# METHIONINE-MORE THAN MILK

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## METABOLIZABLE PROTEIN DURING THE PERIPARTAL (TRANSITION)

Nutritional requirements of dry cows increase as gestation progresses because of the exponential growth of the fetus in late gestation (NRC, 2001). From the cow standpoint, conditions such as increased blood glucocorticoids, lipid mobilization, and fetal size contribute to reducing voluntary DMI close to parturition. In turn, nutrient availability for the cow and fetus decreases (Ingvartsen and Andersen, 2000). Clearly, the amount of protein and consequently metabolizable protein (**MP**) flowing to the intestine from both dietary and microbial sources are often diminished by lower DMI around calving. Therefore, the inability of early postpartal cows to produce sufficient MP to meet mammary and extra-mammary AA requirements, including a significant demand for hepatic gluconeogenesis, promotes active mobilization of tissue protein during the first 2 wk of lactation (Bell et al., 2000; van der Drift et al., 2012). In fact, Bell et al. (2000) estimated that during the first week of lactation cows are up to 600 g/d in negative MP balance, and consequently high-yielding cows will mobilize up to 1,000 g of tissue protein/d. Additionally, van der Drift et al. (2012) evaluated the mobilization of muscle protein by analysis of plasma 3-methylhistidine, an indicator of muscle protein breakdown and concluded that higher mobilization of protein around calving can restrict ketone body production due to higher availability of gluconeogenic precursors.

## INTEREST IN METHIONINE FOR TRANSITION COWS

McCarthy et al. (1968) hypothesized that Methionine (**Met**) deficiency in ruminants may limit hepatic very-low density lipoprotein (**VLDL**) synthesis and be a causative factor of ketosis. Rate of hepatic VLDL synthesis was subsequently demonstrated to be lower in ruminants than monogastrics (Pullen et al., 1990). This inherent feature of ruminants is particularly important at parturition when the homeorhetic adaptations in the animal lead to marked increases in blood non-esterified acids (**NEFA**) which are taken up by liver, hence, increasing the susceptibility for hepatic lipidosis (Grummer, 1993). Several studies since then have assessed the role of Met as a potentially limiting amino acid (**AA**) in the regulation of hepatic fatty acid metabolism. Because of extensive ruminal degradation early work evaluating Met utilized intravenous infusions or a "hydroxyl analog" of Met (Bertics and Grummer, 1999; McCarthy et al., 1968;

Piepenbrink et al., 2004). The analog offers some protection against ruminal metabolism but there are currently other protection technologies that ensure greater level of ruminal "by-pass" as well as high intestinal Met bioavailability (Berthiaume et al., 2006).

Grummer (1993) proposed that utilization of triglyceride (**TAG**) for VLDL synthesis after parturition is impaired when the level of hepatic Met is insufficient. More recent work has established an association between low levels of serum Met during the first 14 days postpartum and severe hepatic lipidosis (Shibano and Kawamura, 2006). The work of Dalbach et al. (2011) demonstrated that it is feasible to increase the serum concentration of Met during the first 2-weeks postpartum by feeding rumen-protected Met. The rate of hepatic metabolism in high-producing cows nearly doubles after parturition (Reynolds et al., 2003), which could be one reason explaining the increase in net liver uptake of Met. In fact, other than Histidine, Met was the only amino acid for which net uptake by liver increased between pre and postpartum (Larsen and Kristensen, 2013). Aspects of Met metabolism in liver via the 1-carbon metabolism and Met-cycle are well-described in monogastrics, and to some extent in classical studies with sheep (Snoswell and Xue, 1987). To our knowledge, however, there is no information on enzyme flux or how flux through the Met-cycle might be altered during the transition period particularly when rumen-protected Met is fed.

Increasing delivery of Met to the liver via supplementation of rumen-protected sources is particularly important for the animal not only because of the key role of Met in milk protein synthesis but also for intra-hepatic VLDL synthesis, production of glutathione and taurine [intracellular antioxidants; (Atmaca, 2004)], and provision of methyl groups (Finkelstein, 1990). At least in non-ruminants, the latter has been demonstrated to be an important aspect of overall Met utilization in liver namely because methylation serves as a way to regulate gene expression, protein function, and RNA processing.

