MANAGEMENT DURING THE DRY PERIOD AND ITS EFFECT ON HEPATIC AND ADIPOSE TISSUE MOLECULAR BIOMARKERS OF METABOLISM AND HEALTH IN GRAZING DAIRY COWS

BY

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DISSERTATION

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

Urbana, Illinois

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ABSTRACT

A successful transition into lactation determines optimum production, reproduction, and health. The peripartum period is characterized by an inflammatory state that, if not controlled, could be detrimental to the cow. The first experiment examined hepatic and adipose gene expression in response to injections of a non-steroidal anti-inflammatory compound (Carprofen) on 1, 3, and 5 d postpartum. Results indicated that after calving both tissues respond to inflammation signals, underscoring its role in the normal homeorhetic adaptations to lactation. The second experiment investigated the effect of prepartal nutrition and its interaction with BCS on hepatic and adipose tissue transcriptome, and the liver one-carbon metabolism and transulfuration pathway. Cows were randomly allocated to one of four groups in a 2 × 2 factorial arrangement: 4.0 or 5.0 BCS prepartum (10-point scale) and dietary energy at 75 or 125% of estimated requirements during the close-up. Tissue biopsies were harvested at -1, 1 and 4 wk relative to parturition. The greater number of hepatic differentially expressed genes in BCS4 cows in response to increased prepartum feed allowance (1071 vs 310, over the entire transition period) indicated a greater responsiveness to prepartum nutrition than optimally-conditioned cows. Thus, overfeeding in late-pregnancy should be limited to underconditioned cows, while cows with optimal BCS should be maintained on an energy-restricted diet. Adipose tissue mRNA and microRNA expression further confirmed this hypothesis, and indicated a relationship between the immune and metabolic response of the adipose tissue underscoring the existence of a “self-regulatory” mechanism. The extensive analysis of the hepatic one-carbon metabolism and related pathways highlighted fundamental differences in the metabolic progression of grazing cows compared to their higher-yield counterpart in TMR-based systems. Results also indicated a

greater flux through these pathways in optimally conditioned cows feed restricted prepartum. The third experiment examined the effect of over-feeding in both close-up and far-off periods on the adipose tissue transcriptome. Far-off over-feeding is usually a standard practice in seasonal grazing systems as, compared with TMR-fed cows, cows are thinner at the end of lactation. Adipose expression data revealed how overfed cows in the far-off period had greater adipogenesis, consistent with their rapid gain in BCS following dry-off, but a lower body fat mobilization in early lactation. The results indicated that neither strategy negatively affected the adaptations to lactation. However, to ensure a favorable transition, cows should be subjected to a small feed restriction in the close-up period, irrespective of far-off nutrition. Overall, results indicated a beneficial involvement of the immune system in the adaptation to lactation, and the possibility to regulate this process through prepartal BCS and nutrition management.

As a result of the three studies, New Zealand farmers, through DairyNZ (the industry organization that represents all New Zealand dairy producers), are now discouraged to apply prophylactic pharmacological intervention early postpartum, in favour of nutritional management during the dry period. Our recommendation is for cows to be properly managed in late lactation and early dry period to attain optimal condition (e.g, BCS 5) by close-up (3 wks from calving). Subsequently cows will benefit from a controlled feed restriction (75–90% of requirements). On the other hand, cows in less than optimal condition (e.g. BCS $\leq$ 4) should be fed to requirements or slightly overfed (110-120% of requirements) before calving. This is an easily implementable strategy based on pasture allocation capable of benefitting the farmer with a minimum cost.

To my Father

ACKNOWLEDGEMENTS

First and foremost, I would like to offer my sincere thanks to my research advisor, Dr. Juan J. Loor, for his guidance, input, and friendship. Furthermore, I would like to extend my gratitude to the members of my doctoral committee, Drs. Walter L. Hurley, Yuan-Xiang Pan, and Fabio Lima. A special thank you to Drs. Jim Drackley and Phil Cardoso for their oversight and participation in many of my research endeavours.

This thesis could have not been completed without the exceptional help of members of DairyNZ Inc., including Drs. John Roche, Susan Meyer, and Mallory Crookenden, and all the staff at Whareroa Farm (Hawera, New Zealand). Further thanks go to all New Zealand dairy farmers through DairyNZ Inc., and the Ministry of Business, Innovation and Employment for their financial support. Support from the Department of Animal Sciences was also appreciated.

I would like to express my appreciation to the past and current members of the Mammalian NutriPhysioGenomics lab for their help and friendship over the course of my PhD: Alea, Zheng, Abdul, Danielle, Ryan, Fernanda, Ahmed, Yusheng, Josh, Johan, Sonia. A special thanks goes also to the past and current member of the Gamma Alpha Graduate Student Society, which I will always remember as my home away from home.

Lastly, I would like to acknowledge with deepest gratitude the constant support from my dearest immediate and extended family. I owe you beyond words can describe.

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CHAPTER 1: INTRODUCTION

The transition period, defined as last 3 wk prepartum through 3 wk postpartum, is one of the most important stages of lactation in dairy cattle, characterized by significant metabolic and immune challenges (Bell, 1995, Drackley, 1999). Because failure to adequately meet these challenges can compromise production, induce metabolic diseases, and increase rates of culling in early lactation (Curtis et al., 1985), the management of the transition cow remains a focal point for dairy producers. Traditional management provides dry cows with a high-fiber/low-energy density ration, increasing the ration energy density and reducing the fiber content in the 3-4 wk prior parturition. This practice originated from the recommendation of Boutflour (1928). However, studies from the last decades have demonstrated that prepartum overfeeding of energy often leads to a wrecked transition (Rukkwamsuk et al., 1999, Holtenius et al., 2003, Dann et al., 2006, Soliman et al., 2007, Janovick et al., 2011, Graugnard et al., 2013, Ji et al., 2014, Khan et al., 2014, Shahzad et al., 2014).

Even though cow adiposity (measured through body condition score, a qualitative measurement) plays an important role in the metabolic response of the animal to lactation and its level is regulated through nutrition, cows with different level of adiposity are generally managed similarly during the prepartum period. The connection of prepartal nutrition and BCS is further strengthened by the fact that similar negative responses to overnutrition have been also observed when cows reached parturition at extreme levels (e.g., too high or too low) of BCS (Waltner et al., 1993, Hayirli et al., 2002, Holtenius et al., 2003, Roche et al., 2005, Roche et al., 2009, Pires et al., 2013, Akbar et al., 2015, Randall et al., 2015).

During the transition period dairy cows experience an inflammatory status (e.g., increased plasma pro-inflammatory cytokines and acute-phase proteins) which magnitude and duration have been linked to increased disease risk and decrease performances (Ohtsuka et al., 2001, Ametaj et al., 2005, Bertoni et al., 2008, Huzzey et al., 2009, Dubuc et al., 2010, Qu et al., 2014). Both nutritional and adiposity management have been proven effective in counteracting the most negative effects of this transient inflammatory status (Drackley and Cardoso, 2014, Kay et al., 2015). Scientist have however tried to use nonsteroidal anti-inflammatory drugs (NSAID) as a possible intervention to modulate the immunological status of the transitioning dairy cow. Successful results using aspirin derived compounds have been published (Bertoni et al., 2004, Trevisi et al., 2005, Trevisi and Bertoni, 2008, Farney et al., 2013a, Farney et al., 2013b), while other drugs have yielded variable outcomes (Newby et al., 2013, Priest et al., 2013, Shock et al., 2018).

The objectives of these studies were to evaluate the effect of (i) carprofen, a commercially available NSAID, or (ii) nutrional interventions (prepartal BCS, energy feeding, and their interaction) on transition cow physiology via molecular characterization of adipose and liver metabolism. These tissues were chosen as important players in the peripartal adaptation to lactation, and as immunoresponsive tissues involved in the network linking immunity and nutrient metabolism. We hypothesized that (i) early NSAID treatment postpartum (following published protocols; Bertoni et al., 2004) would improve lactation performance, reproductive parameters, and overall animal health via molecular mechanism in the adipose and hepatic tissue. Furthermore, we hypothesized that (ii) prepartal nutritional regimen has to be tailored to the cow adiposity level to obtain a favourable transition to lactation, such that the industry standard of

energy overfeeding is suitable for thinner cows, while optimally conditioned cows have to be feed restricted prepartum.

REFERENCES

Akbar, H., T. M. Grala, M. V. 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. Journal of dairy science 98(2):1019-1032.

Ametaj, B. N., B. J. Bradford, G. Bobe, R. A. Nafikov, Y. Lu, J. W. Young, and D. C. Beitz. 2005. Strong relationships between mediators of the acute phase response and fatty liver in dairy cows. Canadian journal of animal science 85(2):165-175.

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

Bertoni, G., E. Trevisi, X. Han, and M. Bionaz. 2008. Effects of inflammatory conditions on liver activity in puerperium period and consequences for performance in dairy cows. Journal of dairy science 91(9):3300-3310.

Bertoni, G., E. Trevisi, and F. Piccioli-Cappelli. 2004. Effects of acetyl-salicylate used in post-calving of dairy cows. Veterinary research communications 28 Suppl 1:217-219.

Boutflour, R. B. 1928. Limiting factors in the feeding and management of milk cows. Pages 15-20 in Report of proceedings of 8th world's dairy congress, London, UK.

Curtis, C. R., H. N. Erb, C. J. Sniffen, R. D. Smith, and D. S. Kronfeld. 1985. Path analysis of dry period nutrition, postpartum metabolic and reproductive disorders, and mastitis in Holstein cows. Journal of dairy science 68(9):2347-2360.

Dann, H. M., N. B. Litherland, J. P. Underwood, M. Bionaz, A. D'Angelo, J. W. McFadden, and J. K. Drackley. 2006. Diets during far-off and close-up dry periods affect periparturient metabolism and lactation in multiparous cows. Journal of dairy science 89(9):3563-3577.

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

Drackley, J. K. and F. C. Cardoso. 2014. Prepartum and postpartum nutritional management to optimize fertility in high-yielding dairy cows in confined TMR systems. Animal: An international journal of animal bioscience 8 Suppl 1:5-14.

Dubuc, J., T. F. Duffield, K. E. Leslie, J. S. Walton, and S. J. LeBlanc. 2010. Risk factors for postpartum uterine diseases in dairy cows. Journal of dairy science 93(12):5764-5771.

Farney, J. K., L. K. Mamedova, J. F. Coetzee, B. KuKanich, L. M. Sordillo, S. K. Stoakes, J. E. Minton, L. C. Hollis, and B. J. Bradford. 2013a. Anti-inflammatory salicylate treatment alters the metabolic adaptations to lactation in dairy cattle. American journal of physiology. Regulatory, integrative and comparative physiology 305(2):R110-117.

Farney, J. K., L. K. Mamedova, J. F. Coetzee, J. E. Minton, L. C. Hollis, and B. J. Bradford. 2013b. Sodium salicylate treatment in early lactation increases whole-lactation milk and milk fat yield in mature dairy cows. Journal of dairy science 96(12):7709-7718.

Graugnard, D. E., K. M. Moyes, E. Trevisi, M. J. Khan, D. Keisler, J. K. Drackley, G. Bertoni, and J. J. Loor. 2013. Liver lipid content and inflammometabolic indices in peripartal dairy cows are altered in response to prepartal energy intake and postpartal intramammary inflammatory challenge. Journal of dairy science 96(2):918-935.

Hayirli, A., R. R. Grummer, E. V. Nordheim, and P. M. Crump. 2002. Animal and dietary factors affecting feed intake during the prefresh transition period in Holsteins. Journal of dairy science 85(12):3430-3443.

Holtenius, K., S. Agenas, C. Delavaud, and Y. Chilliard. 2003. Effects of feeding intensity during the dry period. 2. Metabolic and hormonal responses. Journal of dairy science 86(3):883-891.

Huzzey, J. M., T. F. Duffield, S. J. LeBlanc, D. M. Veira, D. M. Weary, and M. A. G. von Keyserlingk. 2009. Short communication: Haptoglobin as an early indicator of metritis. Journal of dairy science 92(2):621-625.

Janovick, N. A., Y. R. Boisclair, and J. K. Drackley. 2011. Prepartum dietary energy intake affects metabolism and health during the periparturient period in primiparous and multiparous Holstein cows. Journal of dairy science 94(3):1385-1400.

Ji, P., J. K. Drackley, M. J. Khan, and J. J. Loor. 2014. Overfeeding energy upregulates peroxisome proliferator-activated receptor (PPAR)gamma-controlled adipogenic and lipolytic gene networks but does not affect proinflammatory markers in visceral and subcutaneous adipose depots of Holstein cows. Journal of dairy science 97(6):3431-3440.

Kay, J. K., J. J. Loor, A. Heiser, J. McGowan, and J. R. Roche. 2015. Managing the grazing dairy cow through the transition period: A review. Animal production science 55(7):936-942.

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 regulation and systems biology 8:97-111.

Newby, N. C., D. L. Pearl, S. J. LeBlanc, K. E. Leslie, M. A. G. von Keyserlingk, and T. F. Duffield. 2013. Effects of meloxicam on milk production, behavior, and feed intake in dairy cows following assisted calving. Journal of dairy science 96(6):3682-3688.

Ohtsuka, H., M. Koiwa, A. Hatsugaya, K. Kudo, F. Hoshi, N. Itoh, H. Yokota, H. Okada, and S. Kawamura. 2001. Relationship between serum TNF activity and insulin resistance in dairy cows affected with naturally occurring fatty liver. Journal of veterinary medical science 63(9):1021-1025.

Pires, J. A. A., C. Delavaud, Y. Faulconnier, D. Pomies, and Y. Chilliard. 2013. Effects of body condition score at calving on indicators of fat and protein mobilization of periparturient Holstein-Friesian cows. Journal of dairy science 96(10):6423-6439.

Priest, N. V., S. McDougall, C. R. Burke, J. R. Roche, M. Mitchell, K. L. McLeod, S. L. Greenwood, and S. Meier. 2013. The responsiveness of subclinical endometritis to a nonsteroidal antiinflammatory drug in pasture-grazed dairy cows. Journal of dairy science 96(7):4323-4332.

Qu, Y., A. N. Fadden, M. G. Traber, and G. Bobe. 2014. Potential risk indicators of retained placenta and other diseases in multiparous cows. Journal of dairy science 97(7):4151-4165.

Randall, L. V., M. J. Green, M. G. Chagunda, C. Mason, S. C. Archer, L. E. Green, and J. N. Huxley. 2015. Low body condition predisposes cattle to lameness: An 8-year study of one dairy herd. Journal of dairy science 98(6):3766-3777.

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. Journal of dairy science 92(12):5769-5801.

Roche, J. R., E. S. Kolver, and J. K. Kay. 2005. Influence of precalving feed allowance on periparturient metabolic and hormonal responses and milk production in grazing dairy cows. Journal of dairy science 88(2):677-689.

Rukkwamsuk, T., T. Wensing, and M. J. Geelen. 1999. Effect of overfeeding during the dry period on the rate of esterification in adipose tissue of dairy cows during the periparturient period. Journal of dairy science 82(6):1164-1169.

Shahzad, K., M. Bionaz, E. Trevisi, G. Bertoni, S. L. Rodriguez-Zas, and J. J. Loor. 2014. Integrative analyses of hepatic differentially expressed genes and blood biomarkers during the peripartal period between dairy cows overfed or restricted-fed energy prepartum. Plos one 9(6):e99757.

Shock, D. A., D. L. Renaud, S. M. Roche, R. Poliquin, and M. E. Olson. 2018. Evaluating the impact of meloxicam oral suspension administered at parturition on subsequent production, health, and culling in dairy cows: A randomized clinical field trial. Plos One 13(12).

Soliman, M., K. Kimura, M. Ahmed, D. Yamaji, Y. Matsushita, Y. Okamatsu-Ogura, K. Makondo, and M. Saito. 2007. Inverse regulation of leptin mRNA expression by short- and long-chain fatty acids in cultured bovine adipocytes. Domestic animal endocrinology 33(4):400-409.

Trevisi, E. and G. Bertoni. 2008. Attenuation of inflammatory conditions in periparturient dairy cows with acetylsalicylate treatments. Pages 23-57 in Aspirin and Health Research Progress. P. I. P.I. Quinn, ed. Nova Science Publishers, New York, NY.

Trevisi, E., A. Ferrari, I. Archetti, and G. Bertoni. 2005. Anti-inflammatory treatments in calving dairy cows: Effects on haematological and metabolic profiles. Italian journal of animal science 4:203-205.

Waltner, S. S., J. P. McNamara, and J. K. Hillers. 1993. Relationships of body condition score to production variables in high producing Holstein dairy cattle. Journal of dairy science 76(11):3410-3419.

CHAPTER 2: LITERATURE REVIEW

THE TRANSITION PERIOD

Approaching parturition, dairy cows enter the most interesting stage of the lactation cycle, where they undergo tremendous changes during the transition from late gestation to early lactation. Although the length of time classified as the transition period, or periparturient period, has been defined differently by different authors, it is commonly referred to as the last 3 weeks before parturition to 3 weeks after parturition, following the definition by Grummer (1995).

As stated by Goff and Horst (1997), "The transition from the pregnant, non-lactating state to the non-pregnant, lactating state is too often a disastrous experience for the cow. The well-being and profitability of the cow could be greatly enhanced by understanding those factors that account for the high disease incidence in periparturient cows." In this context, the term transition is to underscore the occurring physiological, metabolic, and nutritional changes. It constitutes a turning point in the productive cycle of the dairy cow from one lactation to the next. The manner in which these changes occur and how they are managed are of great importance as they closely relate to lactation performance, clinical and subclinical postpartum diseases, and reproductive performance that can significantly affect profitability. Many reports exist linking metabolic and health problems with a decreased productivity (Simianer et al., 1991, Jones et al., 1994, Emanuelson and Oltenacu, 1998) and with the economic losses associated with these (Drackley, 1999). The occurrence of health problems during the transition period is clearly a major complicating factor for subsequent reproductive performance (Ferguson, 2005), resulting in additional economic losses. Poor transitions also result in milk income losses, with up to 900 to 1800 kg of unrealized milk yield (Wallace et al., 1996). Translating this data to nowadays

production would result in even greater losses. It is, therefore, pertinent to elaborate nutritional strategies to facilitate the passage of the cow through this transition phase; while minimizing health problems and optimizing productivity/ profitability for the remainder of the ensuing lactation.

Homeorhesis and Nutrient Partitioning

The fundamental driver of the physiological changes is to ensure provision of adequate nutrients for the calf, both prenatally and postnatally. In mammals, during a general period, nutrients are utilized by tissues involved in maintenance and growth and for establishing body reserves including energy stores (lipids), glucose reserves (glycogen), and amino acid reserves (labile protein). However, during the transition period two additional tissues are utilizing a substantial portion of maternal nutrients: (i) the developing fetus, and (ii) the lactating mammary gland. One should not underestimate the importance of partitioning nutrients to support pregnancy and lactation, because these physiological states are the essence of survival of the species and, of course, the foundation of the dairy industry. These tissues differ from other body tissues in that they confer no special advantage to the animal. Instead, they make tremendous demands such that the total metabolism of the pregnant or lactating animal must be altered to accommodate these needs. The inability to adjust metabolism quickly enough to meet these needs frequently results in acute and subclinical metabolic disorders in farm animals.

Nature has accorded a high priority to the functions of pregnancy and milk secretion, allowing them to proceed at the expense of other metabolic processes even to the point that a disease state is created. The partitioning of nutrients to various body tissues involves two types of regulation, homeostasis and homeorhesis. Homeostatic control involves maintenance of

physiological equilibrium or constant conditions in the internal environment. Homeorhesis instead, as defined by Bauman and Currie (1980), is "the orchestrated or coordinated changes in metabolism of body tissues necessary to support a physiological state". Homeorhetic regulation involves a coordination of metabolism to insure a uniform flow of nutrients in support of a physiological state. One the most pronounced example of homeorhesis is the modern dairy cow, where initiation of lactation dramatically alters metabolism of many maternal organs in order to supply the mammary gland with the necessary nutrients for milk synthesis. All these physiological assessments happen also just before calving. The fundamental driver of the physiological changes is to ensure provision of adequate nutrients for the calf, both prenatally and postnatally. This can be read as an example of species homeorhesis: the many changes are supporting the continuity of the population, rather than the single animal.

Pregnancy. Pregnancy imposes a substantial cost to the animal, because total requirements for nutrients at the end of pregnancy are about 75% greater than in a non-pregnant animal of the same weight (Bell, 1995). Striking maternal adaptations are required to meet these metabolic requirements and are achieved by regulatory influences arising in the conceptus. Thus, its needs are accorded high priority by the homeorhetic controls it transmits to the dam. The bulk of accumulation of fetal mass occurs late in pregnancy when cattle are in the dry period. The fetus acquires approximately 60% of its birth weight during these last 2 months of gestation, where, to compare the requirement of the dry period to those of the lactation, fetal demands for specific nutrients (glucose and amino acids) are equal to mammary use of nutrients equivalent to about 3 to 6 kg milk/day (Bell, 1995).

Lactation. At the initiation of lactation, marked alterations in the general partitioning of nutrients and metabolism of the whole animal must occur to accommodate demands of the

mammary gland. The nutrient needs of the mammary gland are of such magnitude relative to total metabolism in a high producing dairy cow that the cow should be considered an appendage on the udder rather than the reverse (Brown, 1969).

The table below (Table 2.1) is a partial listing of metabolic changes that occur with initiation of lactation. The listing is by no means complete, and many other important maternal functions also adapt to support lactation. Rather, the list is intended to underline that lactation is not just a function of the mammary gland. Commencement and maintenance of a successful lactation are dependent on alterations in many, perhaps even most, maternal tissues such that nutrients are partitioned to the mammary gland. Thus, regulation of nutrient partitioning by homeorhetic and homeostatic mechanisms is extremely important in insuring a high rate of milk production.

Table 2.1 A partial list of the metabolic changes associated with lactogenesis in ruminants

Physiological Function	**Metabolic Change**	**Tissue involved**
Milk Synthesis	Incremented use of nutrients	Mammary
Lipid Metabolism	Increased lipolysis	Adipose tissue
	Decreased lipogenesis	
Glucose metabolism	Increased gluconeogenesis	Liver
	Increased glycogenolysis	
	Decreased use of glucose and Increased use of lipid as energy source	Body tissues in general
Protein Metabolism	Mobilization of protein reserves	Muscle and other body tissues
Mineral Metabolism	Increased absorption and mobilization of calcium	Kidney, liver, gut and bone

One of the major changes occurs in adipose tissue. Here uptake of nutrients for synthesis of storage lipids is decreased, and lipid reserves are mobilized instead (Drackley, 1999). Another

key nutrient is glucose, and the maximally secreting mammary gland may require up to 80% of the total glucose turnover (Bauman and Currie, 1980). A coordinated response meets this need; rates of gluconeogenesis in the liver are increased dramatically, and presumably glycogen is mobilized. A portion of the increase in liver gluconeogenic rates is from the increased intake when lactation commences (Lindsay, 1971), but total glucose synthesized per day increases even if a constant intake is maintained. The preference of other body tissues for nutrients to be oxidized for energy also is altered to allow partitioning of a greater percentage of glucose to the mammary gland (Bauman and Currie, 1980). At day 30 prepartum, 34% of the total glucose turnover is oxidized to CO2, whereas this decreases to only 8 to 9% by day 7 of lactation (Bennink et al., 1972). Nitrogen balance studies have demonstrated the importance of labile protein reserves in meeting amino acid needs for milk protein and glucose synthesis in early lactation. These reserves are substantial and may comprise 25 to 27% of total body protein in a dairy cow (Botts et al., 1979). Mineral metabolism is another area with extensive changes at onset of lactation, and its changes have been reviewed by Reinhardt et al. (1988) and Horst et al. (1994).

The Dry Matter Intake (DMI) Around Parturition

The most important aspect of transition period is the insufficient dry matter intake (DMI) compared to the energy requirements for lactation and maintenance (Drackley, 1999), resulting in a period of negative energy balance (NEB) (Drackley, 1999, Ingvartsen and Andersen, 2000, Trevisi et al., 2012). Some authors (Ingvartsen and Andersen, 2000, Hayirli et al., 2002) suggested a reduction of DMI of about 30% in the last 3 weeks of gestation and almost 90% of

decrease occur during the 5 to 7 days before calving. Nevertheless, in some experiences this reduction does not occur in all the cows (Trevisi et al., 2002).

The reasons behind the radical decrease in feed intake in dairy cows include a decrease in rumen volume and the hormonal actions that accompany the periparturient period (Bell, 1995, Grummer, 1995). Because of significant fetal growth during the last 60 days of gestation, ruminal capacity decreases by as much as 20%, then starts to increase again within 8 days after calving (Spain and Scheer, 2002). Decreased capacity of the rumen limits the amount of feed the cow can consume, however, it does not totally account for the magnitude of the decrease in dry matter intake that occurs around calving. Factors affecting and regulating feed intake of lactating cows are numerous and complex and span cellular to macroenvironmental levels (Forbes, 1996, Roseler et al., 1997, Allen, 2000). Factors affecting DMI in lactating dairy cows and other ruminants may influence DMI in prefresh transition cows as well. Some can be controlled by humans and include (i) animal factor (i.e., age, BCS, breed, physiological stage, milk yield level), (ii) dietary factors (i.e., ingredient and nutrient compositions of diets, and physical and agronomic characteristics of feeds), (iii) managerial factors (i.e., production, feeding and housing systems), and (iv) climatic factors: i.e., temperature, humidity and wind. Therefore, determination of factors affecting DMI and quantification of their effects are important for developing new feeding strategies during prefresh transition period.

The Energy Metabolism And Energy Balance

Due to the lagging DMI contrapose to the increasing milk production in the first third of the lactation cycle, cows undergo a negative energy balance (NEB), energetically equivalent to approximately 9kg/d milk, and must mobilize body reserves to compensate (Bauman and Currie,

1980). As parturition approaches and the dairy cow transitions into lactation, nutrient demand increases drastically. Requirements for glucose and metabolizable energy increase 2-3 fold from 3 weeks before to 3 weeks after calving (Drackley et al., 2001). This physiological state puts the cow under a very high nutrient demand and forces her to alter her metabolism in order to meet these high energy needs; if the cow is unable to redirect nutrients to the fetus and mammary gland efficiently, she is susceptible to metabolic disorders. Nature has given pregnancy and milk production such high biological priorities that they will continue at the expense of other metabolic processes even if a state of disease is created (Bauman and Currie, 1980).

In order to transition successfully, the cow must undergo several metabolic adaptations. She must increase hepatic gluconeogenesis (glucose generation from non- carbohydrate substances), decrease glucose usage by peripheral tissues, increase fatty acid production from adipose tissues and increase amino acid mobilization from muscle (Bell, 1995). These coordinated processes allow for increased nutrient usage by the mammary gland. Since after calving cows produce more milk than can be energetically produced from the amount of feed they are able to consume, they are forced to rely on body storages. At the onset of lactation, the adipose tissue begins to mobilize body reserves through lipolysis (Bauman and Currie, 1980). Triglycerides (TAG) are mobilized to non-esterified fatty acids (NEFA) though the action of the enzyme hormone sensitive lipase and increase the intracellular fatty acid pool; since there is no stimulus to re-esterify them, they diffuse into the blood. Free fatty acids then bind to serum albumin and circulate to various body tissues (Drackley et al., 2001). They either enter the mammary gland, leading to an increase in milk fat, or are absorbed into the liver. Once in the liver, NEFA can either be (i) oxidized to provide energy in the liver, as the decrease in serum insulin and an increase in glucagon promotes oxidation of fatty acids (Vernon, 2005), (ii) converted back to

triglycerides, or (iii) partially oxidized to ketone bodies and released back into the blood stream to be used for energy by other body tissues (Drackley, 1999, Ingvartsen, 2006, Grummer, 2010). In non-ruminant animals, NEFA conversion to ketone bodies serves as a glucose sparing strategy in time of deficits and similar adaptive processes may occur in transition dairy cows. The heart, kidney, skeletal muscles, mammary glands and gastrointestinal tract can oxidize ketone bodies for energy (Heitmann et al., 1987); further, ketone bodies can be a substrate for fatty acid synthesis in the mammary gland suggesting ketogenesis is a strategy to compensate for insufficient glucose (Drackley, 1999) and a metabolic adaptation to hunger (Ingvartsen, 2006).

Glucose metabolism. At calving, the demand for glucose sharply increases, leaving the cows with a deficit of around 500g/d (Overton et al., 1998). After parturition cows have decreased whole-body oxidation of glucose and increased glucose entry compared with measurements prepartum (Bennink et al., 1972). Glucose conservation is likely driven by the increased concentration of somatotropin (GH) that occurs around parturition (Simmons et al., 1994). Reynolds et al. (2003) measured glucose output by the liver of transition cows and found out that it closely matches glucose demand calculated for the same cows, indicating that metabolic changes take places to support the animal during this period. The discrepancy of nearly 500 g/d between predicted glucose from digestible energy intake and estimated glucose demands must be made up by increased gluconeogenesis from intestinally absorbed amino acids and from endogenous substrates such as amino acids, lactate and glycerol. Esteems of contributions to gluconeogenesis indicates that propionate is responsible for 32 to 73 % of it, amino acids for 10 to 30%, lactate for 15% and glycerol only for small amounts (Seal and Reynolds, 1993). As a precursor, propionate produced during ruminal and hindgut fermentation is quantitatively the most important substrate for gluconeogenesis. Literature suggest that

metabolism of propionate by liver is modulated during the transition period (Overton et al., 1998, Reynolds et al., 2003), indicating that propionate supply and capacity of liver to utilize it for gluconeogenesis are linked closely during times of negative energy balance. Under most conditions, amino acids are not the primary gluconeogenic substrate, but they make a sizable contribution to ruminant gluconeogenesis. Alanine and glutamine are the two that typically make a net contribution to glucose synthesis, as together they account for 40-60% of all amino acids glucogenic potential (Bergman and Heitmann, 1978). During the early postpartal period amino acids may play a particularly significant role as glucogenesis increases. Compared to 21d prepartum, conversion of propionate to glucose increases 119 and 129% at 1 and 21d postpartum (Overton et al., 1998). However, conversion of alanine, increased 198 and 150% at same time point. In time of glucose shortage, the capacity to draw from the organism amino acids pool increases (Overton et al., 1999). The increased capacity in conversion to glucose of glucogenic amino acids might result in more instestinally-absorbed amino acids being converted, but even the skeletal muscle, skin and visceral tissue amino acids pool undergo adaptation to support gluconeogenesis during the periparturient period (Simmons et al., 1994, Bell et al., 2000).

NEFA metabolism. A massive mobilization of NEFAs from adipose tissue during and after parturition in high-yielding dairy cows is the metabolic hallmark of the transition from pregnancy to lactation (Bell, 1995). Once up-taken by the liver, NEFA can be oxidized via β-oxidation in the mitochondria to generate metabolites (acetyl CoA) that can be used to generate energy via the Krebs cycle, or alternatively, undergo β-oxidation in the peroxisomes, which are sub-cellular organelles present in most organs of the body. The main function of peroxisomal oxidation is the shortening of the NEFA chains, for example those larger than 22-24 carbon atoms, preparing them for beta-oxidation by the mitochondrial system (Drackley, 1999). The

ratio of peroxisomal oxidation to total oxidation is correlated positively to liver total lipid, liver TAG and plasma NEFA and negatively to DMI and energy balance (Drackley, 1999). Because peroxisomes do not contain a respiratory chain linked to ATP formation, peroxisomal oxidation is not regulated by energy demands of the cell, thus playing an important role as an "overflow" pathway to oxidize fatty acids during extensive NEFA mobilization. If not oxidized, the excess NEFA are repacked into TAG and exported as very low density lipoprotein (VLDL). However, the bovine liver has a limited capacity to metabolize NEFA into TAG. When the hepatic limit to handle NEFA is reached, the TAG accumulates in the liver, and acetyl CoA that is not utilized in the tricarboxylic acid cycle (TCA) is converted into ketone bodies, such as acetone, acetoacetate and beta-hydroxybutyrate (BHB) (Drackley, 1999). Excessive accumulation of TAG in the liver impairs its normal function, a scenario called fatty liver syndrome. While, when ketones are produced in excess of peripheral tissue's capacity to use them, they accumulate in the bloodstream, and in severe NEB can lead to the disease state known as ketosis. Clinically, dairy cows suffering from these two diseases exhibit reduced feed intake, reduced milk yield, loss of body weight, central nervous system involvement (staggering, lack of coordination, and appearance of staring or blindness) (Mulligan and Doherty, 2008).

THE INFLAMMATORY STATUS OF PERIPARTURIENT COWS

The transition period is doubtless the most important management issue of the whole dairy industry. Most of its many sides and mysteries have been unraveled so far and the comprehension of the metabolic adaptation is greater than 10 year ago, but perhaps one of the most challenging areas for both producers and scientist has been addressing the issues of herd health at the transition from dry to fresh cow (Ingvartsen and Moyes, 2015).

A successful transition is needed to set the stage for a successful lactation and optimum production, reproduction, health and culling. Metabolic disorders and health problems are common during this time and can easily erase the entire profit potential for dairy cows farms (Drackley, 1999). The well-being and profitability of the cow could greatly enhance by understanding those factors that account for high disease incidence in periparturient cows. Around parturition cows experience a period of generalize immunosuppression, from approximately 1 week prepartum to 1 week postpartum (Kehrli et al., 1989, Waller, 2000). This immune dysfunction is not limited to isolated immune variables; rather it is broad in scope and affects multiple functions of various immune cell types (Sordillo and Streicher, 2002). The combined results of immune and metabolic dysfunctions are that dairy cows may be hyposensitive and hyporesponsive to antigens, and therefore more susceptible to infectious disease such as mastitis during the peripaturient period (Mallard et al., 1998).

Even if clear sign of immunosuppression and reduced immune functions in late pregnancy can be observed in late gestation and early lactation, transition cows were shown to display an overt inflammatory response related to pregnancy and lactation (Sordillo et al., 2009), even without signs of microbial infections and/or otherwise determined pathology (Bionaz et al., 2007, Bertoni et al., 2008). This can be observed also in correlation with metabolic stress-related condition in early lactation.

Many doubts remain according to the immunosuppression in the transition period and different studies have shown contradictory data about the function and response of the immune system (Ishikawa et al., 2004, Sander et al., 2011).

Hormonal Effect On Immunity

The causes of the compromised immunological status during the transition period could lay in the nutritional and physiological status of the cow. The dry period, and the periparturient period in particular, is characterize by dramatic changes in the endocrine status (Hayirli et al., 2002). Plasma insulin decreases, and growth hormone increases as the cow progresses from the late gestation to the early lactation, with visible surges of both hormones in plasma concentrations at parturition (Kunz et al., 1985). Plasma thyroxine (T4) gradually increases during late gestation, decreases about 50% at parturition, and then begins to increase again (Kunz et al., 1985). Progesterone concentrations during the dry period are elevated for the sake of pregnancy maintenance, but decline rapidly two days before calving, similarly to estrogen (primarily estrone of placental origin) that increases dramatically in plasma during late gestation, but decreases immediately at calving (Chew et al., 1979). Concentrations of glucocorticoid and prolactin increase on the day of calving and return to near prepartum concentrations the day after (Edgerton and Hafs, 1973).

