MICRORNA INVOLVEMENT IN HEPATIC ADAPTION DURING THE PERIPARTURIENT PERIOD IN HOLSTEIN DAIRY COWS

BY

RYAN ELIZABETH BUCKTROUT

THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Animal Sciences
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2019

Urbana, Illinois

Committee:

Professor Juan Loor, Chair and Director of Research
Professor James Drackley
Assistant Professor Josh McCann

ABSTRACT

During the periparturient period (3 wk before calving to 3 wk after calving) dairy cows commonly experience a negative energy balance due to the increased nutrient requirements for fetal growth and milk production coupled with decreased dry matter intake (DMI). This combination can increase the risk of developing certain immunologic problems and metabolic disorders like oxidative stress, ketosis, and fatty liver. Two experiments were conducted to evaluate 1) hepatic microRNA (miRNA) involvement in periparturient Holstein dairy cows supplemented methionine and 2) how body condition score (BCS) in late pregnancy impacts hepatic biomarkers involved in energy metabolism.

Previous in silico work relevant to fatty liver disease and ketosis revealed miRNAs may play a role in variety of important biological pathways including, fatty acid metabolism (miR-186, -200b and c, -218, -369) and oxidative phosphorylation (miR-101, -142, -186, -200b, -200c, -218, and -369). The main objective of the present study was to verify the expression of these miRNA and examine the relationship they may have in cows supplemented methionine during the peripartal period. Nineteen multiparous Holstein cows were fed experimental treatments consisting of a basal control diet (CON; n = 11) and CON plus Methionine (MET; n = 8). (at a rate of 0.08% on a dry matter basis) (Smartamine M, Adisseo NA). All cows received the same far-off, close-up, and lactation diet. Liver biopsies were performed at −10, 10, and 30 d relative to calving for analysis of miR-218, miR-369 5p, miR-200b, miR-200c, miR-142 3p, miR-142 5p, miR-101, and miR-186 abundance via qPCR after normalization with 3 internal controls. Results indicated that MET cows had less expression of miRNAs that were predicted to downregulate oxidative phosphorylation and fatty acid metabolism, compared to CON. Considering that MET cows had greater overall DMI there is a possibility that they were experiencing less oxidative

stress in the liver, as opposed to CON, but the relationship is still unclear. Further analysis of gene targets and protein expression relevant to miRNAs presented in this study will provide more insight into the biological importance of miRNAs.

In the second study, twenty-six multiparous Holstein cows at 4 wk were divided into 2 groups based on BCS, BCS ≥ 3.50 (n = 13; HiBCS) and BCS ≤ 3.25 (n = 13; LoBCS). In the prepartum, LoBCS cows had greater DMI compared to HiBCS cows ($P = 0.06$). In the postpartum, LoBCS continued to have greater DMI, but HiBCS cows averaged 5.34 kg/d greater in milk yield comparatively. Plasma sampled at d −30, −10, 7, 15, and 30, relative to calving indicated increased concentrations of biomarkers related to negative energy balance in HiBCS cows. In contrast, LoBCS had greater plasma concentrations of biomarkers that have antioxidant properties, thus suggesting a potential relationship with oxidative stress. Liver tissue harvested via biopsy at −15, 7, and 30 d relative to calving and used to evaluate protein abundance via western blotting. We were unable to detect any expression of nuclear factor erythroid 2-like 2 (NFE2L2) or p-NFE2L2 (both are involved in antioxidant signaling pathways) at any time point in liver tissue. However, LoBCS cows had increased abundance of proteins associated with glutathione metabolism, an antioxidant that is capable of preventing overloads of reactive oxygen species (ROS), compared with HiBCS. miR-369 5p, miR-186, and miR-200b, which downregulate fatty acid metabolism and oxidative phosphorylation, had decreased expression in HiBCS cows, which is contrary to the patterns of decreased oxidative stress in LoBCS cows we observed in plasma biomarkers and protein abundance. In conclusion, the differences between production data, blood biomarkers, miRNA expression, and protein abundance underscore a potentially important interaction between energy metabolism in relation to BCS during the periparturient period.

ACKNOWLEDGEMENTS

First, I would like to thank my research advisor, Dr. Juan Loor, for his assistance, support, and friendship throughout the completion of my master's degree. Additionally, I would like to extend my gratitude to my committee members, Dr. James Drackley and Dr. Josh McCann for their insight and guidance.

I owe a tremendous amount of thanks to the past and present members of the Mammalian NutriPhysioGenomics lab for their mentoring and friendship including: Mario, Abdul, Danielle, Yusheng, Ahmed, Fernanda, Hongyu, and Hailei.

Finally, I would like to thank my entire family, especially my mother and father, for their unwavering support throughout my degree. A special thanks to my step-mother for showing me what it meant to be a scientist at a young age. Together, you all have made me who I am today and I could not have done it without you.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AA, Amino Acids; **BCS**, Body Condition Score; **BHB or BHBA**, Beta-Hydroxybutyric Acid; **CoA**, Coenzyme A; **CON**, Control Diet; **d**, day(s); **DIA**, Dynamic Impact Approach; **DIM**, Days in Milk; **DMI**, Dry Matter Intake; **FA**, Fatty Acids; **FRAP**, Ferric Reducing Ability of Plasma; **GAPDH**, Glyceraldehyde 3-Phosphate Dehydrogenase; **GPX3**, Glutathione Peroxidase 3; **GSH**, Glutathione; **GSTA4** Glutathione S-Transferase α 4; **GSTM1**, Glutathione S-Transferase Mu 1; **MET**, Methionine-Supplemented; **miRNA**, microRNA; **NEB**, Negative Energy Balance; **NEFA**, Non Esterified Fatty Acids; **NFE2L2,** Nuclear Factor Erythroid 2-like 2, **NRC**, National Research Council; **PPAR**, Peroxisome Proliferator Activated Receptor; **qPCR**; Quantitative Polymerase Chain Reaction; **ROS**, Reactive Oxygen Species; **TAG**, Triacylglycerol; **TMR**, Total Mixed Ratio; **TRT**, Treatment; **VLDL**, Very Low Density Lipoprotein.

CHAPTER 1: INTRODUCTION

The periparturient period, or transition period (3 wks before calving to 3 wks after calving), indicates the life stage where a dairy cow moves from late pregnancy to early lactation (Grummer, 1995). After parturition, dairy cows are disposed to experience an imbalance between the production of free radicals and their elimination by antioxidants, which is also referred to as oxidative stress (Sordillo and Aitken, 2009). Antioxidants, like glutathione reduce the levels of reactive oxygen species to prevent free radical damage (Bayir, 2005). Additionally, the inflammatory events that afflict cows during early lactation are associated with physiologically stressful conditions and the metabolic overload of the liver to meet the nutrient requirements for maintenance and milk synthesis also referred to as negative energy balance (NEB) (Bertoni, 2015). NEB can lead to hepatic lipidosis which results from excessive accumulation of triacylglycerol (TAG) in the liver, thus impairing normal functions (Bell, 1995, Drackley, 1999). This underscores the role that fatty acid metabolism and oxidative phosphorylation play in dairy cows as they transition from pregnancy to lactation.

There is evidence that when supplementing methionine (a limiting amino acid for milk synthesis) in the diet, cows will have decreased incidences of metabolic disorders and improved immune function (Schwab et al., 1992, Lima et al., 2012, Zhou et al., 2016, Batistel et al., 2017). The supplementation of methionine during the transition period has also improved milk yield, milk protein, and increased antioxidant synthesis (Schmidt et al., 1999, Osorio et al., 2013). All-in-all, methionine supplementation is associated with improved hepatic lipid metabolism in dairy cows (Durand, 1992).

Body condition score (BCS) is a useful measure of animal health as it reflects both the amount of subcutaneous fat in the body and indicates the nutritional status of a dairy cow (Roche

et al., 2009). In the United States, BCS are evaluated on a 5-point scale, where 1 indicates a thin or emaciated cow, while a 5 equates to an over-conditioned obese cow (Roche et al., 2004, Roche et al., 2009). At calving, if a cow has a more optimal BCS this could promote a successful transition period and maximize production in the postpartum (Akbar et al., 2015). Cows with higher BCS are at a greater risk of developing fatty liver disease or ketosis and are under more oxidative stress during the transition period (Reid et al., 1986, Bernabucci et al., 2005, Schulz et al., 2014). Therefore, BCS has a close relationship with metabolic disorders and is a useful tool in dairy cow management.

Another emerging tool to evaluate health of an animal are microRNAs (miRNAs). miRNAs are short non-coding RNAs of 22 nucleotides in length that regulate gene expression post-transcriptionally (Ruby et al., 2007). miRNAs are believed to play a role in fat cell formation (adipogenesis) (Jin et al., 2009, Jin et al., 2010). Additionally, miRNA targets related to adipogenesis and inflammation are involved in miRNA signaling through networks involving transcription factors, suggesting a possible role in the control of inflammation (Arner and Kulyte, 2015).

The main objective of the first study was to verify the expression of miRNA predicted (in silico) to play a role in energy metabolism and examine the relationship they may have in cows supplemented methionine during the peripartal period. The aim of the second study was to further verify the previously mentioned miRNA and identify any associations between body condition score (BCS) in late-pregnancy, plasma biomarkers, and protein abundance linked to energy metabolism.

REFERENCES

Akbar, H., T. M. Grala, M. Vailati Riboni, F. C. Cardoso, G. Verkerk, J. McGowan, K. Macdonald, J. Webster, K. Schutz, S. Meier, L. Matthews, J. R. Roche, and J. J. Loor. 2015. Body condition score at calving affects systemic and hepatic transcriptome indicators of inflammation and nutrient metabolism in grazing dairy cows. J Dairy Sci 98(2):1019-1032.

Arner, P. and A. Kulyte. 2015. MicroRNA regulatory networks in human adipose tissue and obesity. Nat Rev Endocrinol 11(5):276-288.

Batistel, F., J. M. Arroyo, A. Bellingeri, L. Wang, B. Saremi, C. Parys, E. Trevisi, F. C. Cardoso, and J. J. Loor. 2017. Ethyl-cellulose rumen-protected methionine enhances performance during the periparturient period and early lactation in Holstein dairy cows. J Dairy Sci 100(9):7455-7467.

Bayir, H. 2005. Reactive oxygen species. Crit Care Med 33(12 Suppl):S498-501.

Bell, A. W. 1995. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. J Anim Sci 73(9):2804-2819.

Bernabucci, U., B. Ronchi, N. Lacetera, and A. Nardone. 2005. Influence of body condition score on relationships between metabolic status and oxidative stress in periparturient dairy cows. J. Dairy Sci. 88(6):2017-2026.

Bertoni, G. M., Andrea & Trevisi, Erminio. 2015. Immune system, inflammation and nutrition in dairy cattle. Animal Production Science. 55. 10.1071/AN14863.

Drackley, J. K. 1999. ADSA Foundation Scholar Award. Biology of dairy cows during the transition period: the final frontier? J Dairy Sci 82(11):2259-2273.

Durand, D., Y. Chilliard, and D. Bauchart. 1992. Effect of lysine and methionine on in vivo hepatic secretion of VLDL in the high yield- ing dairy cow. J. Dairy Sci 25(Suppl.1)(279).

Grummer, R. R. 1995. Impact of changes in organic nutrient metabolism on feeding the transition dairy cow. J Anim Sci 73(9):2820-2833.

Jin, W., M. V. Dodson, S. S. Moore, J. A. Basarab, and L. L. Guan. 2010. Characterization of microRNA expression in bovine adipose tissues: a potential regulatory mechanism of subcutaneous adipose tissue development. BMC Mol Biol 11:29.

Jin, W., J. R. Grant, P. Stothard, S. S. Moore, and L. L. Guan. 2009. Characterization of bovine miRNAs by sequencing and bioinformatics analysis. BMC Mol Biol 10:90.

Lima, F. S., M. F. Sa Filho, L. F. Greco, and J. E. Santos. 2012. Effects of feeding rumen-protected choline on incidence of diseases and reproduction of dairy cows. Vet J 193(1):140-145.

Osorio, J. S., P. Ji, J. K. Drackley, D. Luchini, and J. J. Loor. 2013. Supplemental Smartamine M or MetaSmart during the transition period benefits postpartal cow performance and blood neutrophil function. J Dairy Sci 96(10):6248-6263.

Reid, I., C. Roberts, R. Treacher, and L. Williams. 1986. Effect of body condition at calving on tissue mobilization, development of fatty liver and blood chemistry of dairy cows. Animal Science 43(1):7-15.

Roche, J. R., P. G. Dillon, C. R. Stockdale, L. H. Baumgard, and M. J. VanBaale. 2004. Relationships among international body condition scoring systems. J Dairy Sci 87(9):3076-3079.

Roche, J. R., N. C. Friggens, J. K. Kay, M. W. Fisher, K. J. Stafford, and D. P. Berry. 2009. Invited review: Body condition score and its association with dairy cow productivity, health, and welfare. J Dairy Sci 92(12):5769-5801.

Ruby, J. G., C. H. Jan, and D. P. Bartel. 2007. Intronic microRNA precursors that bypass Drosha processing. Nature 448(7149):83-86.

Schmidt, J., P. Sipocz, E. Cenkvari, and J. Sipocz. 1999. Use of protected methionine (Mepron M 85) in cattle. Acta Vet Hung 47(4):409-418.

Schulz, K., J. Frahm, U. Meyer, S. Kersten, D. Reiche, J. Rehage, and S. Dänicke. 2014. Effects of prepartal body condition score and peripartal energy supply of dairy cows on postpartal lipolysis, energy balance and ketogenesis: an animal model to investigate subclinical ketosis. JDR 81(3):257-266.

Schwab, C. G., C. K. Bozak, N. L. Whitehouse, and M. M. Mesbah. 1992. Amino acid limitation and flow to duodenum at four stages of lactation. 1. Sequence of lysine and methionine limitation. J Dairy Sci 75(12):3486-3502.

Sordillo, L. M. and S. L. Aitken. 2009. Impact of oxidative stress on the health and immune function of dairy cattle. Vet Immunol Immunopathol 128(1-3):104-109.

Zhou, Z., M. Vailati-Riboni, E. Trevisi, J. K. Drackley, D. N. Luchini, and J. J. Loor. 2016. Better postpartal performance in dairy cows supplemented with rumen-protected methionine compared with choline during the peripartal period. J Dairy Sci 99(11):8716-8732.

CHAPTER 2: LITERATURE REVIEW

The Transition Period

The life stage where dairy cows shift from late pregnancy to early lactation is widely referred to as the transition period or periparturient period. However, the duration a cow spends in this transition is described differently by different authors. Most commonly the period is classified as 3 weeks prior to parturition and 3 weeks after parturition (Grummer, 1995).

The transition period was described perfectly by Goff (1977), as "too often a disastrous experience for the cow". Usually, around 3 weeks before calving a gradual but significant decrease in dry matter intake (DMI) can be observed in dairy cows (Bertics et al., 1992, Drackley, 1999). A potential explanation for decreased DMI is tremendous fetal growth during the last few weeks of gestation. Thus, ruminal capacity can reduce as much as 20%, limiting the amount of feed a cow can consume (Spain, 2002). However, many other factors can influence intake like body condition score (BCS), parity, milk yield, and nutrient composition of the diet (Allen, 2000, Bell et al., 2000). If this trend of DMI proceeds into the lactation period, cows can enter a negative nutrient balance as demands for energy and nutrients increase for colostrum and milk synthesis (Ferguson, 2005). A poor transition from pregnancy to lactation usually results in the loss of 4-9 kg of peak milk yield. In the long run, this could equate to 900-1800 kg of unusable milk (Wallace, 1996). Unfortunately, these losses translate to decreased profits for producers.

Parturition is marked by an increased risk for metabolic disorders (such as milk fever, displaced abomasum, fatty liver, and ketosis), infectious diseases (mastitis and udder edema), and reproductive difficulties (dystocia, retained placenta, uterine infections) making it the most critical physiological stage a cow may face (Drackley, 1999). The occurrence of metabolic

disorders is linked to decreased feed intake and milk production (Bertics et al., 1992). Importance exponentially increases when considering the ramifications disease during the transition period may have on future pregnancies and lactations. Identifying and treating diseases early, potentially as early as the prepartum, may be beneficial in overcoming future production losses (Huzzey et al., 2011). Therefore, the transition period is a crucial time for producers to monitor an animal's health.

Liver and Energy Metabolism

As dairy cows transition from pregnancy to lactation, nutrient supplies become depleted, causing a large amount of non-esterified fatty acids (NEFA) to mobilize from adipose tissue (Bell, 1995). This is primarily due to the increased nutrient requirements for milk production and fetal development, at a time when dry matter intake (DMI) or nutrient supply, is not adequate (Bell, 1995). The disproportion of supply vs. demand leads to a period of negative energy balance (NEB). The liver will take up the mobilized NEFA from adipose for β-oxidation in the mitochondria to create metabolites, like acetyl-CoA, which will be used to generate energy in the Krebs cycle. If not oxidized, the excess NEFA are repackaged into triacylglycerol (TAG) and exported as very low-density lipoprotein (VLDL). However, the liver has a threshold limiting the amount of NEFA that can be metabolized into TAG. Hepatic lipidosis results from excessive accumulation of TAG in the liver which ultimately impairs normal function. When the maximum is reached, the TAG accumulates in the liver, and acetyl CoA that are not utilized are converted into ketone bodies, like beta-hydroxybutyrate (BHB). When ketones are produced in excess of the tissue's ability to use them, they accumulate in the blood, which in severe cases can lead to ketosis (Bell, 1995, Drackley, 1999).

The enzymes that regulate hepatic fatty acid metabolism are subject to hormonal control from insulin and glucagon. A low insulin/glucagon ratio can stimulate lipolysis in adipose tissues and ketogenesis in the liver allowing cows to adapt to different nutritional and physiological conditions (Holtenius and Holtenius, 1996). For example, increased insulin reduces carnitine palmitoyltransferase I (CPTI) activity which will lead to decreased NEFA transport into mitochondria, thereby suppressing ketogenesis (McGarry and Foster, 1980). Although, increased insulin can also promote esterification and more synthesis of triglyceride (Keller et al., 1988).