## RUMEN-PROTECTED METHIONINE AND MILK PRODUCTION

Rumen undegradable protein (**RUP**) is ca. 50% of the total MP and limited research suggests that increasing RUP during late gestation improves subsequence lactation performance (Huyler et al., 1999; Greenfield et al., 2000). Thus, the RUP is important as a source of essential AA (**EAA**), e.g. methionine (Met), for body tissues and are the building blocks of enzymes and hormones of importance in a number of biological functions. Clearly, an adequate profile of EAA in RUP is crucial for a successful transition for both the cow and the unborn calf. Research has determined that Met and Lys in MP are the most-limiting AA in a wide-range of diets for dairy cows (NRC, 2001). In fact, Met is typically first-limiting and supplementation of Met alone improved overall lactation performance in dairy cows (Armentano et al., 1997; Rulquin and Delaby, 1997).

Supplementation of Met during the peripartal period concomitantly increases milk yield, milk protein, and milk fat soon after calving (Ordway et al., 2009; Osorio et al.,

2013). These responses are in large part driven by enhancing Met availability and also by additional flux of Met through the Met cycle in liver which consequently increases the production of downstream compounds such as cysteine (**Cys**; Figure 1). Just as Met, Cys is a sulfur-containing AA and both contribute sulfur bonds during milk protein synthesis in mammary gland. Glutathione is another downstream compound arising in the Met cycle that can supply AA such as Cys to the mammary gland for milk synthesis (Pocius et al., 1981). In fact, Pocius et al. (1981) observed a low uptake of free plasma Cys by mammary gland which indicated that glutathione might be an important source of Cys, since there was a substantial uptake of glutathione by mammary gland. In terms of milk production, we have previously observed an increase of ca. 3.4 kg and 3.9 kg for milk yield (Table 1) and energy corrected milk (**ECM**) in peripartal cows supplemented with Smartamine M (**SM**) or MetaSmart (**MS**) from -21 d to 30 DIM (Osorio et al., 2013), which is consistent with the greater responses in milk yield to postpartum supplementation of SM or MS (St-Pierre and Sylvester, 2005). A recent study from our group (Zhou et al., unpublished) confirmed the benefit of supplemental Smartamine during the transition period in terms of enhancing DMI and milk production (Figure 2).

A large portion of nutrients such as AA and fatty acids that are absorbed in the small intestine are processed/metabolized by the liver. In turn the liver can utilize fatty acids as an energy source (i.e. through beta-oxidation) or esterify them into VLDL that can, in turn, provide peripheral tissues with fatty acids. Other metabolic end-products of liver metabolism including glutathione and glucose also are secreted from liver and during lactation utilized preferentially by the mammary gland. Therefore, some of the effects on milk yield reported with supplemental Met could be related to Met supplementation promoting a better liver function during the transition period.

## LIVER FUNCTION DURING THE TRANSITION PERIOD: LINKS WITH INFLAMMATION AND OXIDATIVE STRESS

It has been recognized in the last decade that the peripartal dairy cow experiences a state of reduced liver function coupled with increased inflammation and oxidative stress (Bionaz et al., 2007; Trevisi et al., 2012). Bilirubin, glutamic-oxaloacetic transaminase (**GOT**), γ-glutamyltransferase (**GGT**) along with albumin and paraoxonase (**PON**) are commonly-used biomarkers of liver status around calving (Bertoni et al., 2008). While the liver is responsible for clearance of bilirubin (Bertoni et al., 2008), higher GOT and GGT are related to liver cell damage (e.g. lysis and necrosis).

The peripartal inflammatory response is characterized by an increase in the production of positive acute-phase proteins (**posAPP**) such as haptoglobin and serum amyloid A (**SAA**), and a concomitant decrease in the production of negative APP (**negAPP**) such as albumin (Bertoni et al., 2008). At the level of liver, the well-established triggers of these responses are the pro-inflammatory cytokines IL-6, IL-1, and TNF-α

(Kindt et al., 2007). On the other hand, oxidative stress is driven by the imbalance between the production of reactive oxygen metabolites (**ROM**) and the neutralizing capacity of antioxidant mechanisms in tissues and in blood. Some of the well-established cellular antioxidants include glutathione, taurine, superoxide dismutase (**SOD**), and vitamins A and E (Bernabucci et al., 2005). When oxidative stress overwhelms cellular antioxidant capacity, the ROM can induce an inflammatory response which is controlled via changes in gene expression of transcription regulators (e.g. STAT3, NFKB).