Estrogens and glucocorticoids are well known immunosuppressive agents (Goff and Horst, 1997). Estrogens have been found in some experiments to stimulate the humoral immune response, but most workers agree they have a strong suppressive effect on cell-mediated immunity (Wyle and Kent, 1977). Glucocorticoids have long been used as powerful immunosuppressive agents (Roth and Kaeberle, 1982). Plasma cortisol concentrations (primarily of maternal adrenal origin) increase from 4 to 8 ng/mL three days before calving to 15 to 30 ng/mL at parturition and the day after calving. The cortisol secretion response is even more pronounced in those cows that develop milk fever (Goff, 2000). Thus, the immunosuppressive effects of the plasma estrogen and cortisol increases observed in the periparturient period would

be likely suspects as causative agents of the immunosuppression observed at calving. However, cortisol is elevated for only hours around calving and therefore its role in prolonged immunosuppression around parturition has been questioned. Although cortisol concentrations are only transiently elevated, changed in glucocorticoid receptor expression driven by changes in estrogen and progesterone at the time of parturition might contribute to immunosuppression for at least several days around calving (Preisler et al., 2000).

Immune Functions And Pregnancy

Apart from the endocrine status, there are significant interactions between the immune system and cells and tissues of the reproductive system that are critical for the maintenance of pregnancy, but are responsible for immune suppression that is associated with increased risk of disease. Sides of both the innate and acquired immune response are compromised, beginning about 1-2 weeks before calving and recovering between 2 and 4 weeks after calving (Kashiwazaki et al., 1985, Kehrli and Goff, 1989, Kehrli et al., 1989, Kehrli et al., 1990). Immunosuppression is manifested in a wide range of immunological dysfunctions, including impaired neutrophil and lymphocyte functions (Kehrli et al., 1989, Mehrzad et al., 2001). As part of the innate immune system, the neutrophil is an essential first responder to infection and is considered vital to effective clearance of bacteria from the mammary gland of the dairy cow (Smith, 2000, Paape et al., 2003, Zychlinsky et al., 2003). Neutrophils have various killing mechanisms to destroy pathogens (Smith, 2000, Segal, 2005). Beside the well-known oxidizing agents produced during the oxidative burst (e.g., ROS), they contain numerous antimicrobial proteins (such as cathelicidins, hydrolases, proteases, lactoferrin, and lysozyme) within granules, that are either released into phagosomes to destroy ingested pathogens, or out of the cell. These

neutrophil functions are suppressed at and around the time of parturition (Kehrli et al., 1989, Mehrzad et al., 2001).

Human studies show significantly less respiratory burst activity in pregnant women (Crouch et al., 1995), maybe due to a different localization, in subcellular areas of pregnant women neutrophils, of two enzyme part of the reduced NAPDH production in the hexosemonophosphate shunt (Kindzelskii et al., 2004). Location of myeloperoxidase (MPO), an enzyme critical to oxidative burst, is also altered in non-pregnant women (cytosol) compared to pregnant women (external to the cell and associated with the cell membrane) (Kindzelskii et al., 2006). Scientists have hypothesized that immune suppression may be an important mechanism in the maintenance of pregnancy and a breakdown of the suppression is a factor in spontaneous abortions (Vince et al., 2001).

Other aspects underline the relationship between the immune and the reproduction system during pregnancy. During pregnancy, we observe and interaction between leukocytes and the corpus luteum: both macrophages and T-cells are found in it. During luteal regression, the number of lymphocytes and macrophages in the tissues increases by both recruitment of cells and proliferation of resident cells (Bauer et al., 2001). Cytokines thought to be expressed by luteal immune cells, but probably released by infiltrating cells, have the ability to inhibit progesterone synthesis by the bovine luteal cells and cause apoptosis of these cells, thus stimulating regression of the corpus luteum (Pate and Landis Keyes, 2001).

Lactation And Immune Status

Studies involving mastectomy of pregnant dairy cows, which removes the impact of milk production, while presumably maintaining endocrine and other changes associated with late

pregnancy and parturition, suggest that lactation plays a significant role in the recovery phase of periparturient immunosuppression, and that the absence of the mammary gland does no affect the manifestation of periparturient immunosuppression, but only affects the duration of the suppression after calving. Kimura et al. (1999) showed that lymphocyte function was significantly different in mastectomized animals compared to normal animals during transition. This demonstrated that the depression of lymphocyte function during the periparturient period could largely be attributed to the metabolic demands of milk production. In contrast to the lymphocytes, neutrophils showed a decrease in function starting about two weeks prior to calving and reaching the low point at the time of calving in both mastectomized and normal cows. However, mastectomized cows quickly recovered neutrophil function (7 days), whereas, normal animals had not recovered neutrophil function after 20 days. Moreover, in intact cows, all T cell subset populations decreased at the time of parturition, while the percentage of monocytes increased (Kimura et al., 2002). Scientist widely agree that the metabolic demands associated with the onset of lactation negatively impacts the composition of circulating peripheral blood mononuclear population of the immune system, associating two metabolic factors, greatly impacted by mastectomy: hypocalcemia and plasma NEFA concentration at parturition.

Stress And The Immune System

Dairy cows must adapt to numerous management challenges during the transition period. Following common practice, the transition from pregnancy to lactation is marked by several social regroupings and changes in diet: from far-off to close-up, then to single stalls for calving, after which cows are brought to the fresh lactating cows group, and finally (after the end of the the transition period) into the mid-late lactation one. Even if the regrouping is needed to ensure a

smother metabolic transition from dry to lactating cow and to closely monitor the cows as they approach parturition, there is evidence that it has negative consequences on both behavior and production. Phillips and Rind (2001) reported that regrouped animals had shorter feeding times, longer standing times, and decreased milk production relative to cows kept in a stable group. When new cows are introduced to a pen the group dynamics change, leading to increased levels of aggression among individuals as social relationships become established (von Keyserlingk et al., 2008). As parturition approaches, cows commonly are moved again to a maternity pen where they are usually isolated from the herd. Social isolation in unfamiliar surroundings has been shown to elicit stress responses in dairy cows in the form of increased heart rate, high cortisol concentrations and increased vocalizations (Rushen et al., 1999). After calving the calf is removed and the cow is moved again.

Stress is not only related to regrouping, its causes are as varied as its manifestations. Other type of stress include heat, NEB, transportation, and pregnancy. Various immune cells, such as neutrophils, T-cells, and dendritic cells, are affected when an animal is stressed (Burton and Kehrli, 1995, Burton et al., 1995, Burton et al., 2005). One of the most well studied molecular effects of stress on the immune system is the effect of cortisol on the expression of the protein L-selectin, which is expressed on the surface of immune cells and is necessary for the transmigration of the cells from the vasculature into the site of infection. Cortisol causes the loss of this protein expression on neutrophils and, thus, the loss of the ability to migrate through the vascular epithelium. The activation of the hypothalamus, pituitary gland and the adrenal gland by a stress response, stimulates those to releases hormones, other than cortisol, such as epinephrine and norepinephrine, all known to have a dramatic effect on the immune system. Examples have

been found in swines, where stressed pigs have significantly fewer white blood cells compared to the dominant one (Sutherland et al., 2006).

The Metabolic Adaptation Influence On The Immune System

An emerging area within transition cow metabolism and management is the consideration of interrelationships with the immune system (Drackley, 1999, Drackley et al., 2001). Periparturient NEB has been implicated in contributing to immunosuppression. However, it alone had little effect on the expression of adhesion molecules on the surface of bovine leukocytes (Perkins et al., 2001). Furthermore, negative energy balance in mid-lactation cows did not affect the clinical symptoms associated with an intramammary endotoxin infusion (Perkins et al., 2002). These results are contrary to previously cited work in periparturient cows where the presence of a mammary gland (vs. mastectomized cows) and its attendant metabolic demands slowed recovery of neutrophil function, suggesting that the metabolic stress of lactation exacerbated periparturient immunosuppression (Kimura et al., 1999). Others have investigated individual metabolic components associated with negative energy balance and have concluded that although hypoglycemia alone is not likely to exacerbate periparturient immunosuppression (Nonnecke et al., 1992), hyperketonemia appears to have multiple negative effects on aspects of immune function (Suriyasathaporn et al., 2000). Ketosis may increase the risk of mastitis in periparturient immunosuppressed cattle because many immune cell types are negatively affected by metabolite levels typical of a ketotic environment (i.e., low concentrations of glucose, and high concentrations of ketone bodies and NEFA). Furthermore, experimental mastitis in ketonemic cows was more severe than mastitis in non-ketonemic cows (Kremer et al., 1993). As

reviewed by Suriyasathaporn et al. (2000), impairment of the udder defense mechanism in cows experiencing negative energy balance seems to be related to hyperketonemia.

In addition to effects of metabolic dysfunction on immunological capacity, it is possible that perturbations of the immune system also may impact the normal adaptations of other aspects of metabolism during the transition period. Studies reported that lactating cows subjected to activation of the immune system via endotoxin administration responded with dramatic changes in circulating concentrations of cortisol, glucagon and insulin in order to maintain glucose homeostasis (Waldron et al., 2003a). Furthermore, immune system activation resulted in decreased concentrations of circulating Ca and P (Waldron et al., 2003b). It is then conceivable that a vigorous immune response during the periparturient period may also predispose cows to the development of secondary metabolic disorder. A significant amount of research focused on the cellular and molecular processes affected by calcium, and vitamins A and D (Mora et al., 2008, Vig and Kinet, 2009). Calcium, and vitamins A and D have a significant effect on the functionality of immune cells. Calcium plays an essential role in intracellular signaling. In immune cells, intracellular calcium regulates many cellular functions including cytokine production, cytokine receptor expression and cell proliferation. Stimulated peripheral mononuclear cells from hypocalcemic cows have a muted intracellular calcium response compared to cows with normal blood calcium levels (Kimura et al., 2006). Furthermore, when stimulated peripheral mononuclear cells from hypocalcemic cows were compared with the same cells obtained from the same cows after intravenous treatment with a calcium solution, a muted intracellular calcium response was demonstrated only when the animals were hypocalcemic (Kimura et al., 2006). A muted intracellular calcium response would have a significant effect on the functional capacity of the cells of the immune system.

The role of insulin in regulating glucose homeostasis and adipose tissue metabolism has received considerable attention relative to periparturient metabolism. In relation to the immune system, nsulin has been shown to be immune supportive in humans and other species. For example, a short term hyperinsulinemic euglycemic clamp resulted in increased concentrations of PMN in blood and increased phagocytic and chemotactic activity of these cells (Walrand et al., 2004, 2006). Nielsen et al. (2003) reported that bovine PMN possess insulin receptors, thus insulin could potentially increase the functional activity of leukocytes. To this end, preliminary data from Røntved et al. (2003) showed that insulin affects phagocytosis capacity of neutrophils during an a 96 h hyperinsulinemic-euglycemic clamp. Furthermore, high levels of insulin did not affect the number of bovine neutrophils in blood or the cells' performance of oxidative burst. It could be speculated that insulin might have a different effect depending on the physiological state of the cow (pregnancy or lactation) due to differences in circulating insulin concentration and insulin resistance in these different classes of animal. However, the same authors did not detect an effect of lactation stage (early vs mid) while testing the insulin effect on neutrophils functionality. In addition to the effect of insulin itself, PPAR-γ is involved in the immune function. In example, PMN are PPAR-γ responsive cells and binding of TZD to the transcription factor PPAR-γ results in direct effects on inflammatory and immune-function genes (Houseknecht et al., 2002). Neutrophils are known to have a preference to engulf refined carbohydrate (glucose, fructose, sucrose, etc.) over bacteria (Sanchez et al., 1973). The neutrophil phagocytic capacity to engulf bacteria is affected when simple sugars are digested and fasting strengthens the neutrophils phagocytic capacity to engulf bacteria. However, the digestion of normal starches has no effect. With these data authors conclude that the function, and not the number of phagocytes, in engulfing bacteria was altered by the ingestion of sugars

(Sanchez et al., 1973). Being insulin involved in the homeostasis of blood glucose, hypothesis could be made about its role in influencing the immune system activity. Data from Revelo and Waldron (2010) support this hypothesis, as in vitro incubation with insulin did not modify neutrophils functionality, contrary to the in vivo data (Røntved et al., 2003). In the same experiment instead, TZD shown anti-inflammatory potential.

PREPARTAL MANAGEMENT OF THE DAIRY COW

At the end of lactation, approximately two months before parturition, dairy cows enter the dry period, a part of their production cycle in which lactation is stopped (hence the name, "dry"). The dry period is a very important moment for the dairy cow. It provides the cow with an opportunity to "rest" from the metabolic demand of milk production, and dedicate its resources for the growing calf, which just started its exponential growth phase (e.g. ~60% of its total growth in mass). Furthermore, it allows the mammary gland to recover from the previous lactation, a process involving the involution of the parenchymal tissue and the regeneration of the mammary epithelial tissue. In fact, without the dry period, milk production per day would be reduced, while transition problem will not disappear (Schukken et al., 2011). Dairy cows with a short dry period (10- to 40-days) produced significantly less milk in the following lactation than cows with a 40- to 60-day dry period (Watters et al., 2008). The dry period offers as well the opportunity to the farmer to closely monitor the status of each animal and ensure a proper transition to the next lactation cycle. Nutritional strategies, feeding management, and control of cows adiposity during this period can impact high-producing dairy cows holistically: health, productivity and fertility.

Nutritional Management

One of the greatest influences and easily controllable factors by the farmer, on metabolic functions and status of the periparturient dairy cow is nutrition, with an emphasis on pre-calving nutrition management. At the beginning of the 20th century Robert Boutflour (1928) recommended producers to "steam up" dairy cows before calving, increasing their energy intake and DMI in the weeks prior parturition. This recommendation still stood decades later, supported by positive correlation between pre-partum NEFA concentration (e.g., the increased energy intake decreased tissue mobilization) and incidence of post-partum metabolic diseases Dyk et al. (1995). To further support Boutflour century-old strategy, a negative association between pre-partum energy intake and peripartum liver TAG accumulation was observed (Bertics et al., 1992). Throughout the years the assumed benefits of Boutflour strategy were believed to result from (i) a stimulation of ruminal papillae development, (ii) an adaptation of the rumen microflora to the higher concentrate offered in the post-partum diet, (iii) a provision of more readily available precursor for gluconeogenesis and thus glucose to the conceptus preventing maternal loss of energy reserves, and (iv) a stimulatory effect on insulin production, suppressing lipolysis and reducing influx of NEFA into the liver, reducing ketosis and steatosis. However, these benefits were disproven in recent years, as reviewed by Roche et al. (2013), Drackley and Cardoso (2014) and Kay et al. (2015).

Years prior to the support of Dyk et al. (1995) and Bertics et al. (1992) findings, Kunz et al. (1985) already started casting doubts on the steaming-up strategy (modernly called close-up diet), reporting that limiting nutrient intakes to requirements of the cows was preferable to overconsumption of energy. This led to a long series of studies showing that controlling energy intake prepartum to near calculated requirements favors a successful transition (Grum et al.,

1996, Dann et al., 2005, Dann et al., 2006, Douglas et al., 2006, Janovick et al., 2011, Graugnard et al., 2012, Ji et al., 2012, Graugnard et al., 2013). Cows even slightly overfed with moderate-energy diets (1.50 to 1.60 Mcal NEL/kg DM) can consume from 40%, up to 80% more net energy for lactation (NEL) than required during both far-off and close-up periods (Dann et al., 2005, Dann et al., 2006, Douglas et al., 2006, Janovick and Drackley, 2010), with no evidence of benefits on their periparturient performances. On the other hand, these studies indicated that the prolonged over-consumption of energy led to a decreased post-calving DMI, negative responses of metabolic indicators (e.g., NEFA, BHBA, liver TAG), and decreased lipogenesis, increased lipolysis and decreased ability of insulin to inhibit lipolysis (Drackley and Cardoso, 2014). Furthermore, the steaming-up strategy led to decrease immune-competence postpartum (Graugnard et al., 2012), supporting field observation that the opposite strategy (a controlled energy dry cow program), decreases health problems throughout transition (Beever, 2006). This line of research has then unequivocally disputed the "benefits" of the Boutflour (1928) strategy, recommending instead a controlled limit-feeding of moderate energy diets, or ad libitum feeding of high-bulk, low-energy rations. (Drackley and Cardoso, 2014). Interestingly, at the time of its recommendations, the steaming up-strategy attacked the already prevailing wisdom that underfeeding cows in the weeks preceding calving was the way to avoid milk fever.

Body Condition Score

The term body condition was first defined by Murray (1919) as the ratio of body fat to non-fat components. During the transition period the dairy cow mobilizes its adipose storages to meet lactation needs, at a time where DMI lags behind the milk production performances. Thus, great importance has been on the quantification of the animal body reserves. Despite popular

misconception, body weight per se is not a great indicator of body reserves, as it relationships with adiposity is affected by multiple factors, including parity, lactation stage, frame size, gestation stage, and breed (Roche et al., 2009). Furthermore, tissue mobilization early postpartum is concomitant to the gradual increase of feed intake, so that actual decreases in adiposity could be masked by the enhanced gastrointestinal fill, nullifying the relationship between body weight and changes in adipose and lean tissue weight (NRC, 2001). Therefore, independent systems to asses dairy cow body condition are required. Multiple subjective systems were introduced in the 1970s and 1980s. Scores were assigned to reflect the apparent degree of animal adiposity, giving them the name of body condition scores (BCS). The first scored introduced (4-point) to the dairy industry was adapted by Lowman et al. (1973) from a beef cattle oriented scoring system. Subsequently, independent systems were developed around the world, based on different scales: a 5-point system in the United States, a 6-point scale in the UK, and 8-point scale in Australia, and a 10-point scale in New Zealand (Roche et al., 2009). A comparison of the systems, with conversion equations, was conducted by Roche et al. (2004). Irrespectively of the scale used, low values reflect thin, emaciated cows, while high values equate to obesity scenarios.

Effectiveness of the scaling system was tested, with strong positive correlation between BCS and the proportion of fat physically dissected from Holsteins cows ($r = 0.75$ or 0.93), or observed body fat ($r = 0.83$) (Wright and Russel, 1984, Otto et al., 1991). However, since BCS assesses the level of subcutaneous fat ($r^2 = 0.89$), it poorly predicts inter- and intramuscular fat ($r^2 = 0.43$) (Wright and Russel, 1984). As the latter account for up to 45% of the body fat, BCS may be less accurate in establishing the adiposity level of thin cows that have little subcutaneous

fat (Ferguson et al., 1994). A further factor affecting BCS accuracy is whether the score was assessed via tactile or visual appraisal of the animal (Roche et al., 2004).

One could think that, since lipomobilization after calving supplies the organism with additional energy to meet the lactation requirement, increased levels of reserves at calving (e.g., greater BCS) will results in greater milk yields. This relationship is true on the lower end of the BCS scale, with peak milk production registered at 3.5 (US 5-point scale), and marginal increments past 3.0 (Waltner et al., 1993, Roche et al., 2007). Additionally, reports show a curvilinear association between BCS and milk production, with impaired lactation performances at the right end of the scale (e.g., decreased milk yield with high calving BCS) (Roche et al., 2009). This reduction can be explained by the negative association between BCS at calving and DMI (Roche et al., 2008), as fatter cows tend to eat less postpartum, compared to thinner animals, thus limiting the animal energy supply. This creates a domino effect: a lower DMI increased the animal NEB, which increases tissue mobilization, which increases liver NEFA overload, leading to metabolic disfunctions such as ketosis and steatosis (Cameron et al., 1998, Buckley et al., 2003, Drackley and Cardoso, 2014). Furthermore, cows that calve at a higher BCS display postpartal impairment of the immune function, increased blood inflammatory markers, and may be more subjected to oxidative stress (Contreras and Sordillo, 2011). These reports ultimately suggest that excessive adiposity contributes to transition problems, with impaired subsequent production, health, and reproduction. However, what constitutes excessive BCS changes from author to author. Optimal BCS at calving has trended downwards over the last decades, and nowadays consensus advices BCS of 3.0-3.5 in US 5-point scale, or 5.0 in the New Zealand 10-point scale for grazing cows (Roche et al., 2009, Drackley and Cardoso, 2014).

NSAID For Inflammation Control

The transition period represents the highest-risk period in the production cycle of dairy cows, with more than 50% of cows estimated to suffer from at last one subclinical disorder. The complexity of the adaptation to lactation have made it difficult for researchers to determine which process are physiological (e.g., homeorhetic), and which are pathological. The subacute inflammatory state typical of this period has been subject of great interest in the past decade, clearly showing that essentially all dairy cows experience some degree of systemic inflammation for a variable number of days after calving (Bradford et al., 2015). As the magnitude and duration of this inflammatory state have been linked to increased disease risk and decrease performances (Ohtsuka et al., 2001, Ametaj et al., 2005, Bertoni et al., 2008, Huzzey et al., 2009, Dubuc et al., 2010, Qu et al., 2014), it is clear why extensive research has been dedicated to its control.

Even thou the interrelated management of prepartal nutrition and BCS at calving have been shown to be effective ways to control and contain the inflammatory response throughout transition, a branch of transition cow research focused on pharmacological intervention as a way to counteract the spike in acute and subacute inflammation postpartum. Nonsteroidal anti-inflammatory drugs (NSAID) have been the preferred choice for their ability to inhibit enzyme cyclo-oxygenase (COX) 1 and/or 2 production of pro-inflammatory mediators (Cashman, 1996). Aspirin, as a broad inhibitor of both COX1 and COX2 was among the first tested. Trevisi et al. (2005) tested in a pilot trial (n = 5 cows/group) the effect of 25g/d of sodium acetylsalicylate for the first 5 days after calving. Treated cows had greater milk yield, a lower BCS loss postpartum, but no significant changes in inflammatory markers. The same authors (Bertoni et al., 2004) then tested the efficacy of an intra muscular injection of lysine acetyl-salicylate in the first five days

of lactation (15 g/d d 1-3, 7.5 g/d d 4-5). They reported better health status (i.e. more favorable indices of inflammation) and a higher feed intake in treated cows, which according to the authors allowed the animals to promptly recovery of common metabolic conditions (e.g., lower NEFA BHBO). Their data also showed better liver activity, a rise in milk yield, and an improvement in fertility. Authors repeated the experiment two more time and concluded that the suggested treatment with intra muscular injection of lysine acetyl-salicylate during the first 5 days of lactation accelerates the recovery from the inflammatory consequences of the transition period, while improving milk yield, fertility, and health conditions (Trevisi and Bertoni, 2008). Overall, even if milk must be dump due to NSAID presence during the treatment (short half-life in the organism of the used NSAID guarantees milk cleanliness when injections are terminated) authors indicates a favorable benefit/cost ratio.

Looking at the success of these results, other have tried to replicate the effects of NSAID administration. Farney et al. (2013b) administered via water 1.95 g/l of sodium salicylate to Holstein cows in the first 7 days post calving. Despite increase in milk fat yield by the third week postpartum, they did not detect overall treatment effects on milk yield, nor intake. They however detected an interaction of treatment and parity on 305-d milk yield, with NSAID decreasing whole lactation performance of primiparous cows, while increasing that of pluriparous (3 or greater) animals. Authors noted as well that salicylate treatment prevented postpartum insulin resistance, likely causing excessive glucose utilization in peripheral tissues and hypoglycemia (Farney et al., 2013a). These results represent, according to the authors, the first evidence that inflammation-associated pathways are involved in homeorhetic adaptations to lactation. To support the need for a certain level of inflammation around parturition, others have also reported increased incidence of retained placenta in cows treated with NSAID (Bradford et al., 2015).

Other NSAID, such as carprofen and meloxicam have been tested in transition dairy cows for control of pain, metritis, or mastitic events. In two separate controlled experiment, either carprofen or meloxicam were unable to stimulate and increased milk production in treated animals (Newby et al., 2013, Priest et al., 2013). However, in a large field trials in Canadians herds, meloxicam increased milk production by ~ 0.65 kg/d. (Shock et al., 2018).

REFERENCES

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

Ametaj, B. N., B. J. Bradford, G. Bobe, R. A. Nafikov, Y. Lu, J. W. Young, and D. C. Beitz. 2005. Strong relationships between mediators of the acute phase response and fatty liver in dairy cows. Canadian journal of animal science 85(2):165-175.

Bauer, M., I. Reibiger, and K. Spanel-Borowski. 2001. Leucocyte proliferation in the bovine corpus luteum. Reproduction 121(2):297-305.

Bauman, D. E. and W. B. Currie. 1980. Partitioning of nutrients during pregnancy and lactation: a review of mechanisms involving homeostasis and homeorhesis. Journal of dairy science 63(9):1514-1529.

Beever, D. E. 2006. The impact of controlled nutrition during the dry period on dairy cow health, fertility and performance. Animal reproduction science 96(3-4):212-226.

Bell, A. W. 1995. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. Journal of animal science 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. The proceedings of the Nutrition Society 59(1):119-126.

Bennink, M. R., R. W. Mellenberger, R. A. Frobish, and D. E. Bauman. 1972. Glucose oxidation and entry rate as affected by the initiation of lactation. Journal of dairy science 55 Suppl 1:712-713.

Bergman, E. N. and R. N. Heitmann. 1978. Metabolism of amino acids by the gut, liver, kidneys, and peripheral tissues. Federation proceedings 37(5):1228-1232.

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. Journal of dairy science 75(7):1914-1922.

Bertoni, G., E. Trevisi, X. Han, and M. Bionaz. 2008. Effects of inflammatory conditions on liver activity in puerperium period and consequences for performance in dairy cows. Journal of dairy science 91(9):3300-3310.

Bertoni, G., E. Trevisi, and F. Piccioli-Cappelli. 2004. Effects of acetyl-salicylate used in post-calving of dairy cows. Veterinary research communications 28 Suppl 1:217-219.

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. Journal of dairy science 90(4):1740-1750.

Botts, R. L., R. W. Hemken, and L. S. Bull. 1979. Protein reserves in the lactating dairy cow. Journal of dairy science 62(3):433-440.

Boutflour, R. B. 1928. Limiting factors in the feeding and management of milk cows. Pages 15-20 in Report of proceedings of 8th world's dairy congress, London, UK.

Bradford, B. J., K. Yuan, J. K. Farney, L. K. Mamedova, and A. J. Carpenter. 2015. Invited review: Inflammation during the transition to lactation: New adventures with an old flame. Journal of dairy science.

Brown, R. 1969. The conversion of nutrients into milk. Proceedings of 3rd nutrition conference for feed manufacturers:23-35.

Buckley, F., K. O'Sullivan, J. F. Mee, R. D. Evans, and P. Dillon. 2003. Relationships among milk yield, body condition, cow weight, and reproduction in spring-calved Holstein-Friesians. Journal of dairy science 86(7):2308-2319.

Burton, J. L. and M. E. Kehrli. 1995. Regulation of neutrophil adhesion molecules and shedding of Staphylococcus aureus in milk of cortisol-treated and dexamethasone-treated cows. American journal of veterinary research 56(8):997-1006.

Burton, J. L., M. E. Kehrli, S. Kapil, and R. L. Horst. 1995. Regulation of L-selectin and Cd18 on bovine neutrophils by glucocorticoids: Effects of cortisol and dexamethasone. Journal of leukocyte biology 57(2):317-325.

Burton, J. L., S. A. Madsen, L. C. Chang, P. S. D. Weber, K. R. Buckham, R. van Dorp, M. C. Hickey, and B. Earley. 2005. Gene expression signatures in neutrophils exposed to glucocorticoids: A new paradigm to help explain "neutrophil dysfunction" in parturient dairy cows. Veterinary immunology and immunopathology 105(3-4):197-219.

Cameron, R. E. B., 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. Journal of dairy science 81(1):132-139.

Cashman, J. N. 1996. The mechanisms of action of NSAIDs in analgesia. Drugs 52 Suppl 5:13-23.

Chew, B. P., R. E. Erb, J. F. Fessler, C. J. Callahan, and P. V. Malven. 1979. Effects of ovariectomy during pregnancy and of prematurely induced parturition on progesterone, estrogens, and calving traits. Journal of dairy science 62(4):557-566.

Contreras, G. A. and L. M. Sordillo. 2011. Lipid mobilization and inflammatory responses during the transition period of dairy cows. Comparative immunology, microbiology, and infectious diseases 34(3):281-289.

Crouch, S. P. M., I. P. Crocker, and J. Fletcher. 1995. The effect of pregnancy on polymorphonuclear leukocyte function. Journal of immunology 155(11):5436-5443.

Dann, H. M., N. B. Litherland, J. P. Underwood, M. Bionaz, A. D'Angelo, J. W. McFadden, and J. K. Drackley. 2006. Diets during far-off and close-up dry periods affect periparturient metabolism and lactation in multiparous cows. Journal of dairy science 89(9):3563-3577.

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. Journal of dairy science 88(9):3249-3264.

Douglas, G. N., T. R. Overton, H. G. Bateman, H. M. Dann, and J. K. Drackley. 2006. Prepartal plane of nutrition, regardless of dietary energy source, affects periparturient metabolism and dry matter intake in Holstein cows. Journal of dairy science 89(6):2141-2157.

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

Drackley, J. K. and F. C. Cardoso. 2014. Prepartum and postpartum nutritional management to optimize fertility in high-yielding dairy cows in confined TMR systems. Animal: an international journal of animal bioscience 8 Suppl 1:5-14.

Drackley, J. K., T. R. Overton, and G. N. Douglas. 2001. Adaptations of glucose and long-chain fatty acid metabolism in liver of dairy cows during the periparturient period. Journal of dairy science 84, Suppl:E100-E112.

Dubuc, J., T. F. Duffield, K. E. Leslie, J. S. Walton, and S. J. LeBlanc. 2010. Risk factors for postpartum uterine diseases in dairy cows. Journal of dairy science 93(12):5764-5771.

Dyk, P. B., R. S. Emery, J. L. Liesman, H. F. Bucholtz, and M. J. VandeHaar. 1995. Prepartum non-esterified fatty acids in plasma are higher in cows developing periparturient health problems. Journal of dairy science 78:264 (Abstr.).

Edgerton, L. A. and H. D. Hafs. 1973. Serum luteinizing hormone, prolactin, glucocorticoid, and progestin in dairy cows from calving to gestation. Journal of dairy science 56(4):451-458.

Emanuelson, U. and P. A. Oltenacu. 1998. Incidences and effects of diseases on the performance of Swedish dairy herds stratified by production. Journal of dairy science 81(9):2376-2382.

Farney, J. K., L. K. Mamedova, J. F. Coetzee, B. KuKanich, L. M. Sordillo, S. K. Stoakes, J. E. Minton, L. C. Hollis, and B. J. Bradford. 2013a. Anti-inflammatory salicylate treatment alters the metabolic adaptations to lactation in dairy cattle. American journal of physiology. Regulatory, integrative and comparative physiology 305(2):R110-117.

Farney, J. K., L. K. Mamedova, J. F. Coetzee, J. E. Minton, L. C. Hollis, and B. J. Bradford. 2013b. Sodium salicylate treatment in early lactation increases whole-lactation milk and milk fat yield in mature dairy cows. Journal of dairy science 96(12):7709-7718.

Ferguson, J. D. 2005. Nutrition and reproduction in dairy herds. Veterinary clinics of North America: Food animal practice 21(2):325-347.

Ferguson, J. D., D. T. Galligan, and N. Thomsen. 1994. Principal descriptors of body condition score in Holstein cows. Journal of dairy science 77(9):2695-2703.

Forbes, J. M. 1996. Integration of regulatory signals controlling forage intake in ruminants. Journal of animal science 74(12):3029-3035.

Goff, J. P. 2000. Pathophysiology of calcium and phosphorus disorders. Veterinary clinics of North America: Food animal practice 16(2):319-337.

Goff, J. P. and R. L. Horst. 1997. Physiological changes at parturition and their relationship to metabolic disorders. Journal of dairy science 80(7):1260-1268.

Graugnard, D. E., M. Bionaz, E. Trevisi, K. M. Moyes, J. L. Salak-Johnson, R. L. Wallace, J. K. Drackley, G. Bertoni, and J. J. Loor. 2012. Blood immunometabolic indices and polymorphonuclear neutrophil function in peripartum dairy cows are altered by level of dietary energy prepartum. Journal of dairy science 95(4):1749-1758.

Graugnard, D. E., K. M. Moyes, E. Trevisi, M. J. Khan, D. Keisler, J. K. Drackley, G. Bertoni, and J. J. Loor. 2013. Liver lipid content and inflammometabolic indices in peripartal dairy cows are altered in response to prepartal energy intake and postpartal intramammary inflammatory challenge. Journal of dairy science 96(2):918-935.

Grum, D. E., J. K. Drackley, R. S. Younker, D. W. LaCount, and J. J. Veenhuizen. 1996. Nutrition during the dry period and hepatic lipid metabolism of periparturient dairy cows. Journal of dairy science 79(10):1850-1864.

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

Grummer, R. R. 2010. Managing the transition cow-emphasis on ketosis and fatty liver syndrome. 2010 Western nutrition conference: Challenging conventional nutrition dogma, Saskatoon, Saskatchewan, Canada, 21-23 September 2010:191-204.

Hayirli, A., R. R. Grummer, E. V. Nordheim, and P. M. Crump. 2002. Animal and dietary factors affecting feed intake during the prefresh transition period in Holsteins. Journal of dairy science 85(12):3430-3443.

Heitmann, R. N., D. J. Dawes, and S. C. Sensenig. 1987. Hepatic ketogenesis and peripheral ketone-body utilization in the ruminant. Journal of nutrition 117(6):1174-1180.

Horst, R. L., J. P. Goff, and T. A. Reinhardt. 1994. Calcium and vitamin D metabolism in the dairy cow. Journal of dairy science 77(7):1936-1951.

Houseknecht, K. L., B. M. Cole, and P. J. Steele. 2002. Peroxisome proliferator-activated receptor gamma (PPARgamma) and its ligands: a review. Domestic animal endocrinology 22(1):1-23.

Huzzey, J. M., T. F. Duffield, S. J. LeBlanc, D. M. Veira, D. M. Weary, and M. A. G. von Keyserlingk. 2009. Short communication: Haptoglobin as an early indicator of metritis. Journal of dairy science 92(2):621-625.

Ingvartsen, K. L. 2006. Feeding- and management-related diseases in the transition cow - Physiological adaptations around calving and strategies to reduce feeding-related diseases. Animal feed science and technology 126(3-4):175-213.

Ingvartsen, K. L. and J. B. Andersen. 2000. Integration of metabolism and intake regulation: A review focusing on periparturient animals. Journal of dairy science 83(7):1573-1597.

Ingvartsen, K. L. and K. M. Moyes. 2015. Factors contributing to immunosuppression in the dairy cow during the periparturient period. Japanese journal of veterinary research 63 Suppl 1:S15-24.

Ishikawa, Y., K. Nakada, K. Hagiwara, R. Kirisawa, H. Iwai, M. Moriyoshi, and Y. Sawamukai. 2004. Changes in interleukin-6 concentration in peripheral blood of pre- and post-partum dairy cattle and its relationship to postpartum reproductive diseases. Journal of veterinary medical science 66(11):1403-1408.