Body Condition Score (BCS)

In previous studies from the early 1990s, body weight alone was the primary tool for measuring fat reserves in a cow. However, body weight does not encompass all indicators of body reserves such as, parity, stage of lactation, frame size, gestation, and breed (Roche et al., 2009). Additionally, increases in body weight can be falsely enhanced by gastrointestinal fill. Body condition score (BCS) is a useful tool as it reflects both the amount of subcutaneous fat in the body and indicates the nutritional status of an animal (Roche et al., 2009). The first scoring system was introduced by Lowman (1973) which was based introduced on a 4-point scale in beef cattle. Today, there are many differences in international scoring systems ranging from a 5-point scale to a 10-point scale so a conversion method was created by Roche et al. (2004). In the United States, BCS are evaluated on a 5-point scale, where 1 indicates a thin or emaciated cow, while a 5 equates to an over-conditioned obese cow (Roche et al., 2004). At calving, if a cow has a more optimal BCS this could promote a successful transition period and maximize production in the postpartum (Akbar et al., 2015). Cows with higher BCS are at a greater risk of developing fatty liver disease or ketosis and are under more oxidative stress during the transition period

(Reid et al., 1986, Bernabucci et al., 2005, Schulz et al., 2014). Thus, BCS has a close relationship with metabolic disorders and is a useful tool in dairy cow management.

Oxidative Phosphorylation

Oxidative phosphorylation begins with an electron transfer chain driven by substrate oxidation, coupled with ATP synthesis through an electrochemical transmembrane gradient (Papa et al., 2012). While this process is essential in creating energy for various biological functions, it also produces reactive oxygen species (ROS) or free radicals (Wallace et al., 1992). ROS is highly reactive and can interact and destroy, DNA, proteins, and lipids. Antioxidants, like glutathione, reduce the levels of ROS to prevent free radical damage (Bayir, 2005). When the rate at which ROS can be removed or repaired becomes imbalanced, oxidative stress and inflammation can begin.

In nonruminants, nuclear factor erythroid 2-like 2 (NFE2L2) (formerly known as Nrf2), is a key transcription factor that controls oxidative stress by increasing the concentration of antioxidant enzymes, a mechanism of critical importance for cellular protection and survival (Kaspar et al., 2009). Glutathione (GSH) is a crucial antioxidant in mammalian cell and the GSH metabolism pathway is one of the target pathways regulated by NFE2L2. In mammary epithelial cell culture, several antioxidants activate *NFE2L2*, causing an increase in the transcription of antioxidants overall (Jin et al., 2016). In summation, this suggests that both NFE2L2 and GSH, play a key role in regulating antioxidant networks.

Methionine

Methionine is a limiting amino acid for milk synthesis in dairy cows making it an important area of focus, especially surrounding parturition (Schwab et al., 1992). Moreover, methionine has a role in processes in the liver like one-carbon metabolism and synthesis of GSH

(Martinov et al., 2010). Previous research supported that methionine supplementation led to a decreased risk of metabolic disorders and improved immune function (Lima et al., 2012, Zhou et al., 2016). The supplementation of rumen-protected methionine during the transition period has also improved milk yield, milk protein, and increased antioxidant synthesis (Schmidt et al., 1999, Osorio et al., 2013). Additionally, methionine supplementation has been associated with overall improved hepatic lipid metabolism in dairy cows (Durand, 1992). A recent study found that in the mammary tissue of cows supplemented methionine, genes related to GSH (GCLC, GCLM, GSR, GPX1, and ME1) metabolism were upregulated, suggesting that amino acids play a role in regulating oxidative stress by controlling GSH metabolism in mammary cells (Han et al., 2018). Most likely because methionine plays a role in one-carbon metabolism in liver and is a source of cysteine, the limiting reagent for synthesis of GSH (Martinov et al., 2010, Zhou et al., 2017).

MicroRNAs (miRNAs)

It is speculated that the human genome encodes around 1000 miRNAs which equate to the regulation of about one-third of all human genes. miRNAs are short non-coding RNAs of 22 nucleotides in length that regulate gene expression post-transcriptionally by binding to the 3' untranslated region of a target mRNA (Ruby et al., 2007). Binding of the miRNA will lead to translational repression, target degradation, and gene silencing (Bartel, 2009).

The role of miRNAs in lipid metabolism was first examined in fruit flies (*Drosophila*), where the deletion of miR-14 resulted in increased levels of triacylglycerol and diacylglycerol (Xu et al., 2003). Since then, more miRNAs were shown to play a role in fat cell formation (adipogenesis). Beef cattle adipose miRNA profiling was recently correlated with fat deposit location and function, supporting the importance of miRNA (miR-378 and miR-143) in

regulating adipocyte metabolism (Jin et al., 2009, Jin et al., 2010). Another study investigated miRNA targets related to adipogenesis and inflammation and found adipocyte metabolic pathways are controlled by miRNA signaling through networks involving transcription factors, suggesting a possible role in the control of inflammation (Arner and Kulyte, 2015).

Furthermore, miRNA research in human clinical trials is an expanding field. Researchers found that miR-122 had an impact on lipid metabolism, but also the destruction of it which increased inflammation, leading to a poor prognosis of liver cancer (Hsu et al., 2012). Similarly, miRNA expression in humans has been associated with increased cytokine development and a greater degree of immune cell infiltration (Kloting et al., 2009). Additionally, miRNAs in serum may be a better biomarker to assess early non-alcoholic fatty liver as opposed to other popular clinical models (Iravani et al., 2018). Though the amount of literature about miRNAs has increased substantially in the past sixteen years, more experimentation (from both clinical trials in humans and animal model research) is needed to understand their involvement in liver metabolism.

REFERENCES

Akbar, H., T. M. Grala, M. Vailati Riboni, F. C. Cardoso, G. Verkerk, J. McGowan, K. Macdonald, J. Webster, K. Schutz, S. Meier, L. Matthews, J. R. Roche, and J. J. Loor. 2015. Body condition score at calving affects systemic and hepatic transcriptome indicators of inflammation and nutrient metabolism in grazing dairy cows. J Dairy Sci 98(2):1019-1032.

Allen, M. S. 2000. Effects of diet on short-term regulation of feed intake by lactating dairy cattle. J Dairy Sci 83(7):1598-1624.

Arner, P. and A. Kulyte. 2015. MicroRNA regulatory networks in human adipose tissue and obesity. Nat Rev Endocrinol 11(5):276-288.

Bartel, D. P. 2009. MicroRNAs: target recognition and regulatory functions. Cell 136(2):215-233.

Bayir, H. 2005. Reactive oxygen species. Crit Care Med 33(12 Suppl):S498-501.

Bell, A. W. 1995. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. J Anim Sci 73(9):2804-2819.

Bell, A. W., W. S. Burhans, and T. R. Overton. 2000. Protein nutrition in late pregnancy, maternal protein reserves and lactation performance in dairy cows. Proc Nutr Soc 59(1):119-126.

Bernabucci, U., B. Ronchi, N. Lacetera, and A. Nardone. 2005. Influence of body condition score on relationships between metabolic status and oxidative stress in periparturient dairy cows. J. Dairy Sci. 88(6):2017-2026.

Bertics, S. J., R. R. Grummer, C. Cadorniga-Valino, and E. E. Stoddard. 1992. Effect of prepartum dry matter intake on liver triglyceride concentration and early lactation. J Dairy Sci 75(7):1914-1922.

Drackley, J. K. 1999. ADSA Foundation Scholar Award. Biology of dairy cows during the transition period: the final frontier? J Dairy Sci 82(11):2259-2273.

Durand, D., Y. Chilliard, and D. Bauchart. 1992. Effect of lysine and methionine on in vivo hepatic secretion of VLDL in the high yield- ing dairy cow. J. Dairy Sci 25(Suppl.1)(279).

Ferguson, J. D. 2005. Nutrition and reproduction in dairy herds. Vet Clin North Am Food Anim Pract 21(2):325-347.

Goff, J. P. a. R. L. H. 1977. Physiological changes at parturition and their relationship to metabolic disorders. Journal of dairy science 80(7):1260-1268.

Grummer, R. R. 1995. Impact of changes in organic nutrient metabolism on feeding the transition dairy cow. J Anim Sci 73(9):2820-2833.

Han, L., F. Batistel, Y. Ma, A. S. M. Alharthi, C. Parys, and J. J. Loor. 2018. Methionine supply alters mammary gland antioxidant gene networks via phosphorylation of nuclear factor erythroid 2-like 2 (NFE2L2) protein in dairy cows during the periparturient period. J Dairy Sci 101(9):8505-8512.

Holtenius, P. and K. Holtenius. 1996. New aspects of ketone bodies in energy metabolism of dairy cows: a review. Zentralbl Veterinarmed A 43(10):579-587.

Hsu, S. H., B. Wang, J. Kota, J. Yu, S. Costinean, H. Kutay, L. Yu, S. Bai, K. La Perle, R. R. Chivukula, H. Mao, M. Wei, K. R. Clark, J. R. Mendell, M. A. Caligiuri, S. T. Jacob, J.

T. Mendell, and K. Ghoshal. 2012. Essential metabolic, anti-inflammatory, and anti-tumorigenic functions of miR-122 in liver. J Clin Invest 122(8):2871-2883.

Huzzey, J. M., D. V. Nydam, R. J. Grant, and T. R. Overton. 2011. Associations of prepartum plasma cortisol, haptoglobin, fecal cortisol metabolites, and nonesterified fatty acids with postpartum health status in Holstein dairy cows. J Dairy Sci 94(12):5878-5889.

Iravani, F., N. Hosseini, and M. Mojarrad. 2018. Role of MicroRNAs in Pathophysiology of Non-alcoholic Fatty Liver Disease and Non-alcoholic Steatohepatitis. Middle East J Dig Dis 10(4):213-219.

Jin, W., M. V. Dodson, S. S. Moore, J. A. Basarab, and L. L. Guan. 2010. Characterization of microRNA expression in bovine adipose tissues: a potential regulatory mechanism of subcutaneous adipose tissue development. BMC Mol Biol 11:29.

Jin, W., J. R. Grant, P. Stothard, S. S. Moore, and L. L. Guan. 2009. Characterization of bovine miRNAs by sequencing and bioinformatics analysis. BMC Mol Biol 10:90.

Jin, X. L., K. Wang, L. Liu, H. Y. Liu, F. Q. Zhao, and J. X. Liu. 2016. Nuclear factor-like factor 2-antioxidant response element signaling activation by tert-butylhydroquinone attenuates acute heat stress in bovine mammary epithelial cells. J Dairy Sci 99(11):9094-9103.

Kaspar, J. W., S. K. Niture, and A. K. Jaiswal. 2009. Nrf2:INrf2 (Keap1) signaling in oxidative stress. Free Radic Biol Med 47(9):1304-1309.

Keller, U., P. P. Gerber, and W. Stauffacher. 1988. Fatty acid-independent inhibition of hepatic ketone body production by insulin in humans. Am J Physiol 254(6 Pt 1):E694-699.

Kloting, N., S. Berthold, P. Kovacs, M. R. Schon, M. Fasshauer, K. Ruschke, M. Stumvoll, and M. Bluher. 2009. MicroRNA expression in human omental and subcutaneous adipose tissue. PLoS One 4(3):e4699.

Lima, F. S., M. F. Sa Filho, L. F. Greco, and J. E. Santos. 2012. Effects of feeding rumen-protected choline on incidence of diseases and reproduction of dairy cows. Vet J 193(1):140-145.

Lowman, B. G., N. Scott, and S. Somerville. 1973. Condition scoring of cattle. East of Scotland College of Agriculture, Bulletin No. 6, Edinburgh, UK.

Martinov, M. V., V. M. Vitvitsky, R. Banerjee, and F. I. Ataullakhanov. 2010. The logic of the hepatic methionine metabolic cycle. Biochim Biophys Acta 1804(1):89-96.

McGarry, J. D. and D. W. Foster. 1980. Regulation of hepatic fatty acid oxidation and ketone body production. Annu Rev Biochem 49:395-420.

Osorio, J. S., P. Ji, J. K. Drackley, D. Luchini, and J. J. Loor. 2013. Supplemental Smartamine M or MetaSmart during the transition period benefits postpartal cow performance and blood neutrophil function. J Dairy Sci 96(10):6248-6263.

Papa, S., P. L. Martino, G. Capitanio, A. Gaballo, D. De Rasmo, A. Signorile, and V. Petruzzella. 2012. The oxidative phosphorylation system in mammalian mitochondria. Adv Exp Med Biol 942:3-37.

Reid, I., C. Roberts, R. Treacher, and L. Williams. 1986. Effect of body condition at calving on tissue mobilization, development of fatty liver and blood chemistry of dairy cows. Animal Science 43(1):7-15.

Roche, J. R., P. G. Dillon, C. R. Stockdale, L. H. Baumgard, and M. J. VanBaale. 2004. Relationships among international body condition scoring systems. J Dairy Sci 87(9):3076-3079.

Roche, J. R., N. C. Friggens, J. K. Kay, M. W. Fisher, K. J. Stafford, and D. P. Berry. 2009. Invited review: Body condition score and its association with dairy cow productivity, health, and welfare. J Dairy Sci 92(12):5769-5801.

Ruby, J. G., C. H. Jan, and D. P. Bartel. 2007. Intronic microRNA precursors that bypass Drosha processing. Nature 448(7149):83-86.

Schmidt, J., P. Sipocz, E. Cenkvari, and J. Sipocz. 1999. Use of protected methionine (Mepron M 85) in cattle. Acta Vet Hung 47(4):409-418.

Schulz, K., J. Frahm, U. Meyer, S. Kersten, D. Reiche, J. Rehage, and S. Dänicke. 2014. Effects of prepartal body condition score and peripartal energy supply of dairy cows on postpartal lipolysis, energy balance and ketogenesis: an animal model to investigate subclinical ketosis. JDR 81(3):257-266.

Schwab, C. G., C. K. Bozak, N. L. Whitehouse, and M. M. Mesbah. 1992. Amino acid limitation and flow to duodenum at four stages of lactation. 1. Sequence of lysine and methionine limitation. J Dairy Sci 75(12):3486-3502.

Spain, J. N. a. W. A. S. 2002. The 100-day contract with the dairy cow: 30 days prepartum to 70 days postpartum. Advances in dairy technology 14:19-42.

Wallace, D. C., J. M. Shoffner, R. L. Watts, J. L. Juncos, and A. Torroni. 1992. Mitochondrial oxidative phosphorylation defects in Parkinson's disease. Ann Neurol 32(1):113-114.

Wallace, R. L., G. C. McCoy, T. R. Overton, and J. H. Clark. 1996. Effect of adverse health events on dry matter consumption, milk production, and body weight loss of dairy cows during early lactation. Journal of dairy science 79 Suppl 1.

Xu, P., S. Y. Vernooy, M. Guo, and B. A. Hay. 2003. The Drosophila microRNA Mir-14 suppresses cell death and is required for normal fat metabolism. Curr Biol 13(9):790-795.

Zhou, Z., T. A. Garrow, X. Dong, D. N. Luchini, and J. J. Loor. 2017. Hepatic Activity and Transcription of Betaine-Homocysteine Methyltransferase, Methionine Synthase, and Cystathionine Synthase in Periparturient Dairy Cows Are Altered to Different Extents by Supply of Methionine and Choline. J Nutr 147(1):11-19.

Zhou, Z., M. Vailati-Riboni, E. Trevisi, J. K. Drackley, D. N. Luchini, and J. J. Loor. 2016. Better postpartal performance in dairy cows supplemented with rumen-protected methionine compared with choline during the peripartal period. J Dairy Sci 99(11):8716-8732.

CHAPTER 3: HEPATIC MICRORNA INVOLVEMENT IN PERIPARTURIENT HOLSTEIN DAIRY COWS SUPPLEMENTED METHIONINE

ABSTRACT

During the peripartal period (3 wk before calving to 3 wk after calving) dairy cows are at risk for entering a negative energy balance which can increase the risk of developing metabolic disorders like ketosis or fatty liver. Methionine supplementation in peripartal diets may be beneficial in combatting diseases dairy cows are prone to during the transition period. Previous in silico work relevant to fatty liver disease and ketosis revealed microRNAs (miRNAs) may play a role in fatty acid metabolism (miR-186, -200b and c, -218, -369), oxidative phosphorylation (miR-101, -142, -186, -200b, -200c, -218, and -369), peroxisome (miR-186, -200b and c), gluconeogenesis (miR-186, -369), peroxisome proliferator-activator receptor (PPAR) signaling pathway (miR-101, -142, -186, -200b and c), insulin signaling pathway (miR-186, -200b, -218, -369), and apoptosis (miR-142, -200b, -369). The main objective of the present study was to verify the expression of these miRNA in liver and examine the relationship they may have in cows supplemented methionine during the peripartal period. Nineteen multiparous Holstein cows were fed experimental treatments consisting of a basal control diet (CON; n = 11) and CON plus Methionine (Smartamine M, Adisseo NA) (MET; n = 8). All cows received the same far-off diet from -50 to -22 d before the expected calving date, close-up diet from -21 d to expected calving, and lactation diet from calving through 30 days in milk (DIM). MET supplementation was adjusted daily from -21 d to 30 DIM at a rate of 0.08% (dry matter basis) of diet dry matter. Liver biopsies were performed at −10, 10, and 30 d relative to calving for analysis of miR-218, miR-369 5p, miR-200b, miR-200c, miR-142 3p, miR-142 5p, miR-101, and miR-186 abundance via qPCR after normalization with 3 internal controls. Data were

subjected to repeated measures ANOVA in SAS using PROC MIXED. The main effects were treatment (CON or MET), time, and their interaction, while cow was the random effect. We observed a significant treatment × time effect for miR-186 ($P \leq 0.05$), associated with less expression in MET compared with CON cows on d -10, which may suggest that MET cows downregulated fatty acid metabolism and oxidative stress. A significant time effect was present in miR-369 5p and miR-200b expression ($P \leq 0.05$) which both were predicted to down regulate oxidative phosphorylation, but the relationship between these miRNAs and the pathways they affect need to be studied more. miR-186 had the most promise for future studies that could determine the role it plays in energy metabolism, as it has the most interaction with overlapping biological pathways. Overall, miRNAs are expressed in the liver of cows supplemented methionine, however analyses of gene targets of these miRNA along with protein expression will provide more insight to the biological relevance.