The fact that albumin is classified as a negAPP implies that hepatic production (the main site in the body) is commonly reduced during the onset of inflammation (Bertoni et al., 2008). Cows with adequate postpartal liver function have albumin concentrations ranging between 33 to 35 g/L (Bionaz et al., 2007; Bertoni et al., 2008). In a recent experiment from our group (Osorio et al., 2014b), albumin concentrations in Met-supplemented cows were above 35 g/L throughout the peripartal period (Figure 3). Although albumin in control (CON) cows was within physiological levels, the decrease in concentration of ~2 g/L from prepartum to 7 d postpartum was similar to that observed by Bionaz et al. (2007) for cows classified as having low liver function. Furthermore, the fact that albumin in CON cows remained lower than prepartal levels for ~2 wk was suggestive of additional stress on the liver during this time.

Concentrations of ceruloplasmin and SAA are likely to increase during inflammatory episodes such as those occurring in the peripartal period (Ceciliani et al., 2012). Supplementing Met during the peripartal period reduced the concentrations of ceruloplasmin and SAA by 10.7% and 30.9% during this period (Table 1). The latter response coupled with a trend ($P$ = 0.13) for lower postpartal IL-6 concentration in Met-supplemented cows than CON could be taken as indication that supplemental Met decreased the synthesis of this proinflammatory cytokine after calving (Osorio et al., 2014b). As such, the production of posAPP such as ceruloplasmin and SAA in liver could have been decreased..

During the transition period cows will normally experience an increase in adipose tissue lipolysis due to changes in hormones such as insulin (decrease) and growth hormone (increase), and consequently blood non-esterified fatty acid (**NEFA**) concentrations increase. Once NEFAs reach the liver these can be oxidized to provide energy to the liver, partially oxidized to produce ketone bodies, or esterified to triglyceride (**TAG**). A major organelle within hepatocytes where NEFA oxidation takes place is the mitochondria, and carnitine is essential for transport of NEFA from cytosol into mitochondria for subsequent β-oxidation (Drackley, 1999). Carnitine is derived endogenously from trimethyllysine (**TML**), in turn TML supply is driven by turnover of proteins containing Lys and Met, where Lys serves as the backbone while Met is the methyl donor for TML synthesis (Carlson et al., 2007). The greater hepatic concentration of carnitine (82.1 vs 37.5 nmol/g of tissue) that was detected in Met-supplemented cows (Table 1) indicates a greater bioavailability of Met to methylate Lys (Osorio et al., 2014b).

As mentioned above, NEFA taken up by the liver can be esterified into TAG and exported into the bloodstream as VLDL, which requires ApoB-100, cholesterol, and phosphatydyl choline (**PC**). Although the latter tended ($P$ = 0.07) to be lower in Met-supplemented cows this could suggest a greater degree of utilization of PC for the assembly and export of VLDL. In fact, we observed a trend for lower plasma concentration of ApoB-100 in CON cows (Osorio et al., 2013) suggesting that that this molecule might have limited the assembly and export of VLDL from liver and consequently contributed to the buildup of PC in CON cows.

Oxidative stress is mainly due to the imbalance between ROM and the neutralizing capacity of antioxidant mechanisms in tissues and in blood. The oxygen radical absorbance capacity (**ORAC**) capacity was 5.9% greater in Met-supplemented cows, which could have been at least in part due to the greater (22.6%) liver glutathione concentration (Table 1) and the greater concentration of vitamins such as retinol and β-carotene (Osorio et al., 2014b). The greater total liver glutathione effect is directly associated with Met supplementation because Met can be incorporated up-stream in the pathway for *de novo* glutathione synthesis (Halsted, 2013).