Janovick, N. A., Y. R. Boisclair, and J. K. Drackley. 2011. Prepartum dietary energy intake affects metabolism and health during the periparturient period in primiparous and multiparous Holstein cows. Journal of dairy science 94(3):1385-1400.

Janovick, N. A. and J. K. Drackley. 2010. Prepartum dietary management of energy intake affects postpartum intake and lactation performance by primiparous and multiparous Holstein cows. Journal of dairy science 93(7):3086-3102.

Ji, P., J. S. Osorio, J. K. Drackley, and J. J. Loor. 2012. Overfeeding a moderate energy diet prepartum does not impair bovine subcutaneous adipose tissue insulin signal transduction and induces marked changes in peripartal gene network expression. Journal of dairy science 95(8):4333-4351.

Jones, W. P., L. B. Hansen, and H. Chesterjones. 1994. Response of health care to selection for milk yield of dairy cattle. Journal of dairy science 77(10):3137-3152.

Kashiwazaki, Y., Y. Maede, and S. Namioka. 1985. Transformation of bovine peripheral-blood lymphocytes in the perinatal period. Japanese journal of veterinary science 47(2):337-339.

Kay, J. K., J. J. Loor, A. Heiser, J. McGowan, and J. R. Roche. 2015. Managing the grazing dairy cow through the transition period: A review. Animal production science 55(7):936-942.

Kehrli, M. E. and J. P. Goff. 1989. Periparturient hypocalcemia in cows: Effects on peripheral-blood neutrophil and lymphocyte function. Journal of dairy science 72(5):1188-1196.

Kehrli, M. E., J. P. Goff, J. A. Harp, J. R. Thurston, and N. L. Norcross. 1990. Effects of preventing periparturient hypocalcemia in cows by parathyroid hormone administration on hematology, conglutinin, immunoglobulin, and shedding of Staphylococcus aureus in milk. Journal of dairy science 73(8):2103-2111.

Kehrli, M. E., Jr., B. J. Nonnecke, and J. A. Roth. 1989. Alterations in bovine neutrophil function during the periparturient period. American journal of veterinary research 50(2):207-214.

Kimura, K., J. P. Goff, and M. E. Kehrli. 1999. Effects of the presence of the mammary gland on expression of neutrophil adhesion molecules and myeloperoxidase activity in periparturient dairy cows. Journal of dairy science 82(11):2385-2392.

Kimura, K., J. P. Goff, M. E. Kehrli, and T. A. Reinhardt. 2002. Decreased neutrophil function as a cause of retained placenta in dairy cattle. Journal of dairy science 85(3):544-550.

Kimura, K., T. A. Reinhardt, and J. P. Goff. 2006. Parturition and hypocalcemia blunts calcium signals in immune cells of dairy cattle. Journal of dairy science 89(7):2588-2595.

Kindzelskii, A. L., A. J. Clark, J. Espinoza, N. Maeda, Y. Aratani, R. Romero, and H. R. Petty. 2006. Myeloperoxidase accumulates at the neutrophil surface and enhances cell metabolism and oxidant release during pregnancy. European journal of immunology 36(6):1619-1628.

Kindzelskii, A. L., T. Ueki, H. Michibata, T. Chaiworapongsa, R. Romero, and H. R. Petty. 2004. 6-phosphogluconate dehydrogenase and glucose-6-phosphate dehydrogenase form a supramolecular complex in human neutrophils that undergoes retrograde trafficking during pregnancy. Journal of immunology 172(10):6373-6381.

Kremer, W. D., E. N. Noordhuizen-Stassen, F. J. Grommers, Y. H. Schukken, R. Heeringa, A. Brand, and C. Burvenich. 1993. Severity of experimental Escherichia coli mastitis in ketonemic and nonketonemic dairy cows. Journal of dairy science 76(11):3428-3436.

Kunz, P. L., J. W. Blum, I. C. Hart, H. Bickel, and J. Landis. 1985. Effects of different energy intakes before and after calving on food intake, performance and blood hormones and metabolites in dairy cows. Animal production 40(Apr):219-231.

Lindsay, D. B. 1971. Changes in the pattern of glucose metabolism in growth, pregnancy and lactation in ruminants. The proceedings of the Nutrition Society 30(3):272-277.

Lowman, B., N. Scott, and S. Somerville. 1973. Condition scoring beef cattle. Edinburgh: East of Scotland College of Agriculture:8.

Mallard, B. A., J. C. Dekkers, M. J. Ireland, K. E. Leslie, S. Sharif, C. L. Vankampen, L. Wagter, and B. N. Wilkie. 1998. Alteration in immune responsiveness during the peripartum period and its ramification on dairy cow and calf health. Journal of dairy science 81(2):585-595.

Mehrzad, J., H. Dosogne, E. Meyer, and C. Burvenich. 2001. Local and systemic effects of endotoxin mastitis on the chemiluminescence of milk and blood neutrophils in dairy cows. Veterinary research 32(2):131-144.

Mora, J. R., M. Iwata, and U. H. von Andrian. 2008. Vitamin effects on the immune system: vitamins A and D take centre stage. Nature reviews immunology 8(9):685-698.

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

Murray, J. A. 1919. Meat production. Journal of agricultural science 9:174-181.

Newby, N. C., D. L. Pearl, S. J. LeBlanc, K. E. Leslie, M. A. G. von Keyserlingk, and T. F. Duffield. 2013. Effects of meloxicam on milk production, behavior, and feed intake in dairy cows following assisted calving. Journal of dairy science 96(6):3682-3688.

Nielsen, L., C. M. Rontved, M. O. Nielsen, L. R. Norup, and K. L. Ingvartsen. 2003. Leukocytes from heifers at different ages express insulin and insulin-like growth factor-1 (IGF-1) receptors. Domestic animal endocrinology 25(2):231-238.

Nonnecke, B. J., S. T. Franklin, and J. W. Young. 1992. Effects of ketones, acetate, and glucose on in vitro immunoglobulin secretion by bovine lymphocytes. Journal of dairy science 75(4):982-990.

NRC. 2001. Nutrient requirements of dairy cattle. 7th revised edition. National Academy Press, Washington, D.C.

Ohtsuka, H., M. Koiwa, A. Hatsugaya, K. Kudo, F. Hoshi, N. Itoh, H. Yokota, H. Okada, and S. Kawamura. 2001. Relationship between serum TNF activity and insulin resistance in dairy cows affected with naturally occurring fatty liver. Journal of veterinary medical science 63(9):1021-1025.

Otto, K. L., J. D. Ferguson, D. G. Fox, and C. J. Sniffen. 1991. Relationship between body condition score and composition of ninth to eleventh rib tissue in Holstein dairy cows. Journal of dairy science 74(3):852-859.

Overton, T. R., J. K. Drackley, C. J. Ottemann-Abbamonte, A. D. Beaulieu, and J. H. Clark. 1998. Metabolic adaptation to experimentally increased glucose demand in ruminants. Journal of animal science 76(11):2938-2946.

Overton, T. R., J. K. Drackley, C. J. Ottemann-Abbamonte, A. D. Beaulieu, L. S. Emmert, and J. H. Clark. 1999. Substrate utilization for hepatic gluconeogenesis is altered by increased glucose demand in ruminants. Journal of animal science 77(7):1940-1951.

Paape, M. J., D. D. Bannerman, X. Zhao, and J. W. Lee. 2003. The bovine neutrophil: Structure and function in blood and milk. Veterinary research 34(5):597-627.

Pate, J. L. and P. Landis Keyes. 2001. Immune cells in the corpus luteum: Friends or foes? Reproduction 122(5):665-676.

Perkins, K. H., M. J. VandeHaar, J. L. Burton, J. S. Liesman, R. J. Erskine, and T. H. Elsasser. 2002. Clinical responses to intramammary endotoxin infusion in dairy cows subjected to feed restriction. Journal of dairy science 85(7):1724-1731.

Perkins, K. H., M. J. VandeHaar, R. J. Tempelman, and J. L. Burton. 2001. Negative energy balance does not decrease expression of leukocyte adhesion or antigen-presenting molecules in cattle. Journal of dairy science 84(2):421-428.

Phillips, C. J. C. and M. I. Rind. 2001. The effects on production and behavior of mixing uniparous and multiparous cows. Journal of dairy science 84(11):2424-2429.

Preisler, M. T., P. S. D. Weber, R. J. Templeman, R. J. Erskine, H. Hunt, and J. L. Burton. 2000. Glucocorticoid receptor down-regulation in neutrophils of periparturient cows. American journal of veterinary research 61(1):14-19.

Priest, N. V., S. McDougall, C. R. Burke, J. R. Roche, M. Mitchell, K. L. McLeod, S. L. Greenwood, and S. Meier. 2013. The responsiveness of subclinical endometritis to a nonsteroidal antiinflammatory drug in pasture-grazed dairy cows. Journal of dairy science 96(7):4323-4332.

Qu, Y., A. N. Fadden, M. G. Traber, and G. Bobe. 2014. Potential risk indicators of retained placenta and other diseases in multiparous cows. Journal of dairy science 97(7):4151-4165.

Reinhardt, T. A., R. L. Horst, and J. P. Goff. 1988. Calcium, phosphorus, and magnesium homeostasis in ruminants. Veterinary clinics of North America: Food animal practice 4(2):331-350.

Revelo, X. S. and M. R. Waldron. 2010. Effects of in vitro insulin and 2,4-thiazolidinedione on the function of neutrophils harvested from blood of cows in different physiological states. Journal of dairy science 93(9):3990-4005.

Reynolds, C. K., P. C. Aikman, B. Lupoli, D. J. Humphries, and D. E. Beever. 2003. Splanchnic metabolism of dairy cows during the transition from late gestation through early lactation. Journal of dairy science 86(4):1201-1217.

Roche, J. R., A. W. Bell, T. R. Overton, and J. J. Loor. 2013. Nutritional management of the transition cow in the 21st century – a paradigm shift in thinking. Animal production science 53:1000–1023.

Roche, J. R., D. P. Berry, J. M. Lee, K. A. Macdonald, and R. C. Boston. 2007. Describing the body condition score change between successive calvings: A novel strategy generalizable to diverse cohorts. Journal of dairy science 90(9):4378-4396.

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. Nutrition research reviews 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. Journal of dairy science 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. Journal of dairy science 92(12):5769-5801.

Røntved, C., L. Norup, M. Mashek, L. Nielsen, and K. Ingvartsen. 2003. Infusion of insulin in dairy cows inhibits neutrophils phagocytosis of Staphyloccocus aureus ex vivo. Acta veterinaria Scandinavica 44 Suppl 1:112.

Roseler, D. K., D. G. Fox, L. E. Chase, A. N. Pell, and W. C. Stone. 1997. Development and evaluation of equations for prediction of feed intake for lactating Holstein dairy cows. Journal of dairy science 80(5):878-893.

Roth, J. A. and M. L. Kaeberle. 1982. Effect of glucocorticoids on the bovine immune system. Journal of the American Veterinary Medical Association 180(8):894-901.

Rushen, J., A. Boissy, E. M. Terlouw, and A. M. de Passille. 1999. Opioid peptides and behavioral and physiological responses of dairy cows to social isolation in unfamiliar surroundings. Journal of animal science 77(11):2918-2924.

Sanchez, A., J. L. Reeser, H. S. Lau, P. Y. Yahiku, R. E. Willard, P. J. Mcmillan, S. Y. Cho, A. R. Magie, and U. D. Register. 1973. Role of sugars in human neutrophilic phagocytosis. American journal of clinical nutrition 26(11):1180-1184.

Sander, A. K., M. Piechotta, G. Schlamberger, H. Bollwein, M. Kaske, A. Sipka, and H. J. Schuberth. 2011. Ex vivo phagocytic overall performance of neutrophilic granulocytes and the relation to plasma insulin-like growth factor-I concentrations in dairy cows during the transition period. Journal of dairy science 94(4):1762-1771.

Schukken, Y., A. Gurjar, and P. Moroni. 2011. Physiology and pathophysiology of the udder during the dry period. Large animal review 17(6):253-259.

Seal, C. J. and C. K. Reynolds. 1993. Nutritional implications of gastrointestinal and liver metabolism in ruminants. Nutrition research reviews 6(1):185-208.

Segal, A. W. 2005. How neutrophils kill microbes. Annual review of immunology 23:197-223.

Shock, D. A., D. L. Renaud, S. M. Roche, R. Poliquin, and M. E. Olson. 2018. Evaluating the impact of meloxicam oral suspension administered at parturition on subsequent production, health, and culling in dairy cows: A randomized clinical field trial. Plos one 13(12).

Simianer, H., H. Solbu, and L. R. Schaeffer. 1991. Estimated genetic correlations between disease and yield traits in dairy cattle. Journal of dairy science 74(12):4358-4365.

Simmons, C. R., W. G. Bergen, M. J. Vandehaar, D. J. Sprecher, C. J. Sniffen, E. P. Stanisiewski, and H. A. Tucker. 1994. Protein and fat metabolism in cows given somavubove before parturition. Journal of dairy science 77(7):1835-1847.

Smith, G. 2000. Neutrophils. Pages 281-296 in Schalm's Veterinary Hematology. B. Feldman, J. Zinkl, and N. Jam, ed. Lippincott Williams and Wilking, Philadelphia.

Sordillo, L. M., G. A. Contreras, and S. L. Aitken. 2009. Metabolic factors affecting the inflammatory response of periparturient dairy cows. Animal health research reviews 10(1):53-63.

Sordillo, L. M. and K. L. Streicher. 2002. Mammary gland immunity and mastitis susceptibility. Journal of mammary gland biology and neoplasia 7(2):135-146.

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

Suriyasathaporn, W., C. Heuer, E. N. Noordhuizen-Stassen, and Y. H. Schukken. 2000. Hyperketonemia and the impairment of udder defense: A review. Veterinary research 31(4):397-412.

Sutherland, M. A., S. R. Niekamp, S. L. Rodriguez-Zas, and J. L. Salak-Johnson. 2006. Impacts of chronic stress and social status on various physiological and performance measures in pigs of different breeds. Journal of animal science 84(3):588-596.

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

Trevisi, E. and G. Bertoni. 2008. Attenuation of inflammatory conditions in periparturient dairy cows with acetylsalicylate treatments. Pages 23-57 in Aspirin and Health Research Progress. P. I. P.I. Quinn, ed. Nova Science Publishers, New York, NY.

Trevisi, E., A. Ferrari, I. Archetti, and G. Bertoni. 2005. Anti-inflammatory treatments in calving dairy cows: Effects on haematological and metabolic profiles. Italian journal of animal science 4:203-205.

Trevisi, E., X. T. Han, F. Piccioli-Cappelli, and G. Bertoni. 2002. Intake reduction before parturition affects milk yield and metabolism in dairy cows. Page 54 in Proceedings of 53rd Annual Meeting EAAP,. Wageningen Acad. Publ., Cairo, Egypt.

Vernon, R. G. 2005. Lipid metabolism during lactation: A review of adipose tissue-liver interactions and the development of fatty liver. Journal of dairy research 72(4):460-469.

Vig, M. and J. P. Kinet. 2009. Calcium signaling in immune cells. Nature immunology 10(1):21-27.

Vince, G., P. Johnson, and P. Gatenby. 2001. Human reproductive immunology. Mosby, London.

von Keyserlingk, M. A. G., D. Olenick, and D. M. Weary. 2008. Acute behavioral effects of regrouping dairy cows. Journal of dairy science 91(3):1011-1016.

Waldron, M. R., T. Nishida, B. J. Nonnecke, and T. R. Overton. 2003a. Effect of lipopolysaccharide on indices of peripheral and hepatic metabolism in lactating cows. Journal of dairy science 86(11):3447-3459.

Waldron, M. R., B. J. Nonnecke, T. Nishida, R. L. Horst, and T. R. Overton. 2003b. Effect of lipopolysaccharide infusion on serum macromineral and vitamin D concentrations in dairy cows. Journal of dairy science 86(11):3440-3446.

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.

Waller, K. P. 2000. Mammary gland immunology around parturition. Influence of stress, nutrition and genetics. Advances in experimental medicine and biology 480:231-245.

Walrand, S., C. Guillet, Y. Boirie, and M. P. Vasson. 2004. In vivo evidences that insulin regulates human polymorphonuclear neutrophil functions. Journal of leukocyte biology 76(6):1104-1110.

Walrand, S., C. Guillet, Y. Boirie, and M. P. Vasson. 2006. Insulin differentially regulates monocyte and polymorphonuclear neutrophil functions in healthy young and elderly humans. Journal of clinical endocrinology and metabolism 91(7):2738-2748.

Waltner, S. S., J. P. McNamara, and J. K. Hillers. 1993. Relationships of body condition score to production variables in high producing Holstein dairy cattle. Journal of dairy science 76(11):3410-3419.

Watters, R. D., J. N. Guenther, A. E. Brickner, R. R. Rastani, P. M. Crump, P. W. Clark, and R. R. Grummer. 2008. Effects of dry period length on milk production and health of dairy cattle. Journal of dairy science 91(7):2595-2603.

Wright, I. A. and A. J. F. Russel. 1984. Partition of fat, body composition and body condition score in mature cows. Animal production 38(Feb):23-32.

Wyle, F. A. and J. R. Kent. 1977. Immunosuppression by sex steroid hormones. The effect upon PHA- and PPD-stimulated lymphocytes. Clinical and experimental immunology 27(3):407-415.

Zychlinsky, A., Y. Weinrauch, and J. Weiss. 2003. Introduction: Forum in immunology on neutrophils. Microbes and infection 5(14):1289-1291.

CHAPTER 3: ADIPOSE AND LIVER GENE EXPRESSION PROFILES IN RESPONSE TO TREATMENT WITH A NONSTEROIDAL ANTIINFLAMMATORY DRUG AFTER CALVING IN GRAZING DAIRY COWS

Manuscript published.

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ABSTRACT

The peripartal or transition period in dairy cattle is often characterized by an inflammatory state that, if not controlled, could be detrimental to production, health, and fertility. Approaches to control the postpartal degree of inflammation include treatments with nonsteroidal antiinflammatory drugs (**NSAID**) postcalving, which have improved cow production and health. To date, most of the research on NSAIDs has been conducted in confinement cows that reach milk production levels substantially greater than those on pasture. Furthermore, there are little data available on the effect of NSAIDs on the mRNA expression of inflammation and metabolism-related genes. Transcription regulation is an important mechanism of inflammation and metabolic control. The present study was conducted to examine hepatic and adipose tissue gene expression in response to injections of a NSAID, carprofen, on 1, 3, and 5 d after calving. Grazing Holstein-Friesian cows from a control group and one treated with carprofen during the first 5 d post-calving were used. Liver and subcutaneous adipose tissue biopsies were harvested at -1, 1, and 2 wk relative to parturition. More than 30 genes associated with fatty acid oxidation, growth hormone/IGF-1 axis, hepatokines, lipoprotein metabolism, gluconeogenesis, and inflammation were analyzed. After calving, data suggest that both tissues

respond to inflammation signals at the onset of lactation. Administration of NSAID led to greater hepatic expression of pyruvate dehydrogenase kinase, isozyme 4 (*PDK4*), which helps regulate gluconeogenesis, and microsomal triglyceride transfer protein (*MTTP*), important for the assembly and secretion of very low density lipoproteins. In adipose tissue, NSAID administration resulted in greater expression of the inflammation-related genes interleukin-1, beta (*IL1B*), interleukin-6 receptor (*IL6R*), toll-like receptor 4 (*TLR4*) and chemokine (C-C motif) ligand 5 (*CCL5*). The data support the role of inflammation as a normal component of the homeorhetic adaptations to lactation and reveal a possible mechanism of the administration of NSAID in transition dairy cows, but do not reflect an effect of carprofen on the extent of the peripartum inflammation.

INTRODUCTION

The transition period, defined as last 3 wk prepartum until 3 wk postpartum, is one of the most important issues in dairy cattle management. A successful transition into lactation is a primary determinant of optimum production, reproduction, and health. Metabolic disorders and health problems are common during this time and can easily erase a farm's profit potential (Drackley, 1999). Postpartal cows are reported to display an overt inflammatory response related to the cessation of pregnancy and the beginning of lactation (Sordillo et al., 2009), even without signs of microbial infections or otherwise determined pathology (Bionaz et al., 2007; Bertoni et al., 2008).

A typical signature of an inflammatory state is the release of pro-inflammatory cytokines, which can cause a myriad of metabolic changes, including anorexia, lipomobilization, reduced insulin sensitivity, and reduced milk yield (Kushibiki et al., 2001; Kushibiki et al., 2003; Bertoni

et al., 2004). Given the detrimental effects of pro-inflammatory cytokines, it has been hypothesized that a reduction in postpartal inflammation might improve the transition into lactation, partly by reducing the accumulation of liver triacylglycerol (**TAG**), promoting gluconeogenesis, and reducing the incidence of disorders. To this end, treatment of early postpartal cows with a nonsteroidal antiinflammatory drug (**NSAID**) has been proposed to facilitate a successful transition through calving; results, however, have been inconsistent (Bertoni et al., 2004; Farney et al., 2013a; Priest et al., 2013).

Bertoni et al. (2004), the first published study utilizing an NSAID (acetyl-salicylate), reported a positive effect of treatment during the first 5 d postpartum on milk production and, also, more favorable indices of inflammatory status (e.g. greater Zn, lower haptoglobin and ceruloplasmin) during the first 4 wk postpartum. In a more recent study with a higher dose of acetyl-salicylate there was no change in milk production; however, data from the study suggested a possible homeorhetic role of inflammatory signals during the peripartal period as components of metabolic adaptations to lactation (Farney et al., 2013a). The roles of molecular mechanisms in coordinating such responses in key metabolic organs, such as adipose and liver, have not, to our knowledge, been studied in dairy cattle.

The objective of the present study was to use adipose and liver tissue from grazing dairy cows receiving an early postpartal administration of an NSAID to control inflammation (Meier et al., 2014) to profile mRNA expression of key metabolic genes associated with inflammation, oxidative stress, adipokines, fatty acid oxidation, ketogenesis, and lipoprotein export.

MATERIALS AND METHODS

Animals, Management, and Sampling

All details of animal management have been reported (Meier et al., 2014). Briefly, cows enrolled in the experiment were part of two seasonal calving dairy herds managed on a single commercial farm (Whareroa Dairy Farm, Hawera, New Zealand). Animals were allocated to one of three groups as they calved within a randomized block design (control: n = 221, earlyNSAID: n = 214, and lateNSAID: n = 204). Only control and earlyNSAID treatment groups were used for gene expression profiling. Cows allocated to earlyNSAID received three subcutaneous injections of 1.4 mg/kg BW of the NSAID carprofen (Carprive LA; Norbrook Ltd., Auckland, New Zealand) on d 1, 3, and 5 postcalving. Adipose and liver tissues were sampled on wk -1, 1, and 2 relative to parturition from 20 cows in each of the two selected groups.

Liver Biopsies. Liver samples were collected by biopsy during the wk before calving (wk -1), and subsequently on wk 1 and 2 post-calving. Briefly, the skin was shaved and disinfected and the area through the skin and body wall were anesthetized with 7 mL of 2% lignocaine (Lopaine 2%, lignocaine hydrochloride 20 mg/mL, Ethical Agents, South Auckland, New Zealand). A stab incision was made through the skin in the right 11th intercostal space at the level of the greater trochanter, through which a 12G × 20 cm biopsy needle was inserted into the liver and approximately 1 g (wet weight) of liver tissue was collected. Liver samples were snap-frozen in liquid nitrogen and stored at -80°C.

Adipose Biopsies. Tissue was harvested during wk -1, 1 and 2 relative to calving as described previously (Grala et al., 2013; 2014). Briefly, subcutaneous adipose tissue was collected posterior to the shoulder blade, and approximately 10 cm down the withers. The site was clipped and cleansed with iodine before administering a local anesthetic (2% lignocaine). A

3 cm incision was made through the skin and 30 to 80 mg of adipose tissue removed by blunt dissection. Biopsies were immediately placed in screw-capped, microcentrifuge tubes, snapfrozen in liquid nitrogen, and stored at −80 °C until RNA extraction.

RNA Extraction, Target Genes, and Real-Time Quantitative PCR (qPCR)

Complete details of these procedures are included in the supplemental material (Appendix A). Briefly, RNA samples were extracted from frozen liver tissue and used for cDNA synthesis, using established protocols in our laboratory (Khan et al., 2014). qPCR performed were SYBR Green-based, using a 7 point standard curve.

Data Processing and Statistical Analysis

After normalization with the geometric mean of the internal control genes, the qPCR data were log-2 transformed prior to statistical analysis to obtain a normal distribution. Statistical analysis was performed with SAS. Normalized, log-2 transformed data were subjected to ANOVA and analyzed using repeated measures ANOVA with PROC MIXED. The statistical model included time (1 and 2 wk postpartum), treatment (**TRT**; control and NSAID), and the time*TRT interaction as fixed effects. Cow, nested within treatment, was the random effect. Data on -1 wk were used as covariate in the model. The SP(POW) was used as the covariate structure. Denominator degrees of freedom were computed by SAS dividing the residual degrees of freedom into between-subject and within subject portions using the DDFM statement. Data were considered significant at a $P \leq 0.05$ using the PDIFF statement in SAS. For ease of interpretation, the data reported in Table 1 and 2 are the log-2 back transformed means that resulted from the statistical analysis.

RESULTS AND DISCUSSION

Hepatic metabolic adaptations. There were no TRT, time (wk), or interaction (TRT*wk) effects ($P > 0.05$) for genes associated with fatty acid oxidation, ketogenesis and the GH/IGF1 axis (Table 3.1). However, *PDK4* transcript abundance was affected by treatment ($P < 0.05$), with a greater expression in NSAID cows. Expression of *PC* decreased gradually over time ($P < 0.05$) regardless of treatment. Expression of *APOB* also was not affected by TRT ($P > 0.05$) and there was no interaction; however, there was an overall TRT effect for *MTTP,* with a greater expression ($P < 0.05$) in cows on NSAID. There was no interaction or TRT effect ($P > 0.05$) for *ANGPTL4* and *FGF21*. Over time the expression of *ANGPTL4* was not affected ($P > 0.05$), while there was a trend ($P = 0.08$) for a time effect for *FGF21* with greater expression at wk 1 than 2.

The lack of change in *FGF21* and *ANGPTL4* expression after calving suggests that cows in the current study underwent a period of "mild" negative energy balance (**NEB**) around parturition, i.e., the more pronounced the NEB the greater the expression of these genes in liver tissue (Schoenberg et al., 2011; Koltes and Spurlock, 2012; Khan et al., 2014). From a mechanistic standpoint, the modest change in these two genes reinforces the idea that the degree of NEB in the present study did not reach a threshold at which PPARα-regulated transcription was activated, i.e. both genes are PPARα targets (Bionaz et al., 2013).

The absence of treatment effect on NEFA level (Meier et al., 2014) could explain the lack of effect of NSAID administration on *PC* expression because there are data indicating that NEFA may stimulate hepatic *PC* gene expression by activating the *PC* promoter I (White et al., 2011). The numerical decrease ($P = 0.09$) in *PC* expression between wk 1 and 2 agrees with previous work with confinement (Greenfield et al., 2000; Loor et al., 2006) and grazing (White et al.,

2012) cows. The NSAID effect on expression of *PDK4* suggests a positive effect on gluconeogenic capacity of liver in NSAID cows despite lack of statistically significance effects between groups in terms of milk production (Meier et al., 2014). The NSAID cows also appeared to be better adapted to the increase in hepatic influx of NEFA typical of the post calving period, e.g. the greater expression of *MTTP* could have increased the rate of lipoprotein synthesis, thus, preventing the accumulation of triglycerides in hepatocytes.

Inflammation mediators in hepatocytes. There was no interaction or treatment effect ($P > 0.05$) for any of the inflammation-related genes. A time effect was detected for the expression of *HP* ($P = 0.0001$), *SAA2* ($P < 0.0001$), *SAA3* ($P = 0.02$) and the expression of the stress-related transcription factor *XBP1* tended to be affected ($P = 0.09$) due to a general decrease in their transcript abundance from wk 1 to 2.

The peripartal period in dairy cows is often characterized by inflammatory conditions that can result in the impairment of optimal milk yield (Bionaz et al., 2007; Bertoni et al., 2008). The inflammatory state is characterized by the induction of positive acute-phase protein (**+APP**) synthesis, mostly detected in blood plasma (e.g., haptoglobin and ceruloplasmin) and the impairment of hepatic synthesis of negative acute-phase proteins (**−APP**), such as albumin and retinol binding protein (Bionaz et al., 2007; Bertoni et al., 2008). As expected, signs of an overt inflammatory state after calving were detected in the present study, i.e. higher expression at wk 1 compared with wk 2 for *HP*, *SAA2* and *SAA3*. The decrease of the +APP genes at wk 2 is suggestive that cows recovered quickly from the post-partal inflammatory state and, possibly, that its intensity was not great enough to be affected by the NSAID treatment. This premise is supported by the lack of changes in the expression of *STAT3* and *NFKB1*, tow key molecules of the inflammatory response (Hodge et al., 2005; Yoshida et al., 2004). Although the blood

concentration of the encoded proteins was not assessed, it is established in non-ruminants that changes in gene expression of APP in liver are closely related with concentrations in circulation (Kushner, 1988).

Overall, as reported, the data indicate that treatment with NSAID did not help alleviate the hepatic inflammatory response; this result contrasts with the reported attenuation effect after early postpartal administration of acetyl-salicylate to confinement cows, another well-known NSAID (Bertoni et al., 2004). Failure for the NSAID, carprofen, to lessen the postcalving inflammatory response could a consequence of an inherently lower inflammatory state among cows of the current study as compared with the higher yielding cows used by Bertoni et al. (2004).

Metabolic adaptation in Adipocytes. As in liver gene expression profiles, the administration of NSAID early postpartum did not affect metabolic adaptation in the adipose tissue. There was no interaction, TRT or time effect ($P > 0.05$) for genes involved in lipid metabolism and adipokine production. Only *LEP* was affected by time ($P < 0.05$) due to a general decrease at wk 2 compared with wk 1 postpartum.

The NSAIDs are well known antagonists of cyclooxygenases (COX), which are responsible for the formation of important biological mediators called prostanoids, including prostaglandins, prostacyclin and thromboxane. Carprofen preferentially inhibits COX-2, interfering only to a very small extent with COX-1 activity (Miciletta et al., 2013). The COXs are enzymes that catalyze the conversion of arachidonic acid to prostaglandin endoperoxide H2, an important inflammation mediator used by COXs themselves to produce prostaglandins (i.e., PGD2, PGE2, PGF2α) (Morita, 2002). In non-ruminants, PGE2 is normally synthesized by the adipose tissue where it inhibits triacylglycerol breakdown (Richelsen, 1992). Thus, blocking its

synthesis early postpartum could be beneficial for cows, allowing them to better face the sudden state of NEB.

Overall, the lack of changes at the transcription level and the modest decrease in body weight and body condition score during the first 2 wk postpartum in the cows studied (Meier et al., 2014) indicate that NSAID administration did not greatly affect lipid mobilization and metabolism. Similar studies using high-producing Holstein cows in confinement (Bertoni et al., 2004, Farney et al., 2013b) reported greater milk production and blood NEFA in responses to the NSAIDs acetyl-salicylate or sodium-salicylate. The difference in response between those studies and the current one could be the specificity of the given NSAID on the COX target, i.e. salicylate can target both COX-1 and COX-2, a characteristic that could enhance its inhibition of prostaglandin synthesis.

Inflammation and the adipose tissue. Apart from tendencies ($P < 0.10$) detected for *CCL2*, *IL6R* and *TLR4* there was no interaction ($P > 0.05$) detected for genes involved in the inflammatory response (Table 3.2). Time had an effect on *TNF* only, as its expression was markedly lower at wk 2 compared with wk 1 postpartum ($P = 0.02$). Expression of *CCL5*, *IL1B*, *IL6R* and *TLR4* was greater ($P < 0.05$) in NSAID cows during wk 1 and 2 post-calving. However, no TRT, time, or interaction effects ($P > 0.05$) were observed for the acute-phase genes *HP* and *SAA3*.

The higher expression of *CCL2*, *TLR4*, and *TNF* in adipose tissue confirmed the existence of a local inflammatory response, underscoring the fact that this tissue is immune responsive, following the systemic inflammatory state postpartum. The upregulation of *CCL2*, a chemoattractant that participates in the process of macrophage recruitment into adipose tissue

could be an important trigger of the adipose tissue inflammation (Kanda et al., 2006). The signals causing the inflammatory response in adipose tissue after calving remain to be identified.

In humans and other animal models, inflammation and its mediators have been linked to insulin resistance (Shoelson et al., 2006; Tilg and Moschen, 2008; Ikeoka and Krusinova, 2009). Insulin resistance is an adaptive mechanism in early lactation to prevent excess use of glucose by body tissues, and direct the greatest amount of glucose towards milk synthesis in the mammary gland (De Koster and Opsomer, 2013). In that context, the induced inflammatory stress postpartum could be helpful for the metabolic adaptations in early lactation that allows the mammary gland to increase the rate of milk synthesis. The greater expression induced by NSAID of genes connected with the inflammatory state (i.e., *CCL5*, *IL1B*, *IL6R*, *TLR4*) could have desensitized the adipose tissue to insulin signaling without the detrimental effect of a strong inflammatory state.

It has been recently hypothesized that the inflammation experienced by cows during the peripartal period could be another homeorhetic adaptation to the lactating state (Farney et al., 2013a), as administration of the NSAID salicylate, postpartum altered the metabolic adaptations to lactation. However, an excessive inflammatory response postpartum can lead to detrimental effects on cow production, fertility and health (Bertoni et al., 2008; Bionaz et al., 2007). A controlled and modulated postpartal inflammatory response should lead to minimal negative effects, while maintaining what seems to be a homeorhetic control mechanism; however, it must be emphasized that administration of NSAID early postpartum has led to inconsistent effects in terms of production outcomes (Bertoni et al., 2004; Farney et al., 2013a,b; Meier et al., 2014). The upregulation of *CCL5* promotes macrophage recruitment and survival in the adipose tissue of non-ruminants (Keophiphath et al., 2010). Recent studies have demonstrated increased

accumulation of these immune cells in adipose tissue of obese rodents and humans, and suggest they may play a role in the development of insulin resistance (Weisberg et al., 2003; Xu et al., 2003). The upregulation of *TLR4* in adipose tissue also could initiate inflammation leading to insulin resistance (Shi et al., 2006; Song et al., 2006). An impairment of insulin signaling in NSAID cows also could have been caused by the greater *IL1B* mRNA expression, leading to a local elevation in concentration of this cytokine. Interleukin-1β is known to induce insulin resistance in adipocytes through down-regulation of insulin receptor substrate-1 expression (Jager et al., 2007). When other indicators (i.e. *HP*, *ALB*, *SAA2*, *SAA3* in liver) suggest an alleviation of the postpartal inflammation, the greater *IL1B* expression could be due to the selective inhibition by carprofen of COX-2. At late stages of inflammatory episodes, COX-2 directs the synthesis of anti-inflammatory cyclopentenone prostaglandins, which are direct inhibitors of IkB kinase (Rossi et al., 2000). IkB kinase activates NF-kB, a nuclear transcription regulator that modulates the expression of several genes, including those involved in the inflammatory response (Schmida and Birbach, 2008).