INTRODUCTION

The periparturient period (3 wk before calving to 3 wk after calving), or transition period, is critically important to the health, production, and profitability of dairy cows (Drackley, 1999). During this time, cows are most susceptible to metabolic disorders and immunologic challenges such as oxidative stress, ketosis, and fatty liver disease. This is primarily due to the increased nutrient requirements for milk production and fetal development, at a time when dry matter intake (DMI) or nutrient supply, is inadequate (Bell, 1995). The disproportion of supply vs. demand leads to a period of negative energy balance (NEB), disrupting fatty acid metabolism. When nutrient supply is depleted, adipose tissue mobilizes non-esterified fatty acids (NEFA) which originate from the hydrolysis of triacylglycerol (TAG). Hepatic lipidosis results from excessive accumulation of TAG in the liver which ultimately impairs normal function. Additionally, when ketones are produced in excess of the peripheral tissue's capacity to use them, they accumulate in the bloodstream, leading to ketosis in severe cases (Bell, 1995, Drackley, 1999). When dairy cows are affected by these two diseases they exhibit clinical symptoms including reduced feed intake, decreased milk yield, body weight loss, staggering, lack of coordination, and blindness (Mulligan and Doherty, 2008).

Previous research exists that links amino acid supplementation to a decreased risk of metabolic disorders, like ketosis and fatty liver disease (Lima et al., 2012, Zhou et al., 2016). Methionine is a limiting amino acid for milk synthesis in dairy cows making it an important area of focus, especially surrounding parturition (Schwab et al., 1992). In previous studies, the supplementation of rumen-protected methionine during the transition period has improved milk yield, milk protein, and postpartum immune function (Schmidt et al., 1999, Osorio et al., 2013). Additionally, methionine supplementation has been associated with improved hepatic lipid

metabolism in dairy cows (Durand, 1992). Therefore, it is possible that supplementation of methionine could reduce elevated fatty acids in the liver, contributing to a decrease in the incidence of fatty liver and ketosis.

microRNAs (miRNAs) are short non-coding RNAs that regulate gene expression extensively (Iravani et al., 2018). In humans, miRNome profiling provided insights into the molecular mechanisms involved in nonalcoholic fatty liver disease (Cheung et al., 2008, Alisi et al., 2011). However, (to our knowledge) the application of miRNome profiling in dairy cows developing fatty liver disease is unknown. To better understand the possible role miRNA play during the onset of fatty liver in transition cows, Vailati-Riboni (2017), applied an in-silico approach to locate a signature of miRNA activation from transcriptomics data. The transcriptome database was created from an experiment by Loor et al. (2007). Briefly, ketosis and fatty liver were induced in cows fed at 50% feed restriction from day 5 until 14 d relative to calving, or until clinical signs of ketosis (anorexia, ataxia, or abnormal behavior) were observed. Liver tissue (n = 7/group) for gene expression profiling was sampled at the onset of signs of clinical ketosis or d 14 postpartum for control cows. Initially, a list of miRNA families and their predicted target genes for *Bos taurus* were downloaded from the Microcosm targets website (v. 5.0). Results for the effect of ketosis were then used to predict miRNA activity from the mRNA expression profiles through 3 approaches described by Arora (2008): Wilcoxon rank test, Ranked Ratio, and Mean absolute expression. After overlapping the results of the 3 approaches, a total of 7 miRNA were predicted as possible components in the transcriptomic response associated with ketosis and fatty liver: miR-101, miR-142, miR-186, miR-200b, miR-200c, miR-218, and miR-369–5p. To identify miRNA functions, the dynamic impact approach (DIA), described by Bionaz et al. (2012), was then used for biological pathway analysis on the compiled differentially

expressed target genes of the predicted miRNA. Pathway analysis relevant to fatty liver revealed these miRNAs may play a role in fatty acid metabolism (miR-186, -200b and c, -218, -369), oxidative phosphorylation (all 8 miRNA), peroxisome (miR-186, -200b and c), gluconeogenesis (miR-186, -369), PPAR signaling pathway (miR-101, -142, -186, -200b and c), insulin signaling pathway (miR-186, -200b, -218, -369), and apoptosis (miR-142, -200b, -369), which is further detailed in Table 3.1.

Overall, the in-silico analysis suggests that specific miRNA may be involved in the etiology of fatty liver through the control of key biological pathways and genes related to this disease (Vailati-Riboni, 2017). Thus, the main objective of the present study was to verify the expression of these miRNA and examine the relationship they may have in cows supplemented methionine during the peripartal period, particularly in fatty acid metabolism and oxidative phosphorylation.

METHODS

Experimental Design and Treatments

All details of animal management were approved by the Animal Care and Use Committee at the University of Illinois (Urbana; protocol no. 14270) and have been reported (Batistel et al., 2017). Briefly, 60 multiparous Holstein cows (average lactation number 3.22 ± 1.11) were assigned to a basal diet (control, n = 30) without additional Methionine or the basal diet with ethyl-cellulose RPM (Met, n = 30; Mepron, Evonik Nutrition and Care GmbH, Hanau-Wolfgang, Germany) in a randomized complete block design. The RPM was top dressed from −28 to 60 d relative to parturition at a rate of 0.09 and 0.10% of DMI of the previous day before and after calving, respectively, to achieve a Lys:Met ratio of 2.8:1. The basal diet had a Lys:Met

ratio of 3.71:1 (prepartum) to 3.78:1 (postpartum). A Lys:Met ratio of 2.8:1 to 2.9:1 has proven beneficial for dairy cow production performance and health, including greater DMI and milk yield, better immune function, and lower incidence of ketosis postpartum (Osorio et al., 2013, Zhou et al., 2016). From −45 to −29 d relative to calving date, all cows were fed the same diet without RPM. Cows were fed once daily in the morning at 120% of expected intake and milked 3 times daily (at 0600, 1400, and 2200 h). Diets were formulated to meet predicted requirements for dairy cows according to NRC (2001) guidelines.

Liver Biopsy

A subset of 10 cows in the control group and 8 cows in the Met group were used for liver biopsies. Cows were selected based on previous 305-d milk yield, BCS, and absence of clinical disease. Liver tissue was harvested via puncture biopsy (Dann et al., 2005) to obtain approximately 4 g (wet weight) of liver. The surgical area was shaved and disinfected and the skin and body wall were anesthetized with 7 mL of 2% lidocaine HCl (VetOne, Boise, ID). Samples were collected at approximately 0800 h on d −10, 10, and 30 ± 2 d relative to parturition. Liver tissue was frozen immediately in liquid N and stored −80°C until further analysis. Health was monitored for 7 d after each procedure.

Total RNA Extraction, Target miRNA, and qPCR

RNA was extracted from tissue using protocols established in our laboratory (Khan et al., 2014). Briefly, liver tissue was weighed (~0.05 g) and immediately placed in 1.2 ml of QIAzol reagent (Qiagen 75842; Qiagen Inc., Valencia, CA) for homogenization. After homogenization, DNase was used to remove any genomic DNA from RNA using RNeasy Mini Kit columns (Qiagen, Hilden, Germany). Concentration was measured using the Nano-Drop ND-1000

spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA), and the Agilent Bioanalyzer system (Agilent 2100 Bioanalyzer, Agilent Technologies, Santa Clara, CA, USA) was used to measure RNA quality. miRNA-specific cDNA synthesis was performed using the Quanta qScript microRNA cDNA Synthesis Kit (Quanta BioSciences, Inc.), following the manufacturer's protocols. The quantitative PCR (qPCR) was SYBR Green-based and performed as described previously by Osorio et al. (2014), using a 7-point standard curve. miRNA selected for expression profiling were miR-218, miR-369 5p, miR-200b, miR-200c, miR-142 3p, miR-142 5p, miR-101, and miR-186 based on the analysis described in Figure 3.1. Internal controls were miR-let-7b, miR-16b and miR-181a. Measured miRNA assay primer sequence information and qPCR performance are included in Supplemental Table A.1. and A.2. (Appendix A). Percentage relative miRNA abundance in relation to the 8 miRNA targets is in Supplemental Figure A.1. (Appendix A).

Statistical Analysis

After normalization with the geometric mean of the internal control genes, miRNA qPCR data were $\log_2$ transformed prior to statistical analysis to obtain a normal distribution. The data were analyzed using the MIXED procedure of SAS v.9.4 (SAS Institute Inc., Cary, NC) according to the following model with repeated measures:

$$Y_{jl} = \mu + M_j + T_l + MT_{jl} + e_{jl},$$

Where Y_{jl} = dependent, continuous variable, μ = overall mean, M_j = fixed effect of treatment (j = CON vs. MET), T_l = fixed effect of time (d -10, 10, and 30 relative to parturition), MT_{jl} = interaction between treatment and time, and e_{jl} = residual error. The subject of the repeated statement was cow, nested within treatment, as the random effect. The Kenward-Roger statement was used for computing the denominator degrees of freedom, while spatial power was used as

the covariance structure since time intervals are not evenly spaced. Data were considered significant at a $P \leq 0.05$ and tendencies at $P \leq 0.10$ using the PDIFF statement in SAS. For ease of interpretation, expression data reported in tables and figures are the $\log_2$ back-transformed LSM that resulted from the statistical analysis. Normality of the residuals was checked with normal probability and box plots, and homogeneity of variances was checked with plots of residuals versus predicted values.

RESULTS AND DISCUSSION

We observed a treatment × time effect for miR-186 due to greater expression in the CON group at d -10 compared with MET (Fig. 3.2). Since miR-186 was predicted to downregulate fatty acid metabolism and oxidative stress (Fig. 3.1), MET cows potentially followed the trend of downregulating energy metabolism. Previously, methionine supplementation has been associated with improved hepatic lipid metabolism in dairy cows (Durand, 1992). Similarly, supplementation with rumen-protected choline and methionine promoted energy balance by increasing postpartal DMI and improved postpartum lactation performance, thereby enhancing antioxidant capacity (Sun et al., 2016). Thus, supplementation of methionine could reduce elevated fatty acids in the liver, contributing to a decrease in the incidence of fatty liver and ketosis. Our original experiment involving 60 cows reported greater DMI in the prepartum and postpartum in MET cows (compared to CON), but no significant differences in the incidence of ketosis in CON and MET cows (Batistel et al., 2017). Though, Zhou et al. (2016), found a tendency for cows supplemented methionine to have less incidence of ketosis during the transition period.

Regarding miR-200b and miR-369 5p expression, we found significant differences in

time ($P \leq 0.05$), but no other differences (Fig. 3.2). Both miR-369 5p and -200b demonstrate an increasing trend through the transition period, with lesser expression at d -10 and greater expression at d 30. This could have resulted from the increasing demand for milk synthesis after parturition, perhaps increasing energy metabolism. Furthermore, these two miRNA were predicted to downregulate oxidative phosphorylation (Fig. 3.1). A recent study found that methionine supplementation alleviated oxidative stress in peripartal dairy cows and upregulated mRNA abundance related to glutathione (GSH) metabolism (Liang et al., 2019). Expression of miR-369 5p was significantly ($P \leq 0.05$) influenced by time, but no differences were detected between the interaction of treatment and time (Fig. 3.2). Another study postulated that miR-369 5p in beef calf muscle tissue could bind to the 3′UTR of fatty acid binding protein 4 (FABP4) to decrease gene expression and thereby inhibit fat cell formation (adipogenesis) (Moisa et al., 2016). This signifies that examining miRNA abundance in tissues other than the liver would give us more insight into their biological impact.

There were no differences observed in regard to treatment or time, and the interaction of treatment and time in miR-218, miR-200c, miR-142-3p, 142-5p, and mir-101, (Fig. 3.2). The lack of significant expression in miR-101 was unexpected since this was the only predicted miRNA to play a role in GSH metabolism (Table 3.1). Glutathione is a frequently studied antioxidant in cells because it maintains redox homeostasis (Forman et al., 2009). Methionine plays a role in one-carbon metabolism in liver and is a source of cysteine, the limiting reagent for synthesis of GSH (Martinov et al., 2010, Zhou et al., 2017). Although, there were no differences in miR-101 expression, miR-186, which was predicted to upregulate cysteine and methionine metabolism (Table 3.1), was expressed more in CON relative to MET at d 10. Since CON cows did not receive methionine they potentially would have upregulated cysteine and methionine

metabolism more so than MET cows. Perhaps, there is a relationship between cysteine and glutathione metabolism in the tested miRNA. However, experimentation is needed to further verify how these miRNAs regulate oxidative phosphorylation and fatty acid metabolism expression in liver or adipose tissue from cows supplemented methionine.

CONCLUSIONS

Overall, a decrease in the expression of miR-186, which is associated with downregulating fatty acid metabolism, in cows supplemented methionine suggests a relationship between pathways and miRNA in liver, compared with control cows. Analyses of gene targets and protein expression relevant to miR-186 and others presented in this study will provide more insight into the biological relevance of the miRNA presented.

FIGURES

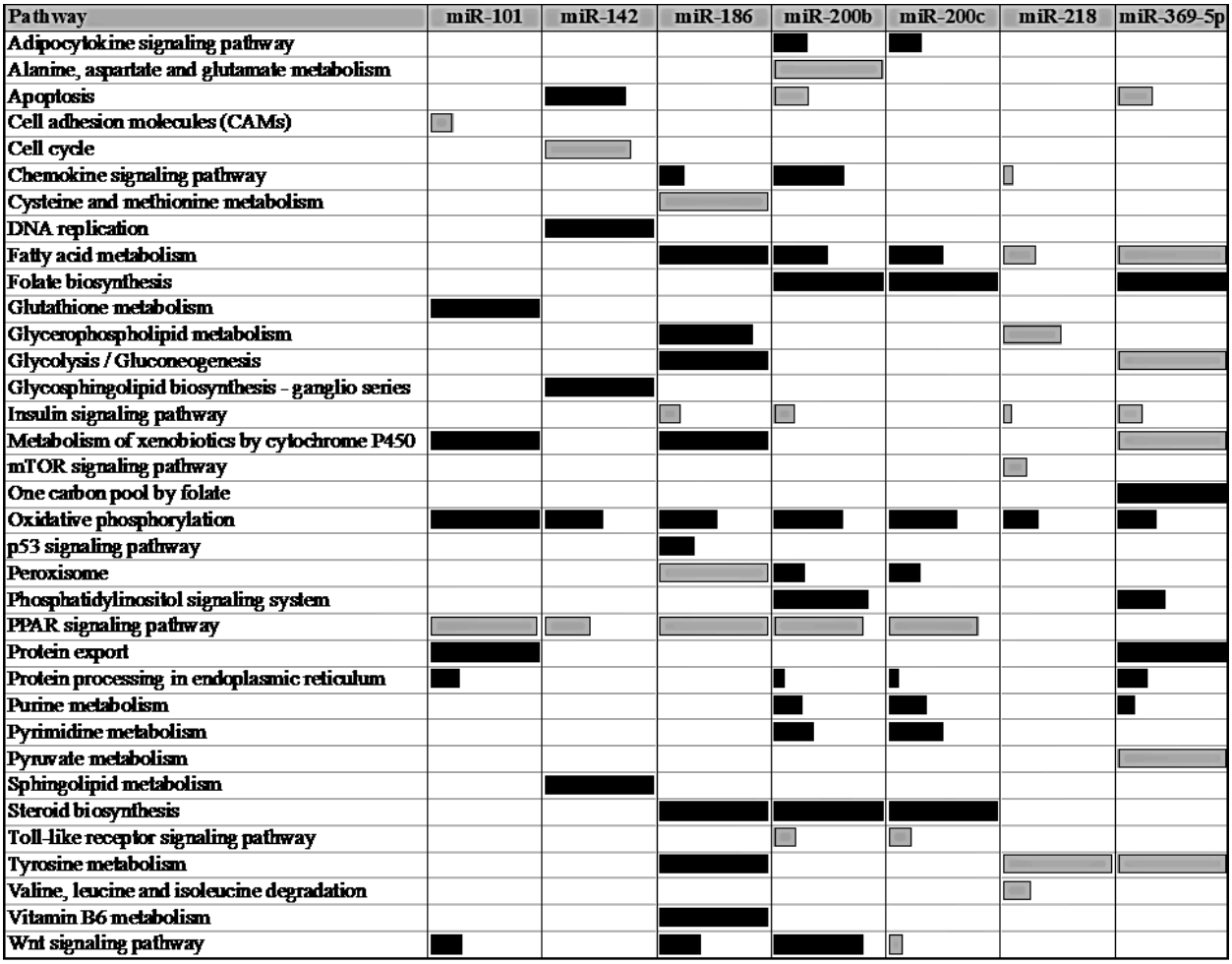


Figure 3.1. Biological importance of pathways involved in the etiology of fatty liver regulated by the activity of miRNAs predicted (in silico) to be activated during the onset of ketosis through feed restriction in liver of peripartal dairy cows. Length of the bar represents the entity impact, while *grey* denotes upregulate and *black* represents downregulation of the pathway. Adapted from Vailati-Riboni (2017)**.**

