Smartamine M and MetaSmart supplementation during the peripartal period alter hepatic expression of gene networks in 1-carbon metabolism, inflammation, oxidative stress, and the growth hormone–insulin-like growth factor 1 axis pathways

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ABSTRACT

Peripartal cows likely require greater amounts of Met not only at the tissue and cell level for methylation reactions but also for milk protein synthesis after calving. Thirty-nine Holstein cows were fed throughout the peripartal period (–21 d to 30 d in milk) a basal control (CON) diet ($n = 14$) with no Met supplementation, CON plus MetaSmart (MS; Adisseo Inc., Antony, France; $n = 12$), or CON plus Smartamine M (SM; Adisseo Inc.; $n = 13$). The Met supplements were adjusted daily and top-dressed over the total mixed ration at a rate of 0.19 or 0.07% (dry matter) of feed for MS or SM. Liver tissue was collected on –10, 7, and 21 d for transcriptome profiling of genes associated with Met and glutathione metabolism as well as components of the inflammation, oxidative stress, growth hormone/insulin-like growth factor-1 axis, and DNA methylation pathways. Data were analyzed using PROC MIXED of SAS (SAS Institute Inc., Cary, NC) with the preplanned contrasts CON versus SM + MS and SM versus MS. The *S*-adenosylhomocysteine hydrolase (*SAHH*) gene was the most abundant among all genes evaluated, with overall greater expression in Met-supplemented cows than CON, and in SM than MS. Expression of Met adenosyltransferase 1A (*MAT1A*) was greater in Met-supplemented cows than CON by 21 d postpartum. A greater overall expression of 5-methyltetrahydrofolate-homocysteine methyltransferase (*MTR*) occurred in Met-supplemented cows than CON. In contrast, the expression of glutathione synthase (*GSS*); glutamate-cysteine ligase, catalytic subunit (*GCLC*); and superoxide dismutase 1, cytosolic (*SOD1*) was lower in Met-supplemented cows than CON. A greater overall expression of nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (*NFKB1*) and greater

upregulation of haptoglobin (*HP*) on d 7 occurred in Met-supplemented cows than CON. Expression of DNA cytosine-5-methyltransferase 3 alpha (*DNMT3A*) was greater but expression of *DNMT1* was lower in Met-supplemented cows than CON. The response observed in *SAHH* reflects its importance to Met supplementation during the peripartum period. Despite greater *HP* expression after calving, the lower expression of glutathione (*GSS* and *GCLC*) metabolism genes and *SOD1* due to Met reflect a lower oxidative stress and mild inflammatory status. The extent to which changes in expression of *DNMT3A* and *DNMT1* result in epigenetic effects partly responsible for the previously observed enhanced performance in Met-supplemented cows remains to be examined. Increasing the supply of Met as SM or MS can affect expression of genes in the Met cycle to various extents and, hence, the supply of methyl donors such as *S*-adenosylmethionine and antioxidants such as glutathione. These compounds likely are in high demand during the peripartum period.

Key words: transition cow, gene expression, methionine

INTRODUCTION

The importance of Met as one of the most-limiting AA for milk protein synthesis in mid-lactation dairy cows has been well established (Schwab et al., 1992; Pisulewski et al., 1996). Similar positive effects on milk protein have been observed soon after calving when supplementing Met during the peripartal period, which leads to a concomitant increase in milk production and milk fat yield (Ordway et al., 2009; Osorio et al., 2013). These responses could be partly driven by supplemental Met helping alleviate the increased demand for methylated compounds with the onset of lactation (Preynat et al., 2009). Increased Met bioavailability in cows supplemented with rumen-protected Met (Graulet et al., 2005) is likely to increase entry of Met into the 1-carbon metabolism cycle (Figure 1) where it is ini-

Received July 29, 2014.

Accepted August 18, 2014.

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tially converted into *S*-adenosylmethionine (**SAM**), the major biological methyl donor (Martinov et al., 2010). Availability of SAM is essential for DNA methylation, an important biological process to regulate gene expression, carried out through the covalent addition of a methyl group from SAM to cytosine within a Cyt-phosphate-Gua (**CpG**) region ("islands") in the DNA. This process is performed by DNA methyltransferases (**DNMT**) such as *DNMT3A* and *DNMT1*, which are responsible for creating methylated CpG patterns in the genome (Kass et al., 1997).

The periparturient dairy cow may experience a state of reduced liver function coupled with increased inflammation, characterized by increased production of positive acute-phase proteins such as ceruloplasmin (**CPL**) and serum amyloid A (**SAA**), and a concomitant decrease in the production of negative acute-phase proteins such as albumin (Bertoni et al., 2008). A decrease in the

inflammatory response has been previously associated with Met supplementation during the periparturient period, where lower periparturient CPL and SAA concentrations coupled with greater albumin concentration (Osorio et al., 2014) was observed.

Oxidative stress occurs when an imbalance exists between the production of reactive oxygen metabolites and neutralizing availability of antioxidants. Some of the well-established antioxidants include glutathione, superoxide dismutase (**SOD**), and vitamins A and E (Bernabucci et al., 2005). Enhancing Met flux through the cycle by Met supplementation could spare intermediate metabolites such as Hcy for use in other pathways such as glutathione metabolism (e.g., we previously observed greater glutathione concentration in the liver of Met-supplemented cows; Osorio et al., 2013).