As previously discussed, other studies reported a greater fat depot mobilization after administration of NSAID early postpartum (Bertoni el al., 2004; Farney et al., 2013a,b). Even if greater plasma NEFA levels were not detected in NSAID cows (Meier et al., 2014), the gene expression data evidence a possible mechanism involving inflammation-induced lipolysis. For instance, the greater *IL6R* expression with NSAID in the present study possibly led to a greater sensitivity of the adipose tissue to IL-6, a pro-inflammatory cytokine, which is known to stimulate lipolysis in humans (Trujillo et al., 2004), rodents (Wallenius et al., 2002), and pigs (Yang et al., 2008), and is also known to enhance insulin resistance in adipocytes (Rotter et al., 2003). Furthermore, a local increase in IL-1β concentration after carprofen injection due to a

greater *IL1B* expression also could have contributed to higher lipid mobilization (Feingold et al., 1992; Doerrler et al., 1994). At least in non-ruminants, an increase in circulating IL-1β is known to stimulate IL-6 production in monocytes (Tosato and Jones, 1990). As *CCL5* was upregulated in NSAID-treated cows and *CCL2* expression was high in both treatment groups after calving, an increase in local monocyte concentrations in adipose tissue could be assumed, hence, leading to a local increase in IL-6 due to a possibly greater level of IL-1β. If these mechanisms exist, they would support the role of NSAID administration postpartum on the modulation of lipid mobilization and its indirect contribution to greater glucose availability to the mammary gland.

CONCLUSIONS

Administration of NSAID in early lactation does not always produce a positive effect on bovine health, fertility, and production. In the present study, few changes at the molecular level were evident after treatment with carprofen early postpartum. The possibility that the cows in the study did not undergo a pronounced inflammatory state in early lactation (e.g., due to differences in production system and genetics) could have masked any positive effects of the treatment. Some insights, however, on the possible interaction between NSAID administration and periparturient cow inflammation and metabolism were apparent. They included the possible upregulation of hepatic gluconeogenesis and local inflammation in adipose tissue as a way to enhance lipolysis and nutrient availability to the mammary gland. Further research is required to better understand the interactions between NSAID and the stress that characterizes metabolic adaptations to lactation.

ACKNOWLEDGEMENTS

This research was supported by New Zealand dairy farmers through DairyNZ Inc. (AN808, AN1202), and the Ministry of Business, Innovation and Employment (Wellington, New Zealand; (UOAX0814, DRCX1201). The authors thank Fonterra and Matt Butler and the farm staff at Whareroa Farm (Hawera, New Zealand), Jacquie Buhler (DairyNZ, Westpac Trust Whareroa Research Centre), and the DairyNZ (Hamilton, New Zealand) technicians involved.

TABLES

Table 3.1. Hepatic gene expression (log2 back-transformed means) during the peripartal period untreated cows (Control) or those treated with a nonsteroidal anti-inflammatory drug (NSAID; 1.4 mg carprofen/kg BW) on d 1, 3, and 5 post-calving.

	Group							
	Control		*NSAID*			*P*[2]		
Gene	**1**[1]	**2**	**1**	**2**	**SEM**	**TRT**	**wk**	**TRT*wk**
Fatty Acid Oxidation, Ketogenesis, VLDL Synthesis and Secretion, and GH/IGF1 Axis								
ACOX1	0.9	1.01	0.98	1.06	0.18	0.65	0.37	0.84
APOB	0.86	0.91	0.89	0.96	0.14	0.64	0.44	0.88
CPT1A	0.86	0.97	0.97	0.95	0.22	0.76	0.73	0.61
GHR	0.93	1.00	0.93	1.07	0.13	0.76	0.14	0.62
HMGSC2	0.92	1.08	0.91	1.01	0.23	0.82	0.37	0.85
IGF1	2.26	2.82	2.04	2.53	0.26	0.62	0.13	0.98
IGFALS[3]	0.84	1.05	0.71	0.85	0.27	0.41	0.15	0.9
IGFBP1	1.07	1.48	1.18	0.98	0.33	0.59	0.69	0.14
MTTP	0.79[$]	0.76	1.06[$]	0.96	0.14	0.04	0.42	0.73
PPARA	0.93	1.00	1.16	1.21	0.23	0.22	0.70	0.92
Carbohydrate Metabolism								
PC	1.21	1.00	1.47	1.11	0.25	0.48	0.09	0.75
PCK1	0.76	0.87	0.85	1.06	0.21	0.25	0.27	0.81
PDK4	0.47	0.59[$]	0.7	0.93[$]	0.25	0.03	0.15	0.9
Hepatokines								
ANGPTL4	0.65	0.62	0.97	0.67	0.29	0.27	0.28	0.40
FGF21[3]	0.61	0.51	0.51[a]	0.24[b]	0.54	0.36	0.08	0.28
Inflammation and Intracellular Stress								
ALB	0.94	1.02	0.88	1.00	0.15	0.73	0.24	0.77
HP	0.24[a]	0.03[b]	0.26[a]	0.07[b]	0.66	0.44	0.0001	0.29
NFKB1	1.08	1.01	1.13	1.10	0.06	0.13	0.28	0.73
SAA2	0.81[a]	0.20[b]	0.47[a]	0.15[b]	0.48	0.34	<.0001	0.52
SAA3[3]	0.4[a]	0.17[b]	0.23	0.14	0.45	0.32	0.02	0.48
STAT3	0.97	0.77	1.09	0.98	0.18	0.22	0.13	0.56
XBP1	0.72	0.58	0.59	0.52	0.17	0.26	0.09	0.66

[1]Week from parturition

[2]TRT = treatment.

[3]Covariate was significant ($P < 0.05$).

[ab]Significant difference ($P < 0.05$) among time points within same group.

[#]Significant difference ($P < 0.05$) between groups at that time point.

Table 3.2. Adipose gene expression (log2 back-transformed means) during the peripartal period in untreated cows (Control) or those treated with a nonsteroidal anti-inflammatory drug (NSAID; 1.4 mg carprofen/kg BW) on d 1, 3, and 5 post-calving.

	Group							
	Control		*NSAID*			*P*[2]		
Gene	**1**[1]	**2**	**1**	**2**	**SEM**	**TRT**	**wk**	**TRT*wk**
Inflammation								
CCL2	2.12[a]	0.82[b]	1.22	1.7	0.51	0.81	0.39	0.08
CCL5	0.76[#]	1.03	1.62[#]	1.49	0.28	0.004	0.60	0.36
HP	1.25	0.78	1.17	0.60	0.62	0.71	0.18	0.82
IL1B[2]	0.39[$]	0.29[#]	1.09[$]	1.04[#]	0.53	0.01	0.63	0.73
IL6[2]	1.41	0.86	1.17	0.85	0.41	0.75	0.12	0.73
IL6R	0.67[#]	0.77[$]	1.42[#]	1.05[$]	0.17	0.0001	0.54	0.09
SAA3[2]	0.47	0.41	0.36	0.28	0.47	0.39	0.47	0.83
TLR4	0.86	0.76[#]	1.06[a]	1.86[b#]	0.31	0.04	0.23	0.07
TNF	1.07[a]	0.54[b]	1.09	0.85	0.32	0.33	0.02	0.25
Lipid Metabolism and Adipokines								
ADIPOQ	0.58	0.38	0.72	0.43	0.56	0.65	0.27	0.90
FASN	0.19	0.14	0.14	0.15	0.53	0.74	0.66	0.41
LEP	0.50	0.17	0.26	0.10	0.95	0.49	0.06	0.87
PPARA	1.30	0.94	1.01	1.07	0.29	0.78	0.51	0.32
PPARG[3]	0.71	0.54	0.94	0.84	0.42	0.15	0.57	0.81
RXRA	1.31	0.96	1.14	1.3	0.21	0.55	0.57	0.16

[1]Week from parturition

[2]TRT = treatment.

[3]Covariate was significant ($P < 0.05$).

[ab]Significant difference ($P < 0.05$) among time points within same group.

[#]Significant difference ($P < 0.05$) between groups at that time point.

REFERENCES

Bertoni, G., E. Trevisi, and F. Piccioli-Cappelli. 2004. Effects of acetyl-salicylate used in post-calving of dairy cows. Veterinary research communications 28:217-219.

Bertoni, G., E. Trevisi, X. Han, and M. Bionaz. 2008. Effects of inflammatory conditions on liver activity in puerperium period and consequences for performance in dairy cows. Journal of dairy science 91:3300-3310.

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. Journal of dairy science 90:1740-1750.

Bionaz, M., S. Chen, M. J. Khan, and J. J. Loor. 2013. Functional role of PPARs in ruminants: Potential targets for fine-tuning metabolism during growth and lactation. PPAR research 2013:1-28.

De Koster, J. D., and G. Opsomer. 2013. Insulin resistance in dairy cows. Veterinary clinics of North America: Food animal practice 29:299-322.

Doerrler, W., K. R. Feingold, and C. Grunfeld. 1994. Cytokines induce catabolic effects in cultured adipocytes by multiple mechanisms. Cytokine 6:478-484.

Drackley, J. K. 1999. Biology of dairy cows during the transition period: The final frontier? Journal of dairy science 82:2259-2273.

Farney, J., L. Mamedova, J. Coetzee, B. KuKanich, L. Sordillo, S. Stoakes, J. E. Minton, L. C. Hollis, and B. J. Bradford. 2013a. Anti-inflammatory salicylate treatment alters the metabolic adaptations to lactation in dairy cattle. American journal of physiology. Regulatory, integrative and comparative physiology 305:110-117.

Farney, J., L. Mamedova, J. Coetzee, J. Minton, L. Hollis, and B. Bradford. 2013b. Sodium salicylate treatment in early lactation increases whole-lactation milk and milk fat yield in mature dairy cows. Journal of dairy science 96:7709-7718.

Feingold, K. R., W. Doerrler, C. A. Dinarello, W. Feirs, and C. Grunfeld. 1992. Stimulation of lipolysis in cultured fat cells by tumor necrosis factor, interleukin-1, and the interferons is blocked by inhibition of prostaglandin synthesis. Endocrinology 130:10-16.

Grala, T. M., J R. Roche, C. V. Phyn, A. G. Rius, R. H. Boyle, R. G. Snell, and J. K. Kay. 2013. Expression of key lipid metabolism genes in adipose tissue is not altered by once-daily milking during a feed restriction of grazing dairy cows. Journal of dairy science 96:7753-7764.

Grala, T. M., J. R. Roche, J. K. Kay, A. G. Rius, H. M. White, S. S. Donkin, M. D. Littlejohn, R. G. Snell, and C. V. Phyn. 2014. The expression of genes involved in hepatic metabolism is altered by temporary changes to milking frequency. Journal of dairy science 97:838-850.

Greenfield, R., M. Cecava, and S. Donkin. 2000. Changes in mRNA expression for gluconeogenic enzymes in liver of dairy cattle during the transition to lactation. Journal of dairy science 83:1228-1236.

Hodge D. R., E. M. Hurt, and W. L. Farrar. 2005. The role of IL-6 and STAT3 in inflammation and cancer. European journal of cancer 41:2502-2512.

Ikeoka, D., and E. Krusinova. 2009. Insulin resistance and lipid metabolism. Revista da associação médica Brasileira 55:234.

Jager, J., T. Grémeaux,, M. Cormont, Y. Le Marchand-Brustel and J. F. Tanti. Interleukin-1beta-induced insulin resistance in adipocytes through down-regulation of insulin receptor substrate-1 expression. Endocrinology 148:241-251.

Kanda, H., S. Tateya, Y. Tamori, K. Kotani, K. Hiasa, R. Kitazawa, S. Kitazawa, H. Miyachi, S. Maeda, K. Egashira, and M. Kasuga. 2006. MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. Journal of clinical investigation 116:1494-1505

Keophiphath, M., C. Rouault, A. Divoux, K. Clément, and D. Lacasa. 2010. CCL5 promotes macrophage recruitment and survival in human adipose tissue. Arteriosclerosis, thrombosis, and vascular biology 30:39-45.

Khan, M. J., C. B. Jacometo, D. E. Graugnard, M. N. Corrêa, E. Schmitt, F. Cardoso, and J. J. Loor. 2014. Overfeeding dairy cattle during late-pregnancy alters hepatic PPARα-regulated pathways including hepatokines: Impact on metabolism and peripheral insulin sensitivity. Gene regulation and systems biology 8:97-111.

Koltes, D., and D. Spurlock. 2012. Adipose tissue angiopoietin-like protein 4 messenger RNA changes with altered energy balance in lactating Holstein cows. Domestic animal endocrinology 43:307-316.

Kushibiki, S., K. Hodate, H. Shingu, Y. Obara, E. Touno, M. Shinoda, and Y. Yokomizo. 2003. Metabolic and lactational responses during recombinant bovine tumor necrosis factor-alpha treatment in lactating cows. Journal of dairy science 86:819-827.

Kushibiki, S., K. Hodate, H. Shingu, Y. Ueda, Y. Mori, T. Itoh, and Y. Yokomizo. 2001. Effects of long-term administration of recombinant bovine tumor necrosis factor-alpha on glucose metabolism and growth hormone secretion in steers. American journal of veterinary research 62:794-798.

Kushner, I. 1988. The acute phase response: An overview. Methods in enzymology 163:373-383.

Loor, J. J., H. M. Dann, N. A. Guretzky, R. E. Everts, R. Oliveira, C. A. Green, N. B. Litherland, S. L. Rodriguez-Zas, H. A. Lewin, and J. K. Drackley. 2006. Plane of nutrition prepartum alters hepatic gene expression and function in dairy cows as assessed by longitudinal transcript and metabolic profiling. Physiological genomics. 27:29-41.

Meier, S., N. Priest, C. Burke, J. Kay, S. McDougall, M. D. Mitchell, C. G. Walker, A. Heiser, J. J. Loor, and J. R. Roche. 2014. Treatment with a nonsteroidal antiinflammatory drug after

calving did not improve milk production, health, or reproduction parameters in pasture-grazed cows. Journal of dairy science 97:1-12.

Miciletta, M., B. Cuniberti, R. Barbero, and G. Re. 2013. In vitro enantioselective pharmacodynamics of Carprofen and Flunixin-meglumine in feedlot cattle. Journal of veterinary pharmacolology and therapeutics 37:43-52.

Morita, I. 2002. Distinct functions of COX-1 and COX-2. Prostaglandin and other lipid mediators 68-69:165-175.

Priest, N. V., S. McDougall, C. R. Burke, J. R. Roche, M. Mitchell, K. L. McLeod, S. L. Greenwood SL and S. Meier. 2013. The responsiveness of subclinical endometritis to a nonsteroidal antiinflammatory drug in pasture-grazed dairy cows. Journal of dairy science 96:4323-4332.

Riechelsen, B. 1992. Release and effects of prostaglandins in adipose tissue. Prostaglandins, leukotrienes, and essential fatty acids 47:171-182

Rossi, A., P. Kapahi, G. Natoli, T. Takahashi, Y. Chen, M. Karin, M., and M. G. Santoro. 2000. Anti-inflammatory cyclopentenone prostaglandins are direct inhibitors of IkappaB kinase. Nature 403:103-118.

Rotter, V., I. Nagaev, and U. Smith. 2003. Interleukin-6 (IL-6) induces insulin resistance in 3T3-L1 adipocytes and is, like IL-8 and tumor necrosis factor-alpha, overexpressed in human fat cells from insulin-resistant subjects. Journal of biological chemistry 278:45777-45784.

Schmid, J., and A. Birbach. 2008. IkappaB kinase beta (IKKbeta/IKK2/IKBKB)--a key molecule in signaling to the transcription factor NF-kappaB. Cytokine and growth factor reviews 19:157-165.

Schoenberg, K., S. Giesy, K. Harvatine, M. Waldron, C. Cheng, A. Kharitonenkov, and Y. R. Boisclair. 2011. Plasma FGF21 is elevated by the intense lipid mobilization of lactation. Endocrinology 152:4652-4661.

Shi, H., M. V. Kokoeva, K. Inouye, I. Tzameli, H. Yin, and J. S. Flier. 2006. TLR4 links innate immunity and fatty acid–induced insulin resistance. Journal of clinical investigation 116:3015-3025.

Shoelson, S. E., J. Lee, and A. B. Goldfine. 2006. Inflammation and insulin resistance. Journal of clinical investigation 116:1793-1801.

Song, M. J., K. H. Kim, J. M. Yoon, and J. B. Kim. 2006. Activation of Toll-like receptor 4 is associated with insulin resistance in adipocytes. Biochemical and biophysical research communications 346:739-745.

Sordillo, L., G. Contreras, and S. Aitken. 2009. Metabolic factors affecting the inflammatory response of periparturient dairy cows. Animal health research reviews 10:53–63.

Tilg, H., and A. R. Moschen. 2008. Insulin resistance, inflammation, and non-alcoholic fatty liver disease. Trends in endocrinology and metabolism. 19:371-379.

Tosato, G., and K. D. Jones. 1990. Interleukin-1 induces interleukin-6 production in peripheral blood monocytes. Blood 75:1305-1310.

Trujillo, M. E., S. Sullivan, I. Harten, S. H. Schneider, A. S. Greenberg, and S. K. Fried. 2004. Interleukin-6 regulates human adipose tissue lipid metabolism and leptin production in vitro. Journal of clinical endocrinology and metabolism 89:5577-5582.

Wallenius, V., K. Wallenius, B. Ahrén, M. Rudling, H. Carlsten, S. L. Dickson, C. Ohlsson, and J. O. Jansson. 2002. Interleukin-6-deficient mice develop mature-onset obesity. Nature medicine 8:75-79.

Weisberg, S. P., D. McCann, M. Desai, M. Rosenbaum, R. L. Leibel, and A. W. Ferante Jr. 2003. Obesity is associated with macrophage accumulation in adipose tissue. Journal of clinical investigation 112:1796-1808.

White, H. M., S. Koser, and S. S. Donkin. 2011. Characterization of bovine pyruvate carboxylase promoter 1 responsiveness to serum from control and feed-restricted cows. Journal of animal science 89:1763-1768.

White, H. M., S. S. Donkin, M. C. Lucy, T. M. Grala, and J. R. Roche. 2012. Short communication: Genetic differences between New Zealand and North American dairy cows alter milk production and gluconeogenic enzyme expression. Journal of dairy science 95:455-459.

Yang, Y., D. Ju, M. Zhang, and G. Yang. 2008. Interleukin-6 stimulates lipolysis in porcine adipocytes. Endocrine 33:261-269.

Yoshida Y., A. Kumar, Y. Koyama, H. Peng, A. Arman, J. A. Boch, and P. E. Auron. 2003. Interleukin 1 activates STAT3/nuclear factor-kappaB cross-talk via a unique TRAF6- and p65-dependent mechanism. Journal of biological chemistry 279:1768-1776.

Xu, H., G. T. Barnes, Q. Yang, G. Tan, D. Yang, C. J. Chou, J. Sole, A. Nichols, J. S. Ross, L. A. Tartaglia, and H. Chen. 2003. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. Journal of clinical investigation 112:1821-1830.

CHAPTER 4: BODY CONDITION SCORE AND PLANE OF NUTRITION PREPARTUM AFFECT ADIPOSE TISSUE TRANSCRIPTOME REGULATORS OF METABOLISM AND INFLAMMATION IN GRAZING DAIRY COWS DURING THE TRANSITION PERIOD

Manuscript published.

Vailati Riboni, M., M. Kanwal, O. Bulgari, 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. 2016. Body condition score and plane of nutrition prepartum affect adipose tissue transcriptome regulators of metabolism and inflammation in grazing dairy cows during the transition period. Journal of Dairy Science, 99(1):758-770.

ABSTRACT

Recent studies demonstrating higher incidence of metabolic disorders after calving have challenged the management practice of increasing dietary energy density during the last ~3 wk prepartum. Despite the knowledge at the whole-animal level, the tissue-level mechanisms that are altered in response to feeding management prepartum remain unclear. Our hypothesis was that prepartum body condition score (BCS) in combination with feeding management play a central role in the peripartum changes associated with energy balance and inflammatory state. Twenty-eight mid-lactation grazing dairy cows of mixed age and breed were randomly allocated to one of four treatment groups in a 2 × 2 factorial arrangement: two prepartum BCS categories (4.0 and 5.0; based on a 10-point scale; BCS4, BCS5), obtained via differential feeding management during late-lactation, and two levels of energy intake during the 3 wk preceding calving (75 and 125% of estimated requirements). Subcutaneous adipose tissue was harvested via biopsy at -1, 1 and 4 wk relative to parturition. Quantitative PCR was used to measure mRNA and microRNA (miRNA) expression of targets related to fatty acid metabolism (lipogenesis, lipolysis), adipokine synthesis, and inflammation. Both, prepartum BCS and

feeding management had a significant effect on mRNA and miRNA expression throughout the peripartum period. Overfed BCS5 cows had the greatest prepartum expression of fatty acid synthase (*FASN*) and an overall greater expression of leptin (*LEP*). BCS5 was associated with greater overall adiponectin (*ADIPOQ*) and peroxisome proliferator-activated receptor gamma (*PPARG*), while overfeeding upregulated expression of proadipogenic miRNA. Higher postpartum expression of chemokine ligand 5 (*CCL5*) and the cytokines interleukin 6 (*IL6*) and tumor necrosis factor (*TNF*) was detected in overfed-BCS5 cows. Feed-restricted BCS4 cows had the highest overall interleukin 1 (*IL1B*) expression. Prepartum feed-restriction resulted in greater chemokine ligand 2 (*CCL2*) expression. Overall, changes in mRNA expression were consistent with the expression pattern of inflammation-related miRNA. These data shed light on molecular mechanisms underlying the effect of prepartum BCS and feeding management on metabolic and inflammatory status of adipose tissue during the peripartum period. Data support the use of a controlled feed restriction prepartum in optimally-conditioned cows, while supporting the use of a higher-level of dietary energy in under-conditioned cows.

INTRODUCTION

Cows in early lactation experience a period of negative energy balance (**NEB**) due to the sudden increase in requirements of the mammary gland for milk production (Drackley, 1999). Nutritional management of the late pregnant non-lactating cow has been examined as a way to improve DMI, energy balance, immunometabolism, and health during the transition period. Traditional management provides “far-off” dry cows with a high-fiber/low-energy density ration, while in the last month of gestation (“close-up” dry period) the ration increases in energy density with a lower fiber content. However, studies from different research groups have demonstrated

that prepartum overfeeding of energy often results in prepartum hyperglycemia and hyperinsulinemia and marked postpartum adipose tissue mobilization (i.e., greater blood NEFA concentration) (Rukkwamsuk et al., 1999, Holtenius et al., 2003, Janovick et al., 2011, Ji et al., 2014a, Khan et al., 2014). In addition, higher-energy close-up diets also have been associated with negative effects in postpartum health indices, underscoring possible detrimental effects of this management approach (Dann et al., 2006, Soliman et al., 2007, Graugnard et al., 2013, Shahzad et al., 2014).

Although a number of studies aimed at understanding metabolic and molecular changes associated with dietary energy intake before calving have been performed, the contributing factors remain poorly understood. One animal factor that could potentially interact with prepartum level of feeding is cow BCS. The BCS provides a qualitative assessment of body fat and, due to its association with production parameters and the chances for a successful lactation (Waltner et al., 1993, Roche et al., 2005, Pires et al., 2013, Randall et al., 2015), it is recognized as an important variable in transition dairy cattle management (Roche et al., 2009). For example, not only "fat" cows are at a greater risk of metabolic disorders postpartum (Hayirli et al., 2002, Holtenius et al., 2003, Randall et al., 2015), but also "thin" cows can be susceptible to failure to transition (Pires et al., 2013, Akbar et al., 2015).

Despite the fact that BCS plays an important role in the metabolic response of the animal to lactation and its level is regulated through nutrition, cows with different level of adiposity are generally managed similarly during the prepartum period. Both BCS and feeding management can have a great impact on cow fat depots. Adipose tissue is an active component of the regulation of animal reserves through production of adipokines (Adamczak and Wiecek, 2013, McGown et al., 2014, Musi and Guardado-Mendoza, 2014). One of its features is the control of

inflammation in a localized manner through recruitment and regulation of the innate immune system, hence, making it an active immunological organ (Grant and Dixit, 2015).

Besides the well-established role of changes in mRNA expression in controlling cellular pathways, recent studies have underscored that microRNA (**miRNA**) also are important for fat cell formation (adipogenesis) and regulation of their metabolic and endocrine functions (Arner and Kulyte, 2015). For example, beef cattle adipose miRNA profiling was recently correlated with fat depot location and function, underscoring the importance of miRNA (e.g., miR-378 and miR-143) in regulating adipocyte metabolism (Jin et al., 2009, Jin et al., 2010), also in response to diet (Romao et al., 2012).

The recognition that cows experience a degree of inflammation around parturition (Bertoni et al., 2008, Trevisi et al., 2012) has led to the hypothesis of a homeorhetic role of inflammation as a physiological adaptation to lactation (Farney et al., 2013, Vailati Riboni et al., 2015). Furthermore, in the context of inflammation, the miRNA signaling through complex networks involving transcription factors has been demonstrated (Arner and Kulyte, 2015). A good example of this is the miRNA regulatory circuits composed by miR-92a, miR-126, and miR-193b that control levels of the chemokine CCL2 in human adipose tissue (Arner et al., 2012). However, regarding inflammation, miRNA not only regulate gene expression, but their expression patterns have been associated with levels of inflammatory molecules such as cytokines and the degree of immune cell infiltration (Kloting et al., 2009).

In the present study, gene and miRNA expression profiling were used to better understand the interaction between pre-calving BCS and plane of nutrition in the metabolism of adipose tissue. Specifically, we were interested in how these factors influence the adipose response to the physiological changes induced by the high metabolic demands of early lactation.

MATERIALS AND METHODS

Animal Management

Complete details of the experimental design are published elsewhere (Roche et al., 2015). Briefly, a group of 150 mid-lactation grazing dairy cows of mixed age and breed were enrolled in the experiment on 21st January 2013. Animals were allocated randomly to one of six treatment groups (25 cows per group) in a 2 × 3 factorial arrangement: two pre-calving BCS categories (4.0 and 5.0, **BCS4** and **BCS5**; based on a 10-point scale, where 1 is emaciated and 10 obese; Roche et al. 2004) and three levels of energy intake during the three wk preceding calving (75, 100 and 125% of estimated requirements; Roche et al. 2005). The different levels of energy intake were obtained by daily manipulation of pasture allowance, adjusting area allocation (m^2/cow) in each group (Roche et al., 2015). Cows were randomly assigned to the six groups, balanced for age, breed (Holstein and Holstein x Jearsey), BCS at the time of enrollment, and expected calving date. For the current study only four groups with a subset of 28 animals (7 cows per group) were considered. These were cows with prepartum BCS4 fed to meet 75 (**B4F75**) or 125 (**B4F125**) % of requirements, and cows with prepartum BCS5 fed to meet 75 (**B5F75**) or 125 (**B5F125**) % of requirements. The subset groups were still balance for breed age and calving date. Average age and days in gestation at enrollment was 6.20 ± 2.18, and 259.32 ± 1.86.

RNA Extraction and Quantitative PCR (qPCR)

Adipose biopsy. Tissue was harvested during wk −1, 1, and 4 relative to parturition as described previously (Grala et al., 2013). Briefly, subcutaneous adipose tissue was collected posterior to the shoulder blade and approximately 10 cm down the withers. The site was clipped and cleansed with iodine before administering a local anesthetic (2% lignocaine). A 3-cm incision was made through the skin and 30 to 80 mg of adipose tissue removed by blunt

dissection. Biopsies were immediately placed in screw-capped, microcentrifuge tubes, snap-frozen in liquid nitrogen, and stored at −80°C until RNA extraction.

Total RNA Extraction, Target Genes, and qPCR. Complete details of these procedures are included in the supplemental material (Appendix B). Briefly, RNA samples were extracted from 0.2 g of frozen adipose tissue and used for cDNA synthesis using established protocols in our laboratory (Khan et al., 2014). The Quanta qScript microRNA cDNA Synthesis Kit (Quanta BioSciences, Inc., Gaithersburg, MD) was used for miRNA following the manufacturer's protocols. The qPCR performed was SYBR Green-based, using a 7-point standard curve. Genes selected for transcript profiling are associated with fatty acid metabolism: fatty acid synthase (*FASN*), peroxisome proliferator-activated receptor gamma (*PPARG*); adipokines: adiponectin (*ADIPOQ*), leptin (*LEP*); and inflammation: chemokine (C-C motif) ligand 2 (*CCL2*), chemokine (C-C motif) ligand 5 (*CCL5*), haptoglobin (*HP*), interleukin-1β (*IL1B*), inteleukin-6 (*IL6*), inteleukin-6 receptor (*IL6R*), retinoid X receptor alpha (*RXRA*), serum amyloid A3 (*SAA3*), toll-like receptor 4 (*TLR4*), and tumor necrosis factor α (*TNF*). miRNA selected for expression profiling (Table 4.1) are associated with immune cell infiltration (miR-26b, miR-126, miR-132, miR-155, miR-193), inflammation and lipolysis (miR-99a, miR-145, miR-221), and positive regulation of adipogenesis (miR-103, miR-143, miR-378).

Blood Collection and Analysis

Blood was sampled by coccygeal venipuncture in correspondence to biopsy, using evacuated blood tubes containing a lithium heparin anticoagulant. Samples were placed immediately on ice and centrifuged within 30 min at 1,500 × *g* for 12 min at 4°C. Following centrifugation, aspirated plasma was stored at −20°C until assayed.

Blood NEFA and BHBA analysis was performed by Gribbles Veterinary Pathology Ltd. Blood metabolites were assayed using colorimetric techniques at 37°C with a Hitachi Modular P800 analyzer (Roche Diagnostics, Indianapolis, IN). Plasma NEFA concentration (mmol/L) was measured using Wako Chemicals (Osaka, Japan) kit NEFA HR2 measuring oxidative condensation of 3-methyl-*N*-ethyl-*N*-β hydroxyethyl aniline with 4-aminoantipyrine, while plasma BHBA (mmol/L) concentrations was assessed using Roche reagent kits measuring the reduction of NAD to NADH during oxidation of d-3-hydroxybutyrate to acetoacetate.

Statistical Analysis

Both expression datasets (mRNA and miRNA) and blood data were analyzed in the same fashion. After normalization with the geometric mean of the internal control genes, the qPCR data were $\log_2$ transformed prior to statistical analysis to obtain a normal distribution, while NEFA and BHBA data were used as is. Statistical analysis was performed with SAS (v 9.3). Normalized, $\log_2$ transformed expression data, and blood metabolites data were subjected to ANOVA and analyzed using repeated measures ANOVA with PROC MIXED. The statistical model included time (**T**; -1, 1 and 4 wk postpartum), BCS (**B**; 4 and 5), feeding (**F**, 75% and 125%) and their interactions (B*T, F*T and B*F*T) as fixed effects. Cow, nested within treatment, was 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. Data were considered significant at a $P \leq 0.05$ using the PDIFF statement in SAS. For ease of interpretation, the expression data reported in Tables 4.3 through 4.6 are the $\log_2$ back-transformed LSM that resulted from the statistical analysis.

RESULTS

NEFA and BHBA

Both NEFA and BHBA were affected by BCS ($P < 0.05$), with a higher overall concentration in BCS5 animals compared to BCS4, independently from prepartum feeding regime (Table 4.2). Feeding instead only affected BHBA concentration, with a greater overall concentration in animal fed 75% compared to 125%. Despite effects were found in both factors, their interaction was not significant in either metabolites (B*F, $P > 0.05$), even thou numerical differences are observable. Time, as expected, was significant for both metabolites.

Gene Expression

Adipokines and fatty acid metabolism. *ADIPOQ* was increased by BCS (B, $P < 0.0001$) due to a greater expression ($P < 0.05$) in BCS5. *LEP* was affected by BCS, pre-calving plane of nutrition and their interaction (B, F, B*F, $P < 0.003$) (Table 4.3). Its expression was greater in B5F75 and B5F125 cows, while B4F75 cows had the lowest expression and B4F125 cows an intermediate expression. Among the adipokines, time was significant only for *LEP* (T, $P < 0.0001$) due to a decrease in expression postpartum (Table 4.4). However, the interaction with BCS ($P < 0.004$) revealed that from 1 to 4 wk BCS4 cows had decreased expression whereas BCS5 cows had similar *LEP* expression ($P < 0.05$).

Among the adipogenic/lipogenic genes, *PPARG* expression was greater ($P < 0.05$) in BCS5 (Table 4.3) compared with BCS4 cows. Further, *PPARG* expression was higher (T, $P < 0.05$) around parturition (-1 and 1 wk) compared with wk 4 of lactation (Table 4.4). There was a triple interaction (B*F*T, $P = 0.02$) for *FASN* expression due to differences among feeding management groups at -1 and 4 wk around parturition coupled with the general decrease in expression postpartum for all cows (Table 4.4). Pre-calving, F125 cows had a greater ($P < 0.05$)

expression (~3-fold) compared with F75 cows regardless of BCS. However, when fed F75, the BCS5 cows had a greater expression compared with BCS4. In contrast, at 4 wk of lactation *FASN* expression was greater ($P < 0.05$) for B4F75 cows compared with B4F125 and B5F75. Compared with all other groups, B5F125 cows had an intermediate level of expression (Table 4.4).

RNA expression of inflammation and immune-related proteins and receptors. Among cytokine-encoding genes, *IL1B* was affected only by the interaction between BCS and feeding management (B*F, $P = 0.05$) due to the greater ($P < 0.05$) expression in B4F75 compared with B4F125 cows (Table 4.3). Cows with BCS5 had an intermediate expression level that did not differ ($P > 0.05$) due to feeding. A three-way interaction was detected (B*F*T, $P < 0.005$) for *IL6* and *TNF* (Table 4). Both genes increased ($P < 0.05$) in expression postpartum across all groups except for B5F75, in which expression of *TNF* was constant over time ($P > 0.05$). *IL6* expression decreased immediately postpartum compared with -1 wk and then peaked at 4 wk of lactation (Table 4.4). Independently of feeding management, prepartum expression of *IL6* was greater ($P < 0.05$) in BCS5 compared with BCS4 cows, while B5F75 cows had the greatest ($P < 0.05$) expression of *TNF*. At 1 wk postpartum, both B4F75 and B5F125 groups had a higher ($P < 0.05$) expression of both *IL6* and *TNF*, but at 4 wk only *TNF* remained higher, while *IL6* expression was greater only in B5F75 cows (Table 4.4).

Both acute-phase protein-related genes *HP* and *SAA3* were affected by time (T, $P < 0.004$), as their expression increased after calving (Table 4.4). A three-way interaction (B*F*T, $P = 0.006$) and an effect of feeding level (F, $P = 0.02$) were detected for *HP*. In all groups *HP* expressions was higher at 1 and 4 wk postpartum, compared to prepartum. In BCS4 animals, disregarding feeding groups, the postpartum expression remained constant. However in BCS5F75 the

expression increased at 4 wk compared to 1wk, while in BCS5F125 it peaked at 1wk to then decrease at 4wk, to an intermediate level compared to the other two time points (Table 4.4). *IL6R* and *TLR4*, both cytokine receptor-encoding genes, were affected by time (T, $P < 0.0001$) due to greater expression postpartum. Both BCS and feeding management (B, F, $P < 0.004$) also affected *IL6R* and *TLR4*, as BCS5 or F75 cows had an overall greater ($P < 0.05$) expression. However, the interaction with time (B*T or F*T) underscored how this difference was only significant ($P < 0.05$) prepartum (Table 4.4). A three-way interaction was detected for *TLR4*, revealing how the response in B5F75 cows due to the higher ($P < 0.05$) expression at -1 wk drove the difference prepartum between management groups. The expression of *TLR4* in B5F75 cows was stable over time and without changes ($P > 0.05$) among time points.