The importance of Met as one of the most-limiting AA for milk yield and components can also be associated with 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) in liver where it is initially converted into S-adenosylmethionine (**SAM**), the major biological methyl-donor (Martinov et al., 2010). This first step in the Met cycle (1-carbon metabolism) is the binding of adenosyl and Met by isoenzymes Met adenosyltransferase I (**MAT1**) or Met adenosyltransferase III (**MATIII**). The *MAT1A* gene encodes both MATI and MATIII isoenzymes in mammals (Martinov et al., 2010). This particular gene was upregulated in Met-supplemented cows compared with CON at 21 d postpartum (Osorio et al., 2014a). This effect in upregulation of *MAT1A* has been previously observed in cultured hepatocytes in response to exogenous Met (Garcia-Trevijano et al., 2000). Thus, part of the mechanism elicited by supplemental Met in postpartal dairy cows is driven by changes in gene expression for key enzymes in the Met cycle. An end-result of such effect is more availability of enzyme protein that could help enhance the flux through the pathway leading to synthesis of several important intermediate metabolites (e.g. SAM, glutathione, taurine, PC).

S-adenosylhomocysteine is the resulting compound after SAM donates its methyl group through transmethylation(**SAH**; Figure 1). Subsequently, S-adenosylhomocysteine hydrolase (**SAHH**) hydrolyzes SAH into adenosine and Homocysteine (**Hcy**). Enzyme activity of SAHH has not, to our knowledge, been studied in ruminants. However, its importance in the methylation cycle in connection with hypermethioninemia is well-established in humans and mice (Baric, 2009). Inhibition of SAHH causes the

accumulation of SAH and consequently suppresses SAM-dependent transmethylation via feedback inhibition (Lee et al., 2011). We observed that among several metabolic genes *SAHH* was the most abundant gene in transition cow liver (Osorio et al., 2014a), 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 in Met-supplemented cows (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 re-methylation by BHMT or MTR can regenerate Met from Hcy by transferring a methyl group from 5-methyltetrahydrofolate (5-MTHF) and betaine (Preynat et al., 2010). We observed that supplementing Met during the peripartal period can increase the expression of *MTR* gene. The greater expression of *SAHH* might have promoted greater production of Hcy also reflected in the greater expression of *MTR* in Met-supplemented cows. Thus, regeneration of Met from Hcy (via MTR) might have been primarily via 5-MTHF (with vitamin $B_{12}$ as a coenzyme). Such scenario also highlights the importance of B vitamins in the metabolism of Met in liver.

As mentioned above, PC is essential for the assembly of VLDL in the liver, and it is synthesized by the transmethylation of a methyl group from SAM to phosphatidylethanolamine (**PE**; Figure 1). This transmethylation is carried out by the enzyme phosphatidylethanolamine methyltransferase (**PEMT**), and the gene coding for this enzyme, *PEMT* tended ($P = 0.10$) to be upregulated in Met-supplemented cows than CON (Osorio et al., 2014a). This partly agrees with our hypothesis that lower liver PC in Met-supplemented cows was due to greater assembly and export of VLDL from the liver into the bloodstream in this group of cows rather than a lower availability or synthesis of this compound.

## RUMEN-PROTECTED MET AND IMMUNE FUNCTION

Besides the positive effect of supplemental Met on milk yield and DMI, the fact that Met can be metabolized to glutathione and taurine, both of which are potent intracellular antioxidants (Atmaca, 2004), led us to hypothesize (Osorio et al., 2013) that enhancing Met supply would have a positive effect on immune function during early lactation. A study with mid-lactation cows reported that supplementation with 30 g/d of rumen-protected Met compared with 0 or 15 g/d led to greater T lymphocyte proliferation in vitro in response to various mitogens (Soder and Holden, 1999). “T” lymphocytes are a type of lymphocyte (a type of white blood cell) that plays a role in cell-mediated immunity. These cells are produced in the bone marrow but leave the bone marrow and mature in the

thymus, hence, the "T". Human lymphocytes seem to have an absolute requirement for Met to proliferate (Hall et al., 1986), which may partly be responsible for the positive effect of supplemental Met on the immune function of monogastrics (Nauss et al., 1982; Tsiagbe et al., 1987). The greater blood neutrophil-killing capacity that we observed postpartum with MetaSmart and Smartamine (Osorio et al., 2013) provides additional evidence of an important role for Met in the immune response during the transition period.