Growth hormone plays an important role during lactation not only to increase milk production but also to

Figure 1. Key genes encoding enzymes of the Met cycle: Met adenosyltransferase 1A (*MAT1A*), phosphatidylethanolamine methyltransferase (*PEMT*), *S*-adenosylhomocysteine hydrolase (*SAHH*), betaine homocysteine methyltransferase (*BHMT* and *BHMT2*), 5-methyltetrahydrofolate-homocysteine methyltransferase (*MTR*), cystathionine β -synthase (*CBS*), and cystathionine β -lyase (*CTH*). PPi = pyrophosphate; Pi = inorganic P; SAM = *S*-adenosylmethionine; PE = phosphatidylethanolamine; PC = phosphatidylcholine; SAH = *S*-adenosylhomocysteine; Ado = adenosyl; THF = tetrahydrofolate; CH₃THF = 5-methyltetrahydrofolate; DMG = dimethylglycine; CSAD = cysteine sulfinic acid decarboxylase; CSA = cysteine sulfinic acid; CDO = cysteine dioxygenase.

stimulate hepatic gluconeogenesis and IGF1 synthesis (Etherton and Bauman, 1998; Feigerlova et al., 2013). Increased growth hormone (GH) concentration has been observed in periparturient dairy cows supplemented with Met (Osorio et al., 2013); therefore, it is plausible that genes related to the GH-IGF1 axis might be modulated by Met supplementation.

The objective of this study was to use gene expression analysis to evaluate potential molecular mechanisms associated with better postparturient performance, reduced periparturient inflammatory response, and greater antioxidant capacity during the periparturient period in cows fed Smartamine (SM; Adisseo Inc., Antony, France) or MetaSmart (MS; Adisseo Inc.; Osorio et al., 2013, 2014). Liver tissue harvested during the periparturient period was used for mRNA expression profiling of genes associated with Met and glutathione metabolism as well as components of inflammatory, GH-IGF1 axis, and DNA methylation pathways (Supplemental Table S1; <http://dx.doi.org/10.3168/jds.2014-8680>).

MATERIALS AND METHODS

Animals and Experimental Design

All procedures were conducted under protocols approved by the University of Illinois Institutional Animal Care and Use Committee (Urbana). Details of the experimental design have been published previously (Osorio et al., 2013). Briefly, 39 Holstein cows entering their second-or-greater lactation were enrolled in the study and were fed experimental treatments consisting of a basal control (CON) diet ($n = 14$) with no Met supplementation, CON plus MS ($n = 12$) at 0.19% of DM, or CON plus SM ($n = 13$) at 0.07% of DM (Osorio et al., 2013). All cows received the same far-off diet (1.24 Mcal/kg of DM; 14.3% CP) from -50 to -21 d before expected calving, a close-up diet (1.54 Mcal/kg of DM; 15.0% CP) from -21 d to calving, and fresh cow diet from calving (1.75 Mcal/kg of DM; 17.5% CP) through 30 DIM. Supplements of Met were top-dressed from -21 to 30 DIM. A subset of cows (CON: $n = 8$; MS: $n = 8$; SM: $n = 9$) from those mentioned above was used to evaluate mRNA expression. Animal husbandry, sampling of ingredients, TMR, BW, BCS, milk weights, sampling for milk composition, and housing of cows pre- and postpartum were as reported previously (Osorio et al., 2013).

Liver Biopsy, RNA Extraction, Quantitative PCR, and Design and Evaluation of Primers

Liver tissue was sampled via puncture biopsy (Dann et al., 2006) from cows under local anesthesia at ap-

proximately 0730 h once prepartum on d -10 (± 3 d), and then postpartum on d 7 and 21. Tissue specimens were stored in liquid N₂ until RNA extraction. Total RNA was extracted from liver samples using established protocols in our laboratory (see supplemental materials; <http://dx.doi.org/10.3168/jds.2014-8680>). Details of quantitative PCR, design, and primer evaluation are presented in the supplemental materials. Percentage relative abundance of mRNA was calculated (Supplemental Table S2; <http://dx.doi.org/10.3168/jds.2014-8680>) to provide additional mechanistic information on the target genes (Bionaz and Loores, 2008).

Statistical Analysis

The gene expression data were analyzed using PROC MIXED of SAS (version 9.3; SAS Institute Inc., Cary, NC). The fixed effects in the model included diet, parity, time, and their interactions. Cow was designated as a random effect. Parity was removed from the model any time this effect was nonsignificant ($P > 0.05$; Supplemental Table S3; <http://dx.doi.org/10.3168/jds.2014-8680>). Unequally spaced data were analyzed using the exponential correlation covariance structure SP for repeated measures.

The SM and MS products differ in the way they could provide extra Met to the cow: Met in SM is physically protected by a pH-sensitive coating to avoid ruminal fermentation, whereas approximately 50% of Met in MS is absorbed through the rumen wall and the other approximately 50% is degraded by rumen microorganisms (Graulet et al., 2005). Therefore, we primarily focused on the contrast MS + SM versus CON, but also evaluated the contrast MS versus SM (Table 1). Mean separations between diets at a given time point were evaluated when trends ($P \leq 0.10$) or significant ($P \leq 0.05$) diet $\times$ time (D $\times$ T) effects were observed. Least squares means separation for significant effects was performed using the PDIFF statement. Statistical significance was declared at $P \leq 0.05$ and tendencies at $P \leq 0.10$.