The expression of *CCL2* was only affected by feeding management (F, $P = 0.0005$) due to higher expression in F75 cows. Expression of *CCL5* had a significant BCS, time and three-way interaction (B, T, B*F*T, $P < 0.0001$) (Tables 4.3 and 4.4). Its expression was overall higher ($P < 0.05$) postpartum and greater ($P < 0.05$) in BCS5 cows independent of feeding, but the greatest ($P < 0.05$) expression was detected postpartum in B5F125 cows.

RXRA was affected ($P < 0.03$) by all factors except time, for which only a tendency was detected ($P = 0.08$) (Tables 4.3 and 4.4). Its expression was overall greater (BCS, F $P < 0.05$) in BCS5 or F125 cows, and this was observed primarily prepartum.

MicroRNA Expression

Proadipogenic miRNA. An effect of time (T, $P < 0.01$) was detected for the expression of miR-378 and miR-103 due to a decrease ($P < 0.05$) postpartum (Table 6). Plane of nutrition affected both miR-378 and miR-143 (F, $P < 0.004$) due to greater expression ($P < 0.05$) in F125 cows (Table 4.5). The expression of miR-143 also had a significant three-way interaction

(B*F*T, $P = 0.03$) due to greater expression over time in B5F125 cows. The expression of miR-103 was affected by BCS alone (B, $P = 0.007$) or by the interaction with feeding (B*F, $P = 0.004$). Its expression was overall greater in BCS5 cows, and even greater when these animals were fed 125% of requirements during the dry period (B5F125).

The expression of miR-103 was greater overall in BCS5 cows (B, $P = 0.007$) and even greater when these animals were fed 125% of requirements during the dry period (B5F125; B*F, $P = 0.004$). The expression of miR-103 was greatest in B5F125 cows (B*F, $P = 0.004$).

Inflammation and lipolysis. Expression of miR-99a (T, B*T, $P < 0.02$) and miR-145 (F*T, $P = 0.04$) decreased ($P < 0.05$) postpartum for BCS4 or F75 animals (Table 4.6). Feeding management affected expression (F, $P = 0.02$) of miR-145 and miR-99a in opposite directions, i.e. F75 increased ($P < 0.05$) miR-145 and decreased ($P < 0.05$) miR-99a relative to F125 cows (Table 4.5). For miR-145 the difference was mainly due to responses in the prepartum period (F*T, P = 0.04). For the expression of miR-221, BCS and feeding interacted differently across the groups (B*F, $P = 0.01$), as its expression was greater ($P < 0.05$) in B4F75 and B5F125 cows.

Adipose infiltration of immune cells. miR-193 was only affected by time (T, $P < 0.0001$), as its expression decreased ($P < 0.05$) gradually from -1 to 4 wk relative to parturition (Table 4.6). The three-way interaction was significant (B*F*T, $P < 0.04$) for miR-155 and miR-26b. For miR-155 this was due to an increase in expression ($P < 0.05$) postpartum and overall greater ($P < 0.05$) expression in BCS4 compared with BCS5 cows, and in F75 compared with F125 cows. Expression of miR-132 and miR-126 was affected by plane of nutrition (F, $P < 0.004$) (Table 4.5). The former was greater ($P < 0.05$) in F75 cows, while the latter was greater in F125 cows (especially prepartum). Both miRNA also were affected by the interaction of BCS with time (B*T, $P < 0.04$). Expression of miR-132 was greater in BCS5 cows, and postpartum its

expression decreased to similar levels than BCS4 cows. Expression of miR-126 changed mainly due to time, with a decrease ($P < 0.05$) in expression immediately postpartum followed by an increase ($P < 0.05$) at 4 wk, at which point it reached prepartum levels only in BCS5 cows. This microRNA also was affected by the interaction of plane of nutrition and time (F*T, $P = 0.01$), mainly due to a higher ($P < 0.05$) expression prepartum in F125 cows, and to a decrease ($P < 0.05$) in expression at 4 wk. In contrast, F75 cows maintained a constant level of expression ($P > 0.05$) throughout the experimental period.

DISCUSSION

Results from the present study enhance the understanding of molecular mechanisms underlying some of the physiological responses associated with both the independent and combined effects of prepartum BCS and feeding management. The relationship between the immune and metabolic response of the adipose tissue was evident both at the mRNA and miRNA expression level, underscoring the existence of a "self-regulatory" mechanism within the adipose depot. Together, the data generated could help develop more mechanistic approaches for utilizing dry period feeding and BCS management to manipulate the physiological response of subcutaneous adipose tissue during the transition period.

Adipocyte metabolism and adipokines. Around parturition, body reserves are mobilized extensively, i.e. there is little anabolism and substantial catabolism (Drackley, 1999). The expression pattern of *FASN* over time exemplifies this adaptation. This enzyme is mainly regulated through expression rather than post-transcriptional mechanisms (Bernard et al., 2008, Bionaz and Loor, 2008); thus, mRNA expression is a suitable indicator of its activity within adipocytes. The marked impact of prepartum overfeeding on *FASN* and the proadipogenic

miRNA studied (miR-378 and miR-143) appear important for driving adipogenesis in adipose tissue. Furthermore, the greater prepartal *FASN* expression when cows of BCS 5 compared with BCS 4 were underfed may indicate their adipose depots were "primed" to deposit fat. This idea is further supported by the effect of BCS on expression of *PPARG* and *RXRA*, two nuclear receptors involved in promoting adipocyte differentiation and hypertrophy (Metzger et al., 2005, Bionaz et al., 2013), and also the expression of miR-103, an upregulator of adipogenesis (Romao et al., 2011, Trajkovski et al., 2011).

As exemplified by the responses in the BCS5 compared with BCS4 cows, the evident association between BCS and fat deposition might be due in part to greater "flexibility" of tissue pre-adipocytes to differentiate rather than enlargement of existing adipocytes (Kawada et al., 2001). The greater expression of *ADIPOQ* in BCS5 cows lends support to this hypothesis. In non-ruminants, adiponectin improves insulin sensitivity and exerts some regulation over fatty acid metabolism (Brochu-Gaudreau et al., 2010). Its expression also is markedly increased during ruminant adipocyte differentiation (Roh et al., 2006). Thus, changes in *ADIPOQ* provide an indication of the degree of pre-adipocyte differentiation (Soliman et al., 2007).

The BCS together with feeding management prepartum also influenced the expression of leptin, another adipokine. Leptin, produced mainly in white adipose tissue, is a protein that in rodents is involved in the regulation of energy intake, storage and expenditure (Chilliard et al., 2005). In cows and heifers, leptin is positively associated with both BCS and nutrient status (Reist et al., 2003, Leon et al., 2004), which explains its greater expression in both BCS5 and overfed cows. However, cow adiposity had a greater role because BCS alone increased prepartum *LEP* expression by 4-fold. Given that leptin could elicit a lipolytic effect (Harris,

2014), its greater expression postpartum along with higher NEFA (Table 2) in cows with BCS5 might be beneficial in terms of helping them cope with the typical NEB of early lactation.

Inflammation and the adipocyte. The mild-to-high systemic inflammation around parturition may impair production (Bertoni et al., 2008). However, recent data suggest that, when controlled, the inflammatory phenomena play a homeorhetic role in the adaptations of the modern dairy cow to transitioning into lactation (Farney et al., 2013, Vailati Riboni et al., 2015). As indicated by the higher *HP* and *SAA3* expression postpartum, cows in the current experiment most probably experienced a degree of peripartum inflammation. Both haptoglobin and serum amyloid A are well known pro-inflammatory biomarkers (Eckersall and Bell, 2010) mainly synthesized by the liver. However, it is well-established in non-ruminants that adipocytes also produce these proteins and they are recognized as adipokines (Trayhurn, 2005). The greater *HP* expression early postpartum in both B4F75 and B5F125 groups compared with their opposite feeding in the same BCS group is indicative of a higher degree of localized inflammation. Thus, avoiding high-dietary energy prepartum (Selim et al., 2014, Shahzad et al., 2014) at an optimal BCS during the dry period (BCS 5), while overfeeding under-conditioned cows during the close-up dry period could help alleviate excessive adipose tissue inflammation after calving. As a result of the lower production of pro-inflammatory mediators, the lipolytic sensitivity of adipose may be reduced. In that context, the peak in *HP* expression detected in optimally-conditioned feed-restricted cows (B5F75) at 4 wk of lactation is noteworthy. Further research is needed to understand the biological mechanisms driving this response.

Apart from the systemic environment, non-ruminant studies revealed that localized inflammation plays an important role in coordinating the physiology of the adipocyte. For instance, infiltration of fat depots by immune cells in obese individuals is one of the triggers of

localized inflammation (Surmi and Hasty, 2008). This occurs via the expression of chemokines (chemoattractant cytokines) such as CCL2/MCP-1 (C-C motif chemokine ligand 2/macrophage chemoattractant protein-1) or CCL5/RANTES (C-C motif chemokine ligand 5/regulated on activation, normal T cell expressed and secreted) (Neels and Olefsky, 2006). Although overfeeding generally increases chemokine expression, both in human and animal models (Lionetti et al., 2009, Ji et al., 2014b), feed-restriction in lactating goats also upregulated *CCL2* in adipose tissue (Faulconnier et al., 2011).

Underfed animals are generally in NEB, and rely heavily on body reserves. Because inflammation induces lipolysis and insulin-resistance in non-ruminant adipocytes (Tilg and Moschen, 2008), the upregulation of *CCL2* could serve as a homeostatic mechanism to reduce utilization of nutrients by adipose depots, hence, directing the greatest amount of glucose toward maintenance or milk synthesis (De Koster and Opsomer, 2013). This idea is supported by the upregulation of miR-155, miR-26b and miR-132 [all of which are markers of macrophage infiltration (Kloting et al., 2009)] in feed-restricted cows independently of BCS. The upregulation of these miRNA postpartum in all cows supports the idea of inflammation as a response to the physiological adaptations to lactation.

In contrast to other markers of immune cell infiltration, there was an additive effect of dry period conditioning on miR-155, as in early lactation the feed-restricted BCS 4 cows had the greatest expression among all groups. Thus, feed-restricting thin cows prepartum could lead to an excess of localized inflammation that might impair the normal adaptation of the tissue to lactation. Based on the treatment effects detected, the expression of miR-126 (upregulated in overfed animals) and miR-193 (no change in relation to feeding management) did not seem to be associated with *CCL2*. In humans, both of these miRNA are involved in the transcriptional

regulation of *CCL2* (Arner et al., 2012). This leads us to speculate that the same mechanism might not be present in bovine adipocytes.

The downregulation of miR-99a along with upregulation of miR-145 especially prepartum in feed-restricted cows offers support to the hypothesis of induced local inflammation via the recruitment of immune cells, likely augmenting tissue lipolytic sensitivity. Because in humans miR-99a abundance is negatively correlated with concentration of free fatty acids within the adipocyte (Kloting et al., 2009) and miR-45 regulates adipocyte lipolysis through different mechanisms (Lorente-Cebrian et al., 2014), their expression patterns imply a higher degree of mobilization in feed-restricted cows, which is partly supported by the higher NEFA prepartum (Roche et al., 2015).

In contrast to feeding level being a primary driver of *CCL2* expression, prepartum cow adiposity (BCS) appeared to be the main effector of *CCL5* expression. Compared with CCL2, CCL5 is a chemokine with a broader spectrum that is not only able to recruit monocytes and promote their survival (Keophiphath et al., 2010), but also mediates the trafficking and homing of T cells, basophils, eosinophils, natural killer cells, dendritic cells and mast cells (Appay and Rowland-Jones, 2001). As in the obesity model (Huber et al., 2008), the effect of BCS 5 on *CCL5* expression could be attributed in part to the higher adiposity, because *CCL2* also was numerically higher at BCS 5. Its expression was further increased postpartum in cows that underwent overfeeding at a BCS of 5 over the pre-calving period.

The nuclear receptor *RXRA* can play a key role in the regulation of innate immunity by modulating expression of other chemokines (e.g. CCL6, CCL9) (Nunez et al., 2010). The fact that its expression followed the same trend as *CCL5*, with increased expression in BCS5 cows and overfed cows especially prepartum, could have contributed to excessive localized

inflammation, greater insulin resistance, and greater lipolytic sensitivity. Such effects could be even greater in cows with BCS higher than 5, as overconditioned cows normally seem to mobilize more body reserves; hence, they are more prone to metabolic disorders like ketosis (Pires et al., 2013; Roche et al., 2013).

Adipose tissue is an active producer of cytokines, primarily by non-fat cells, and their release is enhanced in response to local inflammation (Fain, 2006). In turn, the cytokines released can impact adipose metabolism (Coppack, 2001). Because tumor necrosis factor α is one of the main cytokines produced by infiltrated immune cells, especially macrophages (Weisberg et al., 2003, Xu et al., 2003), and it increases lipolysis (Cawthorn and Sethi, 2008), the greater expression of *TNF* in cows with higher BCS that were overfed indicates they might be most susceptible to having pathological increases in blood NEFA. Interleukin-6, another cytokine known in non-ruminants for its lipolytic effects (Yang et al., 2008), also was upregulated early postpartum in the same animals. Regardless of time around parturition, the upregulation of *IL6R* in BCS 5 cows indicates that they were more susceptible to IL-6. Thus, as proven in overconditioned cows at parturition, an increase in the local sensitivity to IL-6 would lead to excess lipolysis in early lactation and threaten the liver, e.g. increasing triacylglycerol accumulation and impairing its functions (Roche et al., 2009, Akbar et al., 2015). In contrast to overfeeding, the postpartum pro-lipolytic effect of feed-restriction seems to be increased at a lower degree of adiposity. For example, expression of *IL1B*, encoding a cytokine involved in insulin resistance and lipolysis in adipocytes (Lagathu et al., 2006), was markedly increased in feed-restricted BCS 4 cows. This is another example of a prolonged effect of prepartum feeding management into early lactation.

Despite what is known thus far regarding the effect of BCS and plane of nutrition in the dry period, the combination of greater BCS (5) with feed-restriction prepartum seems to affect pre-calving expression of *CCL5*, *TLR4*, and *IL6*. Of particular interest, from the inflammation standpoint, is the lipopolysaccharide receptor TLR4 because in non-ruminants it links innate immunity with fatty acid–induced insulin resistance (Shi et al., 2006). The prepartal response in *CCL5* and *IL6* would be expected to trigger an inflammatory response close to calving, and the upregulation of *TLR4* would induce insulin resistance, both of which favor lipid mobilization mechanisms in cows with optimal BCS. As such, the adaptations in these gene networks serve to prime the adipocytes for the demands of early lactation. It is noteworthy that the transcriptional response of these genes was lost postpartum. Thus, the link between these genes in relationship with BCS and dietary management prepartum needs further research.

CONCLUSIONS

Overfeeding optimally-conditioned cows during the last 3 wk prior to parturition primed adipose tissue for accretion of lipid and a robust localized inflammatory response, which upon parturition increases the probability for metabolic disorders, i.e. localized inflammation renders the adipocyte more susceptible to lipolytic signals that could result in greater flow of NEFA into liver. Similarly, prepartum nutrient restriction of thinner cows enhances the localized pro-inflammatory response of adipocytes, hence, eliciting a similar negative outcome. Overall, the combined data indicate that a regime of nutrient restriction prepartum in optimally-conditioned cows avoids detrimental effects at the adipose tissue level, hence, physiologically priming the cow to the demands of lactation while avoiding a metabolically “lazy” phenotype. Instead

thinner animals seem to benefit from a higher plane of nutrition, with beneficial effects in terms of controlling localized inflammation.

ACKNOWLEDGMENTS

This research was supported by New Zealand dairy farmers through DairyNZ Inc. (RD1403), and the Ministry of Business, Innovation and Employment (Wellington, New Zealand; DRCX1201). The authors thank Jason Philips, Farm Manager, Scott Farm, DairyNZ, and his capable team, as well as Stu Morgan, Kate Watkins, Hamish Hodgson, and Chris Roach for technical support.

TABLES

Table 4.1. Details and functions of the miRNA targets analyzed in the current study.

miRNA	Function and/or expression pattern
Infiltration of immune cells	
miR-26b	Expression is associated with the number of macrophages infiltrating the fat depot Affected by levels of circulating TNF, leptin and resistin
miR-126	Directly inhibits *CCL2* expression
miR-132	Expression levels are associated with the number of macrophages infiltrating fat depots Activates NF-κB signalling and the transcription of *IL8* and *CCL2* Lower expression is associated with increased secretion of IL-6
miR-155	Expression levels are associated with the number of macrophages infiltrating fat depots
miR-193	Indirectly inhibits *CCL2* expression through a network of transcription factors
Inflammation and lipolysis	
miR-99a	Negative correlation with secretion of IL-6 and level of free fatty acids
miR-145	Affects secretion of TNFα, regulating lipolysis
miR-221	Lower expression is associated with high levels of TNFα
Proadipogenic	
miR-103	Regulates expression of *PPARG, PANK1, CAV1, FASN, ADIPOQ* and *FABP4*
miR-143	Regulates expression of *ERK5, SLC2A4, TFAP2A, LIPE, PPARG, CEBPA,* and *FABP4*
miR-378	Targets *PPARG* expression through the MAPK1 pathway

Table 4.2. Effect of prepartum BCS and feeding management on plasma concentration of NEFA and BHBA in dairy cows during the transition period.

							B*F											
	B			F			4		5			*P*-value[1]						
mmol/L	4	5	SE	75%	125%	SE	75%	125%	75%	125%	SE	B	F	B*F	T	B*T	F*T	B*F*T
NEFA	0.50^{x}	0.72^{y}	0,04	0,59	0,62	0,04	0.48^{x}	0.51^{x}	0.70^{y}	0.73^{y}	0,05	0,0004	0,62	0,99	<.0001	0,05	0,26	0,99
BHBA	0.46^{x}	0.60^{y}	0,03	0.57^{x}	0.49^{y}	0,03	0.51^{x,z}	0.42^{x}	0.64^{y}	0.56^{x,z}	0,04	0,004	0,05	0,83	<.0001	0,1	0,01	0,57

[1]T = time (week relative to parturition); B = BCS (1-10 scale); F = level of feeding prepartum relative to requirement (%); B*F*T = interaction of BCS, level of feeding prepartum relative to requirements (%), and time around parturition.
x,y,z = significant difference among groups ($P < 0.05$).

Table 4.3. Effect of prepartum BCS and feeding management on subcutaneous adipose tissue expression (log_2 back-transformed LSM) in dairy cows during the transition period.

					B*F							
	B		F		4		5			*P*-value[1]		
Gene	4	5	75%	125%	75%	125%	75%	125%	SEM[2]	B	F	B*F
Inflammation												
CCL2	2.16	2.82	3.39^{x}	1.79^{y}	2.97	1.57	3.88	2.04	0.44	0.13	0.0005	0.99
CCL5	1.34^{x}	2.88^{y}	1.89	2.04	1.39	1.29	2.56	3.24	0.33	<.0001	0.58	0.28
HP	0.17	0.25	0.28^{x}	0.16^{y}	0.26	0.11	0.30	0.22	0.17	0.13	0.02	0.31
IL1B	0.99	0.93	1.05	0.88	1.21^{x}	0.8^{y}	0.91^{x,y}	0.96^{x,y}	0.10	0.66	0.13	0.05
IL6	0.48^{x}	0.79^{y}	0.70	0.54	0.57	0.40	0.86	0.72	0.10	0.006	0.15	0.63
IL6R	0.82^{x}	1.08^{y}	1.08^{x}	0.82^{y}	0.90	0.75	1.30	0.90	0.11	0.005	0.004	0.32
RXRA	0.74^{x}	1.19^{y}	0.86^{x}	1.02^{y}	0.62^{x}	0.87^{y}	1.19^{z}	1.19^{z}	0.07	<.0001	0.03	0.03
SAA3	0.53	0.39	0.42	0.49	0.52	0.55	0.34	0.43	0.12	0.27	0.61	0.77
TLR4	1.02^{x}	1.25^{y}	1.22^{x}	1.04^{y}	1.09	0.95	1.38	1.14	0.08	0.0005	0.007	0.65
TNF	0.78	0.93	0.87	0.83	0.90	0.68	0.84	1.03	0.10	0.07	0.66	0.01
Adipokines and fatty acid metabolism												
ADIPOQ	0.72^{x}	1.28^{y}	0.87	1.05	0.59	0.88	1.29	1.27	0.11	<.0001	0.09	0.07
FASN	0.15	0.17	0.13^{x}	0.21^{y}	0.12	0.19	0.13	0.23	0.17	0.33	0.0001	0.55
LEP	0.12^{x}	0.65^{y}	0.17^{x}	0.44^{y}	0.05^{x}	0.26^{y}	0.57^{z}	0.74^{z}	0.33	<.0001	<.0001	0.003
PPARG	0.86^{x}	1.08^{y}	0.94	0.99	0.81	0.93	1.10	1.07	0.09	0.008	0.52	0.33

[1] B = BCS (1-10 scale); F = level of feeding prepartum relative to requirements (%); B*F = interaction of BCS and level of feeding prepartum relative to requirements (%).
[2] SEM = greatest standard error of the mean.
x,y,z = significant difference among groups ($P < 0.05$).

Table 4.4. Effect of the interaction among prepartum BCS, prepartum feeding management, and time around parturition on subcutaneous adipose tissue gene expression (log_2 back-transformed LSM) in dairy cows during the transition period.

							B*F*T								
			B*T		F*T		4		5			*P*-values[1]			
Gene	wk	T[1]	4	5	75%	125%	75%	125%	75%	125%	SEM[2]	T	B*T	F*T	B*F*T
Inflammation															
	-1	2.11	1.63	2.72	2.41	1.84	1.57	1.70	3.72	1.99					
CCL2	1	2.48	2.65	2.32	3.58	1.72	4.46	1.57	2.87	1.88	1.57	0.28	0.17	0.23	0.19
	4	2.87	2.32	3.54	4.52	1.82	3.74	1.44	5.46	2.29					
	-1	1.09^{a}	0.69	1.75	1.18	1.01	^{x}0.52^{a}	^{x,z}0.91^{a}	^{y}2.71	^{z}1.12^{a}					
CCL5	1	2.23^{b}	1.71	2.91	2.04	2.43	^{x}2.11^{b}	^{x}1.38^{b}	^{x}1.98	^{y}4.26^{b}	1.53	<.0001	0.25	0.33	<.0001
	4	3.11^{c}	2.05	4.71	2.78	3.47	^{x,y}2.48^{b}	^{x}1.70^{b}	^{y}3.12	^{z}7.10^{b}					
	-1	0.04^{a}	0.04	0.05	0.06	0.03	^{x,y}0.06^{a}	^{x}0.02^{a}	^{y}0.07^{a}	^{x,y}0.04^{a}					
HP	1	0.46^{b}	0.35	0.61	0.49	0.43	^{x,z}0.68^{b}	^{y}0.18^{b}	^{x,y}0.36^{b}	^{z}1.05^{b}	0.46	<.0001	0.83	0.2	0.0006
	4	0.46^{b}	0.40	0.54	0.73	0.29	^{x}0.46^{b}	^{x}0.34^{b}	^{y}1.17^{c}	^{x}0.25^{c}					
	-1	0.93	1.05	0.82	1.03	0.84	1.36	0.81	0.78	0.87					
IL1B	1	0.93	0.85	1.03	1.18	0.73	1.15	0.62	1.22	0.87	0.29	0.7	0.29	0.08	0.81
	4	1.02	1.08	0.96	0.96	1.09	1.15	1.01	0.80	1.16					
	-1	0.53	0.34	0.82	0.62	0.46	^{x,y}0.4^{a}	^{x}0.29^{a}	^{y}0.96^{a}	^{x,y}0.71^{a}					
IL6	1	0.66	0.52	0.83	0.64	0.68	^{x,z}0.76^{b}	^{y}0.36^{b}	^{x,y}0.53^{b}	^{z}1.31^{b}	0.41	0.44	0.16	0.29	0.002
	4	0.66	0.62	0.71	0.86	0.50	^{x,y}0.61^{b}	^{y}0.63^{b}	^{x}1.23^{c}	^{y,z}0.41^{c}					
	-1	0.46^{a}	0.39	0.55	^{x}0.64 ^{a}	^{y}0.33^{a}	0.49	0.31	0.85	0.36					
IL6R	1	1.38^{b}	1.30	1.47	1.52^{b}	1.26^{b}	1.41	1.21	1.64	1.32	0.28	<.0001	0.52	0.011	0.73
	4	1.31^{b}	1.11	1.54	1.30^{b}	1.31^{b}	1.08	1.14	1.58	1.51					
	-1	1.02	^{x}0.66	^{y}1.59^{a}	^{x}0.79	^{y}1.32^{a}	^{x}0.40^{a}	^{y}1.07^{a}	^{z}1.55^{a}	^{z}1.63^{a}					
RXRA	1	0.94	^{x}0.81	^{y}1.08^{b}	0.92	0.96^{b}	^{x}0.79^{b}	^{x}0.83^{a,b}	^{x}1.06^{b}	^{x}1.11^{b}	0.20	0.08	0.0002	0.001	0.01
	4	0.86	^{x}0.75	^{y}0.98^{b}	0.88	0.83^{b}	^{x,y}0.75^{b}	^{y}0.75^{a}	^{x}1.04^{b}	^{x,y}0.93^{b}					
	-1	0.31^{a}	0.32	0.29	0.37	0.25	0.35	0.30	0.40	0.21					
SAA3	1	0.60^{b}	0.71	0.50	0.45	0.79	0.58	0.89	0.36	0.71	0.37	0.004	0.7	0.06	0.4
	4	0.51^{b}	0.65	0.40	0.45	0.58	0.69	0.62	0.29	0.55					
	-1	0.72^{a}	^{x}0.55^{a}	^{y}0.93^{a}	^{x}0.87^{a}	^{y}0.59^{a}	^{x}0.59^{a}	^{x}0.52^{a}	^{y}1.29	^{x}0.67^{a}					
TLR4	1	1.27^{b}	1.24^{b}	1.30^{b}	1.32^{b}	1.22^{b}	1.38^{b}	1.12 ^{b}	1.26	1.33^{b}	0.19	<.0001	0.003	0.04	0.03
	4	1.59^{c}	1.54^{c}	1.63^{c}	1.60^{b}	1.57^{b}	1.60^{b}	1.48 ^{b}	1.60	1.67^{b}					
TNF	-1	0.58^{a}	0.51	0.65	0.68	0.49	^{x,y}0.55^{a}	^{y}0.48^{a}	^{x}0.86	^{y}0.49^{a}	0.27	<.0001	0.87	0.11	0.005
	1	1.10^{b}	1.04	1.16	1.04	1.16	^{x,y}1.24^{b}	^{y}0.88^{b}	^{y}0.88	^{x}1.53^{b}					
	4	0.97^{b}	0.88	1.07	0.92	1.02	^{x,y}1.07^{b}	^{y}0.73^{a,b}	^{y}0.79	^{x}1.44^{b}					

Table 4.4. (cont)

Adipokines and fatty acid metabolism															
	-1	1.13	0.95	1.35	0.99	1.29	0.69	1.32	1.43	1.26					
ADIPOQ	1	0.86	0.63	1.16	0.74	0.99	0.48	0.83	1.14	1.17	0.26	0.07	0.21	0.49	0.24
	4	0.91	0.61	1.34	0.90	0.92	0.61	0.62	1.31	1.37					
	-1	1.47^{a}	1.22	1.78	^{x}0.68^{a}	^{y}3.22^{a}	^{x}0.47^{a}	^{y}3.15^{a}	^{z}0.97^{a}	^{y}3.29^{a}					
FASN	1	0.05^{b}	0.04	0.05	0.04^{b}	0.05^{b}	0.04^{b}	0.05^{b}	0.05^{b}	0.05^{b}	0.78	<.0001	0.38	<.0001	0.02
	4	0.06^{c}	0.07	0.06	0.07^{b}	0.06^{b}	^{x}0.09^{c}	^{y}0.05^{b}	^{y}0.05^{b}	^{x,y}0.08^{b}					
	-1	1.83^{a}	^{x}0.96^{a}	^{y}3.49^{a}	1.03	3.28	0.28	3.26	3.70	3.29					
LEP	1	0.20^{b}	^{x}0.11^{b}	^{y}0.36^{b}	0.14	0.29	0.07	0.17	0.28	0.47	1.32	<.0001	0.004	0.62	0.07
	4	0.06^{c}	^{x}0.02^{c}	^{y}0.21^{b}	0.04	0.09	0.01	0.03	0.17	0.27					
	-1	1.15^{a}	1.03	1.28	1.07	1.23	0.88	1.21	1.30	1.26					
PPARG	1	1.03^{a}	0.93	1.13	1.00	1.05	0.89	0.97	1.13	1.12	0.19	0.0004	0.92	0.72	0.7361
	4	0.77^{b}	0.67	0.88	0.78	0.76	0.67	0.67	0.90	0.86					

[1] T = time (week relative to parturition); B = BCS (1-10 scale); F = level of feeding prepartum relative to requirement (%); B*F*T = interaction of BCS, level of feeding prepartum relative to requirements (%), and time around parturition.
[2] SEM = greatest standard error of the mean.
a,b,c = significant difference among time points, within group ($P < 0.05$).
x,y,z = significant difference among groups within the same week relative to parturition ($P < 0.05$)

Table 4.5. Effect of prepartum BCS and feeding management on subcutaneous adipose tissue microRNA expression (log_2 back-transformed LSM) in dairy cows during the transition period.

					B*F							
	B		F		4		5			*P*-value[1]		
Gene	4	5	75%	125%	75%	125%	75%	125%	SEM[2]	B	F	B*F
Infiltration of immune cells												
miR-26b	1.35	1.52	1.65^{x}	1.24^{y}	1.64	1.11	1.65	1.39	0.17	0.27	0.007	0.30
miR-126	0.72	0.88	0.67^{x}	0.94^{y}	0.66	0.79	0.68	1.13	0.08	0.08	0.004	0.14
miR-132	1.23	1.46	1.55^{x}	1.15^{y}	1.48	1.03	1.63	1.30	0.12	0.07	0.002	0.45
miR-155	1.48^{x}	1.19^{y}	1.74^{x}	1.01^{y}	2.03	1.07	1.50	0.95	0.15	0.04	<.0001	0.37
miR-193	1.05	1.05	1.08	1.03	1.02	1.09	1.15	0.97	0.11	0.99	0.50	0.16
Inflammation and lipolysis												
miR-99a	0.96	0.93	0.85^{x}	1.05^{y}	0.88	1.04	0.82	1.05	0.09	0.70	0.02	0.64
miR-145	1.23	1.40	1.50^{x}	1.15^{y}	1.43	1.06	1.56	1.25	0.12	0.23	0.02	0.70
miR-221	1.24	1.33	1.29	1.28	$1.37^{x,y}$	1.11^{x}	1.20^{x}	1.47^{y}	0.09	0.33	0.93	0.01
Proadipogenic												
miR-103	0.97^{x}	1.16^{y}	1.04	1.08	1.05^{x}	0.90^{x}	1.04^{x}	1.30^{y}	0.07	0.007	0.61	0.004
miR-143	0.94	1.08	0.87^{x}	1.16^{y}	0.86	1.02	0.88	1.32	0.08	0.14	0.004	0.21
miR-378	1.06	0.94	0.80^{x}	1.25^{y}	0.87	1.30	0.73	1.21	0.16	0.19	<.0001	0.58

[1] B = BCS (1-10 scale); F = level of feeding prepartum relative to requirements (%); B*F = interaction of BCS and level of feeding prepartum relative to requirements (%).
[2] SEM = greatest standard error of the mean.
x,y,z = significant difference among groups ($P < 0.05$).

Table 4.6. Effect of the interaction among prepartum BCS, prepartum feeding management, and time around parturition on subcutaneous adipose tissue microRNA expression (log_2 back-transformed LSM) in dairy cows during the transition period.

							B*F*T								
			B*T		F*T		4		5			*P* value			
Gene	wk	T[1]	4	5	75%	125%	75%	125%	75%	125%	SEM[2]	T	B*T	F*T	B*F*T
Infiltration of immune cells															
	-1	0.91[a]	[x]1.10	[y]0.76[a]	1.01	0.82	[x]1.37	[x,y]0.88	[y]0.74[a]	[y]0.77[a]					
miR-26b	1	1.69[b]	1.49	1.92[b]	2.31	1.24	[x]1.78	[x]1.25	[y]2.99[b]	[x]1.24[a]	0.58	<.0001	0.005	0.07	0.04
	4	1.89[b]	[x]1.49	[y]2.39[b]	1.93	1.85	[x,y]1.81	[y]1.22	[x]2.05[b]	[x]2.80[b]					
	-1	0.81[a,b]	0.89[a]	0.73[a]	[x]0.58	[y]1.14[a]	0.72	1.11	0.46	1.16					
miR-126	1	0.93[a]	0.73[a,b]	1.19[b]	[x]0.80	[x]1.09[a]	0.62	0.87	1.03	1.37	0.25	0.007	0.01	0.01	0.28
	4	0.66[b]	0.57 [b]	0.77[a]	0.65	0.67b	0.65	0.50	0.65	0.91					
	-1	1.48	[x]1.17	[y]1.86[a]	1.82	1.20	1.64	0.84	2.02	1.72					
miR-132	1	1.31	1.36	1.28[b]	1.62	1.07	1.77	1.04	1.48	1.10	0.32	0.21	0.04	0.12	0.10
	4	1.23	1.17	1.30[b]	1.27	1.20	1.11	1.24	1.45	1.16					
	-1	0.99[a]	1.01	0.97	1.47	0.67	[x]1.46[a]	[y]0.70[a]	[x]1.49[a,b]	[y]0.63[a]					
miR-155	1	1.30[b]	1.60	1.05	1.65	1.02	[x]2.57[b]	[y]1.00[a]	[y]1.06[a]	[y]1.04[b]	0.44	<.0001	0.24	0.14	0.02
	4	1.82[c]	2.00	1.67	2.19	1.52	[x]2.25[a,b]	[x,y]1.77[b]	[x]2.13[b]	[y]1.30[b]					
	-1	1.63[a]	1.61	1.65	1.68	1.59	1.65	1.57	1.70	1.60					
miR-193	1	1.06[b]	1.18	0.95	1.13	0.99	1.27	1.09	1.01	0.89	0.29	<.0001	0.10	0.66	0.07
	4	0.68[c]	0.62	0.74	0.67	0.69	0.51	0.75	0.88	0.63					
Inflammation and lipolysis															
	-1	1.11[a]	[x]1.36[a]	[y]0.91	0.87	1.41	1.09	1.69	0.70	1.17					
miR-99a	1	0.93[a,b]	0.87[b]	0.99	0.88	0.99	0.77	0.99	1.00	0.99	0.25	0.01	0.01	0.10	0.21
	4	0.81[b]	0.75[b]	0.88	0.79	0.83	0.82	0.68	0.77	1.01					
	-1	1.41	1.26	1.58	[x]1.86[a]	[y]1.07	1.80	0.88	1.93	1.30					
miR-145	1	1.21	1.21	1.22	1.35[b]	1.10	1.45	1.01	1.26	1.19	0.32	0.40	0.62	0.04	0.16
	4	1.31	1.22	1.42	1.33[b]	1.30	1.13	1.32	1.58	1.27					
	-1	1.30	1.13	1.49	1.45	1.16	1.49	0.86	1.41	1.57					
miR-221	1	1.23	1.22	1.25	1.24	1.23	1.42	1.05	1.08	1.45	0.25	0.75	0.14	0.05	0.09
	4	1.31	1.36	1.27	1.18	1.46	1.22	1.52	1.15	1.40					
Proadipogenic															
	-1	1.21[a]	1.08	1.35	1.24	1.17	1.34	0.87	1.16	1.57					
miR-103	1	1.05[a,b]	0.93	1.19	1.04	1.06	0.99	0.87	1.09	1.29	0.20	0.01	0.45	0.42	0.15
	4	0.95[b]	0.91	0.98	0.88	1.02	0.88	0.95	0.88	1.08					
	-1	1.05	1.05	1.05	0.81	1.36	[x,y]0.94	[x,z]1.18[a,b]	[y]0.70[a]	[z]1.58					
miR-143	1	1.03	0.90	1.18	0.93	1.15	[x]0.74	[x,y]1.11[a]	[y]1.18[b]	[y]1.18	0.23	0.44	0.45	0.15	0.03
	4	0.94	0.87	1.01	0.87	1.00	[x,y]0.92	[x]0.81[b]	[x]0.83[a,b]	[y]1.24					
	-1	1.86[a]	2.09	1.65	1.30	2.66	1.62	2.69	1.03	2.63					
miR-378	1	1.01[b]	1.09	0.94	0.82	1.25	0.89	1.34	0.76	1.16	0.43	<.0001	0.50	0.09	0.46
	4	0.53[c]	0.52	0.54	0.47	0.59	0.45	0.61	0.49	0.58					

Table 4.6 (cont)

[1] T = time (week relative to parturition); B = BCS (1-10 scale); F = level of feeding prepartum relative to requirement (%); B*F*T = interaction of BCS, level of feeding prepartum relative to requirements (%), and time around parturition.
[2] SEM = greatest standard error of the mean.
a,b,c = significant difference among time points, within group ($P < 0.05$).
x,y,z = significant difference among groups within the same week relative to parturition ($P < 0.05$).