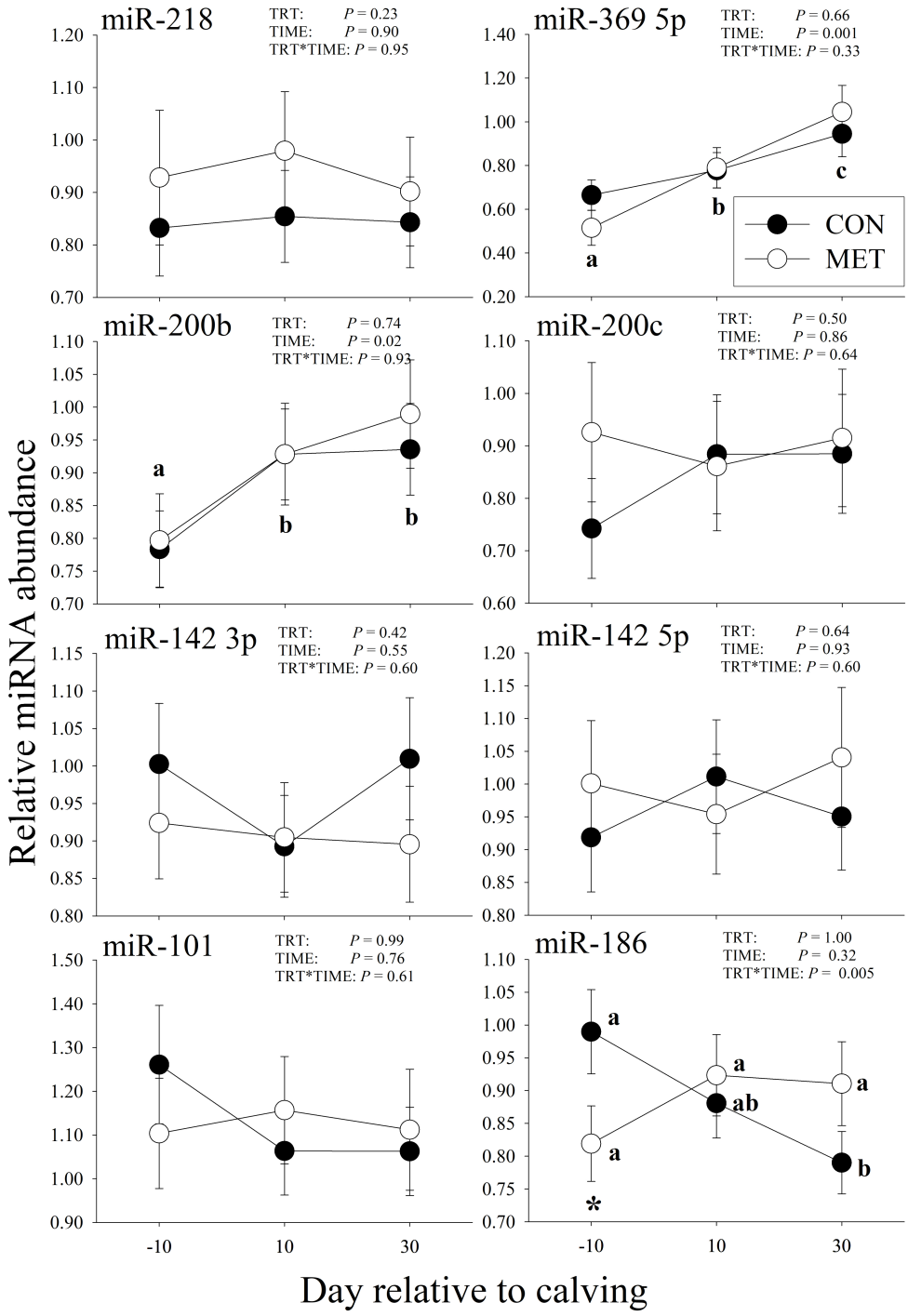


Figure 3.2. Relative miRNA abundance for 8 miRNA targets in CON or MET treatments at -10, 10, and 30 d relative to calving (least square means ± pooled SEM).

*Means between treatments differ at the given time point (TRT x Time $P \leq 0.05$).
[a,b,c]Means within treatment or time differ.

REFERENCES

Alisi, A., L. Da Sacco, G. Bruscalupi, F. Piemonte, N. Panera, R. De Vito, S. Leoni, G. F. Bottazzo, A. Masotti, and V. Nobili. 2011. Mirnome analysis reveals novel molecular determinants in the pathogenesis of diet-induced nonalcoholic fatty liver disease. Lab Invest 91(2):283-293.

Arora, A. S., DA. 2008. Individual mRNA expression profiles reveal the effects of specific microRNAs. Genome Biol 9(5):R82.

Batistel, F., J. M. Arroyo, A. Bellingeri, L. Wang, B. Saremi, C. Parys, E. Trevisi, F. C. Cardoso, and J. J. Loor. 2017. Ethyl-cellulose rumen-protected methionine enhances performance during the periparturient period and early lactation in Holstein dairy cows. J Dairy Sci 100(9):7455-7467.

Bell, A. W. 1995. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. J Anim Sci 73(9):2804-2819.

Bionaz, M., K. Periasamy, S. L. Rodriguez-Zas, W. L. Hurley, and J. J. Loor. 2012. A novel dynamic impact approach (DIA) for functional analysis of time-course omics studies: validation using the bovine mammary transcriptome. PLoS One 7(3):e32455.

Cheung, O., P. Puri, C. Eicken, M. J. Contos, F. Mirshahi, J. W. Maher, J. M. Kellum, H. Min, V. A. Luketic, and A. J. Sanyal. 2008. Nonalcoholic steatohepatitis is associated with altered hepatic MicroRNA expression. Hepatology 48(6):1810-1820.

Dann, H. M., D. E. Morin, G. A. Bollero, M. R. Murphy, and J. K. Drackley. 2005. Prepartum intake, postpartum induction of ketosis, and periparturient disorders affect the metabolic status of dairy cows. J Dairy Sci 88(9):3249-3264.

Drackley, J. K. 1999. ADSA Foundation Scholar Award. Biology of dairy cows during the transition period: the final frontier? J Dairy Sci 82(11):2259-2273.

Durand, D., Y. Chilliard, and D. Bauchart. 1992. Effect of lysine and methionine on in vivo hepatic secretion of VLDL in the high yield- ing dairy cow. J. Dairy Sci 25(Suppl.1)(279).

Forman, H. J., H. Zhang, and A. Rinna. 2009. Glutathione: overview of its protective roles, measurement, and biosynthesis. Mol Aspects Med 30(1-2):1-12.

Iravani, F., N. Hosseini, and M. Mojarrad. 2018. Role of MicroRNAs in Pathophysiology of Non-alcoholic Fatty Liver Disease and Non-alcoholic Steatohepatitis. Middle East J Dig Dis 10(4):213-219.

Khan, M. J., C. B. Jacometo, D. E. Graugnard, M. N. Correa, E. Schmitt, F. Cardoso, and J. J. Loor. 2014. Overfeeding Dairy Cattle During Late-Pregnancy Alters Hepatic PPARalpha-Regulated Pathways Including Hepatokines: Impact on Metabolism and Peripheral Insulin Sensitivity. Gene Regul Syst Bio 8:97-111.

Liang, Y., F. Batistel, C. Parys, and J. J. Loor. 2019. Glutathione metabolism and nuclear factor erythroid 2-like 2 (NFE2L2)-related proteins in adipose tissue are altered by supply of ethyl-cellulose rumen-protected methionine in peripartal Holstein cows. J Dairy Sci 102(6):5530-5541.

Lima, F. S., M. F. Sa Filho, L. F. Greco, and J. E. Santos. 2012. Effects of feeding rumen-protected choline on incidence of diseases and reproduction of dairy cows. Vet J 193(1):140-145.

Loor, J. J., R. E. Everts, M. Bionaz, H. M. Dann, D. E. Morin, R. Oliveira, S. L. Rodriguez-Zas, J. K. Drackley, and H. A. Lewin. 2007. Nutrition-induced ketosis alters metabolic and

signaling gene networks in liver of periparturient dairy cows. Physiol Genomics 32(1):105-116.

Martinov, M. V., V. M. Vitvitsky, R. Banerjee, and F. I. Ataullakhanov. 2010. The logic of the hepatic methionine metabolic cycle. Biochim Biophys Acta 1804(1):89-96.

Moisa, S. J., D. W. Shike, L. Shoup, and J. J. Loor. 2016. Maternal Plane of Nutrition During Late-Gestation and Weaning Age Alter Steer Calf Longissimus Muscle Adipogenic MicroRNA and Target Gene Expression. Lipids 51(1):123-138.

Mulligan, F. J. and M. L. Doherty. 2008. Production diseases of the transition cow. Vet J 176(1):3-9.

NRC. 2001. Nutrient Requirements of Dairy Cattle. 7th ed. Natl. Acad. Press, Washington, DC.

Osorio, J. S., P. Ji, J. K. Drackley, D. Luchini, and J. J. Loor. 2013. Supplemental Smartamine M or MetaSmart during the transition period benefits postpartal cow performance and blood neutrophil function. J Dairy Sci 96(10):6248-6263.

Osorio, J. S., P. Ji, J. K. Drackley, D. Luchini, and J. J. Loor. 2014. 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. J Dairy Sci 97(12):7451-7464.

Schmidt, J., P. Sipocz, E. Cenkvari, and J. Sipocz. 1999. Use of protected methionine (Mepron M 85) in cattle. Acta Vet Hung 47(4):409-418.

Schwab, C. G., C. K. Bozak, N. L. Whitehouse, and M. M. Mesbah. 1992. Amino acid limitation and flow to duodenum at four stages of lactation. 1. Sequence of lysine and methionine limitation. J Dairy Sci 75(12):3486-3502.

Sun, F., Y. Cao, C. Cai, S. Li, C. Yu, and J. Yao. 2016. Regulation of Nutritional Metabolism in Transition Dairy Cows: Energy Homeostasis and Health in Response to Post-Ruminal Choline and Methionine. PLoS One 11(8):e0160659.

Vailati-Riboni, M. P., V.; & Loor, J. J. 2017. The omics side of fatty liver: A holistic approach for a commonly occurring peripartal disease. . Periparturient Diseases of Dairy Cows: A Systems Biology Approach Springer International Publishing:223-246.

Zhou, Z., T. A. Garrow, X. Dong, D. N. Luchini, and J. J. Loor. 2017. Hepatic Activity and Transcription of Betaine-Homocysteine Methyltransferase, Methionine Synthase, and Cystathionine Synthase in Periparturient Dairy Cows Are Altered to Different Extents by Supply of Methionine and Choline. J Nutr 147(1):11-19.

Zhou, Z., M. Vailati-Riboni, E. Trevisi, J. K. Drackley, D. N. Luchini, and J. J. Loor. 2016. Better postpartal performance in dairy cows supplemented with rumen-protected methionine compared with choline during the peripartal period. J Dairy Sci 99(11):8716-8732.

CHAPTER 4: BODY CONDITION SCORE IN LATE PREGNANCY IMPACTS HEPATIC BIOMARKERS OF ENERGY METABOLISM

ABSTRACT

We aimed to verify the expression miRNA in liver and evaluate any relationships they may have with oxidative phosphorylation and fatty acid metabolism. Additionally, our objective was to identify any associations between body condition score (BCS) in late-pregnancy, plasma biomarkers, and protein abundance linked to energy metabolism. Twenty-six multiparous Holstein dairy cows (n= 13/BCS group) were retrospectively selected according to BCS at 4 wk prepartum and divided into two treatments: HiBCS (BCS ≥ 3.50) or LoBCS (BCS ≤ 3.25). In the prepartum, LoBCS cows had greater DMI compared to HiBCS cows ($P = 0.06$). In the postpartum, LoBCS continued to have greater DMI, but HiBCS cows averaged 5.34 kg/d greater in milk yield comparatively. Plasma sampled at d −30, −10, 7, 15, and 30, relative to calving indicated increased concentrations of biomarkers related to negative energy balance in HiBCS cows. In contrast, LoBCS had greater plasma concentrations of biomarkers that have antioxidant properties, thus suggesting a potential relationship with oxidative stress. Liver tissue harvested via biopsy at −15, 7, and 30 d relative to calving and used to evaluate protein abundance via western blotting. LoBCS cows had increased abundance of proteins associated with glutathione metabolism, an antioxidant that is capable of preventing overloads of reactive oxygen species (ROS), compared with HiBCS. miR-369 5p, miR-186, and miR-200b, which downregulate fatty acid metabolism and oxidative phosphorylation, had decreased expression in the liver of HiBCS cows, which is contrary to the patterns of decreased oxidative stress in LoBCS cows we observed in plasma biomarkers and protein abundance. In conclusion, the differences between production data, blood biomarkers, miRNA expression, and protein abundance underscore a potentially

important interaction between energy metabolism in relation to BCS during the periparturient period.

INTRODUCTION

During the periparturient period dairy cows mobilize their adipose stores to meet lactation needs, at a time where dry matter intake (DMI) lags behind milk production, oftentimes creating a negative energy balance (NEB) and disrupting fatty acid metabolism (Drackley, 1999). When nutrient supply is depleted, adipose tissue mobilizes non-esterified fatty acids (NEFA) which originate from the hydrolysis of triacylglycerol (TAG) (Bell, 1995). Hepatic lipidosis results from excessive accumulation of TAG in the liver which ultimately impairs normal function.

Oxidative phosphorylation is essential for creating energy for various biological functions. However, it also produces reactive oxygen species (ROS) or free radicals, which can be destructive to an animal (Wallace et al., 1992). When the rate at which ROS can be removed or repaired becomes imbalanced, oxidative stress and inflammation begin. In nonruminants, nuclear factor erythroid 2-like 2 (NFE2L2) (formerly known as Nrf2), is a key transcription factor that controls oxidative stress by increasing the concentration of antioxidant enzymes, a mechanism of critical importance for cellular protection and survival (Kaspar et al., 2009). Glutathione (GSH), an antioxidant, reduces the levels of ROS to prevent free radical damage and its metabolism is one of the target pathways regulated by NFE2L2 (Bayir, 2005). In mammary epithelial cell culture, several antioxidants activate NFE2L2, causing an increase in the transcription of antioxidants overall (Jin et al., 2016). In summation, this suggests that both NFE2L2 and GSH, play a key role in regulating antioxidant networks.

Importance has been placed on the quantification of the animal's adipose reserves as a measure of animal health. Body condition score (BCS) is a useful tool as it evaluates both the amount of subcutaneous fat in the body and indicates the nutritional status of an animal (Roche et al., 2009). In the United States, BCS are evaluated on a 5-point scale, where 1 indicates a thin or emaciated cow, while a 5 equates to an over-conditioned obese cow (Roche et al., 2004). High BCS cows are at a greater risk of developing metabolic disorders and impaired immune function, compared to cows with lower body condition score cows (Reid et al., 1986, Bernabucci et al., 2005b, Schulz et al., 2014). Therefore, BCS has a close relationship with metabolic disorders and is a useful tool in dairy cow management.

microRNAs (miRNAs) are short non-coding RNAs that regulate gene expression extensively (Iravani et al., 2018). Previous in silico work relevant to fatty liver disease and ketosis revealed miRNAs may play a role in fatty acid metabolism (miR-186, -200b, -369), oxidative phosphorylation (miR-186, -200b, -369), peroxisome (miR-186, -200b), gluconeogenesis (miR-186, -369), peroxisome proliferator-activator receptor (PPAR) signaling pathway (miR-186, -200b), insulin signaling pathway (miR-186, -200b, -369), and apoptosis (miR-200b, -369) (Vailati-Riboni, 2017). We aimed to verify the expression of these miRNA in liver and evaluate any relationships they may have with oxidative phosphorylation and fatty acid metabolism. Additionally, our objective was to identify any associations between body condition score (BCS) in late-pregnancy, plasma biomarkers, and protein abundance linked to energy metabolism.

METHODS

Experimental Design and Treatments

All procedures for this study were conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee at the University of Illinois (Urbana; protocol no. 17168). Twenty-six multiparous Holstein dairy cows (n= 13/BCS group) were retrospectively selected from a larger study that had 4 experimental treatments related to 1-carbon metabolism. In the present study, cows were divided according to BCS at 4 wk prepartum and into two treatments: HiBCS (BCS $\geq$ 3.50, 3.75± 0.22, mean ± SD) or LoBCS (BCS $\leq$ 3.25, 3.07± 0.09, mean ± SD). BCS was evaluated weekly (-4 wk to 4 wk) by the mean score of 3 trained observers on a 5-point scale. All cows had ad libitum access to the same diet; from -45 to -29 d cows were fed a typical Midwestern far-off diet, from −28 d to calving cows received a close-up diet (1.37 Mcal/kg of DM, 8.45% RDP, and 6.05% RUP), and from calving until 30 DIM they received a lactation diet (1.65 Mcal/kg of DM, 11% RDP, and 6% RUP). More detailed ingredient and nutrient composition can be found in Table 4.1 and 4.2. Cows were fed as a total mixed ration (TMR) once daily (0700 h). Dry cows were housed in a free-stall barn with an individual Calan gate feeding system (American Calan, Northwood, NH, USA) and they had access to sand-bedded free stalls. After calving, cows were housed in a tie-stall barn and were fed a common lactation TMR in their individual feed bunks once daily in the morning, and milked 3 times daily (at 0500, 1300, 2300 h). DMI and milk yield were recorded daily. Diets were formulated to meet predicted requirements for dairy cows according to (NRC, 2001)

Blood Collection and Analysis

Blood was sampled from the coccygeal vein in the morning before cows had access to feed at d -30, -10, 7, 15, and 30 relative to calving. 13 cows in each treatment were used for analysis. Samples were collected into vacutainer tubes containing lithium heparin (BD Vacutainer, BD and Co., Franklin Lakes, NJ) and placed on ice. Plasma was obtained by

centrifugation at 2,000 x g for 15 min at 4°C and aliquots stored at -80°C until further analysis. Glucose, haptoglobin, myeloperoxidase, and ROM and γ-glutamyl transferase were analyzed using the IL Test purchased from Instrumentation Laboratory Spa (Werfen Co., Milan, Italy) in the ILAB 600 clinical auto analyzer (Instrumentation Laboratory, Lexington, MA). Fatty acids and BHB were measured using kits from Wako Chemicals (Richmond, VA) and Randox Laboratories Ltd. (Crumlin, United Kingdom), respectively, following the procedures described previously (Bionaz et al., 2007, Trevisi et al., 2012).

Liver Biopsy

Liver tissue was sampled from 13 cows in each group on d -15, 7, and 30, relative to calving. A similar technique described previously by Vailati Riboni et al. (2015), was used to perform the biopsy. Briefly, the skin was shaved and disinfected and the skin and body wall were anesthetized with 7 mL of 2% lidocaine HCL (VetOne, Boise, ID). A stab incision was made through the skin in the right 11th intercostal space, through which an 18-gauge by 10.2-cm bone marrow probe (Monoject-8881-247087; Medtronic, Minneapolis, MN) was inserted into the liver and used to obtain approximately 4 g (wet weight) of liver. No more than three separate penetrations with the biopsy probe were performed. Samples were snap frozen in liquid nitrogen, and subsequently stored at -80°C. Health was monitored for 7 d after each procedure.

