatment interaction ( $P < 0.0001$ ; Figure 1A). On day 0, there were no differences ( $P \geq 0.47$ ) in serum vitamin  $B_{12}$  concentrations across nutritional treatment. Across all days, serum vitamin  $B_{12}$  concentrations were not different ( $P \geq 0.48$ ) between the 0XNEG and 0XPOS treatments, and the 0.5X, 1X, and 2X were always greater ( $P < 0.05$ ) than both the 0XNEG and 0XPOS from day 2 to day 14 of the study (Figure 1A). On day 2, 5, 9, and 12, both the 1X and 2X treatments had greater ( $P \leq 0.04$ ) concentrations of vitamin  $B_{12}$  compared with the 0.5X. On day 7, 2X was greater than 0.5X ( $P = 0.03$ ) with 1X being intermediate and equal ( $P \geq 0.10$ ) to both 0.5X and 2X. Lastly, on day 14, 1X was greater than 0.5X ( $P < 0.01$ ) with the 2X treatment being intermediate and equal ( $P \geq 0.06$ ) to both the 0.5X and 1X treatments (Figure 1A).

Serum folate was influenced by the day  $\times$  treatment interaction ( $P = 0.0004$ ; Figure 1B). There were no differences in serum folate concentrations among treatments on day 0 and 14 of the study ( $P \geq 0.07$ ). On day 2, the concentration of folate was greater ( $P \leq 0.02$ ) in serum of the 2X heifers compared with the 0.5X, 0XPOS, and 0XNEG with 1X being intermediate and equal ( $P \geq 0.15$ ) to all other treatments (Figure 1B). On day 5, the concentration of folate was greater ( $P \leq 0.02$ ) in serum of the 2X-treated heifers compared with all other treatments. On day 7, the concentration of folate was greater ( $P = 0.05$ ) in serum of the 2X-treated heifers compared with 0XNEG with 0XPOS, 0.5X, and 1X being intermediate and equal ( $P \geq 0.14$ ) to both 0XNEG and the 2X-treated heifers. On day 9, the concentration of folate was greater ( $P \leq 0.01$ ) in serum of the 2X-treated heifers compared with all other treatments (Figure 1B). Furthermore, the concentration of folate was greater ( $P = 0.04$ ) in serum of the 1X-treated heifers compared with 0XNEG with 0XPOS and 0.5X being intermediate and equal ( $P \geq 0.23$ ) to both the 0XNEG- and 1X-treated heifers. Lastly, on day 12, the concentration of folate was greater ( $P \leq 0.05$ ) in serum of the 2X-treated heifers compared with all other treatments (Figure 1B).



**Figure 1.** The serum concentrations of vitamin B<sub>12</sub> (A), folate (B), and folate normalized to day 0 concentrations (C) as influenced by day of the estrous cycle, and methyl donor treatments. Probability values for Vitamin B<sub>12</sub>: Day:  $P < 0.0001$ , Trt:  $P < 0.0001$ , Day  $\times$  Trt:  $P < 0.0001$ . Probability values for Folate: Day:  $P < 0.0001$ , Trt:  $P = 0.008$ , and Day  $\times$  Trt:  $P = 0.0004$ . Panel C is solely for visualization of AUC data and does not have statistical analysis directly applied to it. 0XNEG: TMR and sham injections of saline at day 0 and 7 of the estrous cycle, 0XPOS: TMR, rumen protected methionine fed at 0.08% of the diet DM, rumen protected choline fed at 60 g/d, and sham injections of saline at day 0 and 7 of the estrous cycle; 0.5X, TMR, MET, CHOL, 5 mg vitamin B<sub>12</sub>, and 80 mg folate at day 0 and 7 of the estrous cycle; 1X, TMR, MET, CHOL, 10 mg vitamin B<sub>12</sub>, and 160 mg folate at day 0 and 7 of the estrous cycle; 2X, TMR, MET, CHOL, 20 mg vitamin B<sub>12</sub>, and 320 mg folate at day 0 and 7 of the estrous cycle.