REFERENCES

Adamczak, M. and A. Wiecek. 2013. The adipose tissue as an endocrine organ. Seminars in nephrology 33(1):2-13.

Akbar, H., T. M. Grala, M. V. 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. Journal of dairy science 98(2):1019-1032.

Appay, V. and S. L. Rowland-Jones. 2001. RANTES: a versatile and controversial chemokine. Trends in immunology 22(2):83-87.

Arner, E., N. Mejhert, A. Kulyte, P. J. Balwierz, M. Pachkov, M. Cormont, S. Lorente-Cebrian, A. Ehrlund, J. Laurencikiene, P. Heden, K. Dahlman-Wright, J. F. Tanti, Y. Hayashizaki, M. Ryden, I. Dahlman, E. van Nimwegen, C. O. Daub, and P. Arner. 2012. Adipose tissue microRNAs as regulators of CCL2 production in human obesity. Diabetes 61(8):1986-1993.

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

Bernard, L., C. Leroux, and Y. Chilliard. 2008. Expression and nutritional regulation of lipogenic genes in the ruminant lactating mammary gland. Advances in experimental medicine and biology 606:67-108.

Bertoni, G., E. Trevisi, X. Han, and M. Bionaz. 2008. Effects of inflammatory conditions on liver activity in puerperium period and consequences for performance in dairy cows. Journal of dairy science 91(9):3300-3310.

Bionaz, M., G. J. Hausman, J. J. Loor, and S. Mandard. 2013. Physiological and nutritional roles of PPAR across species. PPAR research 2013:807156.

Bionaz, M. and J. J. Loor. 2008. Gene networks driving bovine milk fat synthesis during the lactation cycle. BMC genomics 9:366.

Brochu-Gaudreau, K., C. Rehfeldt, R. Blouin, V. Bordignon, B. D. Murphy, and M. F. Palin. 2010. Adiponectin action from head to toe. Endocrine 37(1):11-32.

Cawthorn, W. P. and J. K. Sethi. 2008. TNF-alpha and adipocyte biology. FEBS letters 582(1):117-131.

Chilliard, Y., C. Delavaud, and M. Bonnet. 2005. Leptin expression in ruminants: nutritional and physiological regulations in relation with energy metabolism. Domestic animal endocrinology 29(1):3-22.

Coppack, S. W. 2001. Pro-inflammatory cytokines and adipose tissue. Proceedings of the Nutrition Society 60(3):349-356.

Dann, H. M., N. B. Litherland, J. P. Underwood, M. Bionaz, A. D'Angelo, J. W. McFadden, and J. K. Drackley. 2006. Diets during far-off and close-up dry periods affect periparturient metabolism and lactation in multiparous cows. Journal of dairy science 89(9):3563-3577.

De Koster, J. D. and G. Opsomer. 2013. Insulin resistance in dairy cows. Veterinary clinics of North America: Food animal practice 29(2):299-322.

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

Eckersall, P. D. and R. Bell. 2010. Acute phase proteins: Biomarkers of infection and inflammation in veterinary medicine. Veterinary journal 185(1):23-27.

Fain, J. N. 2006. Release of interleukins and other inflammatory cytokines by human adipose tissue is enhanced in obesity and primarily due to the nonfat cells. Vitamins and hormones 74:443-477.

Farney, J. K., L. K. Mamedova, J. F. Coetzee, B. KuKanich, L. M. Sordillo, S. K. Stoakes, J. E. Minton, L. C. Hollis, and B. J. Bradford. 2013. Anti-inflammatory salicylate treatment alters the metabolic adaptations to lactation in dairy cattle. American journal of physiology. Regulatory, integrative and comparative physiology 305(2):R110-117.

Faulconnier, Y., Y. Chilliard, M. B. Torbati, and C. Leroux. 2011. The transcriptomic profiles of adipose tissues are modified by feed deprivation in lactating goats. Comparative biochemistry and physiology. Part D, Genomics & proteomics 6(2):139-149.

Grala, T. M., J. R. Roche, C. V. Phyn, A. G. Rius, R. H. Boyle, R. G. Snell, and J. K. Kay. 2013. Expression of key lipid metabolism genes in adipose tissue is not altered by once-daily milking during a feed restriction of grazing dairy cows. Journal of dairy science 96(12):7753-7764.

Grant, R. W. and V. D. Dixit. 2015. Adipose Tissue as an Immunological Organ. Obesity 23(3):512-518.

Graugnard, D. E., K. M. Moyes, E. Trevisi, M. J. Khan, D. Keisler, J. K. Drackley, G. Bertoni, and J. J. Loor. 2013. Liver lipid content and inflammometabolic indices in peripartal dairy cows are altered in response to prepartal energy intake and postpartal intramammary inflammatory challenge. Journal of dairy science 96(2):918-935.

Harris, R. B. 2014. Direct and indirect effects of leptin on adipocyte metabolism. Biochimica et biophysica acta 1842(3):414-423.

Hayirli, A., R. R. Grummer, E. V. Nordheim, and P. M. Crump. 2002. Animal and dietary factors affecting feed intake during the prefresh transition period in Holsteins. Journal of dairy science 85(12):3430-3443.

Holtenius, K., S. Agenas, C. Delavaud, and Y. Chilliard. 2003. Effects of feeding intensity during the dry period. 2. Metabolic and hormonal responses. Journal of dairy science 86(3):883-891.

Huber, J., F. W. Kiefer, M. Zeyda, B. Ludvik, G. R. Silberhumer, G. Prager, G. J. Zlabinger, and T. M. Stulnig. 2008. CC chemokine and CC chemokine receptor profiles in visceral and subcutaneous adipose tissue are altered in human obesity. Journal of clinical endocrinology and metabolism 93(8):3215-3221.

Janovick, N. A., Y. R. Boisclair, and J. K. Drackley. 2011. Prepartum dietary energy intake affects metabolism and health during the periparturient period in primiparous and multiparous Holstein cows. Journal of dairy science 94(3):1385-1400.

Ji, P., J. K. Drackley, M. J. Khan, and J. J. Loor. 2014a. Overfeeding energy upregulates peroxisome proliferator-activated receptor (PPAR)gamma-controlled adipogenic and lipolytic gene networks but does not affect proinflammatory markers in visceral and subcutaneous adipose depots of Holstein cows. Journal of dairy science 97(6):3431-3440.

Ji, P., J. K. Drackley, M. J. Khan, and J. J. Loor. 2014b. Overfeeding energy upregulates peroxisome proliferator-activated receptor (PPAR)gamma-controlled adipogenic and lipolytic gene networks but does not affect proinflammatory markers in visceral and subcutaneous adipose depots of Holstein cows. Journal of dairy science 97(6):3431-3440.

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 molecular biology 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 molecular biology 10:90.

Kawada, T., N. Takahashi, and T. Fushiki. 2001. Biochemical and physiological characteristics of fat cell. Journal of Nutritional Science and Vitaminology 47(1):1-12.

Keophiphath, M., C. Rouault, A. Divoux, K. Clement, and D. Lacasa. 2010. CCL5 promotes macrophage recruitment and survival in human adipose tissue. Arteriosclerosis, thrombosis, and vascular biology 30(1):39-45.

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 regulation and systems biology 8:97-111.

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.

Lagathu, C., L. Yvan-Charvet, J. P. Bastard, M. Maachi, A. Quignard-Boulange, J. Capeau, and M. Caron. 2006. Long-term treatment with interleukin-1beta induces insulin resistance in murine and human adipocytes. Diabetologia 49(9):2162-2173.

Leon, H. V., J. Hernandez-Ceron, D. H. Keislert, and C. G. Gutierrez. 2004. Plasma concentrations of leptin, insulin-like growth factor-I, and insulin in relation to changes in body condition score in heifers. Journal of animal science 82(2):445-451.

Lionetti, L., M. P. Mollica, A. Lombardi, G. Cavaliere, G. Gifuni, and A. Barletta. 2009. From chronic overnutrition to insulin resistance: The role of fat-storing capacity and inflammation. Nutrition, metabolism & cardiovascular diseases 19(2):146-152.

Lorente-Cebrian, S., N. Mejhert, A. Kulyte, J. Laurencikiene, G. Astrom, P. Heden, M. Ryden, and P. Arner. 2014. MicroRNAs regulate human adipocyte lipolysis: Effects of miR-145 are linked to TNF-alpha. Plos one 9(1):e86800.

McGown, C., A. Birerdinc, and Z. M. Younossi. 2014. Adipose tissue as an endocrine organ. Clinics in liver disease 18(1):41-58.

Metzger, D., T. Imai, M. Jiang, R. Takukawa, B. Desvergne, W. Wahli, and P. Chambon. 2005. Functional role of RXRs and PPARgamma in mature adipocytes. Prostaglandins, leukotrienes, and essential fatty acids 73(1):51-58.

Musi, N. and R. Guardado-Mendoza. 2014. Adipose tissue as an endocrine organ. Cellular endocrinology in health and disease:229-237.

Neels, J. G. and J. M. Olefsky. 2006. Inflamed fat: What starts the fire? Journal of clinical investigation 116(1):33-35.

Nunez, V., D. Alameda, D. Rico, R. Mota, P. Gonzalo, M. Cedenilla, T. Fischer, L. Bosca, C. K. Glass, A. G. Arroyo, and M. Ricote. 2010. Retinoid X receptor alpha controls innate inflammatory responses through the up-regulation of chemokine expression. Proceedings of the National Academy of Sciences of the United States of America 107(23):10626-10631.

Pires, J. A. A., C. Delavaud, Y. Faulconnier, D. Pomies, and Y. Chilliard. 2013. Effects of body condition score at calving on indicators of fat and protein mobilization of periparturient Holstein-Friesian cows. Journal of dairy science 96(10):6423-6439.

Randall, L. V., M. J. Green, M. G. Chagunda, C. Mason, S. C. Archer, L. E. Green, and J. N. Huxley. 2015. Low body condition predisposes cattle to lameness: An 8-year study of one dairy herd. Journal of dairy science 98(6):3766-3777.

Reist, M., D. Erdin, D. von Euw, K. Tschuemperlin, H. Leuenberger, C. Delavaud, Y. Chilliard, H. M. Hammon, N. Kuenzi, and J. W. Blum. 2003. Concentrate feeding strategy in lactating dairy cows: Metabolic and endocrine changes with emphasis on leptin. Journal of dairy science 86(5):1690-1706.

Roche, J. R., P. G. Dillon, C. R. Stockdale, L. H. Baumgard, and M. J. VanBaale. 2004. Relationships among international body condition scoring systems. Journal of dairy science 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. Journal of dairy science 92(12):5769-5801.

Roche, J. R., E. S. Kolver, and J. K. Kay. 2005. Influence of precalving feed allowance on periparturient metabolic and hormonal responses and milk production in grazing dairy cows. Journal of dairy science 88(2):677-689.

Roche, J. R., S. Meier, A. Heiser, M. D. Mitchell, C. G. Walker, M. A. Crookenden, M. Vailati Riboni, J. J. Loor, and J. K. Kay. 2015. Effects of pre-calving body condition score and prepartum feeding level on production, reproduction, and health parameters in pasture-based transition dairy cows. Journal of dairy science 98(10):7164-7182.

Roh, S.-G., D. Hishikawa, Y.-H. Hong, and S. Sasaki. 2006. Control of adipogenesis in ruminants. Animal Science Journal 77(5):472-477.

Romao, J. M., W. Jin, M. V. Dodson, G. J. Hausman, S. S. Moore, and L. L. Guan. 2011. MicroRNA regulation in mammalian adipogenesis. Experimental biology and medicine 236(9):997-1004.

Romao, J. M., W. Jin, M. He, T. McAllister, and L. L. Guan. 2012. Altered microRNA expression in bovine subcutaneous and visceral adipose tissues from cattle under different diet. Plos one 7(7):e40605.

Rukkwamsuk, T., T. Wensing, and M. J. Geelen. 1999. Effect of overfeeding during the dry period on the rate of esterification in adipose tissue of dairy cows during the periparturient period. Journal of dairy science 82(6):1164-1169.

Selim, S., S. Salin, J. Taponen, A. Vanhatalo, T. Kokkonen, and K. T. Elo. 2014. Prepartal dietary energy alters transcriptional adaptations of the liver and subcutaneous adipose tissue of dairy cows during the transition period. Physiological genomics 46(9):328-337.

Shahzad, K., M. Bionaz, E. Trevisi, G. Bertoni, S. L. Rodriguez-Zas, and J. J. Loor. 2014. Integrative analyses of hepatic differentially expressed genes and blood biomarkers during the peripartal period between dairy cows overfed or restricted-fed energy prepartum. Plos one 9(6):e99757.

Shi, H., M. V. Kokoeva, K. Inouye, I. Tzameli, H. Yin, and J. S. Flier. 2006. TLR4 links innate immunity and fatty acid-induced insulin resistance. Journal of clinical investigation 116(11):3015-3025.

Soliman, M., K. Kimura, M. Ahmed, D. Yamaji, Y. Matsushita, Y. Okamatsu-Ogura, K. Makondo, and M. Saito. 2007. Inverse regulation of leptin mRNA expression by short- and long-chain fatty acids in cultured bovine adipocytes. Domestic animal endocrinology 33(4):400-409.

Surmi, B. K. and A. H. Hasty. 2008. Macrophage infiltration into adipose tissue: Initiation, propagation and remodeling. Future lipidology 3(5):545-556.

Tilg, H. and A. R. Moschen. 2008. Insulin resistance, inflammation, and non-alcoholic fatty liver disease. Trends in endocrinology and metabolism 19(10):371-379.

Trajkovski, M., J. Hausser, J. Soutschek, B. Bhat, A. Akin, M. Zavolan, M. H. Heim, and M. Stoffel. 2011. MicroRNAs 103 and 107 regulate insulin sensitivity. Nature 474(7353):649-653.

Trayhurn, P. 2005. Endocrine and signalling role of adipose tissue: New perspectives on fat. Acta veterinaria Scandinavica 184(4):285-293.

Trevisi, E., M. Amadori, S. Cogrossi, E. Razzuoli, and G. Bertoni. 2012. Metabolic stress and inflammatory response in high-yielding, periparturient dairy cows. Research in veterinary science 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. Journal of dairy science 98(5):3079-3085.

Waltner, S. S., J. P. McNamara, and J. K. Hillers. 1993. Relationships of body condition score to production variables in high producing Holstein dairy cattle. Journal of dairy science 76(11):3410-3419.

Weisberg, S. P., D. McCann, M. Desai, M. Rosenbaum, R. L. Leibel, and A. W. Ferrante, Jr. 2003. Obesity is associated with macrophage accumulation in adipose tissue. Journal of clinical investigation 112(12):1796-1808.

Xu, H. Y., G. T. Barnes, Q. Yang, G. Tan, D. S. Yang, C. J. Chou, J. Sole, A. Nichols, J. S. Ross, L. A. Tartaglia, and H. Chen. 2003. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. Journal of clinical investigation 112(12):1821-1830.

Yang, Y., D. Ju, M. Zhang, and G. Yang. 2008. Interleukin-6 stimulates lipolysis in porcine adipocytes. Endocrine 33(3):261-269.

CHAPTER 5: PREPARTUM BODY CONDITION SCORE AND PLANE OF NUTRITION AFFECT THE HEPATIC TRANSCRIPTOME DURING THE TRANSITION PERIOD IN GRAZING DAIRY COWS

Manuscript published.

Vailati Riboni, M., S. Meier, C. Burke, J.K. Kay, M.D. Mitchell, C.G. Walker, M.A. Crookenden, A. Heiser, S.L. Rodriguez Zas, J.R. Roche, and J.J. Loor. 2016. Prepartum body condition score and plane of nutrition affect the hepatic transcriptome during the transition period in grazing dairy cows. BMC Genomics, 17:854.

ABSTRACT

Background

A transcriptomic approach was used to evaluate potential interactions between prepartum body condition score (BCS) and feeding management in the weeks before calving on hepatic metabolism during the periparturient period.

Methods

Thirty-two mid-lactation grazing dairy cows of mixed age and breed were randomly allocated to one of four treatment groups in a 2 × 2 factorial arrangement: two prepartum BCS categories [4.0 (thin, BCS4) and 5.0 (optimal, BCS5); based on a 10-point scale], and two levels of energy intake during the 3 wk preceding calving (75 and 125% of estimated requirements). Liver samples were obtained at -7, 7, and 28 d relative to parturition and subsequent RNA was hybridized to the Agilent 44K Bovine (V2) Microarray chip. The Dynamic Impact Approach was used for pathway analysis, and Ingenuity Pathway Analysis was used for gene network analysis.

Results

The greater number of differentially expressed genes in BCS4 cows in response to prepartum feed allowance (1071 vs 310, over the entire transition period) indicates that these animals were

more responsive to prepartum nutrition management than optimally-conditioned cows. However, independent of prepartum BCS, pathway analysis revealed that prepartal feeding level had a marked effect on carbohydrate, amino acid, lipid, and glycan metabolism. Altered carbohydrate and amino acid metabolism suggest a greater and more prolonged negative energy balance postpartum in BCS5 cows overfed prepartum. This is supported by opposite effects of prepartum feeding in BCS4 compared with BCS5 cows in pathways encompassing amino acid, vitamin, and co-factor metabolism. The prepartum feed restriction ameliorates the metabolic adaptation to the onset of lactation in BCS5 cows, while detrimentally affecting BCS4 cows, which seem to better adapt when overfed. Alterations in the glycosaminoglycans synthesis pathway support this idea, indicating better hepatic health status in feed-restricted BCS5 and overfed BCS4 cows. Furthermore, IPA network analysis suggests liver damage in feed-restricted thin cows, likely due to metabolic overload.

Conclusion

Overall, the data support the hypothesis that overfeeding in late-pregnancy should be limited to underconditioned cows, while cows with optimal degree of body condition should be maintained on an energy-restricted diet.

BACKGROUND

Nutritional management of the late pregnant dairy cow has been examined as a way to improve cow DMI and health during the transition period. The aim is to counteract the negative energy balance (**NEB**) that dairy cows experience postpartum, and ease their transition into lactation. Traditionally, management practices provided dry cows with a high-fiber/low-energy density ration ("far-off" diet), switching to a low-fiber/higher-energy density ration for the last

month of gestation ("close-up" diet). Different studies, however, have thus far demonstrated that this "close-up" approach often leads to prepartum hyperglycemia and hyperinsulinemia and greater blood non-esterified fatty acid (**NEFA**) concentrations (i.e., excess lipid mobilization postpartum) (Holtenius et al., 2003, Roche et al., 2005, Janovick et al., 2011, Roche et al., 2015). The move to "close-up" diets with greater energy density may also elicit detrimental effects on the postpartum health of the cow (Dann et al., 2006, Graugnard et al., 2013, Shahzad et al., 2014).

Similar outcomes in the postpartum period have been associated with the level of body condition score (**BCS**) at calving. Altered plasma concentrations of biomarkers of metabolic and inflammation were detected in some studies in overconditioned cows (Hayirli et al., 2002, Holtenius et al., 2003, Roche et al., 2013, Randall et al., 2015) and in "thin", underconditioned cows (Pires et al., 2013, Roche et al., 2013, Akbar et al., 2015), indicating that both groups are at greater risk of developing metabolic or health disorders during transition. Thin cows experience more problems in a pasture-system (the present study) than in a TMR-based system, where the risk of overconditioning during the dry period is far greater (Roche et al., 2007, Roche et al., 2009). For these reasons, assessment of BCS prepartum can provide a qualitative evaluation of the chances for an optimal transition, which, in turn, is closely associated with optimal production and the chances for a successful lactation (Roche et al., 2009).

Despite the fact that both prepartum BCS and plane of nutrition play an important role in the metabolic response of the animal to lactation, cows are generally managed similarly prepartum. During the transition period, the high metabolic demands required for milk synthesis rely on homeorethic mechanisms in multiple tissues, all of which converge on the metabolic ability of the liver (Drackley, 1999). Previous studies have demonstrated the ability of "omics"

approaches for evaluating the effects of calving BCS (Akbar et al., 2014b, c) and feeding management (Loor et al., 2006, Shahzad et al., 2014) on the metabolic activity of the liver during the transition period. Therefore, in the present study, liver transcriptome profiling through microarray technology was undertaken to investigate the hypothesis that an interaction between precalving BCS and prepartum feeding management exists, and that it can affect the hepatic adaptations to lactation in grazing dairy cows.

METHODS

Experimental Design And Sample Collection

Complete details of the experimental design are available elsewhere (Roche et al., 2015). Briefly, 150 mixed breed mid-lactation grazing dairy cows were balanced for age, breed, BCS at the time of enrollment, and expected calving date. Cows were randomly allocated to one of six treatment groups (25 cows per group) in a 2 × 3 factorial arrangement: two pre-calving BCS categories (4.0 and 5.0, **BCS4** and **BCS5**; based on a 10-point scale, where 1 is emaciated and 10 obese; Roche et al., 2004) and three levels of energy intake during the three wk preceding calving (75, 100, and 125% of estimated requirements; Roche et al., 2005). The different groups were obtained by daily manipulation of pasture allowance (Roche et al., 2015).

A subset of 32 animals with the complete set of biopsies and plasma samples (8 cows in each of four treatment groups) was used for transcriptomic analysis. These were cows with prepartum BCS4 fed to meet 75 (**B4F75**) or 125 (**B4F125**) % of requirements, and cows with prepartum BCS5 fed to meet 75 (**B5F75**) or 125 (**B5F125**) % of requirements. The average cow age was 6.4 ± 2.3, 5.4 ± 2.3, 6.3 ± 2.6, and 4.8 ± 1.5 years for B4F75, B4F125, B5F75, and B5F125, respectively. Breed-wise, pure bred New Zealand Holsteins were 4, 2, 6, and 3, for

B4F75, B4F125, B5F75, and B5F125, respectively, with the remaining cows in each group being a mix-bred Jersey with an average percentage of New Zealand Holstein blood of 86 ± 10, 75 ± 19, 69 ± 24, and 47 ± 31 %, for B4F75, B4F125, B5F75, and B5F125, respectively.

The intermediate groups in both BCS classes (B4F100 and B5F100) were omitted from the present analysis as feeding to the exact requirements is rarely achievable in field conditions. In practice, multiple factors (e.g., pasture management and allocation, animal competition and social interaction) cause cows being mostly overfed or underfed.

Liver tissue was harvested via percutaneous biopsy under local anesthesia at -7, 7, and 28 d relative to parturition. Tissue samples were immediately snap frozen in liquid nitrogen and stored at -80°C until further analysis. Blood was sampled on the same days, prior to the biopsy, by coccygeal venipuncture using evacuated blood tubes containing a lithium heparin anticoagulant, and processed for plasma collection and determination of NEFA and BHBA. Assay information is reported in Supplemental file 1 (Appendix C).

RNA Extraction And Microarray Performance

Complete information about the procedures is reported in Supplemental file 1 (Appendix C).

Statistical Analysis

Statistical analysis for transcriptomic data was performed using SAS v9.3 (SAS Institute Inc.). Data from a total of 48 microarrays were adjusted for dye and array effects (Lowess Normalization and array centering). A MIXED model with repeated measures was then fitted to the normalized $\log_2$-tranformed adjusted ratios using Proc MIXED, according to the following model:

$$Y_{ijklm} = \mu + T_i + B_j + F_k + TB_{ij} + TF_{ik} + BF_{jk} + TBF_{ijk} + C_l + e_{ijklm}$$

The model for Y_{ijklm} (dye-array adjusted expression) included the overall mean (μ), the fixed effects of time (T_i, −7, 7, and 28 d), prepartum BCS (B_j, 4 and 5), prepartum feeding management (F_k, 75 and 125%), and their interactions (TB_{ij}, TF_{ik}, BF_{jk}, TBF_{ijk}). Cow (C_l) was considered an uncorrelated random effect, and e_{ijklm} represent the residual error. The raw P-values were adjusted for the number of genes tested using Benjamini and Hochberg's false discovery rate (**FDR**; Benjamini and Hochberg, 1995) to account for multiple comparisons.

Blood data were subjected to ANOVA in SAS (v9.3) and analyzed with PROC MIXED, fitting the same statistical model as used for transcriptome data. Time, BCS, feeding, and their interactions were considered as fixed effects, while cow, nested within treatment, was 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. Data were considered significant at $P \leq 0.05$ using the PDIFF statement.

Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Analysis

The dynamic impact approach (**DIA**) was used for **KEGG** pathway analysis of differentially expressed genes (**DEG**). The DIA calculates the overall effect (relevance of a given pathway) and flux (direction of effect), thus allowing evaluation of transcriptome profiles in a more holistic fashion. The detailed methodology of DIA is described elsewhere (Bionaz et al., 2012). Briefly, the whole dataset with Entrez gene ID, fold-change (**FC**) (≤ -1.5 and ≥ 1.5), and raw *P*-value (≤ 0.01) was uploaded to DIA. For the analyses, a minimum of 30% annotated genes on the microarray versus the whole genome (Bionaz et al., 2012) was selected.

Transcription Regulators and Gene Network Analysis

Ingenuity pathway analysis (**IPA**) software (Qiagen) was used to analyze the upstream transcription regulators and their connections with other downstream genes that were

differentially expressed. For this purpose, a list of DEG, with the same thresholds used for DIA analysis, was uploaded into IPA. However, for IPA analysis, the time effect was not included to focus on overall interaction of BCS and prepartum feeding management along the whole transition period.

Verification of Microarray Results

To verify some of the key findings from the microarray and bioinformatics analysis, the expression levels of 11 genes, including eight and three DEG for the comparisons B4F125vsB4F75 and B5F125vsB5F75, respectively, were analyzed using real-time quantitative PCR. Because of the higher number of detected DEG, validation was performed only on samples obtained at 7 days postpartum. Complete information about the procedures is reported in Supplemental file 1 (Appendix C), together with the list of analyzed genes and respective pathways annotated via DIA. Per each comparison a fold-change was calculated from the normalized relative mRNA abundance obtained from each gene with its own standard curve, and compared with the fold-change from the microarray analysis. A Pearson correlation using the proc CORR procedure in SAS (v9.3) was then run to establish similarity between the two analyses. Results are reported in Figure 5.6.

RESULTS AND DISCUSSION

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 adiposity are generally managed similarly during the prepartum period. In light of this, the present manuscript focuses on the pre- and postpartum (-7, 7, and 28 d) metabolic effect of different prepartum feeding regimes within BCS groups (B4F125 vs B4F75, and B5F125 vs B5F75). Time was not considered in the

discussion of transcription regulator and gene network analysis; instead, the focus was on the overall effect of BCS and feeding management, using the same comparisons as in the DIA analysis. Differentially expressed genes were determined as reported previously (Piantoni et al., 2012, Akbar et al., 2014a), applying first a stringent *P* value cut-off of $P \leq 0.01$ to the data, and then an FC threshold of ≤ -1.5 and ≥ 1.5. Validation for this approach, which does not take into account multiple testing corrections, was reported previously (Guo et al., 2006, Shi et al., 2008). The resulting number of DEG is reported in Table 5.1. The five main areas among the KEGG categories and subcategories that were enriched with DEG are reported in Figure 5.1. Among these, the following discussion will only concern the 'metabolism' category, with a focus on the 25 metabolic pathways that were most-impacted by treatment (Figure 5.2 and 3), emphasizing the effect of prepartum diet in combination with prepartum BCS. The term "impact" refers to the biological importance of a given pathway as a function of the change in expression of genes composing the pathway (proportion of DEG and their magnitude) in response to a treatment, condition, or change in physiological state (Bionaz et al., 2012). Consequently, the direction of the impact, or flux, characterizes the average change in expression as up-regulation/activation, down-regulation/inhibition, or no change.

The effect of body condition score

The hepatic transcriptome of BCS4 compared with BCS5 cows was more impacted by prepartum feeding management in the early postpartum (7 d), i.e. during the homeorethic metabolic shift that the cow experiences in response to the onset of lactation. According to established management standards (Roche et al., 2009), BCS5 cows are considered optimally-conditioned at parturition, while BCS4 cows are underconditioned. Thus, the fact that the number of DEG detected due to different prepartum feeding management was on average 3×

higher in BCS4 than BCS5 cows underscores how transition cow nutrition could be more important in underconditioned animals.

In both BCS4 and BCS5 cows, feeding at 125% of requirements upregulated the prepartum metabolism-related transcriptome (Flux value in Figure 5.1). However, as the change in flux indicates, immediately postpartum (7 d) BCS4 cows benefitted from the greater feed allowance (125% vs. 75%), because there was an overall activation of metabolic pathways. In contrast, when BCS5 cows were overfed during the dry period, the same pathways were inhibited early post-partum (Figure 5.2). This indicates that a greater feed allowance prepartum might have elicited a non-desirable effect on optimally-conditioned cows, rendering them "metabolically-lazy" during the metabolically-demanding transition to lactation.

Carbohydrate metabolism. Bell (1995) estimated a marked increase in demand for glucose during the transition period. Because glucose and related metabolites constitute the fulcrum of 'Carbohydrate metabolism' in the cow, an impact (or overall effect based on changes in gene expression) on this pathway was expected and evident right after parturition regardless of BCS (Figure 5.1). However, only BCS4 cows had a greater impact (or significant change) of feeding management on 'Carbohydrate metabolism', with half of the subcategories impacted at 7 d (Supplemental Table C.7).

Among the most-impacted pathways (i.e. of high biological relevance based on transcriptome), 'Pyruvate metabolism' was activated at 7 d postpartum when cows were fed 75% of requirements before calving, regardless of BCS, although the timing of changes to volatile fatty acid (**VFA**) metabolism was BCS-dependent. Metabolism of propionate and butyrate was impacted shortly after parturition (7 d) in BCS4, and at the end of the transition period (28 d) in BCS5 cows. For both BCS groups, overfeeding prepartum led to the activation of VFA

pathways. When cows were underfed prepartum in both BCS4 and 5, the high impact and flux of 'Pyruvate metabolism' early postpartum were due to the high expression of the cytosolic form of malic enzyme (*ME1*) (Supplemental Table C.2 and C.5). Malic enzyme activity supplies NADPH for fatty acid biosynthesis, particularly in adipose tissue (Laliotis et al., 2010); however, its role in bovine liver is unclear, as the liver is not a lipogenic organ in this species (Bell, 1979). Furthermore, the idea that 'Pyruvate metabolism' serves to provide precursors for gluconeogenesis through oxaloacetate is inconsistent with the positive flux (activation) of the 'Gluconeogenesis/Glycolysis' in the BCS4 cows. In fact, data indicated an induction of this pathway in B4F125 cows. Thus, we hypothesize that, independently of BCS, the impact on 'Pyruvate metabolism' due to changes in *ME1* expression could be related to the more pronounced postpartum NEB in cows overfed prepartum. This is supported, at least in part, by the lower activity of liver malic enzyme during starvation in ruminants (Martin et al., 1973).

However, when interpreting the expression profile of BCS4 cows, the fact that VFA production is tightly linked with DMI (greater intake, greater VFA production) and that both 'Propanoate metabolism' and 'Butanoate metabolism' early postpartum were upregulated in B4F125 cows seems to argue against our hypothesis of a more pronounced postpartum NEB in generally overfed cows prepartum. In fact, these data (pathways of VFA metabolism, Gluconeogenesis and Fatty acid biosynthesis) oppositely indicate a greater postpartum DMI, which would lead to a less pronounced NEB in underconditioned cows when overfed rather than feed restricted during the transition period. The increase in impact of the VFA pathways in BCS5 at 28 d cows, instead, agrees with a more prolonged NEB when overfeeding prepartum (Roche et al., 2015).

***Lipid metabolism*.** Despite the lower adiposity in leaner than optimally-conditioned cows (Figure 5.1), 'lipid metabolism' was more impacted as a consequence of prepartum nutrition management in BCS4 than BCS5 cows. Furthermore, the changes in flux early postpartum indicated that feeding BCS4 cows at 125% of requirements had an activation effect, but in BCS5 cows it had a slight inhibitory effect. Also, because plasma NEFA were overall higher in BCS5 cows compared with BCS4 (Table 5.2), the slight inhibition of lipid metabolism in BCS5 cows resulting from prepartum overfeeding could compromise the ability of the liver to handle mobilized fatty acids in early lactation. In contrast, despite the lower hepatic flux of NEFA, prepartum overfeeding seems to have 'prepared' the liver of under-conditioned cows by matching post-partum lipolysis with an up-regulation of lipid metabolism.

Within the top impacted pathways (Figure 5.2 and 3), none overlapped between the two BCS categories underscoring that prepartum nutrition management had a unique effect that was dependent on degree of adiposity. A complete discussion for each comparison of the pathways involved in the "Lipid Metabolism" category is available in Supplemental File 1 (Appendix C).