RESULTS

Met Metabolism

Main effects of diet, time, and interactions for genes associated with Met metabolism are presented in Figure 2. The mRNA expression of S-adenosylhomocysteine hydrolase (SAHH) and Met adenosyltransferase 1A (MAT1A) was affected ($P \leq 0.05$) by the D $\times$ T effect, whereas a trend ($P < 0.10$) was observed for cysteine sulfinic acid decarboxylase (CSAD). In contrast, expression of betaine homocysteine methyltransferase

Table 1. Contrast comparison of relative mRNA expression ($\log_2$ scale) of genes related to Met and glutathione metabolism, inflammation, oxidative stress, growth hormone (GH)-IGF1 axis, and DNA methylation in cows fed MetaSmart (MS; Adisseo Inc., Antony, France) versus Smartamine M (SM; Adisseo Inc.) during the periparturient period

Gene	Treatment			SEM	Contrast ¹		
	MS	SM	Overall		−10	7	21
Met metabolism							
<i>SAHH</i>	0.44	0.51	0.04	0.20	0.07	0.06	0.03
<i>BHMT</i>	0.01	0.06	0.10	0.70	0.29	0.62	0.94
<i>BHMT2</i>	−1.26	−1.55	0.08	0.01	0.09	0.88	0.03
<i>CBS</i>	0.70	0.64	0.08	0.54	0.40	0.94	0.62
<i>MTR</i>	0.04	0.04	0.002	0.71	0.43	0.79	0.67
<i>MAT1A</i>	0.60	0.66	0.07	0.50	0.08	0.42	0.29
<i>PEMT</i>	0.31	0.33	0.01	0.29	0.64	0.07	0.60
<i>CSAD</i>	0.24	0.25	0.03	0.84	0.60	0.90	0.96
<i>CTH</i>	0.47	0.46	0.02	0.74	0.80	0.66	0.44
Glutathione metabolism							
<i>GSR</i>	−1.75	−1.92	0.07	0.08	0.30	0.61	0.02
<i>GSS</i>	0.46	0.46	0.01	0.89	0.99	0.99	0.86
<i>GPX1</i>	1.43	1.53	0.08	0.33	0.68	0.71	0.02
<i>GCLC</i>	−4.09	−4.22	0.08	0.24	0.21	0.83	0.57
Inflammatory response							
<i>NFKB1</i>	2.24	2.63	0.11	0.01	0.01	0.51	0.09
<i>CP</i>	1.16	1.23	0.11	0.62	0.56	0.47	0.83
<i>HP</i>	−2.38	−2.24	0.66	0.88	0.87	0.35	0.65
<i>SAA2</i>	−6.21	−5.98	0.30	0.59	0.69	0.75	0.81
<i>STAT3</i>	0.56	0.49	0.11	0.68	0.32	0.26	0.43
Oxidative stress							
<i>SOD1</i>	1.85	1.72	0.05	0.09	0.65	0.79	<0.01
<i>SOD2</i>	2.13	2.00	0.09	0.30	0.07	0.97	0.44
GH-IGF1 axis							
<i>STAT5B</i>	2.42	2.52	0.08	0.34	0.05	0.09	0.16
<i>SOCS2</i>	1.03	1.15	0.16	0.60	0.72	0.11	0.02
<i>IGF1</i>	2.12	2.67	0.11	<0.01	0.08	0.58	<0.01
DNA methylation							
<i>DNMT1</i>	−5.70	−5.70	0.08	0.97	0.14	0.37	0.47
<i>DNMT3A</i>	1.47	1.22	0.14	0.23	0.08	0.66	0.43

¹Contrast MS versus SM throughout experiment (overall), as well as at −10, 7, and 21 d relative to parturition.

(*BHMT*)2, 5-methyltetrahydrofolate-homocysteine methyltransferase (*MTR*), phosphatidylethanolamine methyltransferase (*PEMT*), *BHMT*, cystathionine β -synthase (*CBS*), and cystathionine β -lyase (*CTH*) was not affected ($P \geq 0.10$) by $D \times T$. Overall expression of *SAHH* was greater ($P = 0.02$) in Met-supplemented than in CON cows, mainly at 7 d ($P = 0.03$). Expression of *SAHH* was greater in SM compared with CON at −10 d ($P = 0.05$). Additionally, *SAHH* expression was greater in SM than MS at 21 d ($P = 0.03$), whereas a trend for greater *SAHH* expression in SM than MS was observed at −10 d ($P = 0.07$) and 7 d ($P = 0.06$).

The expression of *MAT1A* was greater in Met-supplemented cows than CON at 21 d ($P = 0.04$), whereas *CSAD* expression was lower ($P = 0.01$) in Met-supplemented cows than CON at the same time point (Figure 2). The overall expression of *BHMT2* was lower ($P = 0.02$) in Met-supplemented cows than CON. In contrast to *BHMT2*, *MTR* expression was greater ($P = 0.04$) in Met-supplemented cows than CON. The overall expression of *BHMT2* was lower ($P = 0.01$) in SM

than MS cows (Table 1). A trend existed for greater ($P = 0.10$) expression of *PEMT* in Met-supplemented cows than CON. Although *BHMT2* expression remained unchanged ($P > 0.10$) over time, expression of *BHMT* increased ($P < 0.01$) over time; however, its expression was not affected by dietary treatments. Expression of *CBS* and *CTH* was not affected by diet or time.

Glutathione Metabolism

Main effects of diet, time, and interactions for genes associated with glutathione metabolism are presented in Figure 3. A $D \times T$ effect ($P = 0.05$) on glutamate-cysteine ligase, catalytic subunit (*GCLC*) expression was observed, where lower expression was observed in Met-supplemented cows at 7 ($P = 0.04$) and 21 d ($P = 0.02$) postpartum. This lower expression is consistent with a trend ($P = 0.06$) for overall lower expression of *GCLC* in Met-supplemented cows than CON.