Western Blot Analysis

Total protein was extracted from (~0.05 g) of liver tissue using a tissue protein extraction reagent (catalog no. 78510; Thermo Fisher Scientific) containing Halt protease and phosphatase inhibitor cocktail (100×, catalog no. 78442; Thermo Fisher Scientific). The concentration of total protein was determined using a Nano-Drop ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA). Protein samples were denatured by heating at 95°C for 5

min before loading 75 μg of protein into each lane of a 4–20% SDS-PAGE gel (catalog no. 4561096; Bio-Rad, Hercules, CA). Reactions were run for 10 min at 180 V, and then for 45 to 60 min at 110 V. After activating a polyvinylidene fluoride membrane (catalog no. 1620261; Bio-Rad) with methanol for 1 min, the protein sample was transferred to the membrane in a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (catalog no. 170-3940; Bio-Rad). Membranes were then blocked in 1× Tris-buffered saline (TBST) containing 5% nonfat milk for 2 h at room temperature. The membranes were then incubated in 1× TBST containing primary antibodies to glutathione peroxidase 3 (GPX3), glutathione S-transferase mu 1 (GSTM1), glutathione S-transferase α 4 (GSTA4), NFE2L2, and phospho-NFE2L2 (Ser40) [catalog number and dilution ratio are included in Supplemental Table B.1. (Appendix B)] overnight at 4°C. The membranes were then washed 6 times with 1× TBST and incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibodies (catalog no. 7074S; dilution 1:2,000; Cell Signaling Technology, Danvers, MA) for 1 h at room temperature. Subsequently, the membranes were washed 6 times with 1× TBST and then incubated with enhanced chemiluminescence reagent (catalog no. 170-5060; Bio-Rad) for 3 min in the dark prior to image acquisition. GAPDH (catalog no. 2118S; Cell Signaling Technology) was used as the internal control. Images were acquired using the ChemiDOC MP Imaging System (Bio-Rad). The intensities of the bands were measured with Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD). Specific target protein band density values were normalized to GAPDH density values. Representative blots are included in Supplemental Figure B.1. (Appendix B).

Total RNA Extraction, Target miRNA, and qPCR

RNA was extracted from tissue using protocols established in our laboratory (Khan et al., 2014). Briefly, liver tissue was weighed (~0.05 g) and immediately placed in 1.2 ml of QIAzol

reagent (Qiagen 75842; Qiagen Inc., Valencia, CA) for homogenization. After homogenization, DNase was used to remove any genomic DNA from RNA using RNeasy Mini Kit columns (Qiagen, Hilden, Germany). Concentration was measured using the Nano-Drop ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA), and the Agilent Bioanalyzer system (Agilent 2100 Bioanalyzer, Agilent Technologies, Santa Clara, CA, USA) was used to measure RNA quality. miRNA-specific cDNA synthesis was performed using the Quanta qScript microRNA cDNA Synthesis Kit (Quanta BioSciences, Inc.), following the manufacturer's protocols. The quantitative PCR (qPCR) was SYBR Green-based and performed as described previously by Osorio et al. (2014), using a 7-point standard curve. miRNA selected for expression profiling were miR-369 5p, miR-186, and miR-200b, because previous research found they are involved in a variety of biological pathways, verified by being expressed in liver tissue (Bucktrout, unpublished 2018, Vailati-Riboni et al., 2015). The final data were normalized using the geometric mean of, miR-let-7b, miR-16b and miR-181a, which served as the internal controls. Measured miRNA assay primer sequence information and qPCR performance are included in Supplemental Table B.2. and B.3. (Appendix B). Percentage relative miRNA abundance in relation to the 3 miRNA targets is in Supplemental Figure B.2. (Appendix B)

Statistical Analysis

Data were $\log_2$ transformed prior to statistical analysis to obtain a normal distribution. Statistical analysis was performed using the MIXED procedure of SAS v.9.4 (SAS Institute Inc., Cary, NC) according to the following model with repeated measures:

$$Y_{jl} = \mu + M_j + T_l + MT_{jl} + e_{jl},$$

Where Y_{jl} = dependent, continuous variable, μ = overall mean, M_j = fixed effect of treatment (j = HiBCS vs. LoBCS), T_l = fixed effect of time, MT_{jl} = interaction between treatment and time, and

e_{jl} = residual error. Cow, nested within treatment, was the random effect. The production data were analyzed separately for the prepartum (d -28 to -1) and postpartum (from 1 to 30 d postpartum) periods. The exponential correlation covariance structure [SP(POW)] for repeated measures was used in analysis where time intervals are not evenly spaced such as blood biomarkers, miRNA abundance, and protein abundance. The covariance structure [(AR)(1)] was used for analysis when time was evenly spaced such as, DMI and milk yield. The Kenward-Roger statement was used for computing the denominator degrees of freedom. Data were considered significant at a $P \leq 0.05$ and tendencies at $P \leq 0.10$ using the PDIFF statement in SAS. For ease of interpretation, data reported in tables and figures are the $\log_2$ back-transformed LSM that resulted from the statistical analysis. Normality of the residuals was checked with normal probability and box plots, and homogeneity of variances was checked with plots of residuals versus predicted values.

RESULTS AND DISCUSSION

Dry Matter Intake and Milk Production

In the prepartum, LoBCS cows had greater DMI compared to HiBCS (P =0.06) (Fig. 4.1). Overall, both groups of cow's DMI gradually declined in the 3 weeks before parturition. HiBCS had a steeper decline in DMI, perhaps suggesting they were more inclined to enter NEB after pregnancy, compared with LoBCS. Many authors report a decrease in DMI before calving, most likely due cows entering NEB, reduced rumen volume, and hormonal responses to pregnancy (Zhou et al., 2016, Batistel et al., 2017, Alharthi et al., 2018). Despite the fact that BCS is linked with the metabolic response to lactation and its level is regulated through nutrition, cows with different levels of adipose deposits are generally managed similarly during the

prepartum period. For a producer to divide cows based on BCS before calving and design different diets based on energy requirements seems unpractical. However, it would be interesting to examine if the incidence of metabolic disorders in the postpartum decrease.

In the immediate 2 weeks after calving, LoBCS cows had greater DMI compared to HiBCS cows ($P \leq 0.05$) (Fig. 4.2). As both treatments approached around twenty DIM, there were no major differences in DMI through the remainder of the transition period. This is consistent with the literature since cows with higher body condition scores tend to eat less postpartum, thus receive less energy supply compared to low body condition score cows (Roche et al., 2008). Thereby causing a ripple effect to increase tissue mobilization of NEFA to the liver, leading to a greater risk of developing metabolic disorders like ketosis and fatty liver in the postpartum (Cameron et al., 1998).

Overall milk production was 5.34 kg/d greater ($P \leq 0.05$) in HiBCS compared with LoBCS cows (Fig. 4.3). The effect of BCS on milk yield in the literature is conflicting. Many authors report a negative relationship between BCS and milk yield (Roche et al., 2009). Though HiBCS had less intake before and after calving, they produced more milk suggesting they were in an even greater NEB than predicted. Another study found that high BCS cows (BCS = 4.00 ± 0.20) had greater milk yield compared with medium BCS cows (BCS = 3.25 ± 0.25) from calving to 180 DIM (Zahrazadeh et al., 2018). Similarly, cows grouped by high BCS (5.5) and medium BCS (4.5) had greater milk yield than low BCS cows (3.5) surrounding parturition (Akbar et al., 2015). Although, Zhao et al. (2019), found that there is no difference in milk yield among medium BCS (3.0-3.25), high BCS (3.5-3.75), higher BCS (4.0-4.25), and highest BCS (4.5-5.0) cows during the first 100 DIM. However, it is likely that the differences between studies are caused by the length of DIM observed and different BCS cut-offs for high vs. low.

Future studies could explore a treatment design that span high, optimal, and low BCS intervals.

Plasma Biomarkers

Cows in HiBCS had greater overall plasma concentrations of biomarkers relevant to energy balance such as NEFA ($P \leq 0.05$) and a tendency for increased BHBA ($P = 0.07$), compared to LoBCS (Table 4.3). It is well established that cows with higher BCS and greater BCS loss have increased concentrations of NEFA and BHBA, making them sensitive to oxidative stress (Rukkwamsuk et al., 1998, Bernabucci et al., 2005a). We observed a similar trend whereby, HiBCS before calving were still greater conditioned than LoBCS throughout -4 to 4 wk (Fig. 4.5). However, HiBCS had around 0.44 greater BCS loss compared to LoBCS cows when comparing only wk -4 to wk 4 (Fig. 4.6). Furthermore, myeloperoxidase, a biomarker involved in inflammation, had greater plasma concentrations in HiBCS ($P \leq 0.05$) (Table 4.3). Our results are consistent with another study that found energy partitioning between peripheral tissues likely increases plasma NEFA and lipid peroxidation, but may reduce the antioxidant capacity of high producing dairy cows (Castillo et al., 2005). The higher concentration of biomarkers that contribute to energy balance and inflammation in HiBCS cows may suggest that they are experiencing a NEB.

LoBCS had greater overall plasma concentrations of an inflammation biomarker, alkaline phosphatase, including greater concentrations at d -30 and -10 relative to calving compared to HiBCS cows ($P \leq 0.05$) (Fig. 4.4). However, myeloperoxidase, another biomarker involved in inflammation, had greater plasma concentrations in HiBCS ($P \leq 0.05$) (Table 4.3). It is plausible that both HiBCS and LoBCS have greater concentrations of inflammation biomarkers because of the increased energy demands surrounding calving.

β-carotene is a lipid soluble antioxidant that plays an important role in health and immunity in transition cows (Spears and Weiss, 2008). We found that β-carotene ($P \leq 0.05$) had greater overall plasma concentrations in LoBCS vs. HiBCS, and concentrations were greatest at d -30 and -10 relative to calving ($P \leq 0.05$) compared with HiBCs cows (Fig. 4.4). Tocopherol plasma concentrations were greater ($P \leq 0.05$) in LoBCS vs. HiBCS cows, marked by greater concentrations at d -30 and 15 in LoBCS cows (Fig. 4.4). β-carotene and tocopherol work in synergy as antioxidants to reduce oxidative stress (Ryan et al., 2010). FRAP is another indicator of antioxidant, or reducing ability, in oxidative stress (Benzie and Strain, 1996). We observed that LoBCS cows FRAP concentration had a tendency ($P = 0.06$) to be greater at 30 d relative to calving compared with HiBCS (Fig. 4.4). This suggests that compared to HiBCS cows, LoBCS were under less oxidative stress.

Protein Abundance

We were unable to detect any protein abundance of nuclear factor erythroid 2-like 2 (NFE2L2) or p-NFE2L2 (both are involved in antioxidant signaling pathways) at any time point or treatment in liver tissue as represented by blots in Supplemental Figure B.1 (Appendix B). This could be attributed to the fact that in western blotting we use antibodies that are guaranteed to react in human, mouse and rat models, but are only predicted to react in cows. Most work involving NFE2L2 in cows is focused on the gene networks in mammary tissue (Han et al., 2018, Ma et al., 2018). In adipose tissue genes related to NFE2L2 are highly upregulated in liver tissue (Liang et al., 2019).

The enzymes GSTM1, GSTA4, and GPX3 play essential roles in inactivating pro-oxidant products such as H_2O_2 and oxygen radicals in various tissues (Lee et al., 2008, Curtis et al.,

2010). Protein abundance of GSTM1 had a significant treatment by time interaction, namely due to greater abundance in LoBCS cows at d -15 relative to calving, compared to HiBCS ($P \leq 0.05$) (Fig. 4.7). Furthermore, GSTM1 overall abundance tended to be greater in LoBCS at d-15 and 7, but not 30. ($P = 0.08$) (Fig. 4.7). Though the protein abundance of GSTA4 did not have a significant relationship between treatment and time, LoBCS had overall greater abundance compared with HiBCS ($P \leq 0.05$) (Fig. 4.7). The glutathione-S-transferase enzyme family includes GSTM1 and GSTA4 which are considered to be important for detoxification of electrophiles via GSH conjugation (Strange et al., 2001). LoBCS cows had greater abundance of GPX3 compared to HiBCS ($P \leq 0.05$) (Fig. 4.7). In obese mice, GPX3 protein expression was downregulated which was speculated to increase ROS accumulation, leading to oxidative stress and metabolic disorders (Lee et al., 2008). Overall, protein abundance of 4 GSH-related targets increased in LoBCS liver, suggesting a decrease in oxidative stress, as opposed to HiBCS.

miRNA Expression

miR-186 expression had no detectable changes in the relationship between treatment and time effect ($P = 0.43$) (Fig. 4.8). This miRNA was predicted to play a role in the downregulation of fatty acid metabolism, which is relevant to cows with varying BCS during parturition as they try to meet their energy needs. Furthermore, these results were unexpected given that our previous unpublished work found decreased miR-186 expression in cows supplemented rumen-protected methionine, thus implying less fatty acid metabolism in the liver, compared to control cows. Although no detectable changes were found in the liver for this miRNA, adipose tissue expression could be an area for future study. Another study in beef cattle found that miR-186 was expressed at higher levels in adipose tissue from low backfat thickness animals, compared to high backfat thickness animals, suggesting that miRNA may have a role in adipogenesis and/or

lipid deposition in tissue (Jin et al., 2010). This underscores the need for more experimentation on miRNAs involved in energy metabolism in cows with varying levels of adipose deposits (BCS).

Abundance of miR-200b had a treatment by time effect ($P \leq 0.05$) due to a marked upregulation between −15 and 7 d followed by increased abundance at 30 d in cows with LoBCS (Fig. 4.8). miR-200b was predicted to downregulate both fatty acid metabolism and oxidative phosphorylation (Vailati-Riboni, 2017). miR-369 5p was predicted to play a role in upregulating fatty acid metabolism and downregulating oxidative phosphorylation (Vailati-Riboni, 2017). Expression of miR-369 5p was lower ($P \leq 0.05$) overall in cows with HiBCS (Fig. 4.8). Overall, miR-369 5p and -200b were both predicted to downregulate fatty acid *metabolism*. Some would infer that since they were downregulated in HiBCS liver, the tissue potentially has less fatty acids, which could suggest less incidence of NEB or oxidative phosphorylation. However, fatty acid metabolism encompasses both the degradation and synthesis of fatty acids. A cow could have a significant capacity for fat mobilization and still have no incidence of the negative health effects we observe in dairy cows surrounding parturition and vice versa.

Oxidative phosphorylation is essential in energy metabolism, but it increases ROS which can damage DNA, proteins, and lipids. Antioxidants, like glutathione, reduce the levels of ROS to prevent free radical damage (Bayir, 2005). When the rate at which ROS can be removed or repaired becomes imbalanced, oxidative stress begins. Intuitively speaking, when oxidative phosphorylation is downregulated in HiBCS they may be under less oxidative stress. However, the findings that miR-369 5p and -200b expression suggest are inconsistent with the other parameters examined in the present study, as we observed protein abundance and plasma biomarkers suggest a decrease in oxidative stress in LoBCS compared to HiBCS cows. Overall,

research on miRNA biomarkers is still a developing field, especially considering the vast majority of studies are in non-ruminant models. It is also important to recall that the miRNA functions in biological pathways were predicted in silico, which holds many limitations compared with in vivo studies. This underscores the importance of more experimentation examining how miRNAs work in regulating biological processes like fatty acid metabolism, as some results are conflicting.

CONCLUSIONS

Cows with less BCS had increased intake in the postpartum and immediately after calving compared to high BCS cows. Additionally, plasma biomarkers and protein abundance may suggest LoBCS are under less oxidative stress than HIBCS cows. Predicted miRNA potentially play a role in energy metabolism. Future in vitro studies using hepatocytes may help us to better understand the role and mechanisms for these responses.

TABLES AND FIGURES

Table 4.1. Ingredient composition of diets fed to HiBCS and LoBCS cows during close-up (−28 d to calving), and early lactation (calving to 30 d) periods.

Ingredient (% of DM)	Close-up	Lactation
Corn silage	37.47	41.18
Ground shelled corn	-	23.95
Wheat straw	36.60	2.30
Canola Meal	11.67	3.25
Soybean meal	6.30	13.12
Alfalfa hay	-	8.70
Soychlor	3.37	-
Corn gluten	2.80	2.50
Mineral Mix	0.53	4.1
Calcium sulfate	0.53	-
ProvAAL2 AADvantage	0.47	0.73

Table 4.2. Nutrient composition of prepartal and postpartal diets fed to HiBCS and LoBCS cows during the peripartal period.

Chemical component	Close-up	Lactation
NEl Mcal/kg	1.37	1.65
CP (% of DM)	14.50	17.00
RUP (% of DM)	6.05	6.00
RDP (% of DM)	8.45	11.00
NDF Forage (% of DM)	43.30	21.50
ADF (% of DM)	33.80	16.76
aNDFom (% of DM)	49.21	27.01
Starch (% of DM)	13.75	30.00
Sugar (% of DM)	5.00	4.76
Ca (% of DM)	0.66	1.00
P (% of DM)	0.33	0.35
Salt (% of DM)	0.10	0.25
Na (% of DM)	0.12	0.45
Cl (% of DM)	0.78	0.68
Mg (% of DM)	0.45	0.38
K (% of DM)	1.36	1.45
S (% of DM)	0.33	0.20

Table 4.3. Least square means and pooled SEM for plasma biomarkers in cows grouped into HiBCS and LoBCS at -30, -10, 7, 15, and 30 d relative to calving.