Net AUC data for both vitamin B<sub>12</sub> and folate are presented in Table 2. Figure 1C is prepared for visualization of folate data with day 0 as a baseline. Serum vitamin B<sub>12</sub> net AUC was greater ( $P < 0.01$ ) for the first week (day 1 to 7) in the 1X- and 2X-treated heifers compared with the 0.5X heifers, which

were greater than the 0XNEG- and 0XPOS-treated heifers. There was also a quadratic increase ( $P < 0.01$ ) in net AUC for vitamin B<sub>12</sub> with increasing supplementation from the 0XPOS to 2X supplementation levels. There was no difference ( $P = 0.77$ ) between the 0XNEG and 0XPOS treatments which

**Table 2.** Area under the curve analysis of vitamin B<sub>12</sub> and folate through the first 14 d of the estrous cycle

Vitamin <sup>3</sup>	Time <sup>4</sup>	Treatment <sup>1</sup>					SEM <sup>5</sup>	Probability Values <sup>2</sup>				
		0XNEG	0XPOS	0.5X	1X	2X		Trt	Neg vs. Pos	Met + Chol vs. Vitamin	Linear	Quadratic
B <sub>12</sub>	1st Week	86.3a	38.1a	1300.3b	1932.2c	2216.9c	124.1	<0.01	<0.01	<0.01	<0.01	0.52
	2nd Week	313.8a	358.8a	2728.9b	3631.2c	3908.3c	193.8	<0.01	<0.01	<0.01	<0.01	0.18
	Total	310.9a	247.6a	3512.1b	4851.9c	5319.2c	233.4	<0.01	<0.01	<0.01	<0.01	0.18
Folate	1st Week	-1.13a	-6.05a	4.83a	3.75a	16.89b	4.92	0.04	0.03	0.01	0.91	0.39
	2nd Week	-3.31	-13.47	6.09	3.04	21.50	9.14	0.13	0.05	0.03	0.77	0.40
	Total	-0.35a	-15.55a	13.86a	9.28a	37.45b	10.74	0.03	0.02	0.01	0.72	0.29

<sup>1</sup>0XNEG, TMR and sham injections of saline at day 0 and 7 of the estrous cycle; 0XPOS, TMR, rumen protected methionine fed at 0.08% of the diet DM, rumen protected choline fed at 60 g/d, and sham injections of saline at day 0 and 7 of the estrous cycle; 0.5X, TMR, MET, CHOL, 5 mg vitamin B<sub>12</sub> and 80 mg folate at day 0 and 7 of the estrous cycle; 1X, TMR, MET, CHOL, 10 mg vitamin B<sub>12</sub> and 160 mg folate at day 0 and 7 of the estrous cycle; 2X, TMR, MET, CHOL, 20 mg vitamin B<sub>12</sub> and 320 mg folate at day 0 and 7 of the estrous cycle.

<sup>2</sup>Probability values for: Trt, main effect of treatment. Probability values for contrast statements: Neg vs. Pos, 0XNEG vs. 0XPOS; Met + Chol vs. Vitamin, 0XPOS vs. 0.5X, 1X, and 2X; Linear, Quadratic, and Cubic. Polynomial contrasts with increasing folate level of 0XPOS to 2X.

<sup>3</sup>Vitamin B<sub>12</sub> (Cyanocobalamin). Folic Acid (Vitamin B<sub>9</sub>).

<sup>4</sup>Area under the curve measurements for: 1st Week, area under the curve for the first week of the estrous cycle (day 0 to 7), 2nd Week, area under the curve for day 7 to 14 of the using d 0 as the baseline value. The average standard error of the mean for a specific measurement.

<sup>5</sup>a cMeans within row without a common superscript differ by a main effect of treatment.

received injections of saline. The second week AUC followed a similar trend as the first week, being greater ( $P < 0.01$ ) in the 1X- and 2X-treated heifers compared with the 0.5X treated heifers which were greater than the 0XNEG- and 0XPOS-treated heifers. Furthermore, there was a quadratic ( $P < 0.01$ ) increase in net AUC with increasing supplementation level, and no difference ( $P = 0.88$ ) between the 0XNEG and 0XPOS treatments.

Serum folate net AUC during the first week was greater ( $P = 0.04$ ) in the 2X compared with all other treatments. Furthermore, there was a linear ( $P = 0.01$ ) increase in net AUC during the first week with increasing supplementation level from the 0XPOS to the 2X treatments, with no difference ( $P = 0.41$ ) in folate AUC between the 0XNEG and 0XPOS treatments. There were no differences ( $P = 0.13$ ) in net AUC for folate in the second week; however, there was a linear increase ( $P = 0.03$ ) in net folate AUC. The total net AUC was greater ( $P = 0.03$ ) in the 2X treatment compared with all other treatments. Moreover, there was a linear increase ( $P = 0.01$ ) in total net AUC with increasing supplementation from 0XPOS to 2X.