A trend existed for a $D \times T$ effect ($P = 0.07$) on glutathione peroxidase (*GPX1*) expression, where greater

Figure 2. Expression of genes related to the Met cycle in cows supplemented with MetaSmart (MS; Adisseo Inc., Antony, France) or Smartamine M (SM; Adisseo Inc.) at 0.19 or 0.07% of DM throughout the peripartur period. The P -values for main effect of treatment (diet), time, and interaction diet $\times$ time (D $\times$ T) as well as the contrast MS + SM versus control (CON; when the contrast effect is $P \leq 0.10$) are shown. Mean separations between diets at a given time point were evaluated when a D $\times$ T effect ($P \leq 0.10$) was observed via contrasts denoted as significant ($P \leq 0.05$) as follows: MS + SM versus CON (*) and SM versus CON (**). Values are means, with SE represented by vertical bars. SAHH = *S*-adenosylhomocysteine hydrolase; MAT1A = Met adenosyltransferase 1A; CSAD = cysteine sulfinic acid decarboxylase; BHMT = betaine homocysteine methyltransferase; MTR = 5-methyltetrahydrofolate-homocysteine methyltransferase; PEMT = phosphatidylethanolamine methyltransferase; CBS = cystathionine β -synthase; CTH = cystathionine β -lyase.

($P = 0.02$) expression was observed at 21 d in cows fed SM than MS. Similar to *GCLC*, glutathione synthase (*GSS*) expression was lower ($P = 0.02$) in Met-supplemented cows than CON. A trend ($P = 0.09$) existed for a diet effect on glutathione reductase (*GSR*) expression, where SM cows had lower ($P = 0.04$) expression than CON.

Inflammation

Main effects of diet, time, and interactions on genes associated with inflammation are presented in Figure 4. A trend ($P = 0.09$) existed for a D $\times$ T effect on haptoglobin (*HP*) expression, where Met-supplemented

cows had greater ($P = 0.03$) expression of *HP* at 7 d postpartum than CON cows. Overall expression of nuclear factor of kappa light polypeptide gene enhancer in B-cells (*NFKB1*) was greater ($P = 0.02$) in Met-supplemented cows than CON. Similarly, expression of *NFKB1* was greater ($P = 0.01$) in SM cows than MS (Table 1). Expression of *CP* and *SAA2* decreased ($P < 0.01$) over time, regardless of treatment. The D $\times$ T effect on signal transducer and activator of transcription (*STAT3*) was primarily due to a trend ($P = 0.06$) for greater expression in MS cows than CON at 7 d postpartum as well as changes over time in Met-supplemented cows; for instance, *STAT3* in MS reached the greatest ($P < 0.02$) expression at 7 d, whereas a

trend ($P < 0.07$) for the lowest *STAT3* expression in SM was at 21 d. Expression of *STAT3* in CON cows remained unchanged over time ($P > 0.39$).

Oxidative Stress

Main effects of diet, time, and interactions for genes associated with oxidative stress are presented in Figure 5. No $D \times T$ effect existed for the expression of *SOD1* and *SOD2*. However, a diet effect ($P = 0.05$) on *SOD1* expression was observed that was reflected in a trend ($P = 0.06$) for lower expression in Met-supplemented cows than CON.

GH Signaling

Main effects of diet, time, and interactions for genes associated with GH signaling are presented in Figure 6. A $D \times T$ effect ($P < 0.03$) existed for all genes related to GH signaling. The $D \times T$ effect on *STAT5B* could be associated with greater ($P = 0.01$) expression in Met-supplemented cows than CON at 7 d postpartum. Although the expression of *STAT5B* in SM cows was greater than CON at -10 d ($P = 0.05$), the MS cows had a lower ($P < 0.05$) expression than CON at 21 d. Within the Met-supplemented cows, a greater expression of *STAT5B* in SM was observed compared with MS at -10 d ($P = 0.05$; Table 1). The $D \times T$ effect on suppressor of cytokine signaling 2 (*SOCS2*) expression was mainly associated with the trend ($P = 0.07$) for greater expression in MS cows than CON at 7 d, whereas a lower ($P = 0.02$) expression of *SOCS2* in MS cows than SM was observed at 21 d postpartum.

The $D \times T$ effect on *IGF1* could be related to greater ($P = 0.01$) expression in Met-supplemented cows than CON at -10 d as well as greater ($P = 0.03$) expression of *IGF* in MS cows than CON at 21 d postpartum. Within the Met-supplemented cows, greater ($P < 0.01$) overall expression of *IGF1* was observed in SM than MS cows, which could be associated with greater ($P < 0.01$) expression of *IGF1* in SM than MS cows at 21 d and a trend ($P = 0.08$) for the same effect of greater *IGF1* in SM over MS cows at -10 d (Table 1).

DNA Methylation

Main effects of diet, time, and interactions for genes associated with DNA methylation are presented in Figure 7. A $D \times T$ effect on *DNMT3A* ($P = 0.05$) was observed. The $D \times T$ effect on *DNMT3A* was mainly associated with a greater ($P < 0.01$) expression in Met-supplemented cows than CON at -10 d prepartum. The latter prepartal effect could be related to the overall greater ($P = 0.02$) expression of *DNMT3A* in Met-sup-

Figure 3. Expression of genes related to the glutathione cycle in cows supplemented with MetaSmart (MS; Adisseo Inc., Antony, France) or Smartamine M (SM; Adisseo Inc.) at 0.19 or 0.07% of DM throughout the peripartal period. The P -values for main effect of treatment (diet), time, and interaction diet $\times$ time ($D \times T$) as well as the contrast MS + SM versus control (CON; when the contrast effect is $P \leq 0.10$) are shown. Mean separations between diets at a given time point were evaluated when a $D \times T$ effect ($P \leq 0.10$) was observed via contrasts denoted as significant ($P \leq 0.05$) as follows: MS + SM versus CON (*), and tendencies ($P \leq 0.10$) were as follows: MS + SM versus CON (#) and SM versus CON (###). Values are means, with SE represented by vertical bars. *GCLC* = glutamate-cysteine ligase, catalytic subunit; *GSS* = glutathione synthase; *GPX1* = glutathione peroxidase; *GSR* = glutathione reductase.