***Amino acid metabolism*.** Although cows mobilize less energy from muscle protein than from fat reserves, muscle protein mobilization occurs mainly in the first few weeks after calving (Tamminga et al., 1997, Knegsel et al., 2007). Muscle catabolism is of great importance during NEB, not only as a "provider" of amino acids for milk protein synthesis, but also of gluconeogenic precursors to the liver (Reynolds et al., 2003).

In both BCS groups, 'amino acid (**AA**) metabolism' was among the most-impacted categories (Figure 5.1), as cows experienced an activation of the related pathways all through the transition period. However, when feeding management was considered, clear differences emerged between groups. In fact, prepartum and early postpartum, the fluxes indicated a greater

activation of AA metabolism in BCS4 cows when fed 125% of requirements, while in BCS5 cows it occurred when fed at 75%. At 28 d postpartum, feeding at 125% seemed to activate this pathway in both BCS groups.

Independently of BCS, the 'Alanine, Aspartate, and Glutamate metabolism' pathway was impacted after parturition. Because this pathway revolves around the citric acid cycle, as the three amino acids are all gluconeogenic, its link with muscle protein catabolism is logical. This is supported by published data on free amino acid concentrations in muscle (and similar trends in plasma), where Ala, Asp and Glu experience an average ~2-fold increase at 3 wk postpartum compared with 1 wk prepartum (Meijer et al., 1995).

The 'β-Alanine metabolism' pathway is closely linked with 'Alanine, Aspartate, and Glutamate metabolism', because L-Asp can be converted to β-Alanine. The impact and flux of the two pathways, in fact, matched very closely (Figure 5.2 and 3). β-Alanine could be used to synthesize carnosine. Among its biological activities, carnosine can scavenge reactive oxygen and nitrogen species (Hipkiss, 2009). In liver tissue it can also act as an antioxidant by increasing vitamin E and Zn-Superoxide dismutase activity (Aydin et al., 2010). Thus, a putative increase in hepatic concentration of carnosine in B5F125 cows at 28 d might be connected to a greater need of antioxidants due to a possible heightened state of oxidative stress. Unlike B5F125 cows, a possible increase of carnosine in B4F125 cows supports the hypothesis discussed in subsequent sections (B4F125 vs B4F75 – Amino acid metabolism) of a concerted activation of protective mechanisms to account for the responses in pathways related to glutathione, taurine and hypotaurine, and selenoamino acid metabolism.

***Glycan biosynthesis and metabolism*.** Together with 'Carbohydrate' and 'Amino acid metabolism', this subcategory contains most of the top impacted metabolic pathways. Glycans

are carbohydrates linked with lipid and protein moieties to form glycolipids and glycoproteins that can act as signaling molecules (Etzler and Esko, 2009). Despite previous work with dairy cows (Bionaz et al., 2012, Shahzad et al., 2014, Shahzad et al., 2015) demonstrating substantial changes in several glycan-related pathways in liver tissue during the transition period, not much attention has been placed on glycan metabolism. However, a review of the literature highlighted that there has been a marked increase in glycomic studies in humans (Bertozzi and Sasisekharan, 2009), highlighting their importance. Further research will soon be required to understand the role of glycans in dairy cow liver during the transition period, as, in fact, glycan modifications of intracellular and extracellular proteins have critical functions in almost all biological pathways (Cummings and Pierce, 2014).

The most-impacted glycan pathways in our experiment included both biosynthesis [Glycosaminoglycan **(GAG)**, Glycosylphosphatidylinositol (**GPI**)-anchor, O- and N-Glycan] and degradation (Glycosaminoglycan, other glycans) (Figure 5.2 and 3). However, the degradation pathways were impacted in response to feeding management only in BCS5 cows. Glycosaminoglycans are the most abundant heteropolysaccharides in the body (Sasisekharan et al., 2006). Based on core disaccharide structures, GAG are classified into heparin sulfate, chondroitin sulfate, keratin sulfate, and hyaluronan (Sasisekharan et al., 2006). Sulfated GAG are key players in both molecular and cellular events of the regulation of inflammation (Pomin, 2015). Most importantly, they display anti-inflammatory functions in humans (Pomin, 2015). The synthesis of all three sulfated classes was affected by feeding management in both BCS4 and BCS5 cows. However, feeding at 125% of requirements activated the synthesis pathways in BCS4 cows, while it inhibited it in BCS5 cows. Furthermore, the overfeeding approach induced the degradation of GAG in BCS5 cows.

When you consider the three classes of sulfated glycosaminoglycans, data indicated that increasing dietary allowance prepartum in BCS4 cows induced GAG synthesis throughout the transition period. Because the transition period is characterized by a systemic level of inflammation (Trevisi et al., 2012), from sub-acute to acute, an increased synthesis of anti-inflammatory compounds could be beneficial and help reduce any detrimental effect while maintaining its physiological homeorhetic properties. In contrast, overfeeding seemed to reduce sulfated GAG synthesis in optimally-conditioned cows (BCS 5), supporting the idea that a restricted feeding prepartum might be a better option for fatter cows.

Ingenuity pathway analysis. Using the same DIA thresholds and cutoffs comparing overfeeding with feed restriction within BCS groups, the IPA analysis identified 16 and 44 transcription regulators in BCS5 and BCS4. After considering only those with FC ≥ |1.5|, there was 1 identified transcription regulator for B5F125 compared with B5F75, and 5 for B4F125 compared with B4F75, with 2 and 32 downstream DEG affected. Prepartum overfeeding led to the downregulation of the 6 transcription factors, 5 and 1 for BCS4 and BCS5 respectively, which are somewhat connected with cell cycle and proliferation and tissue development. In BCS5 cows, the small network around *CUX1* is ambiguous as the two affected downstream genes (*ECT2* and *WNT6*) are both involved in cell proliferation and liver regeneration, but have opposite effects on the regulation of these processes. Thus, it was concluded that feeding level did not affect hepatocyte transcriptome regulators in optimally-conditioned cows. However, in BCS4 cows, probably due to the greater number of DEG, the network around the transcription regulators *ERG*, *LHX1*, *MYOD1*, *SIM1*, and *SOX2* is more consistent in its direction; hence, more defined regulatory patterns could be discerned. More detailed information about the

upstream analysis is reported in Table 5.3, while the graphic networks are in Figure 5.4 (B4F125vsB4F75) and 5 (B5F125vsB5F75).

B4F125 vs B4F75

Carbohydrate metabolism. 'Galactose metabolism' was affected postpartum by feeding management, with an increase in impact from 7 to 28 d postpartum. Flux was positive (activation) at 7 d and negative (inhibition) at 28 d, underscoring an interaction between prepartum feeding and time postpartum. At 28 d *LALBA* (Lactalbumin, Alpha-) was responsible for the negative flux. Because LALBA is essential for the synthesis of lactose, the fact that lactose production takes place only in mammary cells complicates the interpretation of 'Galactose metabolism' and its impact on liver. However, early postpartum (7 d), *PFKL* (Phosphofructokinase) was responsible for the pathway activation. Among 'Galactose metabolism' related genes, *PFKL* leads to the formation of tagatose-6P, which, in rat hepatocytes has been proven to protect against pro-oxidant-induced cell injury (Valeri et al., 1997). This, again, supports the hypothesis, discussed in subsequent sections (B4F125 vs B4F75 – Amino acid metabolism), that there is an activation of protective mechanisms against oxidative damage in B4F125 cows.

Amino acid metabolism. Unique to the BCS4 group was the activation one week after parturition of three amino acid-related pathways involved in antioxidant status (Figure 5.2). 'Glutathione metabolism' and 'Selenoamino acid metabolism' were both induced in BCS4 cows fed 75%, while 'Taurine and Hypotaurine metabolism' was induced when cows were fed 125% of requirements. Among the DEG with a FC $\geq |3|$ (Supplemental Table C.2), *GPX3* (Glutathione Peroxidase 3) and *MARS* (methionyl-tRNA synthetase) were the main drivers for the impact and

flux of the first two pathways. Although *MARS* in the 'Selenoamino acid metabolism' catalyzes the incorporation of Se into proteins as seleno-Met (**SeMet**), this form of Met functions mainly for storage of selenium, which, once released during turnover, can be incorporated by the same pathway into seleno-Cys, a fundamental component of GPX3. Because glutathione peroxidase protects the organism from oxidative damage, we hypothesize that the induction of *GPX3* and the glutathione and selenoamino acid pathways in B4F75 cows reflects greater oxidative stress conditions postpartum when cows were feed restricted pre-calving, i.e. this management approach is likely not suitable for underconditioned cows. Even if mainly a storage form of Se, SeMet has been demonstrated to protect bovine mammary epithelial cells from H_2O_2-induced apoptosis and increase proliferation and cell viability under conditions of oxidative stress (Miranda et al., 2011). A similar mechanism could be present in hepatocytes.

In contrast to feeding 75% of requirements, feeding 125% may have strengthened hepatic antioxidant capacity by increasing the taurine and hypotaurine pool. In fact *GAD2* (glutamate decarboxylase 2; Supplemental table C.2) was upregulated in B4F125 cows, leading to the possible formation of the two antioxidants. By modulating gene expression, taurine has a beneficial effect on liver health, as it alleviates hepatic TAG accumulation (at least in non-ruminants). It acts by enhancing the expression of *MTTP* (microsomal triglyceride transfer protein), which improves hepatic lipid efflux. It also can enhance *PPARA* (peroxisome proliferator-activated receptor α) expression, and, in turn, increase hepatic mitochondrial and peroxisomal lipid (Bonfleur et al., 2015). In our dataset of affected genes, only *MTTP* was significantly up-regulated (FC = 1.3, $P < 0.01$, data not shown) by overfeeding lean cows.

The main difference between feeding management strategies was that in B4F75, the impact was due to an enzyme (*GPX3*) directly involved in the antioxidant system and production

of SeMet, while in B4F125 the antioxidant pool was increased. Thus, we consider the first scenario a response to stress and the second scenario a "build up" of a defense mechanism. In conclusion, we believe that, overfeeding underconditioned cows prepartum could improve liver health postpartum at least in part by enhancing hepatic handling of tissue-mobilized fatty acids, and also by building a greater antioxidant defense against oxidative stress in the postpartum.

Glycan biosynthesis and metabolism. Both "O-glycans" and "GPI-anchor biosynthesis" were activated early postpartum by the prepartum increased feed allowance. O-glycans play a role in mammalian Notch signaling (Stanley, 2014), which is important for the regulation of the complex interactions between liver cell types involved in tissue repair (Geisler and Strazzabosco, 2015). Because hepatocytes experience an increased work load after calving due to a shift in metabolism (Drackley, 1999), an increase in differentiation and repair through the Notch pathway stimulated by O-glycans might be a mechanism to support high hepatocyte turnover. The fact that a persistent activation of Notch signaling is associated with liver malignancies in mouse models (Geisler and Strazzabosco, 2015) might explain why at 28 d postpartum the impact of the pathway was reduced and the flux became slightly negative, especially in B4F125 cows.

GPI is a glycolipid that can be attached to the C-terminus of a protein during posttranslational modification, forming the so called GPI-anchored proteins (Sangiorgio et al., 2004). In the context of the transition period, a GPI anchor is used by hepatocytes to bring the high-density lipoprotein (**HDL**) binding protein into contact with the blood stream (Dallinga-Thie et al., 2010). HDLs contain little TAG but a large amount of cholesterol; thus, an increase in their uptake could, hypothetically, result in a greater tissue cholesterol concentration. Cholesterol is also a component of very low density lipoproteins (**VLDL**) and its concentration

can regulate liver apolipoprotein B synthesis (ApoB), another indispensable component of VLDL, during hepatic lipid export (Thompson et al., 1996). Although *APOB* was upregulated 1.2-fold it did not reach statistical significance ($P = 0.10$), but it should be kept in mind that synthesis of APOB is primarily regulated at the post-translational level (Davidson and Shelness, 2000). Immediately post-partum cows increase adipose tissue lipolysis (Drackley, 1999) therefore, an increase of VLDL synthesis could help the liver of B4F125 cows repackage mobilized fat, thereby avoiding the detrimental effect of hepatic TAG accumulation.

Metabolism of cofactors and vitamins. The postpartum impact of 'Retinol metabolism' detected in B4F125 cows reinforces the idea (as previously hypothesized) that modest overfeeding in underconditioned cows in the dry period leads to better DMI and, possibly, a lower degree of NEB in early lactation because there were no differences in milk production detected (Roche et al., 2015). The same hypothesis of a better energy status postpartum in B4F125 than B4F75 cows emerges when examining the pattern of 'Nicotinate and nicotinamide metabolism'. Flux through 'Nicotinate and nicotinamide metabolism' leads to the synthesis of some of the most-important mediators of energy metabolism reactions, the nicotinamide adenine dinucleotides (NADs) family, which includes NAD^+, NADH, and the phosphorylated versions $NADP^+$ and NADPH. The sustained upregulation of 'Nicotinate and nicotinamide metabolism' postpartum indicates a beneficial effect of prepartum overfeeding in underconditioned cows, i.e. a greater amount of these mediators would indicate that they are better able to cope with the energetic metabolic demand of early lactation. Among them, NAD^+ might play the most-important role, as it is necessary to maintain the hepatic fatty acid oxidative capacity (Schulz, 1991) and also because the lipogenic capacity of ruminant liver is quite low (Bell, 1979). The

involvement of NADPH in the cellular antioxidant system (Ying, 2008) might also indicate a better oxidative status of B4F125 compared with B4F75 cows.

Alterations in 'Folate biosynthesis', 'One carbon pool by folate', and 'Vitamin B6 metabolism' also revealed a possible positive effect of prepartum overfeeding in underconditioned cows. It is important to note that these three pathways are connected with the Met cycle. Folate, through 5-methyl tetrahydrofolate and vitamin B6, can, in fact, donate the methyl group required by Met synthase to synthesize Met from homocysteine (Selhub, 2002). Furthermore, vitamin B6 is a cofactor of cystathionine β-synthase and cystathionine γ-lyase that lead to the formation of cystathione (Ramakrishnan et al., 2006), a precursor for the antioxidants glutathione, taurine, and hypotaurine. The changes detected in these three pathways seem to indicate a greater flux through the Met cycle, with a potential increase in the availability of Met and antioxidants. This hypothetical effect, through the Met cycle, also could explain the increase in activation of 'Taurine and Hypotaurine metabolism' in early lactation in B4F125 cows.

It is well known that Met, together with Lys, is one of the most-limiting AA in a wide range of dairy diets (NRC, 2001). Methionine is typically first-limiting and its supplementation alone is able to improve not only overall lactation performance, but also improves antioxidant status and overall immune function and inflammation status in transition dairy cows (Osorio et al., 2013, 2014a, Osorio et al., 2014b). Hence, some of the observed beneficial effects of overfeeding underconditioned cows prepartum could be related to an improved Met and antioxidant balance in early lactation.

Ingenuity pathway analysis. Judging by the upregulation in B4F75 cows of *ANGPT1*, *DAG1*, *HDAC6*, *MEIS1*, *RBH38*, and *RYR1* (Figure 5.4), the prepartum feed restriction could have resulted in some type of liver damage, including apoptosis. This idea agrees with the

induction of the 'Apoptosis' pathway in B4F75 cows revealed by the DIA output (Supplemental file 2, Appendix C). In parallel, the liver of these cows seemed to experience an inflammatory state at least in part due to lower *ALB*, greater *CXCR4* and *CD86*, all coupled with the induction of the cholinergic anti-inflammatory pathway (*ACHE, CHRNG*) and together with upregulation of genes associated with oxidative stress (*ATP7A*, *GPX3*, *TMIGD1*). In contrast, as judged by the upregulation of genes involved in growth, proliferation, and differentiation (*BCL9L*, *FMNL2*, *PTPN4*, *SP1*, *ZKSCAN1*) in B4F125 cows, the overfeeding of underconditioned cows prepartum appeared to result in a healthier liver.

To some extent the compromised state of the liver in B4F75 cows is further evidenced from results of the IPA network analysis. The downregulation of *ALB* and *FABP7* suggests a lower ability to handle the mobilized fatty acids during the transition to lactation, perhaps enhancing the susceptibility of the liver to accumulate lipid at times of high NEFA concentration in the circulation (e.g. early postpartum). The upregulation of *PKIB* (CAMP-Dependent Protein Kinase Inhibitor Beta), a potential inhibitor of protein kinase A (**PKA**) (Dalton and Dewey, 2006), is also noteworthy, because PKA is a direct activator of the gluconeogenic pathway in non-ruminant liver (Desvergne et al., 2006, Rui, 2014). Thus, the greater expression of *PKIB* in B4F75 cows could have impaired hepatic gluconeogenesis. In contrast, the lower expression of *PKIB* in B4F125 cows would have helped increase the intracellular glucose pool.

B5F125 vs B5F75

Carbohydrate metabolism. Overfeeding cows in the prepartum period had an effect on 'TCA cycle' and 'Inositol phosphate metabolism'. It is plausible that the activation of the TCA cycle prepartum was a result of the higher dietary energy supply; in comparison, however, the activation early postpartum of 'Inositol phosphate metabolism' might have been related with an

inflammatory state induced by prepartum overfeeding. In fact, this pathway was primarily affected by the upregulation of *IPPK* (inositol-pentakisphosphate 2-kinase) in B5F125 compared with B5F75 cows. The IPPK protein catalyzes the formation of inositol hexakisphosphate (**InsP6**), a ubiquitous and abundant cytosolic inositiol phosphate. In humans, InsP6 primes and stimulates neutrophil respiratory burst (Eggleton et al., 1991, Kitchen et al., 1996); thus, we speculate that this metabolite could participate in the inflammatory response. However, both *in vitro* and *in vivo*, Insp6 inhibits L- and P-selectin function (Cecconi et al., 1994). Thus, if InsP6 produced by the liver is secreted into blood it could impair neutrophil infiltration by downregulating the attachment molecules, thus, leading to reduced neutrophil functions when optimally-conditioned cows are overfed prepartum.

Amino acid metabolism. Contrary to BCS4, in BCS5 cows the 'Alanine, Aspartate, and Glutamate metabolism' pathway was impacted only at the end of the transition period (28 d), and was activated by overfeeding (B5F125). Because BCS5 cows calved with better body condition than BCS4, they would have been better able to cope with the NEB by drawing from the more abundant lipid reserves, as indicated by the higher NEFA concentrations (Table 5.2). The late activation of this amino acid pathway, as hypothesized when discussing VFA metabolism (The effect of body condition – Carbohydrate metabolism), might have been due to a prolonged postpartum NEB in the overfed BCS5 cows, which could lead to mobilization of muscle protein. This hypothesis is supported by data indicating that overconditioned cows have a more pronounced drop in DMI after calving and a more intense NEB compared with cows fed to or slightly below requirements during the dry period (Ji et al., 2014, Khan et al., 2014, Roche et al., 2015).

Among the most-impacted metabolic pathways, the metabolism of Arg and Pro and of the three branched-chained amino acids (**BCAA**), valine, leucine, and isoleucine were highly impacted (Figure 5.3). The impact and flux of the 'Arginine and Proline metabolism' pathway prepartum was caused by the differential expression (FC = -3) of *CKMT1B* (Creatine kinase, mitochondrial 1B), which encodes an enzyme responsible for the formation of phosphocreatine (**PCr**). In normal conditions PCr is then transported to the muscle as energy storage to facilitate a quick muscle response (Wallimann et al., 1992). Because the putative increase of PCr appears to have occurred prepartum in B5F75 cows, feed restriction in optimally-conditioned cows seems to position the cow in a better energy state compared with overfeeding, i.e. the liver would have extra energy to store as PCr in muscle. Furthermore, it can be hypothesized that a local increase of PCr would be useful to the liver as an "energy buffer" in the metabolically-demanding postpartum period. Also, the 'Arginine and Proline Pathway' was induced early postpartum in B5F75 cows compared with B5F125. Both amino acids are gluconeogenic; thus, it seems that prepartum feed-restricted optimally-conditioned cows were in a better energy state compared with overfed cows early in the postpartum. The same pathway is also used to produce putrescine, a polyamine with growth factor-like functions on liver regeneration and DNA synthesis (Fujiwara and Nagoshi, 1998). An increase in its concentration would promote a faster turnover of hepatocytes under the metabolic load of early lactation, possibly resulting in a more active and responsive liver.

The BCAA metabolism pathway was impacted due to differences in the prepartum diet. The main affected gene (FC < -3, Supplemental table C.5) for both the catabolic and anabolic branches of the BCAA pathway at 7 d postpartum was *BCAT1* (Branched Chain Amino-Acid Transaminase 1, Cytosolic), which can catalyze both the deamination and amination of BCAA

and related keto-acids. Recently, a higher postpartum plasma concentration of BCAA (specifically Val and Iso) in TMR-fed dairy cows was linked to greater liver function and a possible better responsiveness of the immune system (Zhou et al., 2016). In the present study, plasma AA data were not collected, but it is plausible that an increase in hepatic BCAA biosynthesis in B5F75 cows led to similar results, indicating how feed restriction would be more appropriate for optimally-conditioned cows. Furthermore, the fact that the 'BCAA degradation' pathway was induced at 28 d in B5F125 cows reflects a prolonged NEB in overfed optimally-conditioned cows, which induced protein mobilization, supporting, again, our claim for prepartum feed restriction in BCS5 cow.

Glycan biosynthesis and metabolism. Feeding BCS5 cows at 125% of requirements prepartum induced an activation of the 'N-glycan biosynthesis' and 'Other glycan degradation' pathways early postpartum. A proposed role of N-Glycan biosynthesis in the liver is to handle the misfolded proteins in the endoplasmic reticulum (**ER**) during periods of stress (Fagioli and Sitia, 2001). As the 'Protein processing in the endoplasmic reticulum' pathway followed the same pattern as N-glycan biosynthesis (Supplemental Table C.7), this might be indicative of a greater degree of ER stress in optimally-conditioned cows fed to 125% of requirements prepartum. Because a similar induction of ER stress was also previously reported as a consequence of prepartum overfeeding (Khan et al., 2014, Shahzad et al., 2014), our data further indicate that a slight feed-restriction might be a better approach for these cows.

The 'Other glycan degradation' pathway was highly-impacted early postpartum due to a relatively small activation in B5F125 versus B5F75 cows. This pathway concerns the degradation of N-glycans and glycosphingolipids. Because pathways concerning glycosphingolipid biosynthesis were not affected (Supplemental Table C.7), it is assumed that

their liver concentration, and not that of N-glycans, which had a positive biosynthesis, decreased. These changes might be biologically relevant in transition cows, because mice fed a high-fat diet, in which glycan synthesis was suppressed (Bijl et al., 2009, Zhao et al., 2009), had a decrease in liver accumulation of triacylglycerol along with a reduced hepatocyte expression of several genes associated with steatosis; this included those involved in lipogenesis, gluconeogenesis, and inflammation. Studies from different research groups have demonstrated that prepartum over-allowance of energy results in prepartum hyperglycemia and hyperinsulinemia and marked postpartum adipose tissue mobilization (i.e., greater blood NEFA concentration) (Rukkwamsuk et al., 1999, Holtenius et al., 2003, Roche et al., 2005, Janovick et al., 2011, Khan et al., 2014, Roche et al., 2015). Thus, the reduction in hepatic levels of glycosphingolipids early postpartum could be a mechanism to counteract the development of lipidosis in B5F125 cows due to the possible accumulation of lipid in the liver.

Metabolism of cofactors and vitamins. The physiological levels of retinol (vitamin A) and its precursor β-carotene are tightly correlated with dietary intake (Daley et al., 2010). Similarly, thiamine (vitamin B1) production depends on the dietary availability of cobalt (Co), and on ruminal microbial activity and microbial biomass, which are connected to DMI. Thus, because F125 cows were allowed a greater availability of pasture, which is naturally rich in β-carotene and a possible source of Co, the activation of ’Retinol metabolism’ and ‘Thiamine metabolism’ in B5F125 cows prepartum could be related to the greater pasture allowance owing to the experimental design.

The postpartum inhibition of ’Pantothenate and CoA biosynthesis’ seems to indicate that the feed restriction in the late dry period could enhance the energy status of optimally-conditioned cows (BCS5). Panthothenate is synthetized in the body as a component of

Coenzyme A (**CoA**), an indispensable cofactor in synthesis and oxidation of fatty acids (Mahler, 1953). Furthermore, CoA is a component of acetyl-CoA, the crossover molecule in cellular metabolism (Pietrocola et al., 2015). With an apparent increase in production of CoA, B5F75 cows would have been able to better handle the mobilized adipose fatty acids, and more effectively coordinate the metabolic shift that occurs at calving.

CONCLUSIONS

As hypothesized, the effect of prepartum plane of nutrition on hepatic function was dependent on the BCS of the cow, underscoring how these management tools need to be evaluated together to optimize the biological adaptations of the cow during the peripartum period. The more pronounced transcriptome changes in underconditioned cows highlighted that they are more sensitive to prepartum feeding level/allocation than optimally-conditioned cows. Similarly, the bioinformatics analysis revealed transcriptome signatures that indicate a greater and potentially more prolonged NEB in overfed optimally-conditioned cows and also in feed-restricted underconditioned cows. Based on gene network analysis, the latter group might be more prone to liver dysfunction. The data indicate a less pronounced mobilization and better handling of NEFA in overfed underconditioned cows. Overall, results indicate that overfeeding in late-pregnancy should be limited to underconditioned cows, while cows with optimal degree of body condition and those with greater than optimal condition should be maintained on a restricted energy diet.

LIST OF ABBREVIATIONS

AA, Amino Acids; **ACHE**, Acetylcholinesterase; **ALB**, Albumin; **ANGPT1**, Angiopoietin 1; **APOB**, Apolipoprotein B; **ATP7A**, ATPase Copper Transporting Alpha; **BCAA**, Branched Chain Amino Acid; **BCAT1**, Branched Chain Amino Acid Transaminase 1; **BCL9L**, B-Cell CLL/Lymphoma 9-Like; **BCS**, Body Condition Score; **BHBA**, Beta-Hydroxybutyric Acid; **CD86**, CD86 Antigen, B-Lymphocyte Antigen B7-2; **CHRNG**, Cholinergic Receptor Nicotinic Gamma Subunit; **CKMT1B**, Creatine Kinase, Mitochondrial 1B; **CoA**, Coenzyme A; **CUX1**, Cut Like Homeobox 1; **CXCR4**, C-X-C Motif Chemokine Receptor 4; **DAG1**, Dystroglycan 1; **DEG**, Differentially Expressed Gene; **DIA**, Dynamic Impact Approach; **DMI**, Dry Matter Intake; **ECT2**, Epithelial Cell Transforming 2; **ER**, Endoplasmatic Reticulum; **ERG**, Erythroblast Transformation-Specific Transcription Factor ERG Variant 10; **FABP7**, Fatty Acid Binding Protein 7; **FC**, Fold Change; **FMNL2**, Formin Like 2; **GAG**, Glycosaminoglycan; **GPI**, Glycosylphosphatidylinositol; **GPX3**, Glutathione Peroxidase 3; **HDAC6**, Histone Deacetylase 6; **HDL**, High Density Lipoprotein; **Insp6**, Inositol Hexakisphosphate; **IPA**, Ingenuity Pathway Analysis; **KEGG**, Kyoto Encyclopedia of Genes and Genome; **LALBA**, Lactalbumin Alpha; **LHX1**, LIM Homeobox 1; **MARS**, Methionyl-TRNA Synthetase; **ME1**, Malic Enzyme 1; **MEIS1**, Meis Homeobox 1; **MTTP**, Microsomal Triglyceride Transfer Protein; **MYOD1**, Myogenic Differentiation 1; **NAD**, Nicotinamide Adenine Dinucleotide; **NEB**, Negative Energy Balance; **NEFA**, Non Esterified Fatty Acids; **PCr**, Phosphocreatine; **PFKL**, Phosphofructokinase, Liver Type; **PKA**, Protein Kinase A; **PKIB**, CAMP-Dependent Protein Kinase Inhibitor Beta; **PPARA**, Peroxisome Proliferator Activated Receptor Alpha; **PTPN4**, Protein Tyrosine Phosphatase, Non-Receptor Type 4; **RBM38**, RNA Binding Motif Protein 38; **RYR1**, Ryanodine Receptor 1; **SeMet**, Seleno-Methionine; **SIM1**, Single-Minded Family BHLH

Transcription Factor 1; **SOX2**, SRY-Box 2; **SP1**, Sp1 Transcription Factor; **TAG**, Triacylglycerol; **TMIGD1**, Transmembrane And Immunoglobulin Domain Containing 1; **TMR**, Total Mixed Ratio; **VFA**, Volatile Fatty Acid; **VLDL**, Very Low Density Lipoprotein; **WNT6**, Wnt Family Member 6; **ZKSCAN1**, Zinc Finger With KRAB And SCAN Domains 1.

DECLARATIONS

Ethics approval

The Ruakura Animal Ethics Committee (Hamilton, New Zealand) approved all animal manipulations in accordance with the New Zealand Animal Welfare Act (1999).

Consent for publication

Non applicable

Availability of supporting data

The arrays data have been submitted to Gene Expression Omnibus database (BioProject ID: PRJNA323499).

Competing interests

The authors declare that they have no competing interests.

Funding

This research was supported by New Zealand dairy farmers through DairyNZ Inc. (RD1403) and the Ministry of Business, Innovation, and Employment (DRCX1201).

Authors' contributions

MVR performed the microarray experiment, run the DIA and IPA analysis, and wrote the main draft of the manuscript, with inputs from JRR and JLL. JRR, with the help of SM, CRB, JKK, MDM, AH, and CGW designed the study and participated in its coordination. MAC

extracted and quality assessed the RNA from the biopsy samples. SLRZ handled the statistical analysis of the array results. All authors read and approved the final manuscript..

Acknowledgements

The authors thank Jason Philips, Farm Manager, Scott Farm, DairyNZ, and his capable team, as well as Stu Morgan, Kate Watkins, Hamish Hodgson, and Chris Roach for technical.

TABLES AND FIGURES

Table 5.1. Number of differentially expressed genes, used for DIA and IPA analysis, for each considered comparison after applying a *P* value cutoff of ≤ 0.01 and a fold change threshold at ≤ / ≥ ± 1.5

Comparison	Wk[1]	Up	Down	Total
DIA				
B4F125 vs B4F75	-7	62	63	125
	7	336	229	565
	28	147	234	381
B5F125 vs B5F75	-7	31	43	74
	7	99	106	205
	28	15	16	31
IPA				
B4F125 vs B4F75	n.a.	69	174	243
B5F125 vs B5F75	n.a.	56	62	118

[1] Time factor was used only for DIA analysis, while IPA analysis focused on the overall effect during the whole transition period.

Table 5.2. Effect of prepartum body condition score (BCS) and feeding management on plasma concentration of NEFA and BHBA in dairy cows during the transition period.

	B		F			*P*-value[1]						
mmol/L	**4**	**5**	**75%**	**125%**	**SE**	**B**	**F**	**B*F**	**T**	**B*T**	**F*T**	**B*F*T**
NEFA	0.50^{x}	0.72^{y}	0.59	0.62	0.04	0.0004	0.62	0.99	<0.0001	0.05	0.26	0.99
BHBA	0.46^{x}	0.60^{y}	0.57^{x}	0.49^{y}	0.03	0.004	0.05	0.83	<0.0001	0.10	0.01	0.57

[1]T = time (week relative to parturition); B = BCS (1-10 scale); F = level of feeding prepartum relative to requirement (%); B*F*T = interaction of BCS, level of feeding prepartum relative to requirements (%), and time around parturition.

x,y,z = significant difference among groups ($P < 0.05$)

Table 5.3. Upstream differentially expressed transcription regulators and their target genes with fold change (FC) ≥ |3|.

Transcription regulator	FC	Target genes and responses within the specific comparison
B4F125 vs B4F75		
SIM1	-1.980	↑*AOX1*, ↑*CBFB*, ↓*CD86*, ↓*GPX3*, ↓*PKIB*, ↑*STRN*, ↓*THEG*
SOX2	-1.766	↑*ALB*, ↓*ANGPT1*, ↑*APCDD1*, ↑*BCL9L*, ↓*CST6*, ↓*CXCR4*, ↓*FABP7*, ↓*MEIS1*
MYOD1	-1.641	↓*ACHE*, ↓*ATP7A*, ↓*CHRNG*, ↓*DAG1*, ↓*RBM38*, ↓*RYR1*, ↑*SP1*
ERG	-1.552	↓*CXCR4*, ↓*EZH2*, ↑*FMNL2*, ↓*HDAC6*, ↓*ITGA6*, ↑ *PTPN4*
LHX1	-1.505	↓*SIM1*, ↑*SOSTDC1*, ↓*TMIGD1*, ↑*ZKSCAN1*
B5F125 vs B5F75		
CUX1	-2.035	↓*ECT2*, ↑*WNT6*

Figure 5.1. Summary of KEGG metabolic subcategories resulting from the DIA analysis in liver of BCS 4 and 5 cows fed 125% of requirements prepartum compared with 75%. For each sampling time, the columns represent the effect (impact) and flux responses. The transparent bars represent the effect value (0 to 30), and the flux columns represent negative (−) and positive (+) flux (−30 to +30) based on the direction of the effect. The negative flux (green bars) indicates an upregulation in B4F75 or B5F75 cows, while the positive flux (red bars) indicates an upregulation in B4F125 or B5F125 cows.

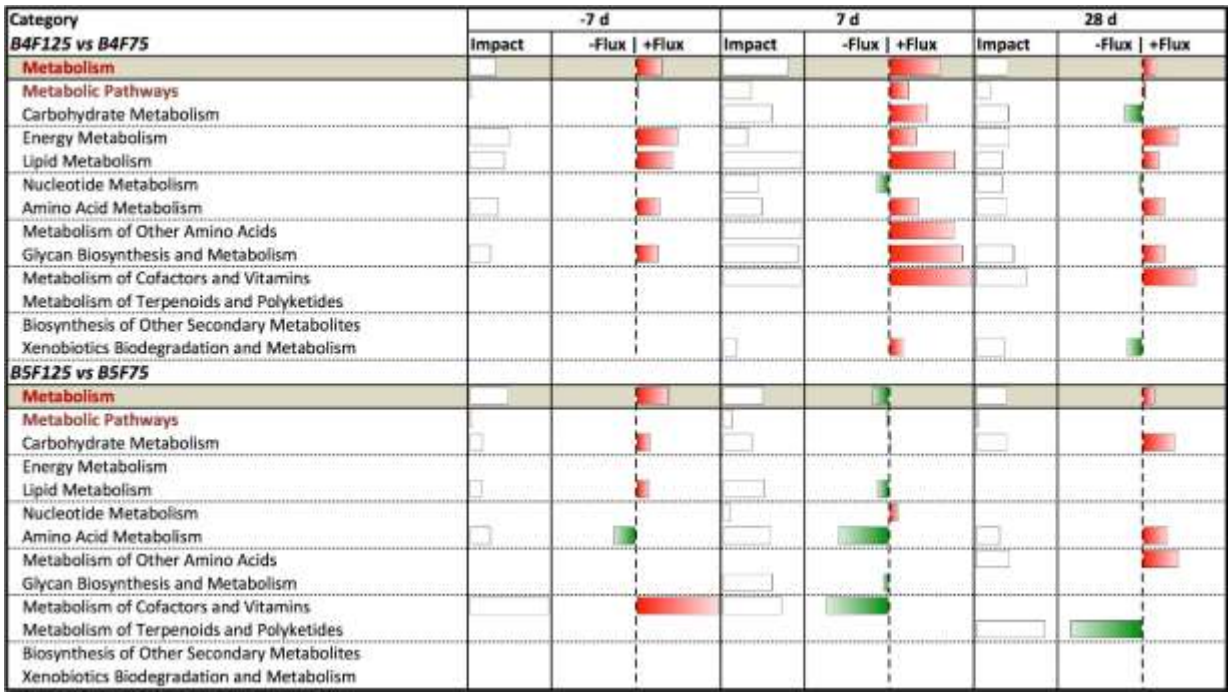


Figure 5.2. Dynamic Impact Approach (DIA) results (Impact and Direction of the Impact) for the most impacted metabolic KEGG pathways (Top 25), grouped in sub-categories of pathways, in BCS 4 cows fed 125% compared with 75% of requirements prepartum. Lines represent the Impact (0-100) along the transition period, while bars show the Direction of the Impact (−100 - +100) (red = upregulation, green = downregulation, in B4F125 vs B4F75).