### Hepatic methionine-folate cycle intermediates

All hepatic methionine-folate cycle analyte data are presented in Table 3. There were no treatment differences ( $P \geq 0.19$ ) in any methionine cycle intermediates or choline in liver at day 14 of the estrous cycle. The concentration of methionine, homocysteine, or the SAM:SAH ratio was not different ( $P \geq 0.13$ ) in any contrasts measured. The concentrations of SAM and SAH tended ( $P = 0.08$  and  $P = 0.07$ , respectively) to increase in liver with increasing supplementation level from 0XPOS to 2X. The concentration of SAH also decreased ( $P = 0.03$ ) in the 0XPOS compared to the 0XNEG treatment. The concentration of choline tended ( $P = 0.09$ ) to be greater in the 0XPOS compared with 0XNEG treated heifers and tended ( $P = 0.08$ ) to be affected by a cubic response from 0XPOS to 2X, where the 0.5X treatment decreased in choline abundance compared with the 0XPOS, and subsequently increased to be equal to the 0XPOS at 1X and 2X.

The concentrations of folate cycle intermediates in liver were affected by treatment. Folate concentrations in liver were greater ( $P < 0.01$ ) in 2X compared with the 0.5X and 1X-treated heifers which were greater than the 0XNEG- and 0XPOS-treated heifers. The concentration of DHF in liver was greater ( $P < 0.01$ ) in 2X-treated heifers compared with the 1X-treated heifers, which were greater than the 0XNEG and 0XPOS, with 0.5X being intermediate and equal to 0XNEG-, 0XPOS-, and the 1X-treated heifers. The concentration of THF was greater ( $P = 0.04$ ) in 2X heifers compared with both the negative and positive control as well as the 0.5X-treated heifers, with 1X-treated heifers being intermediate and equal to all other treatments. The concentration of 5,10-methylene-THF in liver was greater ( $P < 0.01$ ) in the 1X- and 2X-treated heifers compared with the 0XNEG-, 0XPOS-, and 0.5X-treated heifers which were all equal. Finally, the concentration of the physiological methyl donor 5-methyl-THF was greater in the 2X-treated heifers ( $P = 0.01$ ) than the 0XNEG, 0XPOS, and 0.5X. The concentration of 5-methyl-THF was greater ( $P = 0.05$ ) in 1X than 0XNEG, and 0XPOS with 0.5X being intermediate and equal to the others. The concentrations of folate, DHF, 5,10-methylene-THF, and 5-methyl-THF were greater ( $P \leq 0.03$ ) in the folate-treated heifers compared

**Table 3.** Hepatic methionine-folate cycle analyte abundance at day 14 of the synchronized estrous cycle

Analyte	Treatment <sup>1</sup>							SEM <sup>3</sup>	Probability Values <sup>2</sup>			
	0XNEG	0XPOS	0.5X	1X	2X	Trt	Neg vs. Pos		Met + Chol vs. Vitamin	Linear	Quadratic	Cubic
Methionine, ng/mg	76.7	80.9	83.3	82.5	87.3	3.66	0.41	0.44	0.40	0.24	0.82	0.66
S-adenosyl Methionine (SAM), ng/mg	7.52	5.91	5.52	4.99	8.56	1.15	0.19	0.33	0.73	0.08	0.12	0.66
S-adenosyl Homocysteine (SAH), ng/mg	1.09	0.84	0.92	0.95	1.13	0.10	0.27	0.03	0.21	0.07	0.85	0.76
Homocysteine, ng/mg	1.48	1.48	1.34	1.26	1.38	0.10	0.49	0.99	0.17	0.54	0.13	0.87
SAM:SAH	7.08	7.47	5.88	5.54	8.33	1.33	0.57	0.84	0.58	0.52	0.14	0.99
Choline, µmol/g	5642	7559	4741	7025	7315	975	0.22	0.09	0.31	0.63	0.26	0.08
Folate, ng/mg	0.28a	0.35a	1.07b	1.13b	2.03c	0.23	<0.01	0.71	<0.01	<0.01	0.82	0.31
Dihydrofolate, ng/mg	31.7a	35.6a	58.4ab	68.0b	109.8c	10.1	<0.01	0.50	<0.01	<0.01	0.88	0.61
Tetrahydrofolate, ng/mg	0.26a	0.33a	0.33a	0.40ab	0.63b	0.08	0.04	0.24	0.30	0.02	0.44	0.89
5,10-Methylene-THF, ng/mg	0.10a	0.12a	0.90a	2.00b	2.82b	0.32	<0.01	0.92	<0.01	<0.01	0.29	0.53
5-Methyl-THF, ng/mg	1.05a	1.57a	4.20ab	6.63bc	9.35c	1.67	0.01	0.55	0.03	0.01	0.60	0.94