Figure 4. Expression of genes related to inflammation in cows supplemented with MetaSmart (MS; Adisseo Inc., Antony, France) or Smartamine M (SM; Adisseo Inc.) at 0.19 or 0.07% of DM throughout the periparturition period. The P -values for main effect of treatment (diet), time, and interaction diet $\times$ time ($D \times T$) as well as the contrast MS + SM versus control (CON; when the contrast effect is $P \leq 0.10$) are shown. Mean separations between diets at a given time point were evaluated when a $D \times T$ effect ($P \leq 0.10$) was observed via contrasts denoted as significant ($P \leq 0.05$) as follows: MS + SM versus CON (*), and tendencies ($P \leq 0.10$) were as follows: MS versus CON (##). Values are means, with SE represented by vertical bars. HP = haptoglobin; NFKB1 = nuclear factor of kappa light polypeptide gene enhancer in B-cells 1; CP = ceruloplasmin; SAA2 = serum amyloid A2; STAT3 = signal transducer and activator of transcription 3.

plemented cows than CON. In contrast to *DNMT3A*, *DNMT1* expression tended ($P = 0.08$) to be lower in Met-supplemented cows. Expression of *DNMT3A* did not change ($P = 0.38$) over time, but *DNMT1* increased ($P = 0.02$) from -10 to 7 d postpartum at which point it reached a peak in expression followed by a decrease ($P < 0.01$) from 7 to 21 d.

DISCUSSION

In the first of a series of companion manuscripts we reported that supplementation with MS or SM im-

proved milk production at least in part by increasing postpartal DMI and reducing liver lipid accumulation (Osorio et al., 2013). The latter also was associated with a faster recovery toward positive energy balance. Additionally, we observed evidence of a dampened inflammatory response in Met-supplemented cows during the periparturition period (Osorio et al., 2014). The present study deals with mRNA expression profiling in liver tissue as a way to understand better the potential role of molecular mechanisms in coordinating liver function and animal performance during the periparturition period.

Figure 5. Expression of genes related to oxidative stress in cows supplemented with MetaSmart (MS; Adisseo Inc., Antony, France) or Smartamine M (SM; Adisseo Inc.) at 0.19 or 0.07% of DM throughout the periparturition period. The P -values for main effect of treatment (diet), time, and interaction diet $\times$ time ($D \times T$) as well as the contrast MS + SM versus control (CON; when the contrast effect is $P \leq 0.10$) are shown. Values are means, with SE represented by vertical bars. *SOD1* = superoxide dismutase 1, cytosolic; *SOD2* = superoxide dismutase 2, mitochondrial.

Met Metabolism

The first step in the Met cycle (1-carbon metabolism) is the binding of adenosyl by Met adenosyltransferase I (**MATI**) or Met adenosyltransferase III (**MATIII**) in the liver and Met to form SAM (Martinov et al., 2010). The *MAT1A* gene encodes both MATI and MATIII isoenzymes mammals (Martinov et al., 2010). The enzyme MATI is inhibited by SAM, whereas MATIII is activated by it. The expression of *MAT1A* was upregulated in a dose-dependent fashion in cultured hepatocytes in response to exogenous SAM, and this effect was mimicked with Met (Garcia-Trevijano et al., 2000). That response helps explain the upregulation

of *MAT1A* over time in Met-supplemented cows. Additional data suggest that *MAT1A* expression could be stimulated by other factors such as glucocorticoids and cyclic AMP, as well as downregulated by hypoxia and tumor necrosis factor α , among others. These factors that can modify *MAT1A* expression are also considered to play important physiological and inflammatory roles during the periparturition period (Bertoni et al., 2008); as such, they might have partly diminished the Met supplementation effect on the preparturition expression of this gene, thus partly explaining the lack of evident effect of supplemental Met on reducing liver triacylglycerol concentration postpartum (Osorio et al., 2013).

Activity of the enzyme SAHH has not, to our knowledge, been studied in ruminants. However, its importance in the methylation cycle in connection with hypermethioninemia, by hydrolyzing SAH to adenosine and Hcy, is well established in humans and mice (Barić, 2009). Because SAHH is a substrate-dependent enzyme, it might have played an important role for both the availability of SAM and Hcy in our study. Inhibition of SAHH causes the accumulation of SAH and, consequently, suppresses SAM-dependent transmethylation via feedback inhibition (Lee et al., 2011).

We observed that *SAHH* was the most abundant gene (Supplemental Table S2; <http://dx.doi.org/10.3168/jds.2014-8680>), which underscores its importance under the conditions and experimental design. On the other hand, Hcy could serve as an important substrate in the synthesis of proteins such as glutathione, which supports our observation of greater liver glutathione concentration in Met-supplemented cows as well as greater antioxidant capacity in those cows, both of which would be beneficial in terms of overall liver function and health and allowing cows to perform better (Osorio et al., 2013). It is possible that cellular SAH concentration could have built up after transmethylation of SAM due to Met supplementation; thus, this effect might have triggered the greater mRNA expression of *SAHH* in Met-supplemented cows.