	Treatment			Time						*P*-values		
Parameter[1]	HiBCS	LoBCS	SEM	-30	-10	7	15	30	SEM	Treatment	Time	Trt*time
Energy Balance												
β-hydroxybutyrate, mmol/L	0.68	0.59	0.04	0.44	0.46	0.87	0.73	0.79	0.06	0.07	<.01	0.76
Glucose, mmol/L	4.12	4.09	0.05	4.54	4.50	3.74	3.84	3.88	0.07	0.70	<.01	0.79
NEFA, mmol/L	0.50[a]	0.38[b]	0.04	0.11	0.26	0.99	0.80	0.66	0.10	0.03	<.01	0.37
Liver Function												
AST, U/L	92.78	103.98	4.74	88.71	75.21	123.38	115.82	95.88	6.13	0.09	<.01	0.86
Bilirubin, µmol/L	2.77	2.23	0.42	0.54	1.11	7.57	5.47	3.84	1.16	0.31	<.01	0.98
Cholesterol, mmol/L	2.78	3.20	0.20	3.23	2.77	2.02	2.89	4.03	0.16	0.14	<.01	0.62
GGT, U/L	22.68	21.32	1.34	4.47	4.26	4.26	4.63	4.68	0.09	0.46	<.01	0.61
Paraoxonase, U/mL	80.98	84.07	4.33	93.92	86.50	66.54	77.00	88.66	4.06	0.61	<.01	0.60
Inflammation												
Alkaline phosphatase, U/L	46.69[b]	58.43[a]	4.18	50.98	50.25	56.58	53.69	49.95	3.54	0.04	0.05	0.01*
Albumin, g/L	36.30	36.11	0.35	36.61	36.13	35.53	36.14	36.62	0.37	0.71	0.06	0.51
Ceruloplasmin, µmol/L	2.94	2.92	0.08	2.50	2.46	3.23	3.26	3.18	0.08	0.89	<.01	0.52
Haptoglobin, g/L	0.32	0.30	0.02	0.27	0.28	0.50	0.33	0.24	0.04	0.33	<.01	0.79
Myeloperoxidase, U/L	512.57[a]	473.12[b]	12.10	463.90	490.09	536.69	508.69	464.85	15.69	0.03	<.01	0.25
Oxidative Stress												
FRAP, µmol/L	117.06	121.84	2.50	114.28	107.55	127.50	126.82	122.24	3.55	0.18	<.01	0.06*
ROM, H2O2/100 mL	14.67	14.47	0.31	13.47	13.01	15.76	15.53	15.08	0.38	0.64	<.01	0.80
β-carotene, mg/100 mL	0.20[b]	0.26[a]	0.02	0.35	0.29	0.14	0.15	0.21	0.02	0.03	<.01	0.01*
Retinol, µg/mL	27.25	30.08	1.74	33.69	28.39	22.04	26.76	32.45	1.66	0.26	<.01	0.30
Tocopherol, µg/mL	3.20[b]	3.83[a]	0.20	4.12	3.36	2.37	3.20	4.50	0.22	0.03	<.01	<.01*

*Means between treatments differ at a specific time point (TRT x Time $P \leq 0.05$, Tendency $P \leq 0.10$) (See Fig. 4.4).

[a,b] Means between treatments differ (TRT $P \leq 0.05$).

[1]NEFA = non-esterified fatty acids, AST = aspartate aminotransferase, GGT = γ-glutamyl transferase, FRAP = ferric-reducing ability of plasma, ROM = reactive oxygen metabolites

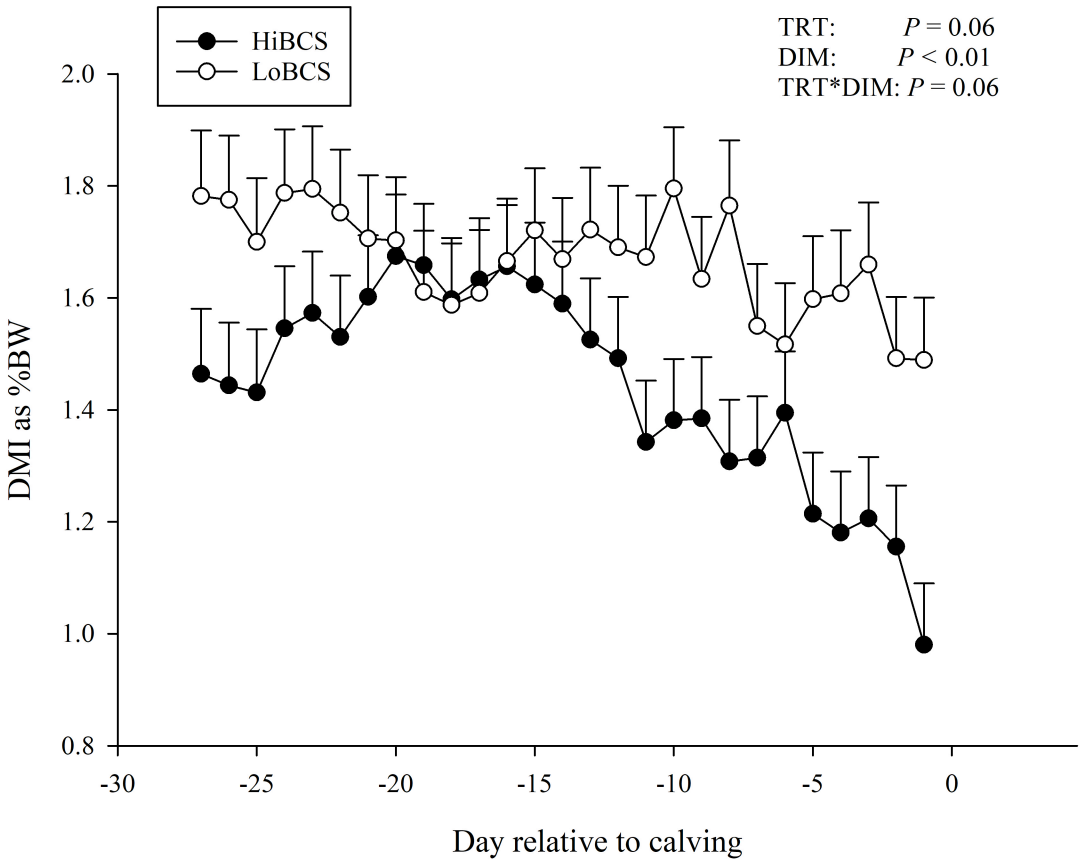


Figure 4.1. Dry matter intake (DMI) as % body weight (BW) (least square means ± pooled SEM) of HiBCS and LoBCS cows in the prepartum (-28 d to parturition).

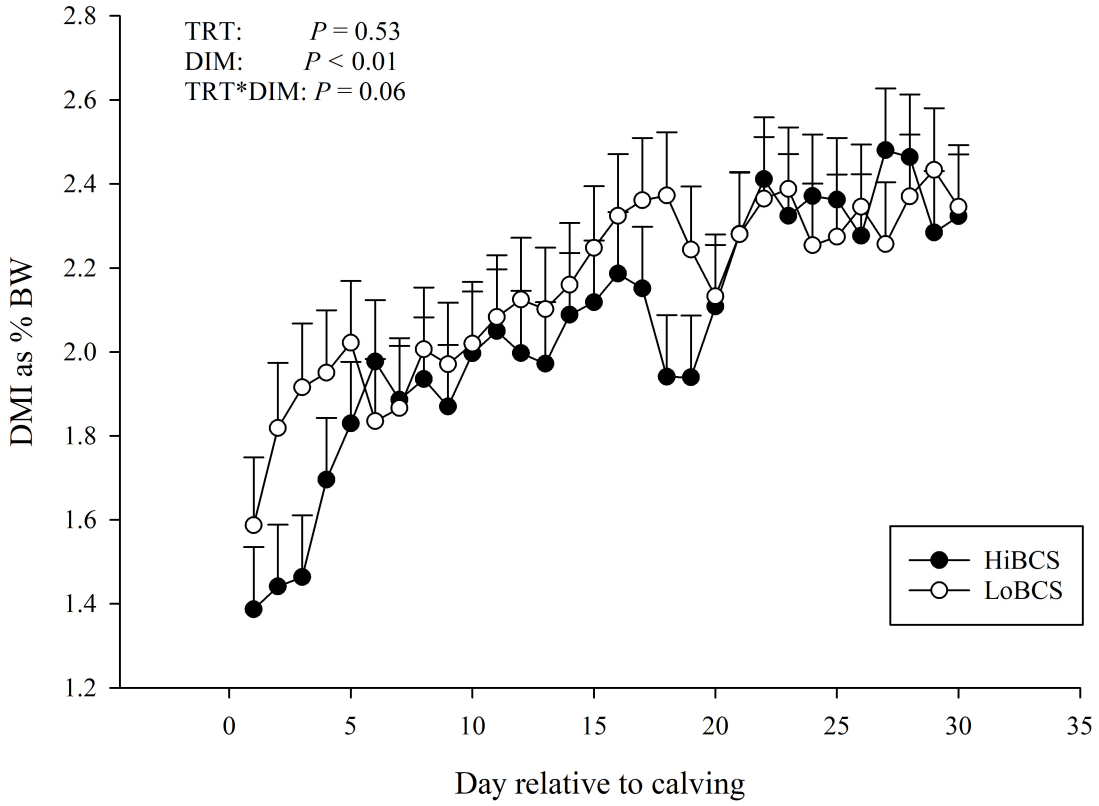


Figure 4.2. Dry matter intake (DMI) as % body weight (BW) (least square means ± pooled SEM) of HiBCS and LoBCS cows during early lactation (1 to 30 days relative to calving).

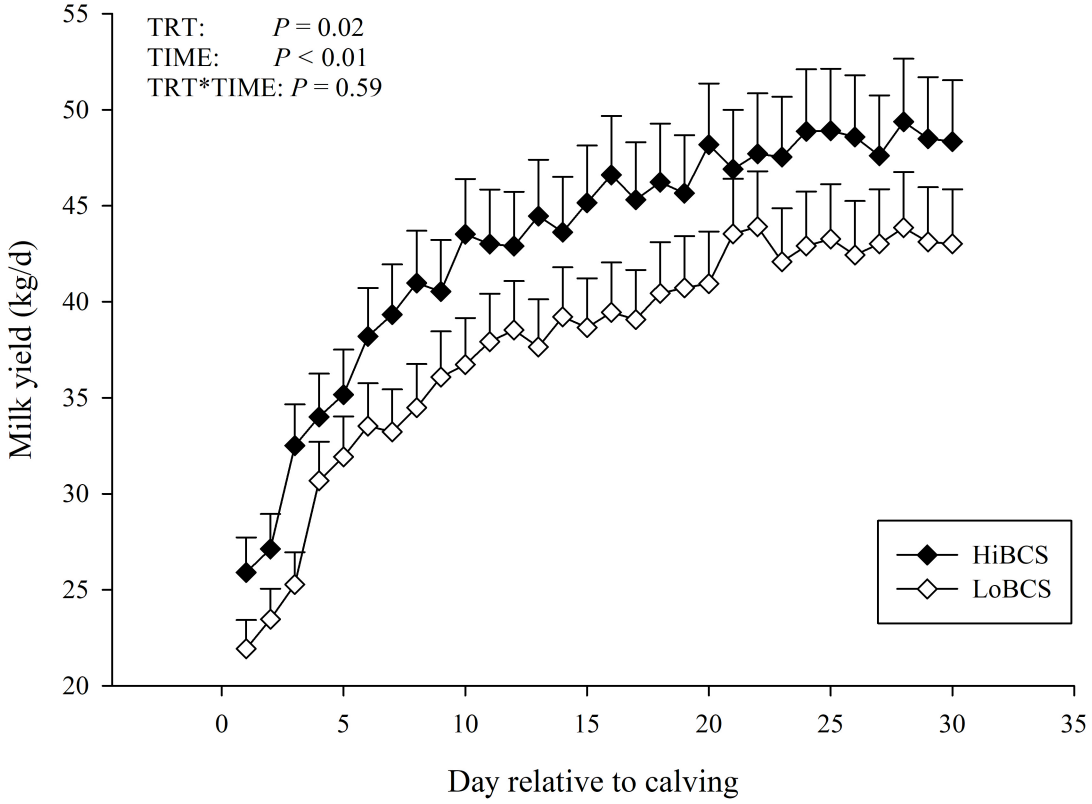


Figure 4.3. Milk yield (kg/d) (least square means ± pooled SEM) of HiBCS and LoBCS cows from 1 to 30 days relative to parturition.

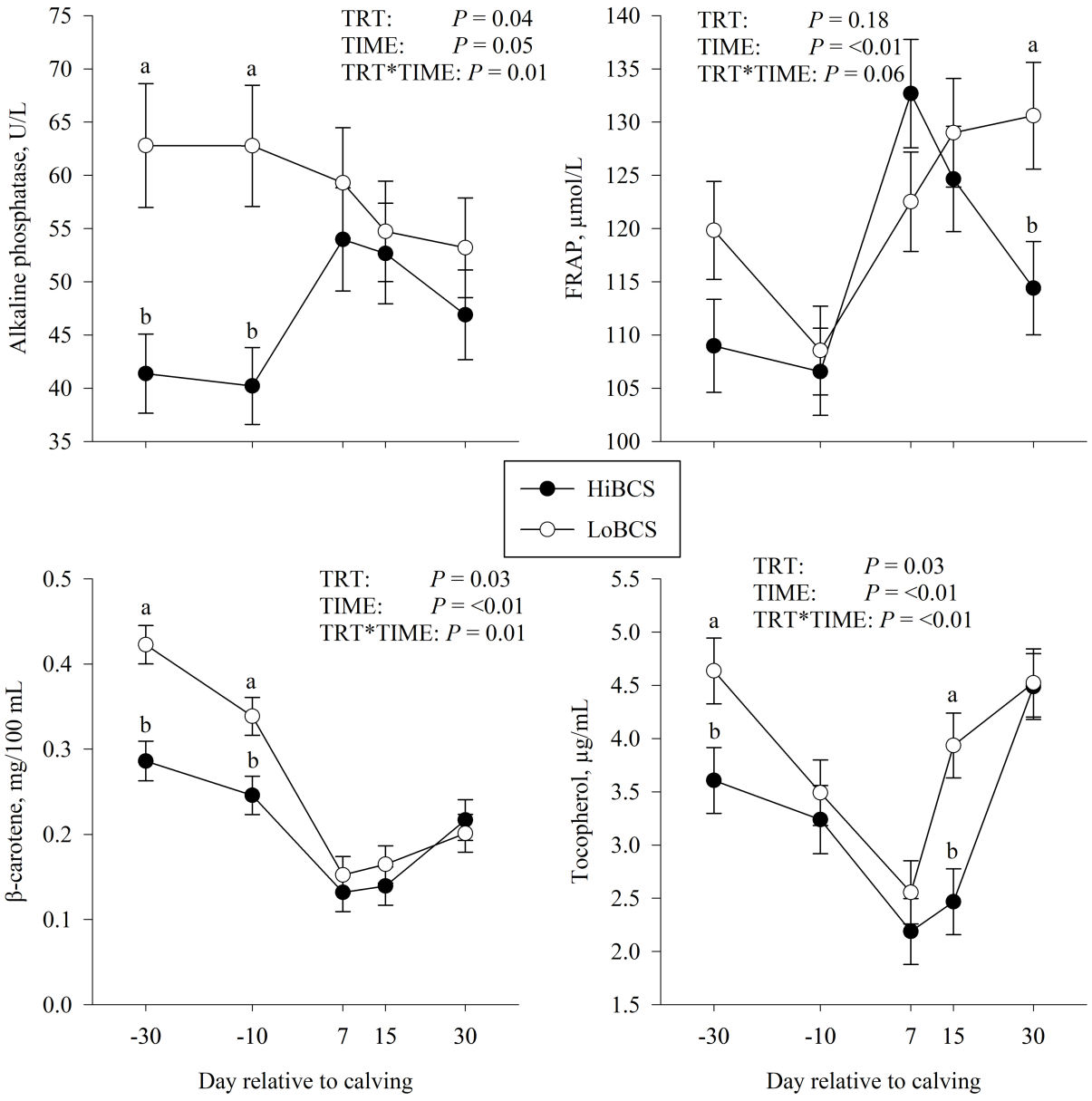


Figure 4.4 Plasma concentrations of indicators of inflammation (alkaline phosphatase) and oxidative stress (FRAP[1], β-carotene, and tocopherol) in HiBCS vs. LoBCS cows at -30, -10, 7, 15, and 30 d relative to parturition (least square means ± pooled SEM).

[a,b] Means between treatments differ at the given time point (TRT x Time $P \leq 0.05$, Tendency $P \leq 0.10$).
[1]FRAP = ferric-reducing ability of plasma

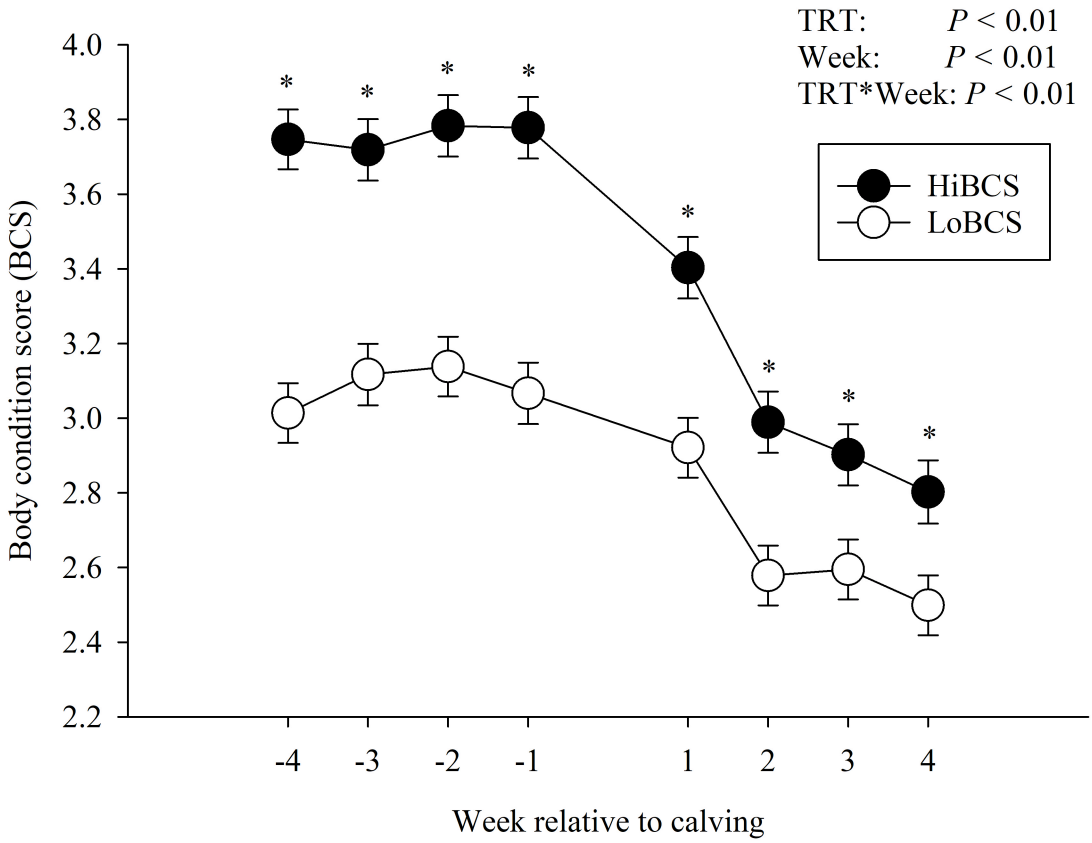


Figure 4.5. Body condition scores (BCS) from -4 wk to 4 wk relative to calving in HiBCS and LoBCS cows.