<sup>1</sup>0XNEG, TMR and sham injections of saline at day 0 and 7 of the estrous cycle; 0XPOS, TMR, rumen protected methionine fed at 0.08% of the diet DM, rumen protected choline fed at 60 g/d, and sham injections of saline at day 0 and 7 of the estrous cycle; 0.5X, TMR, MET, CHOL, 5 mg vitamin B<sub>12</sub> and 80 mg folate at day 0 and 7 of the estrous cycle; 1X, TMR, MET, CHOL, 10 mg vitamin B<sub>12</sub> and 160 mg folate at day 0 and 7 of the estrous cycle; 2X, TMR, MET, CHOL, 20 mg vitamin B<sub>12</sub> and 320 mg folate at day 0 and 7 of the estrous cycle.

<sup>2</sup>Probability values for: Trt, main effect of treatment. Probability values for contrast statements: Neg vs. Pos, 0XNEG vs. 0XPOS; Met + Chol vs. Vitamin, 0XPOS vs. 0.5X, 1X, and 2X; Linear, Quadratic, and Cubic. Polynomial contrasts with increasing folate level of 0XPOS to 2X.

<sup>3</sup>The average standard error of the mean for a specific measurement.

a-c Means within row without a common superscript differ by a main effect of treatment.

with the 0XPOS and increased linearly ( $P \leq 0.01$ ) with increasing folate supplementation from 0XPOS to 2X-treated heifers. The concentration of THF in liver was not different ( $P = 0.30$ ) between the 0XPOS-treated heifers and the folate-treated heifers, but THF increased linearly ( $P = 0.02$ ) with increasing folate supplementation from 0XPOS to 2X.

### Serum methionine cycle intermediates

All day 14 serum analyte data are presented in Table 4. Because of the homeostasis in methionine cycle analyte abundance in liver tissue, methionine cycle analytes were measured in serum at day 14 of the estrous cycle. The concentration of methionine was increased ( $P = 0.04$ ) in 0.5X- and 2X-treated heifers compared with 0XNEG with 0XPOS, with 1X being intermediate and equal to all other treatments. The concentration of SAM was not affected ( $P = 0.18$ ) by nutritional treatment. The concentration of SAH tended ( $P = 0.06$ ) to be greater in the 0.5X-treated heifers compared with 0XNEG. The concentration of homocysteine in serum was not affected ( $P = 0.98$ ) by treatment at day 14 of the estrous cycle. Finally, the SAM:SAH ratio was greater ( $P = 0.05$ ) in the 0XNEG-, 0XPOS-, and 2X-treated heifers compared with the 0.5X-treated heifers with the 1X-treated heifers being intermediate and equal to all other treatments. Concentration differences in methionine and homocysteine were not detected by any contrasts investigated ( $P \geq 0.12$ ). The concentration of SAM tended ( $P = 0.06$ ) to be affected quadratically, decreasing from 0XPOS to 0.5X and increasing from 1X- to 2X-treated heifers. In contrast, the concentration of SAH tended ( $P = 0.07$ ) to be affected cubically such that the concentration in 0.5X-treated heifers was increased compared to 0XPOS and subsequently decreased in 1X and 2X which were equal to the 0XPOS heifers. Lastly, the SAM:SAH ratio tended ( $P = 0.08$ ) to decrease with folate and B<sub>12</sub> supplementation compared to heifers receiving only methionine and choline. Furthermore, the SAM:SAH ratio was affected quadratically ( $P = 0.03$ ) such that it decreased from 0XPOS to 0.5X and increased from 0.5X to 1X and further increased to 2X to be equivalent to the concentration of the 0XPOS treated heifers.