Homocysteine is a sulfur-containing AA that upon remethylation by BHMT or MTR can regenerate Met from Hcy by transferring a methyl group from 5-methyltetrahydrofolate and betaine (Preynat et al., 2010). *BHMT2* encodes a protein 73% identical in AA sequence to BHMT, but its function remains unclear. In fact, protein expression of *BHMT2* has not been reported (Li et al., 2008). In the present study, supplementation with Met mainly affected *BHMT2* and *MTR* expression, with no effects on *BHMT*, but *BHMT* was the fifth most abundant gene above *BHMT2* and *MTR* (Supplemental Table S2; <http://dx.doi.org/10.3168/jds.2014-8680>). The greater expression of *SAHH* might have promoted greater production of Hcy also reflected

Figure 6. Expression of genes related to the growth hormone (GH)-IGF1 axis in cows supplemented with MetaSmart (MS; Adisseo Inc., Antony, France) or Smartamine M (SM; Adisseo Inc.) at 0.19 or 0.07% of DM throughout the periparturition period. The P -values for main effect of treatment (diet), time, and interaction diet $\times$ time (D $\times$ T) as well as the contrast MS + SM versus control (CON; when the contrast effect is $P \leq 0.10$) are shown. Mean separations between diets at a given time point were evaluated when a D $\times$ T effect ($P \leq 0.10$) was observed via contrasts denoted as significant ($P \leq 0.05$) as follows: MS + SM versus CON (*), MS versus CON (**), and SM versus CON (***), and tendencies ($P \leq 0.10$) were as follows: MS versus CON (##) and SM versus CON (###). Values are means, with SE represented by vertical bars. *STAT5B* = signal transducer and activator of transcription 5B; *SOCS2* = suppressor of cytokine signaling 2.

in the greater expression of *MTR* in Met-supplemented cows. Thus, regeneration of Met from Hcy (via *MTR*) might have been primarily via 5-methyltetrahydrofolate (with vitamin B₁₂ as a coenzyme).

Ruminal microbes are the main source of vitamin B₁₂ for the cow (NRC, 2001). Rumen functionality at calving is reduced not only due to decreased ruminal capacity associated with rapid fetal development during late gestation, but also due to the adjustment to a lactation diet, which is reflected in the lower availability of vitamin B₁₂ (Girard et al., 2005; Girard and Matte, 2005). Therefore, the effect on *MTR* expression is suggestive of lower enzyme activity after calving, partly due to lower vitamin B₁₂ availability. Under such circumstances, it is likely that after calving cows need to rely more on BHMT than *MTR* for regeneration of Met from Hcy, which is consistent with the concomitant increase in *BHMT* mRNA expression and the decrease in *MTR* after calving, regardless of treatment.

The Hcy not converted into Met can be used to synthesize cystathionine and subsequently cysteine via the enzymes CBS and CTH (Figure 1). The lack of change in expression of *CBS* and *CTH* to Met supplementation (Figure 2) suggests that similar flux of Hcy into cysteine existed across dietary treatments and time. The fate of cysteine metabolism at the branch point of cysteine sulfinic acid between sulfate and taurine biosynthetic pathways is regulated in nonruminants by CSAD activity, where decreased activity of this enzyme leads to the biosynthesis of taurine (Jerkins et al., 1998). Feeding high-protein diets to rats resulted in a decrease in the *CSAD* expression and, consequently, lower *CSAD* enzyme activity (Jerkins et al., 1998). We observed similar results in Met-supplemented cows, with lower expression of *CSAD* by 21 d postpartum. Such response could indicate an increase in the biosynthesis of taurine, which also is a potent antioxidant.

Glutathione Metabolism

The importance of antioxidant activity through enzymes such as glutathione peroxidase during the periparturition period is well established (Spears and Weiss, 2008). The activity of this enzyme decreases after calving (Weiss et al., 1990). Glutathione is the most abundant nonprotein thiol that defends against oxidative stress. The enzyme glutamate-cysteine ligase (*GCL*) is rate-limiting in the synthesis of glutathione, and it is a heterodimeric complex consisting of a catalytic (*GCLC*) and modifier (*GCLM*) subunit. The last step in the synthesis of glutathione is carried out by GSS, which binds glycine to the γ -glutamylcysteine complex preformed by *GCLC* (Franklin et al., 2009).

Figure 7. Expression of genes related to the DNA methylation in cows supplemented with MetaSmart (MS; Adisseo Inc., Antony, France) or Smartamine M (SM; Adisseo Inc.) at 0.19 or 0.07% of DM throughout the periparturition period. The P -values for main effect of treatment (diet), time, and interaction diet $\times$ time ($D \times T$) as well as the contrast MS + SM versus control (CON; when the contrast effect is $P \leq 0.10$) are shown. Mean separations between diets at a given time point were evaluated when a $D \times T$ effect ($P \leq 0.10$) was observed via contrasts denoted as significant ($P \leq 0.05$) as follows: MS + SM vs. CON (*). Values are means, with SE represented by vertical bars. *DNMT3A* = DNA cytosine-5-methyltransferase 3 alpha; *DNMT1* = DNA cytosine-5-methyltransferase 1.

Increased Met bioavailability through Met supplementation can indirectly increase the production of total hepatic glutathione through the transsulfuration pathway (Osorio et al., 2013). Increased concentration of glutathione has been associated with a feedback inhibition effect on GCL, and this effect might be translated into a downregulation of *GCLC*, which would agree with our finding in Met-supplemented cows (Figure 3). It is noteworthy that expression of *GSR* (primarily between SM vs. CON), *GCLC*, and *GSS* was lower in Met-supplemented cows, and oxidative stress is among the many conditions that could increase GCL activity and *GCLC* mRNA expression (Lu, 2013). Therefore,

it is plausible that the decreased expression observed for *GCLC*, *GSS*, and *GSR* was associated with lower oxidative stress in Met-supplemented cows. This idea is supported by the lower overall oxidative stress status in those cows (Osorio et al., 2014), which in turn might have dampened the inflammatory response, resulting in greater DMI and milk production (Osorio et al., 2013).