Whether the greater phagocytosis of whole blood that we observed was a result of more cells or a more pronounced oxidative burst response (or both) was not established in that study. However, in a follow-up study feeding Smartamine (Zhou et al., unpublished) between -21 and 30 days relative to parturition we detected both greater blood neutrophil phagocytosis and oxidative burst from day 1 post-calving through day 28 postpartum (Figure 4). The data suggest that the greater immune response with Smartamine was unrelated to DMI (Figure 3) because phagocytosis and oxidative burst had little fluctuation in those cows during the study. In contrast, cows in the control had a decrease in oxidative burst during the first week postpartum followed by an increase at day 28. These data seem to underscore the importance of supplementing rumen-protected Met ahead of parturition as a way to enhance the intracellular pool of anti-oxidants (e.g. glutathione, taurine) in tissues such as liver and also immune cells.

Taken together, the data generated to date suggest that supplemental Met metabolism to anti-oxidants can reduce the oxidative stress response at calving in a way that diminishes its contribution to the pro-inflammatory environment characteristic of the post-partal period. In that scenario, pro-inflammatory cytokines are less likely to contribute to reducing DMI, thus, allowing cows to increase DMI faster and achieve greater rates of milk synthesis. Additional data from this study at the level of blood, tissue, and milk are currently being generated and will help understand better the mechanisms whereby supplemental rumen-protected Met benefits post-partal dairy cow performance and health.

**Table 1.** Production and blood and liver tissue biomarker concentrations in cows fed a control or rumen-protected Met diet (MetaSmart or Smartamine) from -21 through 30 days around parturition (Osorio et al., 2013; 2014b).

| | Diet | | | P-value[1] | |
|---|---|---|---|---|---|
| Item | Control | MetaSmart | Smartamine | Diet | Met |
| DMI, kg/d | 13.3 | 15.2 | 15.6 | .18 | .06 |
| Milk yield, kg/d | 35.7 | 38.1 | 40.0 | .15 | .08 |
| ECM, kg/d | 41.0 | 44.8 | 45.0 | .09 | .03 |
| Blood | | | | | |
| Albumin, g/L | 35.1 | 36.1 | 35.7 | .28 | .15 |
| Ceruloplasmin, μmol/L | 3.02 | 2.68 | 2.71 | .03 | .009 |
| Serum amyloid A, μg/mL | 61 | 40.7 | 43.5 | .17 | .06 |
| ORAC, mol/L | 11.9 | 12.9 | 12.4 | .05 | .04 |
| Liver tissue | | | | | |
| Carnitine, nmol/g of tissue | 37.5 | 98.2 | 66.0 | .01 | <.01 |
| PC, μM/g of tissue | 10.6 | 7.7 | 9.1 | .15 | .07 |
| Glutathione, mM | 1.27 | 1.55 | 1.73 | .09 | .04 |