### Methionine-folate cycle enzyme transcript abundance

Enzyme transcript abundance data can be found in Table 5. The transcript abundance of *DNMT1*, *DNMT3A*, *DNMT3B*, *MAT1A*, *MTHFR*, and *PRMT1* were not affected ( $P \geq 0.12$ ) by nutritional treatment. Transcript abundance of *MAT2A* was greater ( $P = 0.01$ ) in 2X-treated heifers compared with 0XNEG, 0.5X, and 1X treated heifers. Additionally, *MAT2A* transcript abundance was greater in 0XNEG and 0XPOS compared with 0.5X, with 1X being intermediate and equal to 0XNEG, 0XPOS, and 0.5X. The transcript abundance of *MAT2B* was greatest ( $P < 0.01$ ) in 2X, intermediate in 0XNEG and 0XPOS, and lower in 0.5X. The transcript abundance of the 1X treatment was intermediate and equal to both the 0XNEG, 0XPOS, and 0.5X treatments. The transcript abundance of *MTR* tended ( $P = 0.08$ ) to be greater in the 2X-treated heifers compared with the 0.5X- and 1X-treated heifers. Finally, the transcript abundance of *MTRR* tended ( $P = 0.07$ ) to be greater in the 2X treatment compared with all other treatments. The abundance of *DNMT1* was affected quadratically ( $P = 0.02$ ), where the transcript abundance

Table 4. Serum methionine cycle analyte abundance at day 14 of the synchronized estrous cycle

Analyte	Treatment <sup>1</sup>			SEM <sup>3</sup>	Probability Values <sup>2</sup>							
	0XNEG	0XPOS	0.5X		1X	2X	Trt	Neg vs. Pos	Met + Chol vs. Vitamin	Linear	Quadratic	Cubic
Methionine, ng/mL	3178a	3688ab	4150b	3635ab	4398b	267	0.04	0.30	0.23	0.12	0.52	0.14
S-adenosyl Methionine, ng/mL	20.5	21.2	17.8	18.8	20.4	1.07	0.18	0.55	0.11	0.95	0.06	0.26
S-adenosyl Homocysteine, ng/mL	5.51	6.10	7.48	6.42	6.07	0.45	0.06	0.36	0.29	0.44	0.15	0.07
Homocysteine, ng/mL	568	560	572	558	589	38.3	0.98	0.90	0.74	0.57	0.79	0.70
SAM:SAH	3.48b	3.52b	2.50a	3.02ab	3.37b	0.25	0.05	0.91	0.08	0.69	0.03	0.07

<sup>1</sup>0XNEG, TMR and sham injections of saline at day 0 and 7 of the estrous cycle; 0XPOS, TMR, rumen protected methionine fed at 0.08% of the diet DM, rumen protected choline fed at 60 g/d, and sham injections of saline at day 0 and 7 of the estrous cycle; 0.5X, TMR, MET, CHOL, 5 mg vitamin B<sub>12</sub> and 80 mg folate at day 0 and 7 of the estrous cycle; 1X, TMR, MET, CHOL, 10 mg vitamin B<sub>12</sub> and 160 mg folate at day 0 and 7 of the estrous cycle; 2X, TMR, MET, CHOL, 20 mg vitamin B<sub>12</sub> and 320 mg folate at day 0 and 7 of the estrous cycle.

<sup>2</sup>Probability values for: Trt, main effect of treatment. Probability values for contrast statements: Neg vs. Pos, 0XNEG vs. 0XPOS; Met + Chol vs. Vitamin, 0XPOS vs. 0.5X, 1X, and 2X; Linear, Quadratic, and Cubic. Polynomial contrasts with increasing folate level of 0XPOS to 2X.

<sup>3</sup>The average standard error of the mean for a specific measurement.

a-c Means within row without a common superscript differ by a main effect of treatment.



**Table 5.** Hepatic methionine-folate cycle enzyme transcript abundance at day 14 of the synchronized estrous cycle

Genes <sup>3</sup>	Treatment <sup>1</sup>											
	0XNEG	0XPOS	0.5X	1X	2X	SEM <sup>4</sup>	Trt	Neg vs. Pos	Met + Chol vs. Vitamin	Linear	Quadratic	Cubic
<i>DNNMT1</i>	1.30	1.39	1.27	0.97	1.56	0.16	0.12	0.76	0.49	0.43	0.02	0.29
<i>DNNMT3A</i>	0.68	0.76	0.81	0.69	0.92	0.11	0.55	0.67	0.74	0.37	0.38	0.41
<i>GNNMT</i>	0.98	1.73	0.92	1.25	2.25	0.56	0.42	0.49	0.70	0.33	0.18	0.61
<i>MAT1A</i>	0.73	0.93	0.91	0.73	0.90	0.10	0.36	0.25	0.34	0.64	0.13	0.26
<i>MAT2A</i>	0.93b	1.25bc	0.70a	0.91ab	1.55c	0.18	0.02	0.35	0.31	0.06	<0.01	0.24
<i>MAT2B</i>	1.26b	1.18b	0.85a	1.04ab	1.85c	0.12	<0.01	0.68	0.62	<0.01	<0.01	0.36
<i>MTHFR</i>	0.37	0.26	0.30	0.46	0.45	0.10	0.56	0.47	0.23	0.14	0.55	0.51
<i>MTR</i>	1.61	1.71	1.33	1.34	2.02	0.20	0.08	0.79	0.52	0.12	0.02	0.81
<i>MTRR</i>	1.16	1.41	1.10	1.30	2.07	0.27	0.07	0.53	0.78	0.04	0.11	0.68
<i>PRMT1</i>	0.47	0.72	0.50	0.48	0.61	0.18	0.83	0.40	0.76	0.34	0.84	0.36