Another plausible cause for the effects on *GCLC* and *GSS* was the decreased expression of *CSAD* at 21 d, which could have increased the supply of cysteine for taurine synthesis, while causing a shortage of cysteine for glutathione synthesis. Expression of *GPX1* and *GCLC* decreased over time, which can be related to several factors such as lower cysteine availability due to greater AA requirements for milk protein, and hepatic glutathione depletion due to increased oxidative stress (Lu, 2009).

Inflammation

The *STAT3* transcription factor is a known target gene of IL-6 (Ling et al., 2004). Upon activation, *STAT3* can induce the expression of multiple genes, including *NFKB1* (Loor, 2010). The unchanged expression of *STAT3* in CON cows agrees with the lack of change in serum IL-6 concentration in CON cows between pre- and postpartum periods (Osorio et al., 2014). In contrast, changes over time for *STAT3* expression in Met-supplemented cows could be partly associated with the overall greater response of *NFKB1* (Figure 4). In contrast with CON cows, the decrease in *NFKB1* from -10 d to 21 d in Met-supplemented cows mirrored the lower blood IL-6 concentration during the same time points (Osorio et al., 2014), supporting a less pronounced inflammatory status in response to Met supplementation. The overall decreased expression of *NFKB1* over time is consistent with previous work from our group (Grauagnard et al., 2013) and indicates a gradual decline in the inflammatory status of healthy cows as lactation progresses.

Acute-Phase Response

A common event during the acute-phase response is the greater hepatic synthesis of acute-phase proteins such as CPL, HP, and SAA (Kindt et al., 2007). No dietary effects were observed for serum HP and, in fact, SAA concentration was lower in Met-supplemented cows (Osorio et al., 2014). However, from a transcriptional standpoint, the greater *HP* in Met-supplemented cows at 7 d followed by a return to comparable expression to those in CON cows at 21 d suggests that cows were undergoing an inflammatory condition early on that was eventually resolved. The discrepancy between

hepatic mRNA expression and actual blood protein concentration of these acute-phase proteins suggests the existence of posttranscriptional regulation. For instance, oxidative stress and, in particular, accumulation of H_2O_2 has been associated with greater *CP* mRNA decay by acting at the 3' untranslated region and consequently modifying the overall expression of this gene and production of the protein (Tapryal et al., 2009).

The data suggest that a mild inflammatory status within the liver along with moderate accumulation of triacylglycerol did not prevent Met-supplemented cows from achieving greater DMI and milk production (Osorio et al., 2013). Recent work from our group has demonstrated that prepartal nutritional management can prime the liver to better face the postpartal metabolic and inflammatory challenges through alterations in gene networks controlled by transcription regulators. Such types of adaptations remain to be studied in cows fed rumen-protected Met.

Oxidative Stress

Between the 2 isozymes of SOD (*SOD1* and *SOD2*), *SOD1* was the only one affected by Met supplementation (Figure 5). This suggests that any effect of Met supplementation was partly compartmentalized to the cytosol (*SOD1*) rather than mitochondria (*SOD2*). Although downregulation of *SOD1* has been related to negative energy balance in blood neutrophils (Moyes et al., 2010), such effect might not apply to the liver, as an upregulation of this gene in dairy cows has been observed 7 d postcalving compared with 21 d before calving (Gessner et al., 2013). In fact, the apparent downregulation of *SOD1* in SM cows from -10 to 21 d cannot be directly associated with negative energy balance because the same group of cows had an approximately 40% increase in energy balance at 21 d from calving; in contrast, CON cows had an approximately 20% decrease during this period (Osorio et al., 2013).

The fact that *SOD1* expression at 7 d postpartum was upregulated in the study of Gessner et al. (2013) and remained unchanged in the current experiment could be related mainly to the prepartal time comparison, as Gessner et al. (2013) compared expression at 7 versus -21 d rather than 7 versus -10 d as in the present study. Similar to *GCLC*, *GSS*, and *GSR*, the lower *SOD1* in Met-supplemented cows is more likely associated with either a reduced oxidative stress environment or the potential for this group of cows relying on other antioxidants such as ascorbic acid, taurine, tocopherol, and retinol. The greater retinol concentration in SM cows at 21 d postpartum (Osorio et al., 2014) supports this idea.

GH Signaling

High GH concentration not only stimulates milk production but also enhances and sustains gluconeogenesis in the liver and lipolysis in adipocytes (Eherton and Bauman, 1998). In agreement with those effects, we previously observed greater postpartal plasma GH concentration in Met-supplemented cows coupled with greater milk production (Osorio et al., 2013). Although Met-supplemented cows had greater plasma GH concentration, the greater *IGF1* at 21 d in SM but not in MS cows suggested that the GH-IGF1 axis was regulated differently. For instance, MS cows had greater GH and NEFA concentrations at 7 d postpartum (Osorio et al., 2013), a pattern associated with lower GH receptor A (*GHR1A*) and *IGF1* (Lucy, 2008).

The *GHR1A* is the main transcript of the GH receptor in the liver, and its expression decreases around parturition (Lucy, 2008). This effect could partly explain the unresponsive *IGF1* expression to high plasma GH in Met-supplemented cows. Although this is not consistent with greater expression of *STAT5B* at 7 d in Met-supplemented cows, its expression followed the pattern of *IGF1* by decreasing over time, regardless of treatment. Under normal conditions, *STAT5B* after being phosphorylated by Janus kinase (JAK) is mainly responsible for the upregulation of *IGF1*. Although *STAT5B* has been proven in nonruminants to play a central role in the GH-IGF1 signaling cascade, it can be activated by multiple cytokines involved in immunity (Feigerlova et al., 2013).