* indicates a significant treatment by week effect ($P \leq 0.05$).

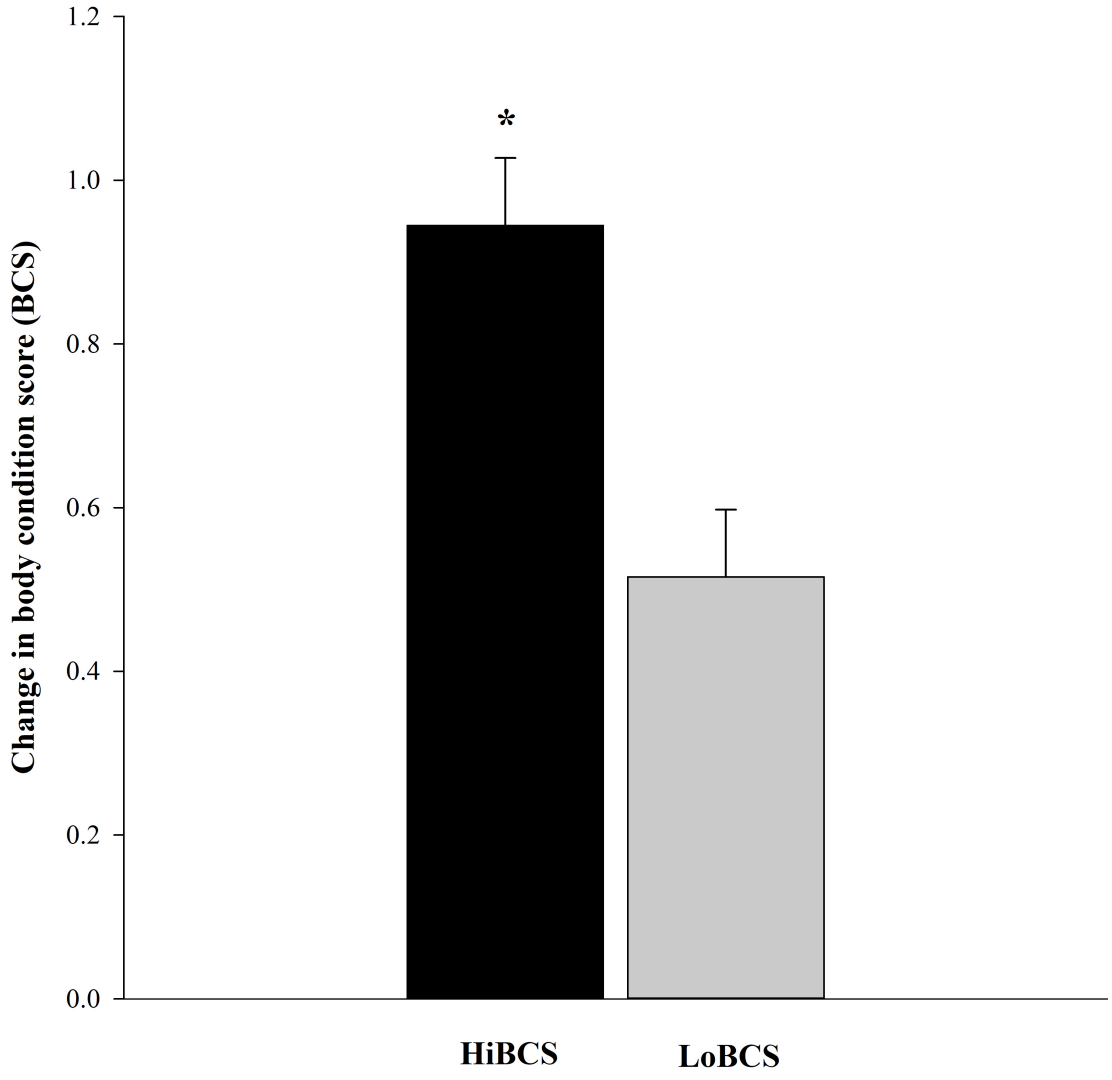


Figure 4.6. Change in body condition score (BCS) on a 5-point scale from -4 wk to 4 wk relative to calving in HiBCS and LoBCS cows.

* indicates significant differences ($P \leq 0.05$).

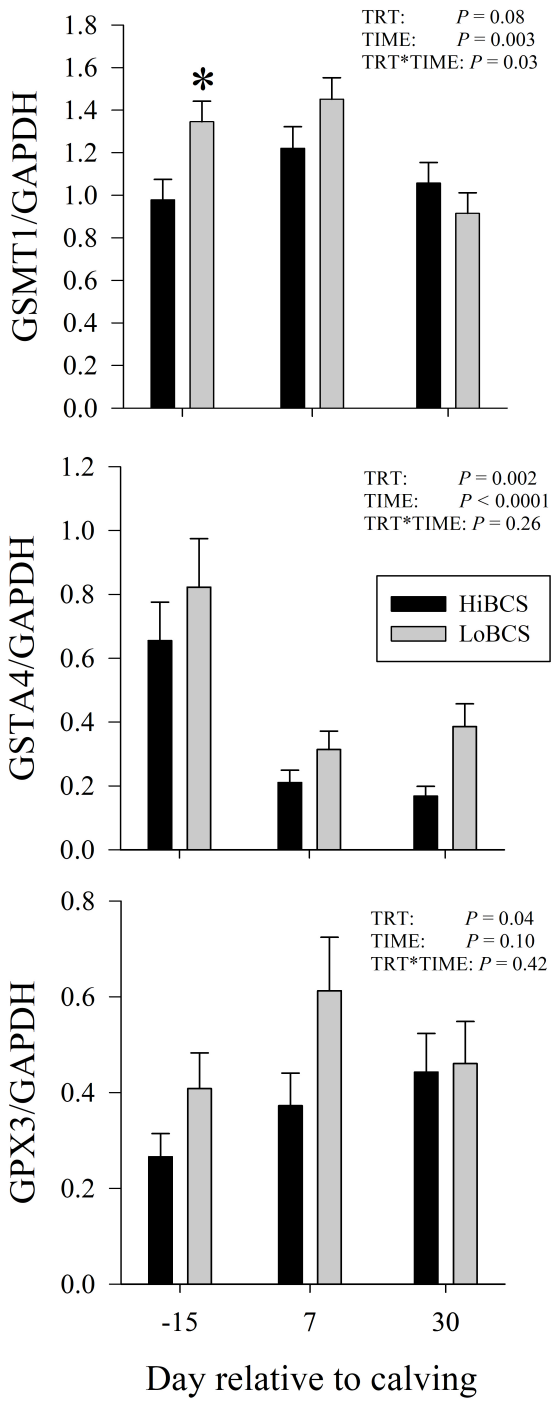


Figure 4.7. Protein abundance (relative to GAPDH) of glutathione metabolism-related enzymes (GSTM1 = glutathione S-transferase mu 1; GSTA4 = glutathione S-transferase α 4; GPX3 = glutathione peroxidase 3) in liver tissue harvested from HiBCS and LoBCS cows at -15, 7, and 30 d relative to parturition (least square means ± pooled SEM).

*Means between treatments differ at the given time point (TRT x Time $P \leq 0.05$).

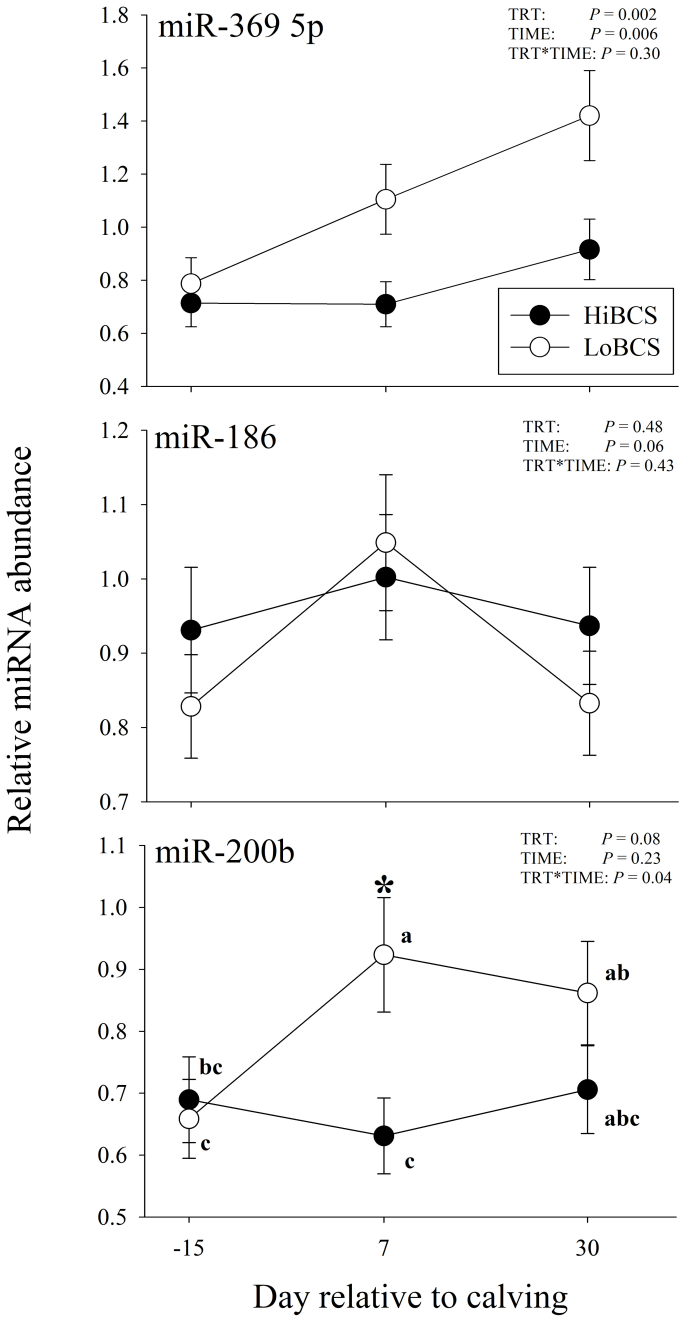


Figure 4.8. Relative miRNA abundance for 3 miRNA targets in HiBCS and LoBCS cows at -15, 7, and 30 d relative to calving (least square means ± pooled SEM).

*Means between treatments differ at the given time point (TRT x Time $P \leq 0.05$).
[a,b,c]Means within treatment differ.

REFERENCES

Akbar, H., T. M. Grala, M. Vailati Riboni, F. C. Cardoso, G. Verkerk, J. McGowan, K. Macdonald, J. Webster, K. Schutz, S. Meier, L. Matthews, J. R. Roche, and J. J. Loor. 2015. Body condition score at calving affects systemic and hepatic transcriptome indicators of inflammation and nutrient metabolism in grazing dairy cows. J Dairy Sci 98(2):1019-1032.

Alharthi, A., Z. Zhou, V. Lopreiato, E. Trevisi, and J. J. Loor. 2018. Body condition score prior to parturition is associated with plasma and adipose tissue biomarkers of lipid metabolism and inflammation in Holstein cows. J Anim Sci Biotechnol 9:12.

Batistel, F., J. M. Arroyo, A. Bellingeri, L. Wang, B. Saremi, C. Parys, E. Trevisi, F. C. Cardoso, and J. J. Loor. 2017. Ethyl-cellulose rumen-protected methionine enhances performance during the periparturient period and early lactation in Holstein dairy cows. J Dairy Sci 100(9):7455-7467.

Bayir, H. 2005. Reactive oxygen species. Crit Care Med 33(12 Suppl):S498-501.

Bell, A. W. 1995. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. J Anim Sci 73(9):2804-2819.

Benzie, I. F. and J. J. Strain. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal Biochem 239(1):70-76.

Bernabucci, U., B. Ronchi, N. Lacetera, and A. Nardone. 2005a. Influence of body condition score on relationships between metabolic status and oxidative stress in periparturient dairy cows. J Dairy Sci 88(6):2017-2026.

Bernabucci, U., B. Ronchi, N. Lacetera, and A. Nardone. 2005b. Influence of body condition score on relationships between metabolic status and oxidative stress in periparturient dairy cows. J. Dairy Sci. 88(6):2017-2026.

Bionaz, M., E. Trevisi, L. Calamari, F. Librandi, A. Ferrari, and G. Bertoni. 2007. Plasma paraoxonase, health, inflammatory conditions, and liver function in transition dairy cows. J Dairy Sci 90(4):1740-1750.

Cameron, R. E., P. B. Dyk, T. H. Herdt, J. B. Kaneene, R. Miller, H. F. Bucholtz, J. S. Liesman, M. J. Vandehaar, and R. S. Emery. 1998. Dry cow diet, management, and energy balance as risk factors for displaced abomasum in high producing dairy herds. J Dairy Sci 81(1):132-139.

Castillo, C., J. Hernandez, A. Bravo, M. Lopez-Alonso, V. Pereira, and J. L. Benedito. 2005. Oxidative status during late pregnancy and early lactation in dairy cows. Vet J 169(2):286-292.

Curtis, J. M., P. A. Grimsrud, W. S. Wright, X. Xu, R. E. Foncea, D. W. Graham, J. R. Brestoff, B. M. Wiczer, O. Ilkayeva, K. Cianflone, D. E. Muoio, E. A. Arriaga, and D. A. Bernlohr. 2010. Downregulation of adipose glutathione S-transferase A4 leads to increased protein carbonylation, oxidative stress, and mitochondrial dysfunction. Diabetes 59(5):1132-1142.

Drackley, J. K. 1999. ADSA Foundation Scholar Award. Biology of dairy cows during the transition period: the final frontier? J Dairy Sci 82(11):2259-2273.

Han, L., F. Batistel, Y. Ma, A. S. M. Alharthi, C. Parys, and J. J. Loor. 2018. Methionine supply alters mammary gland antioxidant gene networks via phosphorylation of nuclear factor

erythroid 2-like 2 (NFE2L2) protein in dairy cows during the periparturient period. J Dairy Sci 101(9):8505-8512.

Iravani, F., N. Hosseini, and M. Mojarrad. 2018. Role of MicroRNAs in Pathophysiology of Non-alcoholic Fatty Liver Disease and Non-alcoholic Steatohepatitis. Middle East J Dig Dis 10(4):213-219.

Jin, W., M. V. Dodson, S. S. Moore, J. A. Basarab, and L. L. Guan. 2010. Characterization of microRNA expression in bovine adipose tissues: a potential regulatory mechanism of subcutaneous adipose tissue development. BMC Mol Biol 11:29.

Jin, X. L., K. Wang, L. Liu, H. Y. Liu, F. Q. Zhao, and J. X. Liu. 2016. Nuclear factor-like factor 2-antioxidant response element signaling activation by tert-butylhydroquinone attenuates acute heat stress in bovine mammary epithelial cells. J Dairy Sci 99(11):9094-9103.

Kaspar, J. W., S. K. Niture, and A. K. Jaiswal. 2009. Nrf2:INrf2 (Keap1) signaling in oxidative stress. Free Radic Biol Med 47(9):1304-1309.

Khan, M. J., C. B. Jacometo, D. E. Graugnard, M. N. Correa, E. Schmitt, F. Cardoso, and J. J. Loor. 2014. Overfeeding Dairy Cattle During Late-Pregnancy Alters Hepatic PPARalpha-Regulated Pathways Including Hepatokines: Impact on Metabolism and Peripheral Insulin Sensitivity. Gene Regul Syst Bio 8:97-111.

Lee, Y. S., A. Y. Kim, J. W. Choi, M. Kim, S. Yasue, H. J. Son, H. Masuzaki, K. S. Park, and J. B. Kim. 2008. Dysregulation of adipose glutathione peroxidase 3 in obesity contributes to local and systemic oxidative stress. Mol Endocrinol 22(9):2176-2189.

Liang, Y., F. Batistel, C. Parys, and J. J. Loor. 2019. Glutathione metabolism and nuclear factor erythroid 2-like 2 (NFE2L2)-related proteins in adipose tissue are altered by supply of

ethyl-cellulose rumen-protected methionine in peripartal Holstein cows. J Dairy Sci 102(6):5530-5541.

Ma, Y. F., Z. H. Wu, M. Gao, and J. J. Loor. 2018. Nuclear factor erythroid 2-related factor 2 antioxidant response element pathways protect bovine mammary epithelial cells against H2O2-induced oxidative damage in vitro. J Dairy Sci 101(6):5329-5344.

NRC. 2001. Nutrient Requirements of Dairy Cattle. 7th ed. Natl. Acad. Press, Washington, DC.

Osorio, J. S., P. Ji, J. K. Drackley, D. Luchini, and J. J. Loor. 2014. 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. J Dairy Sci 97(12):7451-7464.

Reid, I., C. Roberts, R. Treacher, and L. Williams. 1986. Effect of body condition at calving on tissue mobilization, development of fatty liver and blood chemistry of dairy cows. Animal Science 43(1):7-15.

Roche, J. R., D. Blache, J. K. Kay, D. R. Miller, A. J. Sheahan, and D. W. Miller. 2008. Neuroendocrine and physiological regulation of intake with particular reference to domesticated ruminant animals. Nutr Res Rev 21(2):207-234.