<sup>1</sup>0XNEG, TMR, and sham injections of saline at day 0 and 7 of the estrous cycle; 0XPOS, TMR, rumen protected methionine fed at 0.08% of the diet DM, rumen protected choline fed at 60 g/d, and sham injections of saline at day 0 and 7 of the estrous cycle; 0.5X, TMR, MET, CHOL, 5 mg vitamin B<sub>12</sub> and 80 mg folate at day 0 and 7 of the estrous cycle; 1X, TMR, MET, CHOL, 10 mg vitamin B<sub>12</sub> and 160 mg folate at day 0 and 7 of the estrous cycle; 2X, TMR, MET, CHOL, 20 mg vitamin B<sub>12</sub> and 320 mg folate at d 0 and 7 of the estrous cycle.

<sup>2</sup>Probability values for: Trt, main effect of treatment. Probability values for contrast statements: Neg vs. Pos, 0XNEG vs. 0XPOS; Met + Chol vs. Vitamin, 0XPOS vs. 0.5X, 1X, and 2X; Linear, Quadratic, and Cubic. Polynomial contrasts with increasing folate level of 0XPOS to 2X.

<sup>3</sup>Genes are *DNNMT1*, DNA methyltransferase 1; *DNNMT3A*, DNA methyltransferase 3A; *GNNMT*, glycine N-methyltransferase; *MAT1A*, methionine adenosyltransferase 1A; *MAT2A*, methionine adenosyltransferase 2A; *MAT2B*, methionine adenosyltransferase 2B; *MTHFR*, methylenetetrahydrofolate reductase; *MTR*, 5-methyltetrahydrofolate-homocysteine methyltransferase; *MTRR*, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase; *PRMT1*, protein arginine methyltransferase 1.

<sup>4</sup>The average standard error of the mean for a specific measurement.

<sup>a-c</sup>Means without a common superscript differ by a main effect of treatment.

decreased from 0XPOS to 1X and increased from 1X to 2X. The transcript abundance of *MAT2A*, *MAT2B*, and *MTR* was affected quadratically ( $P \leq 0.02$ ) such that the abundance decreased from 0XPOS to 0.5X and 1X, with a subsequent increase to 2X. Finally, the transcript abundance of *MTRR* increased linearly ( $P = 0.04$ ) with increasing supplementation of folate and vitamin B<sub>12</sub>.

## Discussion

It is well established that cattle with functioning rumens have microbiota that are very active in the synthesis of B-vitamins including choline, folic acid, and vitamin B<sub>12</sub> (McDowell, 2000), and that under normal conditions, the synthesis of these B vitamins should be adequate for normal metabolic functions. In the case of vitamin B<sub>12</sub>, its synthesis requires the presence of cobalt, which is at the center of its corrin ring structure, and in some areas of the United States, New Zealand, and Australia, the concentrations of cobalt in consumed forages may be inadequate for microbial vitamin B<sub>12</sub> synthesis in the rumen (National Academies of Sciences, Engineering, and Medicine, 2016). Furthermore, there are times within the normal beef cattle production cycle, such as during drought, that culminates in limited forage quantity and/or forage quality, resulting in nutrient restriction and decreases in circulating methionine concentrations (Crouse et al., 2019). Syring et al., (2022) reported that restricting beef heifers to 60% of CON intake resulted in increased folate and vitamin B<sub>12</sub> concentrations in allantoinic fluid, which may be a compensatory mechanism to maintain fetal growth and the fetal epigenome during times of aberrant maternal nutrition. Reports using ruminant models demonstrated the negative responses to OCM restriction on offspring performance (Sinclair et al., 2007) such as altered fat metabolism and accretion, and insulin resistance in male offspring of methyl deficient ewes. Contrarily, recent studies have demonstrated the positive responses to maternal OCM supplementation on beef calf development when supplemented during the periconceptual period of gestation or in the culture medium during in vitro fertilization (Liu et al., 2020; Estrada-Cortés et al., 2021; Silva et al., 2021) such as increased rate of gain and gain:feed (Silva et al., 2021), and increased birth and adjusted weaning weights (Estrada-Cortés et al., 2021). It should be noted that while positive responses to late gestation OCM supplementation to the cow or calf have been documented in dairy cattle (Preynat et al., 2009a; Batistel et al., 2017; Alharthi et al., 2018, 2019; Batistel et al., 2019; Palombo et al., 2021), there are discrepancies in the data as to the benefit of supplementation of methionine in beef cattle. Feeding methionine hydroxy analogues to beef cows prior to and after calving increased weaning weights in studies from Varner (1974), Varner et al., (1975) and Thomas and Langford (1978) but failed to affect calf weaning weights in studies reported from Clanton and England (1980) and Clements et al., (2017). It is important to note when comparing these studies that metabolic stressors such as lactation, nutrient intake and forage quality, breeding/calving season, and limiting/meeting/exceeding protein requirements also play an important role in the responses to methionine supplementation. Furthermore, there are multiple methionine supplement sources and the type (methionine hydroxy analog or rumen protection) influences the bioavailability which should be accounted for prior to supplementation. A recent focus in beef cattle has been placed on early-pregnancy (Liu et al.,