[1]Met = control vs. MetaSmart + Smartamine

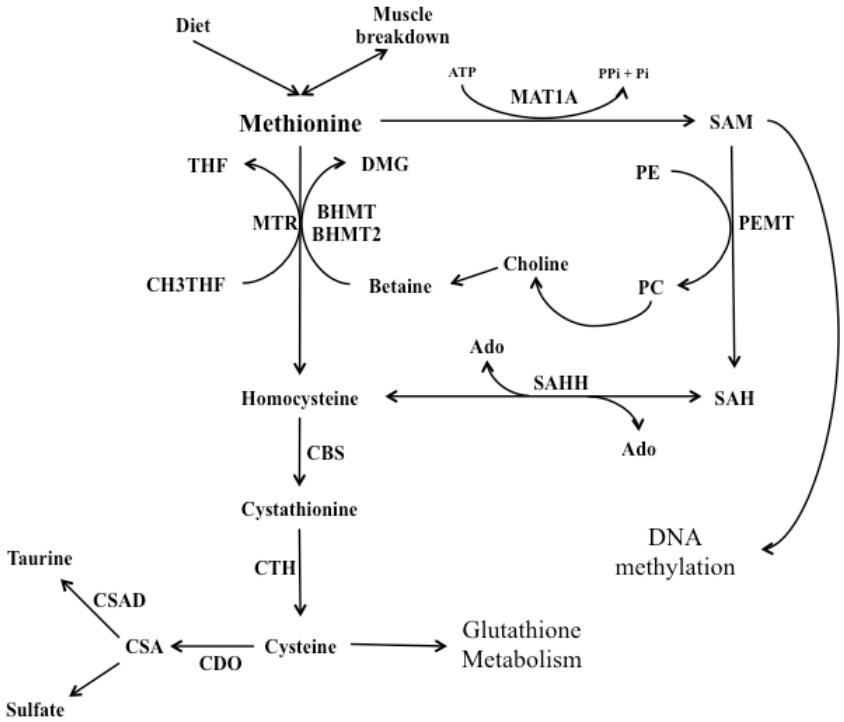


**Figure 1.** Key genes encoding enzymes of the Met cycle: Met adenosyltransferase 1A (*MAT1A*), phosphatidylethanolamine methyltransferase (*PEMT*), S-adenosylhomocysteine hydrolase (*SAHH*), betaine homocysteine methyltransferase (*BHMT* and *BHMT2*), 5-methyltetrahydrofolate-homocysteine methyltransferase (*MTR*), cystathionine β-synthase (*CBS*), cystathionine β-lyase (*CTH*). SAM = S-adenosylmethionine, PE = phosphatidylethanolamine, PC = phosphatidylcholine, SAH = S-adenosylhomocysteine, Ado = adenosyl, THF = tetrahydrofolate, $CH_3THF$ = 5-methyl-tetrahydrofolate, DMG = dimethyl-glycine.

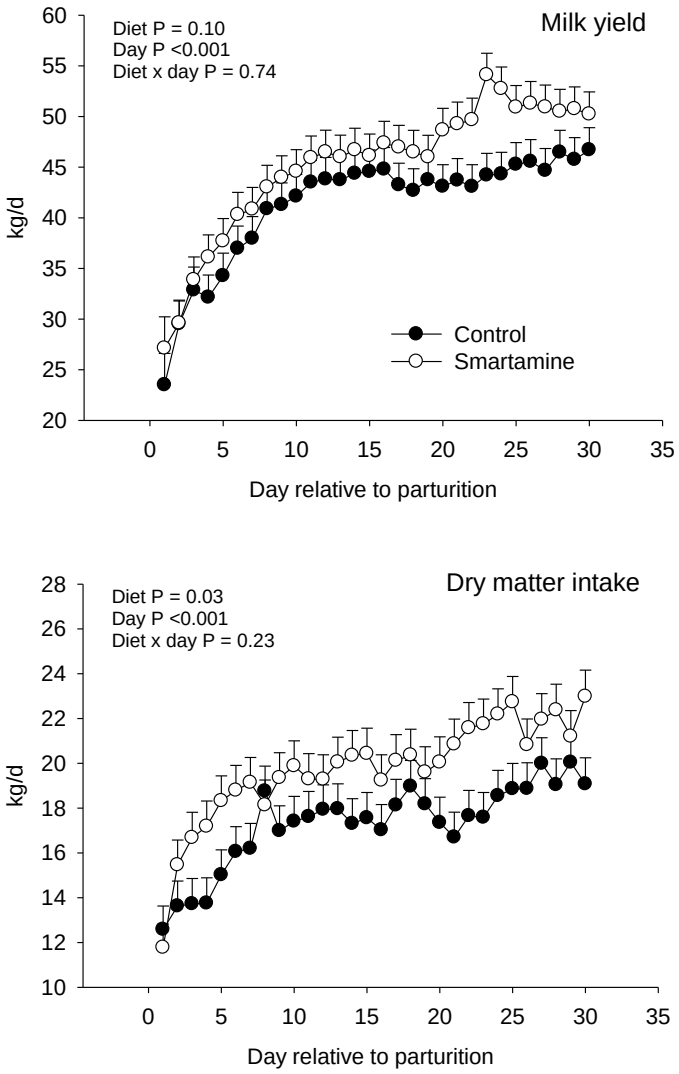


**Figure 2.** Milk yield and dry matter intake during the first 30 days post-calving in cows fed a control diet (n = 20) or the control diet supplemented with Smartamine (n = 20) from -21 through 30 days relative to parturition (Zhou et al., unpublished).