Roche, J. R., P. G. Dillon, C. R. Stockdale, L. H. Baumgard, and M. J. VanBaale. 2004. Relationships among international body condition scoring systems. J Dairy Sci 87(9):3076-3079.

Roche, J. R., N. C. Friggens, J. K. Kay, M. W. Fisher, K. J. Stafford, and D. P. Berry. 2009. Invited review: Body condition score and its association with dairy cow productivity, health, and welfare. J Dairy Sci 92(12):5769-5801.

Rukkwamsuk, T., T. Wensing, and M. J. Geelen. 1998. Effect of overfeeding during the dry period on regulation of adipose tissue metabolism in dairy cows during the periparturient period. J Dairy Sci 81(11):2904-2911.

Ryan, M. J., H. J. Dudash, M. Docherty, K. B. Geronilla, B. A. Baker, G. G. Haff, R. G. Cutlip, and S. E. Alway. 2010. Vitamin E and C supplementation reduces oxidative stress, improves antioxidant enzymes and positive muscle work in chronically loaded muscles of aged rats. Exp Gerontol 45(11):882-895.

Schulz, K., J. Frahm, U. Meyer, S. Kersten, D. Reiche, J. Rehage, and S. Dänicke. 2014. Effects of prepartal body condition score and peripartal energy supply of dairy cows on postpartal lipolysis, energy balance and ketogenesis: an animal model to investigate subclinical ketosis. JDR 81(3):257-266.

Spears, J. W. and W. P. Weiss. 2008. Role of antioxidants and trace elements in health and immunity of transition dairy cows. The Veterinary Journal 176(1):70-76.

Strange, R. C., M. A. Spiteri, S. Ramachandran, and A. A. Fryer. 2001. Glutathione-S-transferase family of enzymes. Mutat Res 482(1-2):21-26.

Trevisi, E., M. Amadori, S. Cogrossi, E. Razzuoli, and G. Bertoni. 2012. Metabolic stress and inflammatory response in high-yielding, periparturient dairy cows. Res Vet Sci 93(2):695-704.

Vailati Riboni, M., S. Meier, N. V. Priest, C. R. Burke, J. K. Kay, S. McDougall, M. D. Mitchell, C. G. Walker, M. Crookenden, A. Heiser, J. R. Roche, and J. J. Loor. 2015. Adipose and liver gene expression profiles in response to treatment with a nonsteroidal antiinflammatory drug after calving in grazing dairy cows. J Dairy Sci 98(5):3079-3085.

Vailati-Riboni, M. P., V.; & Loor, J. J. 2017. The omics side of fatty liver: A holistic approach for a commonly occurring peripartal disease. . Periparturient Diseases of Dairy Cows: A Systems Biology Approach Springer International Publishing:223-246.

Wallace, D. C., J. M. Shoffner, R. L. Watts, J. L. Juncos, and A. Torroni. 1992. Mitochondrial oxidative phosphorylation defects in Parkinson's disease. Ann Neurol 32(1):113-114.

Zahrazadeh, M., A. Riasi, H. Farhangfar, and S. A. Mahyari. 2018. Effects of close-up body condition score and selenium-vitamin E injection on lactation performance, blood metabolites, and oxidative status in high-producing dairy cows. J. Dairy Sci. 101(11):10495-10504.

Zhao, W., X. Chen, J. Xiao, X. H. Chen, X. F. Zhang, T. Wang, Y.-G. Zhen, and G. X. Qin. 2019. Prepartum body condition score affects milk yield, lipid metabolism, and oxidation status of Holstein cows. Asian-Australas J Anim Sci.

Zhou, Z., M. Vailati-Riboni, E. Trevisi, J. K. Drackley, D. N. Luchini, and J. J. Loor. 2016. Better postpartal performance in dairy cows supplemented with rumen-protected methionine compared with choline during the peripartal period. J Dairy Sci 99(11):8716-8732.

APPENDIX A
CHAPTER 3-SUPPLEMENTAL MATERIAL

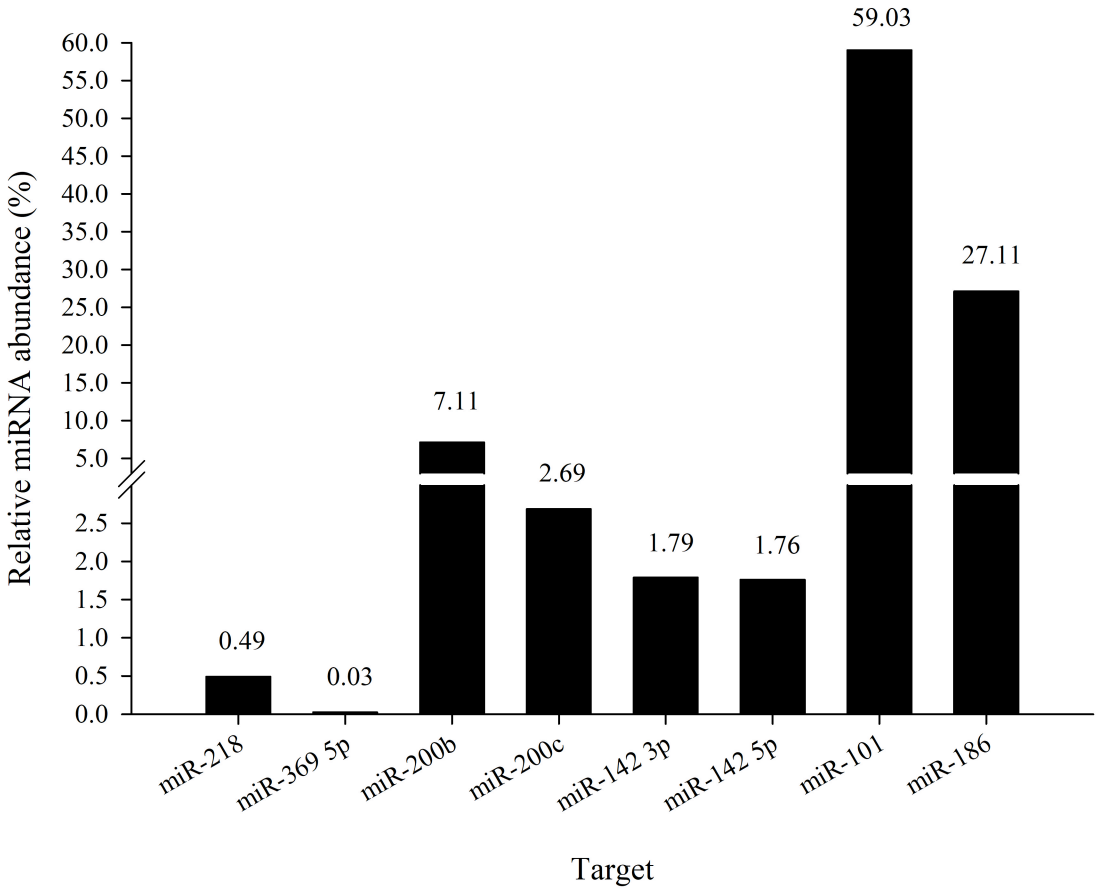
Supplemental Table A.1. Measured microRNA assay primer sequence information. A PerfeCTa Universal PCR Primer (Quanta BioSciences, Inc.) was coupled to the assay primer for expression analysis via qPCR.

miRNAs	**Sequence**
miR-218	TTGTGCTTGATCTAACCATGTG
miR-369 5p	ATCGACCGTGTTATATTCGC
miR-200b	TAATACTGCCTGGTAATGATG
miR-200c	TAATACTGCCGGGTAATGATGGA
miR-142 3p	AGTGTTTCCTACTTTATGGATG
miR-142 5p	CATAAAGTAGAAAGCACTAC
miR-101	TACAGTACTGTGATAACTGAA
miR-186	CAAAGAATTCTCCTTTTGGGCT

Supplemental Table A.2. qPCR performance of measured miRNAs.

Target	Median Ct[1]	Median ΔCt[2]	Slope[3]	(R^2)[4]	Efficiency[5]
miR-218	24.96	6.04	-3.367	0.995	1.982
miR-369 5p	29.28	10.28	-3.363	0.993	1.983
miR-200b	21.17	2.18	-3.437	0.999	1.954
miR-200c	22.46	3.50	-3.315	0.999	2.003
miR-142 3p	23.24	4.16	-3.375	0.999	1.978
miR-142 5p	23.16	4.20	-3.382	0.995	1.976
miR-101	18.06	-0.91	-3.207	0.997	2.050
miR-186	19.20	0.18	-3.477	0.998	1.939

[1] The median is calculated considering all time points and all cows.
[2] The median of ΔCt is calculated as [Ct gene – geometrical mean of Ct internal controls] for each time point and each cow.
[3] Slope of the standard curve.
[4] R^2 stands for the coefficient of determination of the standard curve.
[5] Efficiency is calculated as [$10^{(-1 / \text{Slope})}$].



Supplemental Figure A.1. Percentage relative miRNA abundance[1] in relation to 8 miRNA targets.

[1]relative miRNA abundance = 1/ Efficiency $^{\text{Median }\Delta Ct}$,1/EΔCt = relative miRNA abundance/∑relative mRNA abundance.

APPENDIX B
CHAPTER 4-SUPPLEMENTAL MATERIAL

Supplemental Table B.1. Antibody symbol, catalog number, company, dilution ratio, and antibody name for antibodies measured in liver tissue from periparturient dairy cows.

Antibody	Catalog Number	Company	Dilution ratio	Antibody Name
GSTM1	ARP41769_P050	Aviva Systems Biology	1:250	Glutathione S-transferase Mu 1
GSTA4	ARP48555_P050	Aviva Systems Biology	1:250	Glutathione S-transferase A4
GPX3	ARP41491_P050	Aviva Systems Biology	1:250	Glutathione peroxidase 3
NFE2L2	137550	Abcam	1:1000, 1:750, 1:500, 1:250	Nuclear factor erythroid 2-like 2
Phospho-NFE2L2(Ser40)	PA5-67520	Invitrogen	1:1000, 1:750, 1:500, 1:250	Phosphorylated nuclear factor erythroid 2-like 2

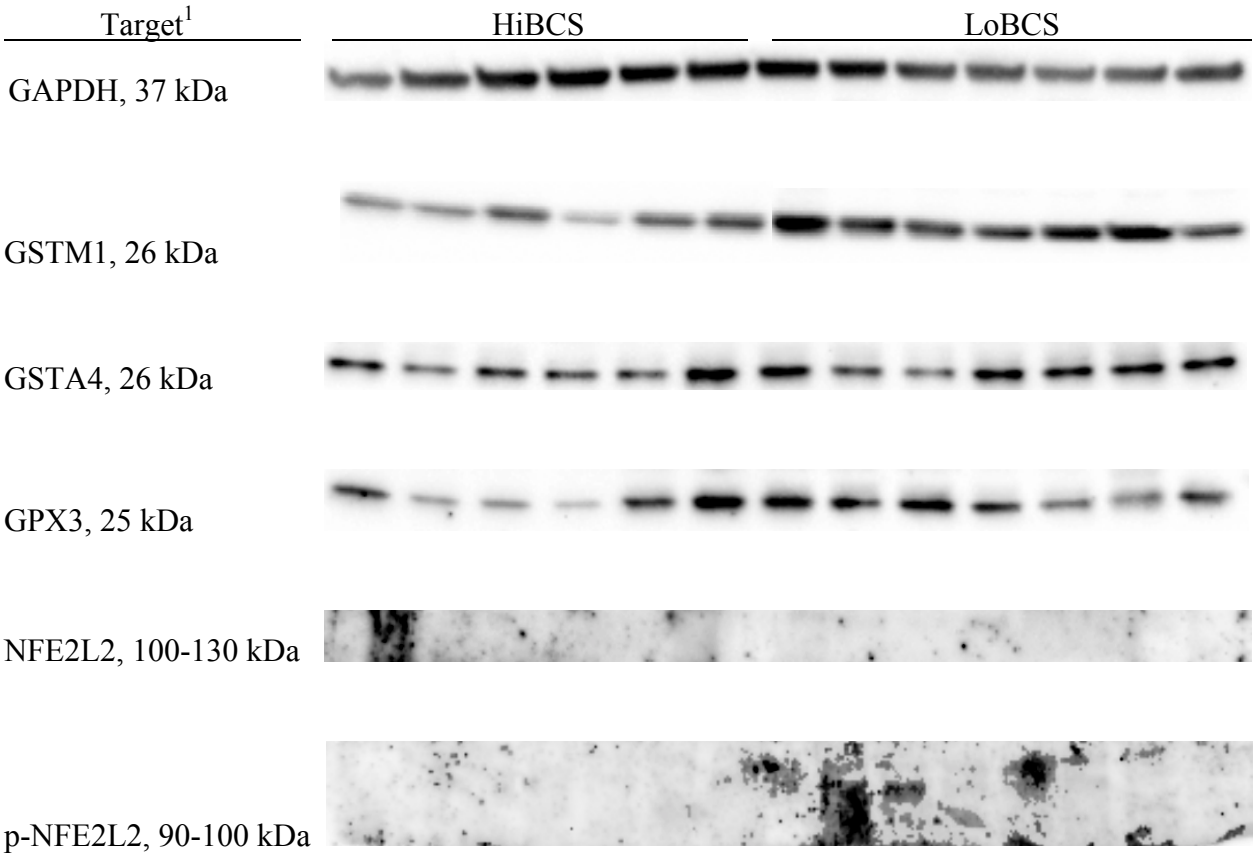
Supplemental Table B.2. Measured microRNA assay primer sequence information. A PerfeCTa Universal PCR Primer (Quanta BioSciences, Inc.) was coupled to the assay primer for expression analysis via qPCR.

miRNAs	**Sequence**
miR-369 5p	ATCGACCGTGTTATATTCGC
miR-186	CAAAGAATTCTCCTTTTGGGCT
miR-200b	TAATACTGCCTGGTAATGATG
miR-144	TACAGTATAGATGATGTACTAG

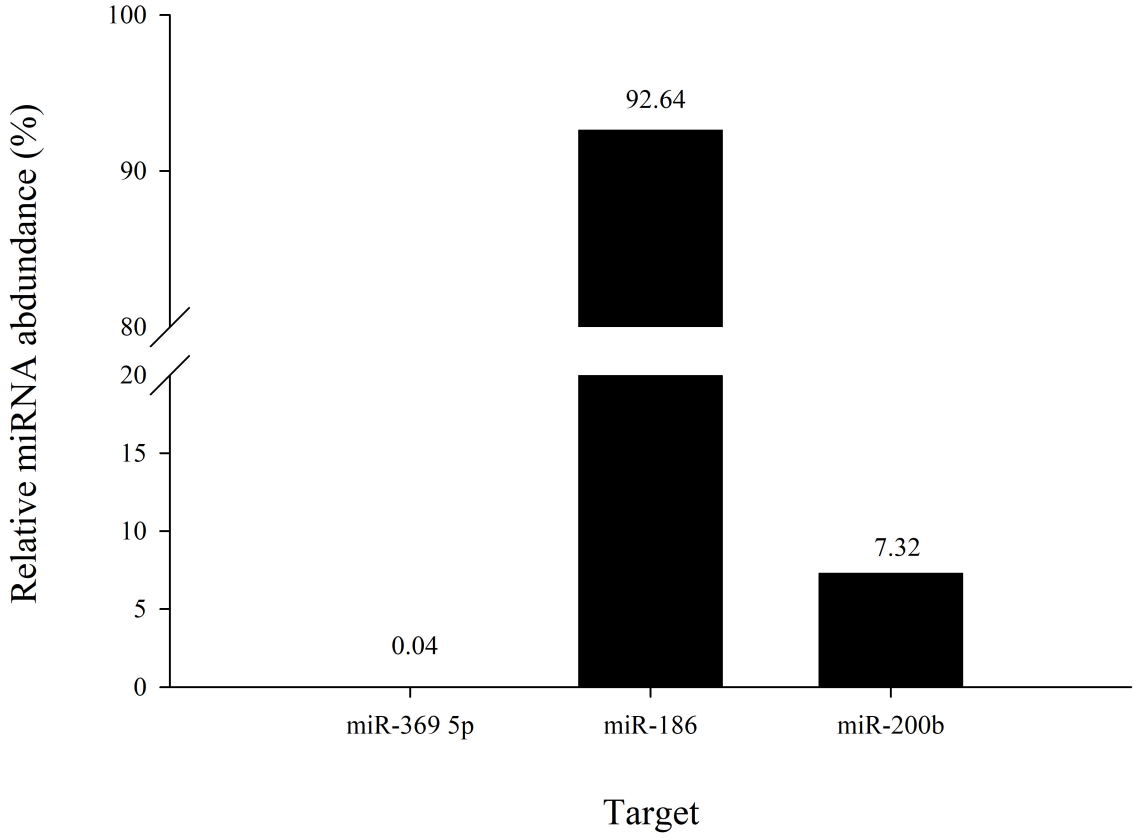
Supplemental Table B.3. qPCR performance of measured microRNAs.

Target	Median Ct[1]	Median ΔCt[2]	Slope[3]	(R^2)[4]	Efficiency[5]
miR-369 5p	30.27	11.09	-3.387	0.985	1.974
miR-186	18.92	-0.28	-3.474	0.998	1.940
miR-200b	22.72	3.42	-3.353	0.991	1.987

[1] The median is calculated considering all time points and all cows.
[2] The median of ΔCt is calculated as [Ct gene – geometrical mean of Ct internal controls] for each time point and each cow.
[3] Slope of the standard curve.
[4] R^2 stands for the coefficient of determination of the standard curve.
[5] Efficiency is calculated as $[10^{(-1 / Slope)}]$.



Supplemental Figure B.1. Representative blots with band size information. NFE2L2 and p-NFE2L2 were undetected at all time points.



Supplemental Figure B.2. Percentage relative miRNA abundance[1] in relation to 3 miRNA targets.

[1]relative miRNA abundance = 1/ Efficiency $^{\text{Median } \Delta Ct}$,1/EΔCt = relative miRNA abundance/∑relative mRNA abundance.