2020; Estrada-Cortés et al., 2021; Silva et al., 2021), a time when there is epigenetic reprogramming that is taking place during early gestation and thus substantiates the specific need for OCM during this time (Dobbs et al., 2013). Therefore, these critical events and timing of nutrient supplementation require the additional investigation of OCM supplementation during early gestation as well as defining the optimal supplementation dose of OCM during early pregnancy.

Data presented in this manuscript demonstrate that the concentrations of folate, vitamin B<sub>12</sub>, and methionine-folate cycle intermediates in serum and liver tissue in response to supplemental B<sub>12</sub>, folate, methionine, and choline are dose dependent, and that not all supplementation levels yield positive responses to increasing the circulating concentrations of methyl donors. It is important to note that while choline and methionine are stored in the body either via phospholipids or proteins, water soluble vitamins such as folate and vitamin B<sub>12</sub> are not stored in substantial quantities. Therefore, these data support the injection of folate and B<sub>12</sub> at 160 to 320 mg folate and 10 to 20 mg of vitamin B<sub>12</sub> to yearling beef heifers fed rumen protected methionine and choline at the manufacturer recommended intakes (Smartamine inclusion: 0.08% of DMI; Reashure inclusion: 60 g/d) to maintain increased circulating concentrations of OCM particularly folate and vitamin B<sub>12</sub>.

Regardless of injection dosage, vitamin B<sub>12</sub> concentration was greater in all supplemented heifers compared with negative and positive controls for both concentrations and area under the curve. The concentration of folate, however, was not increased until 320 mg of folate was injected weekly. This is likely due to the different roles of vitamin B<sub>12</sub> and folate in metabolism with vitamin B<sub>12</sub> being a cofactor for enzymes such as methionine synthase and methylmalonyl-CoA-mutase and folate being an intermediate in one-carbon metabolism and nucleotide synthesis (McDowell, 2000; Kräutler, 2012; Clare et al., 2019; Aggett et al., 2020). Similar to previously published work in dairy cattle (Preynat et al., 2009a), our work demonstrated that supplementation of methionine without vitamin B<sub>12</sub> and folate injections resulted in decreased folate concentrations in circulation. Increases in SAM result in the downregulation of MTHFR enzyme (Selhub, 1999; Lucock, 2000; Clare et al., 2019) and thus a decrease in circulating 5-methyl-THF, which is the major form of folates found in circulation of humans (Selhub, 1999) and cattle (Preynat et al., 2009b). Therefore, the coordinated increase in both methionine and SAM as presented herein in coordination with the decrease in circulating folates in the 0XPOS-treated heifers is supported from previous research.

The concentrations of methionine cycle intermediates in liver at day 14 of the estrous cycle did not differ by treatment. A recent publication from Clare et al. (2021) determined that in tissues with betaine homocysteine methyltransferase, such as liver, the net flux of methionine would be maintained regardless of substrate supply. Therefore, the lack of differences seen in hepatic methionine cycle intermediates in this study was to be expected. Furthermore, all folate cycle intermediates measured were increased in folate supplemented heifers compared with the saline injected heifers. Like the circulating total folates in serum, only the 2X treatment was greater in all folate metabolites measured in liver suggesting a similarity between hepatic and circulating folate responses.