The concomitant increase in *SOCS2* expression with a decrease in *STAT5B* and *IGF1* expression over time suggests that a regulatory action by *SOCS2* took place. In fact, the expression of *SOCS2* in MS cows compared with CON mimicked to some extent that of *STAT5B*, without altering *IGF1*. Additionally, greater *STAT5B* and *IGF1* in SM cows than CON at -10 d could be associated with the nadir in expression of *SOCS2* at that time point, thus suggesting that before calving an activated *STAT5B* via Met supplementation was less likely inhibited by *SOCS2*. Interestingly, such response did not translate into a greater blood concentration of IGF in SM cows at this time (Osorio et al., 2013); therefore, additional mechanisms that regulate not only translation of *IGF1* gene into protein but also the actual protein expression can be downregulated by affecting the half-life of IGF1 through binding proteins such as acid labile subunit or insulin-like growth factor-binding proteins (Piechotta et al., 2014).

DNA Methylation

Methylation of DNA has an essential regulatory function in the tissue- and stage-specific modulation of

genes. This mechanism is carried out through the covalent addition of a methyl group to cytosine within the context of the CpG dinucleotide, and has profound effects on the mammalian genome (Robertson and Jones, 2000). Methylation of DNA via a non-sequence-specific binding site relies on methyl-CpG recognition and, in this way, the methylation is independent of the DNA sequence (Kass et al., 1997). The DNA methyltransferases are known to methylate cytosines in DNA and consequently create methylated CpG patterns in the mammalian genome. Components such as methyl CpG-binding protein 2 (MeCP2) bind to these methylated CpG to mediate transcription (Sansom et al., 2007). Several DNMT are known to date (e.g., DNMT1b, DNMT2, and DNMT3b) that could methylate DNA (Zhang and Liu, 2010). Although *DNMT3A* did not change over time, the expression of *DNMT1* reached a peak expression at 7 d postpartum (Figure 7), regardless of treatment, and suggests that DNMT might respond differently throughout the transition period from pregnancy to lactation.

The actual mechanisms regarding DNA methylation during stress conditions such as the periparturition period in dairy cows remain unknown. The greater overall expression of *DNMT3A* in Met-supplemented cows, especially before calving (Figure 7), suggests that preparturition conditions allowed the activation of this DNMT for DNA methylation via Met supplementation and, consequently, an epigenetic effect might have taken place in the liver of Met-supplemented cows. The repercussions of such an effect in regulating gene expression and the eventual effects at the immunological and metabolic level on the overall cow's preparedness to enter lactation remain unknown and should be further investigated.

In contrast with our results, rats fed a diet deficient in L-Met and devoid of folic acid and choline for at least 9 wk had an elevated level of hepatic *DNMT3A* mRNA (Ghoshal et al., 2006). Among the reasons for different results between Ghoshal et al. (2006) and the current experiment is the vast change in hormonal environment that dairy cows experience during the periparturition period as a means to increase the supply of nutrients to the mammary gland for milk production. In fact, the greater demand for MP after calving (Bell et al., 2000) might be linked to the decrease in postparturition *DNMT3A* expression. Thus, the use of Met for DNA methylation might fall into a lower priority due to the greater demand of Met for milk production.

In contrast to *DNMT3A*, *DNMT1* seemed to be activated soon after calving and even more puzzling is the fact that this DNMT was downregulated in Met-supplemented cows. The latter agrees with results observed by Ghoshal et al. (2006), where rats fed a diet defi-

cient in Met and devoid of folic acid and choline led to greater hepatic *DNMT1* mRNA. Besides the difference between *DNMT3A* and *DNMT1* found in the current experiment, it has been previously observed in vitro and in vivo that these DNMT may, in fact, have different functions during the DNA methylation process (Hsieh, 2005). For instance, *DNMT3A* along with *DNMT3B* identify unmethylated CpG regions within the DNA and initiate de novo methyltransferase, whereas the activity of *DNMT1* is primarily methylation of remaining unmethylated cytosines within those CpG regions previously methylated by *DNMT3A* (Hsieh, 2005).

If these functions can be extrapolated to our data, it could mean that although identification of DNA unmethylated CpG regions was constant (*DNMT3A* was unchanged over time) during the periparturition period, it is likely that further DNA methylation was carried out soon after calving via the increase in *DNMT1*. Interestingly, the preparturition upregulation of *DNMT3A* in Met-supplemented coupled with the postparturition upregulation of *DNMT1* in all cows suggests that Met-supplemented cows undergo a more robust alteration at the molecular level. The extent of this epigenetic effect on the previously observed (Osorio et al., 2013, 2014) enhanced performance in Met-supplemented cows remains unknown.

CONCLUSIONS

Our findings provide evidence that the supplementation of MS or SM to cows during the periparturition period can affect the hepatic expression of Met, glutathione metabolism, inflammation, oxidative stress, and DNA methylation-related genes. Flux through the Met and 1-carbon metabolism cycles in response to SM and MS was mainly via changes in expression of *SAHH*, *GCLC*, *MTR*, *SOD1*, and *DNMT3A*. As such, alterations in *SAHH* might have promoted Hcy synthesis and its use for glutathione or regeneration of Met. The high *SAHH* mRNA abundance likely reflects the importance of this gene to 1-carbon metabolism cycling in the liver. Production of glutathione could be increased by Met supplementation. Sustained supply of Met within the liver during the periparturition period could increase synthesis of antioxidants (e.g., glutathione and taurine) and also alter the tissue-wide DNA methylation status. As such, inflammation, oxidative stress and genome-wide transcription of genes could be altered. The functional implications of changes in DNA methylation warrant further study.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Adisseo (Commeny, France) for partial financial support of this

research. The authors thank Travis Michels and Mike Katterhenry of the University of Illinois Dairy Research Unit (Urbana) staff for help with animal management.

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