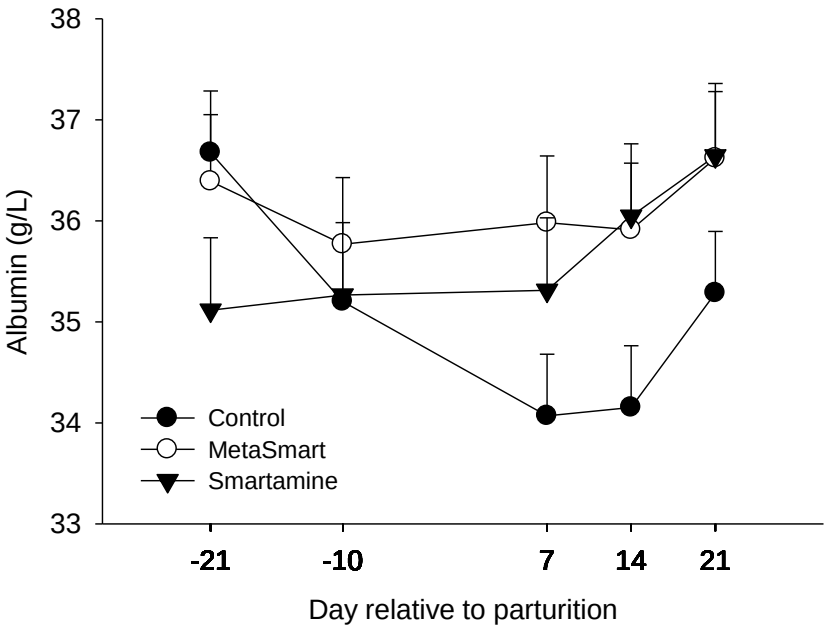


**Figure 3.** Blood albumin concentration during the transition period in cows fed supplemental Smartamine or MetaSmart from -21 d through 30 days around parturition (Osorio et al., 2014b).

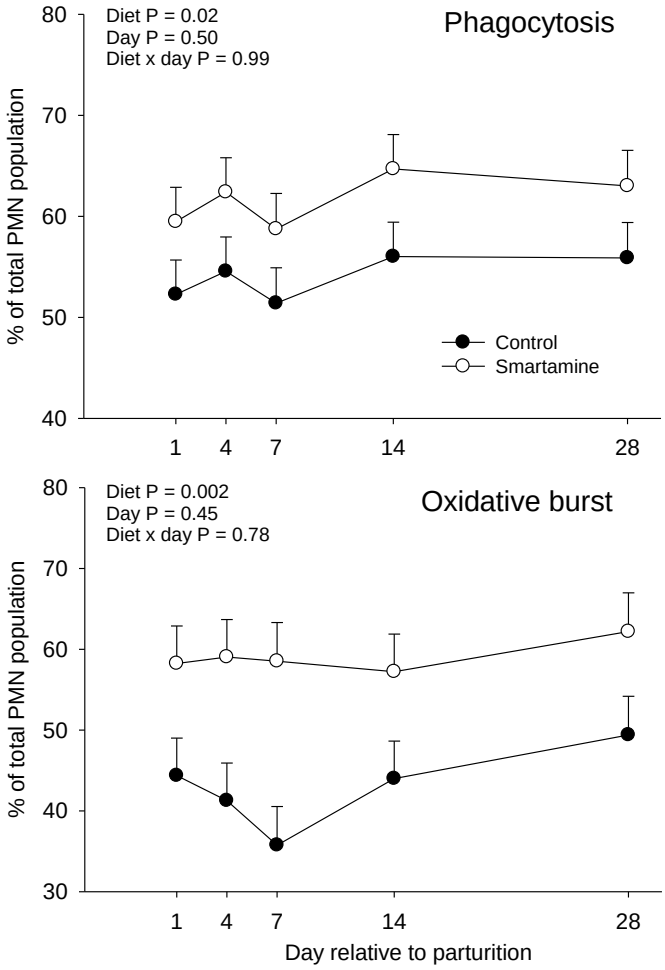


**Figure 3.** Blood neutrophil (PMN) phagocytosis and oxidative burst during the first 30 days post-calving in cows fed a control diet (n = 20) or the control diet supplemented with Smartamine (n = 20) from -21 through 30 days relative to parturition (Zhou et al., unpublished).

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