Serum methionine cycle metabolites in the present study demonstrate that there are doses of folate and vitamin B<sub>12</sub>

that alter the methylation potential in beef heifers. Methylation potential is the ability of cells to methylate DNA, RNA, or proteins and is measured by the ratio of SAM:SAH, that are the substrates and products, respectively, for methyltransferases. A decreased SAM:SAH ratio is associated with decreased methylation potential and hypomethylated DNA, primarily driven by increased SAH (Caudill et al., 2001). The tendency to increase serum SAH and decrease the SAM:SAH ratio in heifers in the present study demonstrates a decreased methylation potential in heifers receiving the 0.5X treatment.

Recently, Crouse et al. (2022) reported cubic effects on embryonic cell growth, mitochondrial respiration, and differential methylation in bovine embryonic fibroblasts cultured with a similar model of doubling OCM in the media. These cubic effects seen both in vitro (increase in mitochondrial respiration and cell growth from control to 2.5X, decrease at 5X, and subsequent increase at 10X) and in vivo (decrease in SAM:SAH ratio in 0.5X) support the interconnected nature and stoichiometry of the methionine-folate cycle and the need to establish an optimal substrate level for each OCM in relationship to the others to keep the balance of substrates and products in each cycle and their connected pathways. It should also be noted that the concentration of circulating homocysteine was not altered by supplementing OCM in the current study. This may be due to the basal diet already meeting protein requirements and the fact that this study did not aim to induce a methyl deficiency either directly via the basal diet, or indirectly by the inclusion of methyl substrate consumers or inhibitors as has been previously demonstrated (Sinclair et al., 2007; Ardalan et al., 2020).

To further understand the effects of OCM supplementation on concentrations of circulating OCM, hepatic methionine-folate cycle enzyme transcript abundance was measured. Interestingly, enzymes responsible for the synthesis of SAM as well as the folate cycle remethylation of homocysteine to methionine decreased in the 0.5X treatment and subsequently increased to be equal to or greater than the 0XPOS treated heifers by the 2X supplemented group. The differences in transcript abundance of *MAT2A* and *MAT2B* accompany a similar quadratic response in the concentrations of SAM decreasing from 0XPOS to 0.5X and subsequently increasing to the 2X supplemented level. Methionine adenosyl transferase 2A is one of the enzymes that synthesizes SAM from methionine whereas *MAT2B* encodes a regulatory subunit for *MAT2A* (Nordgren et al., 2011). The change in *MAT2B* transcript abundance in Crouse et al., (2022) and in the current report demonstrates similar effects between in vitro culture of an immortalized cell line of bovine embryonic fibroblasts and in vivo derived tissues from this study. Increases in SAM typically result in inhibition of *MTHFR* and activate the transsulfuration pathway to decrease the amount of 5-mTHF available for the remethylation of homocysteine to methionine but also remove homocysteine via transsulfuration (Neidhart, 2016; Clare et al., 2019). While there were no changes in the magnitude of SAM due to nutritional treatment, our data support these findings with no change in *MTHFR* transcript abundance either. The decrease in *MTR* and *MTRR* at 0.5X do not follow the same pattern as the increase in their substrate concentrations. Further work in cattle would need to be conducted to determine the regulatory effects of methionine-folate intermediates on enzyme

transcript abundance as well as to determine the enzyme function in the presence of increased or decreased substrate.

It is important to note that data from this study was conducted in cyclic heifers, and that further work using these doses are needed to determine whether dietary and injectable OCM may be used in ruminants to alter the conceptus or fetal epigenomes and lead to developmental programming responses. Furthermore, not all concentrations of vitamin B<sub>12</sub> and folic acid used in this study yielded positive responses in terms of methyl donor availability/metabolism, and that more folate and vitamin B<sub>12</sub> may not be better for the conceptus. Therefore, further mechanistic studies should be conducted with heifers/cows at different physiological stages to better understand the stoichiometric balance of methyl donors in the methionine-folate cycle in beef cattle thereby more appropriately supplementing cattle of different breeds and at different physiological stages in an effort to improve growth, reproductive performance, and developmental programming outcomes.

In conclusion, these data support the injection of 20 mg of vitamin B<sub>12</sub> and 320 mg of folic acid injected once weekly when cyclic heifers are fed rumen protected methionine and rumen protected choline at manufacturer recommended feed intakes to increase circulating concentrations of B<sub>12</sub> and folate. These doses set the basis upon which future studies can be based when feeding and injecting one-carbon metabolites.

## 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.

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