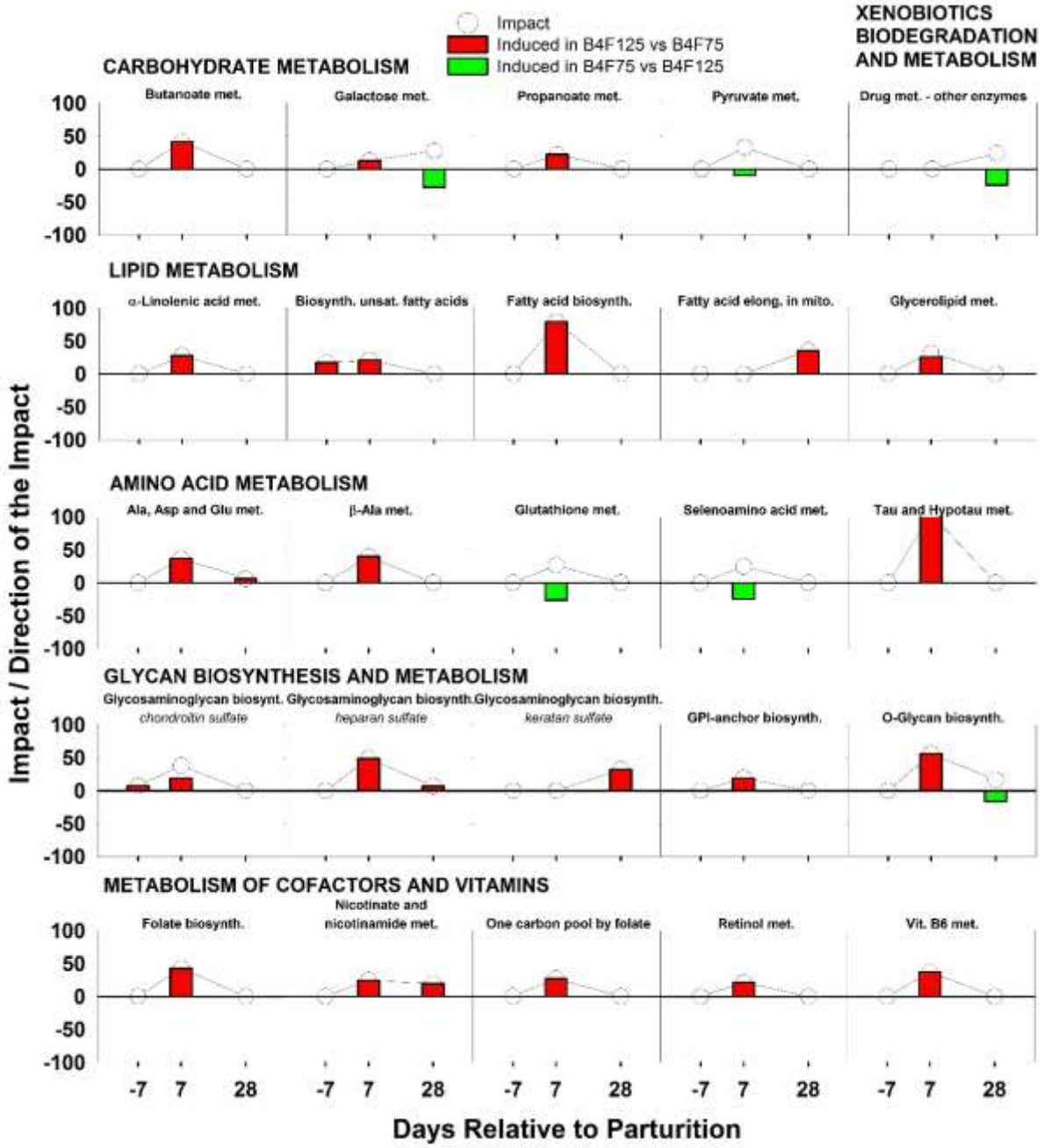


Figure 5.3. Dynamic Impact Approach (DIA) results (Impact and Direction of the Impact) for the most impacted metabolic KEGG pathways (Top 23), grouped in sub-categories of pathways, in BCS 5 cows fed 125% compared with 75% of requirements prepartum. Lines represent the Impact (0-50) along the transition period, while bars show the Direction of the Impact (−50 - +50) (red = upregulation, green = downregulation, in B5F125 vs B5F75).

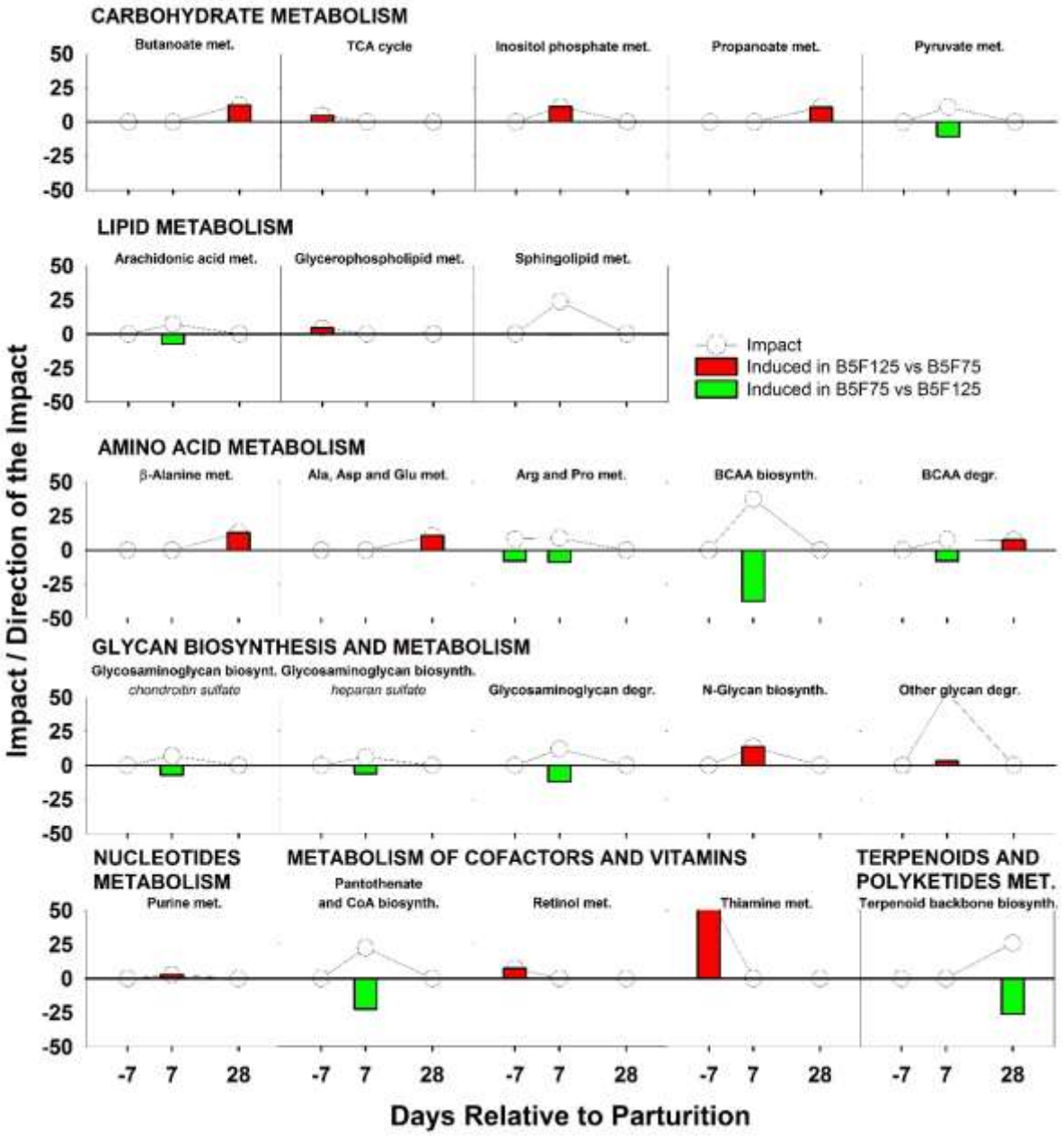


Figure 5.4. Ingenuity Pathway Analysis (IPA) upstream network analysis of differentially expressed genes in liver of B4F125 cows compared to B4F75. Upstream regulators are located at the center of the network, and the downstream target genes are located at the periphery. The arrow represents the direction of the target molecule. The red color represents upregulation of genes in B4F125, and green color represents upregulation of genes in B4F75. The detailed IPA legend can be found in Supplemental figure C.1.

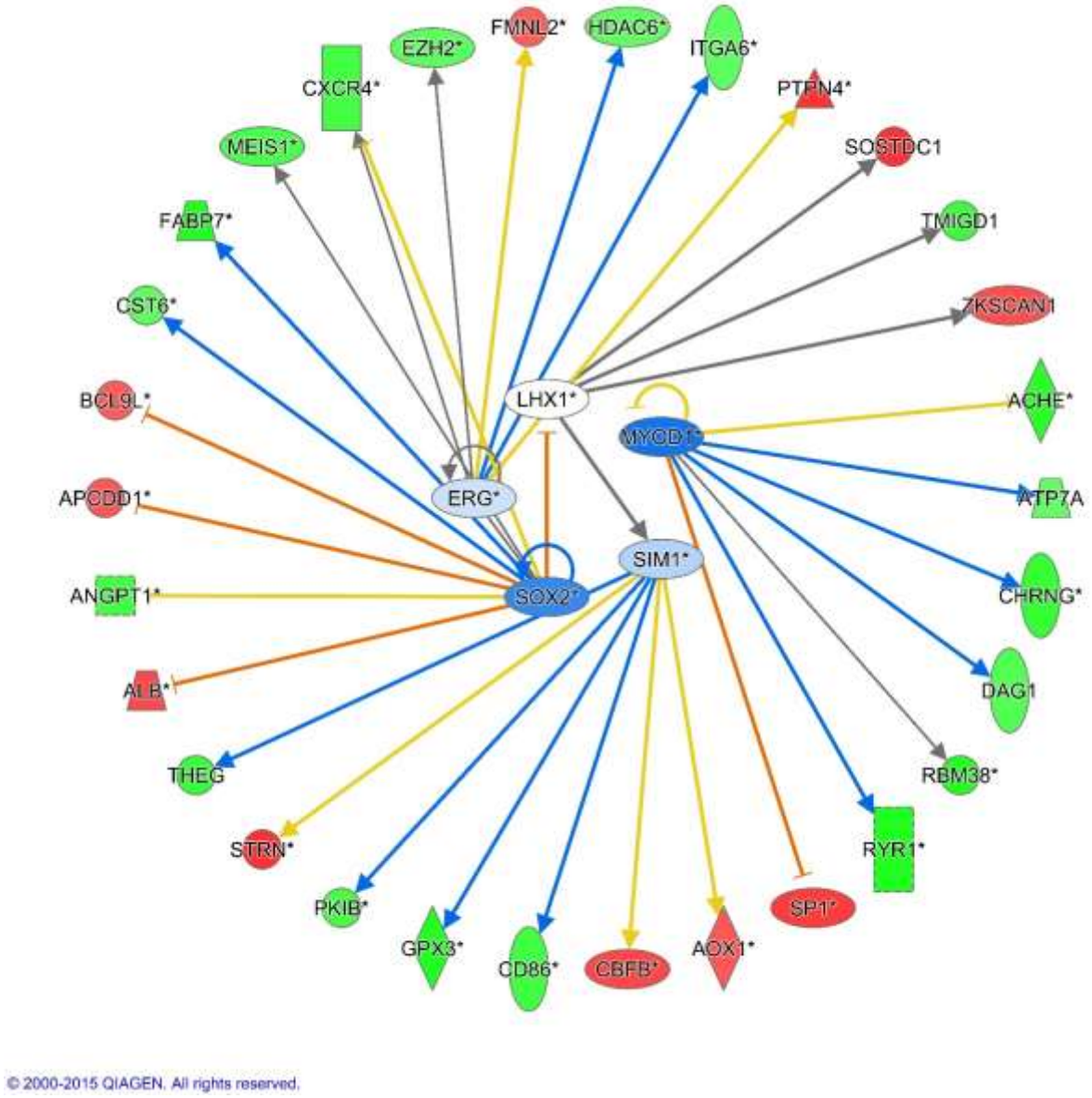


Figure 5.5. Ingenuity Pathway Analysis (IPA) upstream network analysis of differentially expressed genes in liver of B5F125 cows compared to B5F75. Upstream regulators are located at the center of the network, and the downstream target genes are located at the periphery. The arrow represents the direction of the target molecule. The red color represents upregulation of genes in B5F125, and green color represents upregulation of genes in B5F75. The detailed IPA legend can be found in Supplemental figure C.1

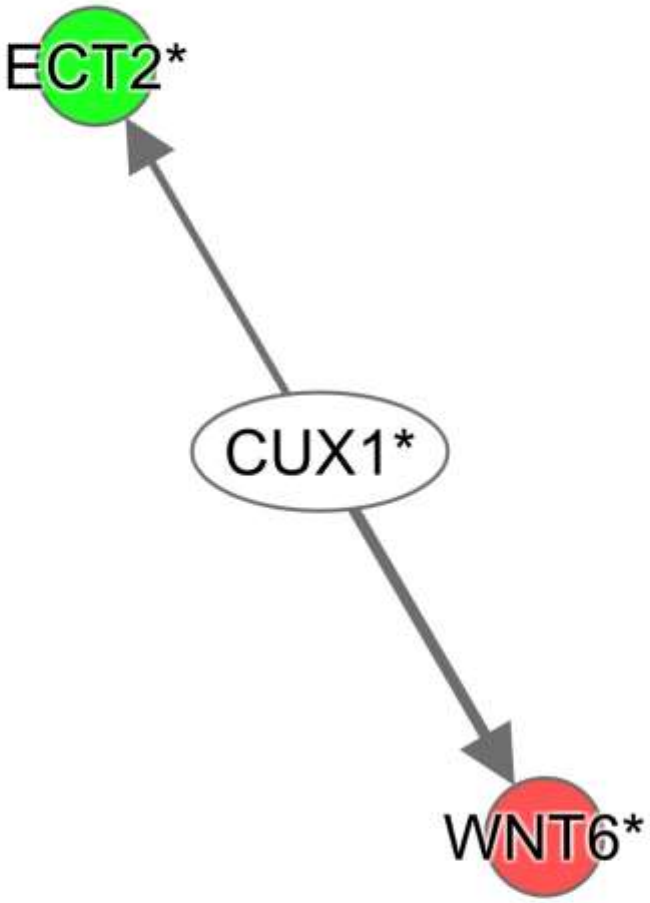
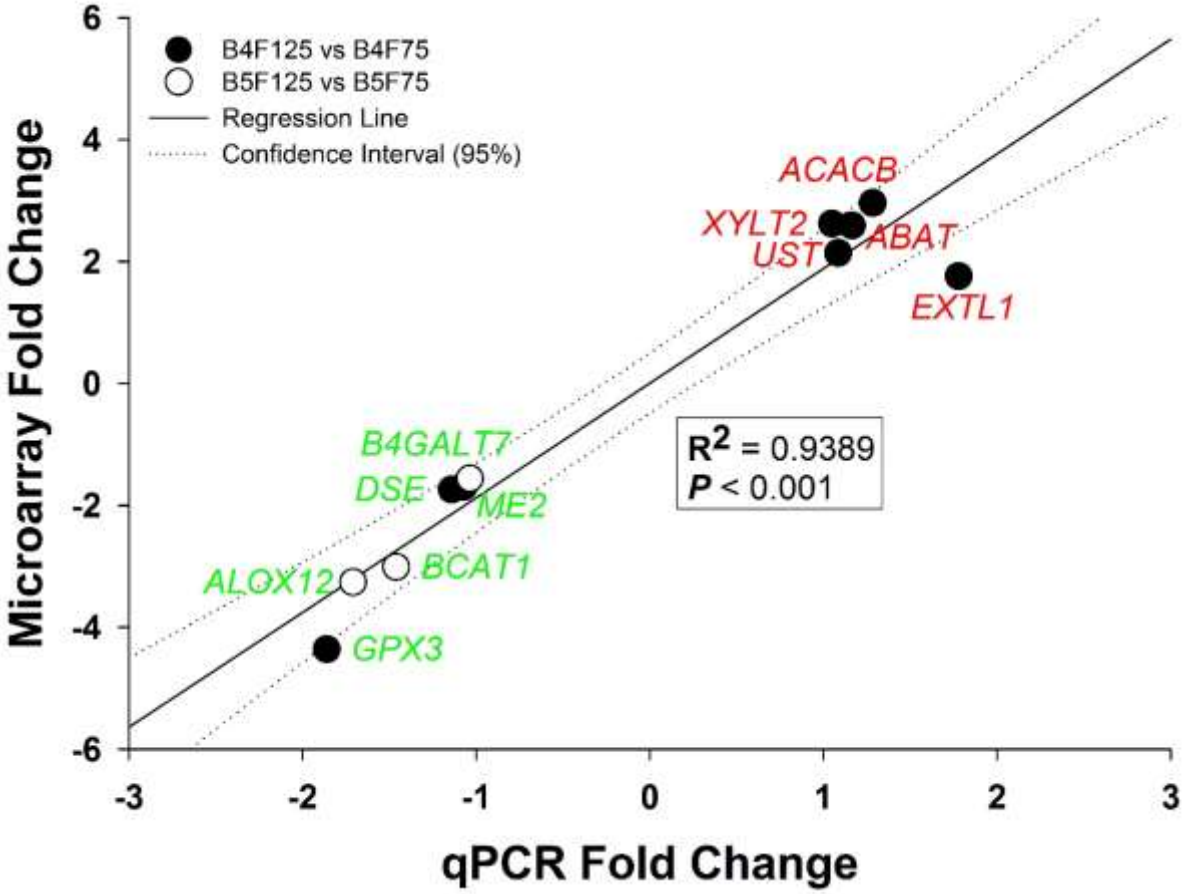


Figure 5.6. Correlation between qPCR and microarray expression fold change results. Upregulated genes are highlighted in red, while downregulated genes are in green. Black circles represent genes validated for the comparison B4F125vsB4F75, while white circles represent genes validated for the comparisons B5F125vsB5F75.



REFERENCES

Akbar, H., F. C. Cardoso, S. Meier, C. Burke, S. McDougall, M. Mitchell, C. Walker, S. L. Rodriguez-Zas, R. E. Everts, H. A. Lewin, J. R. Roche, and J. J. Loor. 2014a. Postpartal subclinical endometritis alters transcriptome profiles in liver and adipose tissue of dairy cows. Bioinformatics and biology insights 8:45-63.

Akbar, H., T. M. Grala, M. V. 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. Journal of dairy science 98(2):1019-1032.

Akbar, H., Z. Zhou, K. Macdonald, K. E. Schütz, G. Verkerk, J. R. Webster, S. L. Rodriguez Zas, J. R. Roche, and J. J. Loor. 2014b. Body condition score at calving alters the hepatic transcriptome in grazing dairy cattle. in Joint Annual Meeting. Vol. 97, E-Supplement 1. Journal of dairy science, Kansas City, MO.

Akbar, H., Z. Zhou, K. Macdonald, K. E. Schütz, G. Verkerk, J. R. Webster, S. L. Rodriguez Zas, J. R. Roche, and J. J. Loor. 2014c. Differences in hepatic transcriptional regulatory networks due to body condition score at calving in grazing dairy cattle. in Joint Annual Meeting. Vol. 92, E-Supplement 1. Journal of dairy science, Kansas City, MO.

Aydin, A. F., C. Kucukgergin, G. Ozdemirler-Erata, N. Kocak-Toker, and M. Uysal. 2010. The effect of carnosine treatment on prooxidant-antioxidant balance in liver, heart and brain tissues of male aged rats. Biogerontology 11(1):103-109.

Bell, A. W. 1979. Lipid metabolism in liver and selected tissues and in the whole body of ruminant animals. Progress in lipid research 18(3):117-164.

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

Bertozzi, C. R. and R. Sasisekharan. 2009. Glycomics. in Essentials of Glycobiology. 2nd ed. A. Varki, R. D. Cummings, J. D. Esko, H. H. Freeze, P. Stanley, C. R. Bertozzi, G. W. Hart, and M. E. Etzler, ed, Cold Spring Harbor (NY).

Bijl, N., M. Sokolovic, C. Vrins, M. Langeveld, P. D. Moerland, R. Ottenhoff, C. P. van Roomen, N. Claessen, R. G. Boot, J. Aten, A. K. Groen, J. M. Aerts, and M. van Eijk. 2009. Modulation of glycosphingolipid metabolism significantly improves hepatic insulin sensitivity and reverses hepatic steatosis in mice. Hepatology 50(5):1431-1441.

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.

Bonfleur, M. L., P. C. Borck, R. A. Ribeiro, L. C. Caetano, G. M. Soares, E. M. Carneiro, and S. L. Balbo. 2015. Improvement in the expression of hepatic genes involved in fatty acid metabolism in obese rats supplemented with taurine. Life sciences 135:15-21.

Cecconi, O., R. M. Nelson, W. G. Roberts, K. Hanasaki, G. Mannori, C. Schultz, T. R. Ulich, A. Aruffo, and M. P. Bevilacqua. 1994. Inositol polyanions. Noncarbohydrate inhibitors of L- and P-selectin that block inflammation. Journal of biological chemistry 269(21):15060-15066.

Cummings, R. D. and J. M. Pierce. 2014. The challenge and promise of glycomics. Chemistry and biology 21(1):1-15.

Daley, C. A., A. Abbott, P. S. Doyle, G. A. Nader, and S. Larson. 2010. A review of fatty acid profiles and antioxidant content in grass-fed and grain-fed beef. Nutrition journal 9:10.

Dallinga-Thie, G. M., R. Franssen, H. L. Mooij, M. E. Visser, H. C. Hassing, F. Peelman, J. J. Kastelein, M. Peterfy, and M. Nieuwdorp. 2010. The metabolism of triglyceride-rich lipoproteins revisited: New players, new insight. Atherosclerosis 211(1):1-8.

Dalton, G. D. and W. L. Dewey. 2006. Protein kinase inhibitor peptide (PKI): A family of endogenous neuropeptides that modulate neuronal cAMP-dependent protein kinase function. Neuropeptides 40(1):23-34.

Dann, H. M., N. B. Litherland, J. P. Underwood, M. Bionaz, A. D'Angelo, J. W. McFadden, and J. K. Drackley. 2006. Diets during far-off and close-up dry periods affect periparturient metabolism and lactation in multiparous cows. Journal of dairy science 89(9):3563-3577.

Davidson, N. O. and G. S. Shelness. 2000. Apolipoprotein B: mRNA editing, lipoprotein assembly, and presecretory degradation. Annual review of nutrition 20:169-193.

Desvergne, B., L. Michalik, and W. Wahli. 2006. Transcriptional regulation of metabolism. Physiological reviews 86(2):465-514.

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

Eggleton, P., J. Penhallow, and N. Crawford. 1991. Priming action of inositol hexakisphosphate (InsP6) on the stimulated respiratory burst in human neutrophils. Biochimica et biophysica acta 1094(3):309-316.

Etzler, M. E. and J. D. Esko. 2009. Free Glycans as Signaling Molecules. in Essentials of Glycobiology. 2nd ed. A. Varki, R. D. Cummings, J. D. Esko, H. H. Freeze, P. Stanley, C. R. Bertozzi, G. W. Hart, and M. E. Etzler, ed, Cold Spring Harbor (NY).

Fagioli, C. and R. Sitia. 2001. Glycoprotein quality control in the endoplasmic reticulum. Mannose trimming by endoplasmic reticulum mannosidase I times the proteasomal degradation of unassembled immunoglobulin subunits. Journal of biological chemistry 276(16):12885-12892.

Fujiwara, K. and S. Nagoshi. 1998. Biological activity of growth factors in vivo. Pages 163-182 in Liver Growth and Repair. A. Strain and A. Diehl, ed. Springer Netherlands.

Geisler, F. and M. Strazzabosco. 2015. Emerging roles of Notch signaling in liver disease. Hepatology 61(1):382-392.

Graugnard, D. E., K. M. Moyes, E. Trevisi, M. J. Khan, D. Keisler, J. K. Drackley, G. Bertoni, and J. J. Loor. 2013. Liver lipid content and inflammometabolic indices in peripartal dairy cows are altered in response to prepartal energy intake and postpartal intramammary inflammatory challenge. Journal of dairy science 96(2):918-935.

Guo, L., E. K. Lobenhofer, C. Wang, R. Shippy, S. C. Harris, L. Zhang, N. Mei, T. Chen, D. Herman, F. M. Goodsaid, P. Hurban, K. L. Phillips, J. Xu, X. Deng, Y. A. Sun, W. Tong, Y. P. Dragan, and L. Shi. 2006. Rat toxicogenomic study reveals analytical consistency across microarray platforms. Nature biotechnology 24(9):1162-1169.

Hayirli, A., R. R. Grummer, E. V. Nordheim, and P. M. Crump. 2002. Animal and dietary factors affecting feed intake during the prefresh transition period in Holsteins. Journal of dairy science 85(12):3430-3443.

Hipkiss, A. R. 2009. Carnosine and its possible roles in nutrition and health. Advances in food and nutrition research 57:87-154.

Holtenius, K., S. Agenas, C. Delavaud, and Y. Chilliard. 2003. Effects of feeding intensity during the dry period. 2. Metabolic and hormonal responses. Journal of dairy science 86(3):883-891.

Janovick, N. A., Y. R. Boisclair, and J. K. Drackley. 2011. Prepartum dietary energy intake affects metabolism and health during the periparturient period in primiparous and multiparous Holstein cows. Journal of dairy science 94(3):1385-1400.

Ji, P., J. K. Drackley, M. J. Khan, and J. J. Loor. 2014. Overfeeding energy upregulates peroxisome proliferator-activated receptor (PPAR)gamma-controlled adipogenic and lipolytic gene networks but does not affect proinflammatory markers in visceral and subcutaneous adipose depots of Holstein cows. Journal of dairy science 97(6):3431-3440.

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 regulation and systems biology 8:97-111.

Kitchen, E., A. M. Condliffe, A. G. Rossi, C. Haslett, and E. R. Chilvers. 1996. Characterization of inositol hexakisphosphate (InsP6)-mediated priming in human neutrophils: Lack of extracellular [3H]-InsP6 receptors. British journal of pharmacology 117(5):979-985.

Knegsel, A. T. M. v., H. v. d. Brand, J. Dijkstra, W. M. v. Straalen, M. J. W. Heetkamp, S. Tamminga, B. Kemp, H. v. den Brand, A. T. M. van Knegsel, H. van den Brand, and W. M. van Straalen. 2007. Dietary energy source in dairy cows in early lactation: Energy partitioning and milk composition. Journal of dairy science 90(3):1467-1476.

Laliotis, G. P., I. Bizelis, and E. Rogdakis. 2010. Comparative approach of the de novo fatty acid synthesis (lipogenesis) between ruminant and non ruminant mammalian species: From biochemical level to the main regulatory lipogenic genes. Current genomics 11(3):168-183.

Loor, J. J., H. M. Dann, N. A. Guretzky, R. E. Everts, R. Oliveira, C. A. Green, N. B. Litherland, S. L. Rodriguez-Zas, H. A. Lewin, and J. K. Drackley. 2006. Plane of nutrition prepartum alters hepatic gene expression and function in dairy cows as assessed by longitudinal transcript and metabolic profiling. Physiological genomics 27(1):29-41.

Mahler, H. R. 1953. Role of coenzyme A in fatty acid metabolism. Federation proceedings 12(3):694-702.

Martin, R. J., L. L. Wilson, R. L. Cowan, and J. D. Sink. 1973. Effects of fasting and diet on enzyme profiles in ovine liver and adipose tissue. Journal of animal science 36(1):101-106.

Meijer, G. A., J. Van der Meulen, J. G. Bakker, C. J. Van der Koelen, and A. M. Van Vuuren. 1995. Free amino acids in plasma and muscle of high yielding dairy cows in early lactation. Journal of dairy science 78(5):1131-1141.

Miranda, S. G., N. G. Purdie, V. R. Osborne, B. L. Coomber, and J. P. Cant. 2011. Selenomethionine increases proliferation and reduces apoptosis in bovine mammary epithelial cells under oxidative stress. Journal of dairy science 94(1):165-173.

NRC. 2001. Nutrient requirements of dairy cattle. 7th revised edition. National Academy Press, Washington, D.C.

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. Journal of dairy science 96(10):6248-6263.

Osorio, J. S., P. Ji, J. K. Drackley, D. Luchini, and J. J. Loor. 2014a. 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. Journal of dairy science 97(12):7451-7464.

Osorio, J. S., E. Trevisi, P. Ji, J. K. Drackley, D. Luchini, G. Bertoni, and J. J. Loor. 2014b. Biomarkers of inflammation, metabolism, and oxidative stress in blood, liver, and milk reveal a better immunometabolic status in peripartal cows supplemented with Smartamine M or MetaSmart. Journal of dairy science 97(12):7437-7450.

Piantoni, P., K. M. Daniels, R. E. Everts, S. L. Rodriguez-Zas, H. A. Lewin, W. L. Hurley, R. M. Akers, and J. J. Loor. 2012. Level of nutrient intake affects mammary gland gene expression profiles in preweaned Holstein heifers. Journal of dairy science 95(5):2550-2561.

Pietrocola, F., L. Galluzzi, J. M. Bravo-San Pedro, F. Madeo, and G. Kroemer. 2015. Acetyl coenzyme A: A central metabolite and second messenger. Cell metabolism 21(6):805-821.

Pires, J. A. A., C. Delavaud, Y. Faulconnier, D. Pomies, and Y. Chilliard. 2013. Effects of body condition score at calving on indicators of fat and protein mobilization of periparturient Holstein-Friesian cows. Journal of dairy science 96(10):6423-6439.

Pomin, V. H. 2015. Sulfated glycans in inflammation. European journal of medicinal chemistry 92:353-369.

Ramakrishnan, S., K. N. Sulochana, S. Lakshmi, R. Selvi, and N. Angayarkanni. 2006. Biochemistry of homocysteine in health and diseases. Indian journal of biochemistry and biophysics 43(5):275-283.

Randall, L. V., M. J. Green, M. G. Chagunda, C. Mason, S. C. Archer, L. E. Green, and J. N. Huxley. 2015. Low body condition predisposes cattle to lameness: An 8-year study of one dairy herd. Journal of dairy science 98(6):3766-3777.

Reynolds, C. K., P. C. Aikman, B. Lupoli, D. J. Humphries, and D. E. Beever. 2003. Splanchnic metabolism of dairy cows during the transition from late gestation through early lactation. Journal of dairy science 86(4):1201-1217.

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. Journal of dairy science 92(12):5769-5801.

Roche, J. R., E. S. Kolver, and J. K. Kay. 2005. Influence of precalving feed allowance on periparturient metabolic and hormonal responses and milk production in grazing dairy cows. Journal of dairy science 88(2):677-689.

Roche, J. R., J. M. Lee, K. A. Macdonald, and D. P. Berry. 2007. Relationships among body condition score, body weight, and milk production variables in pasture-based dairy cows. Journal of dairy science 90(8):3802-3815.

Roche, J. R., K. A. Macdonald, K. E. Schutz, L. R. Matthews, G. A. Verkerk, S. Meier, J. J. Loor, A. R. Rogers, J. McGowan, S. R. Morgan, S. Taukiri, and J. R. Webster. 2013. Calving body condition score affects indicators of health in grazing dairy cows. Journal of dairy science 96(9):5811-5825.

Roche, J. R., S. Meier, A. Heiser, M. D. Mitchell, C. G. Walker, M. A. Crookenden, M. Vailati Riboni, J. J. Loor, and J. K. Kay. 2015. Effects of pre-calving body condition score and prepartum feeding level on production, reproduction, and health parameters in pasture-based transition dairy cows. Journal of dairy science 98(10):7164-7182.

Rui, L. 2014. Energy metabolism in the liver. Comprehensive physiology 4(1):177-197.

Rukkwamsuk, T., T. Wensing, and M. J. Geelen. 1999. Effect of overfeeding during the dry period on the rate of esterification in adipose tissue of dairy cows during the periparturient period. Journal of dairy science 82(6):1164-1169.

Sangiorgio, V., M. Pitto, P. Palestini, and M. Masserini. 2004. GPI-anchored proteins and lipid rafts. Italian journal of biochemistry 53(2):98-111.

Sasisekharan, R., R. Raman, and V. Prabhakar. 2006. Glycomics approach to structure-function relationships of glycosaminoglycans. Annual review of biomedical engineering 8:181-231.

Schulz, H. 1991. Beta oxidation of fatty acids. Biochimica et biophysica acta 1081(2):109-120.

Selhub, J. 2002. Folate, vitamin B12 and vitamin B6 and one carbon metabolism. Journal of nutrition, health and aging 6(1):39-42.

Shahzad, K., H. Akbar, M. Vailati-Riboni, L. Basirico, P. Morera, S. L. Rodriguez-Zas, A. Nardone, U. Bernabucci, and J. J. Loor. 2015. The effect of calving in the summer on the hepatic transcriptome of Holstein cows during the peripartal period. Journal of dairy science 98(8):5401-5413.

Shahzad, K., M. Bionaz, E. Trevisi, G. Bertoni, S. L. Rodriguez-Zas, and J. J. Loor. 2014. Integrative analyses of hepatic differentially expressed genes and blood biomarkers during the peripartal period between dairy cows overfed or restricted-fed energy prepartum. Plos one 9(6):e99757.

Shi, L., W. D. Jones, R. V. Jensen, S. C. Harris, R. G. Perkins, F. M. Goodsaid, L. Guo, L. J. Croner, C. Boysen, H. Fang, F. Qian, S. Amur, W. Bao, C. C. Barbacioru, V. Bertholet, X. M. Cao, T. M. Chu, P. J. Collins, X. H. Fan, F. W. Frueh, J. C. Fuscoe, X. Guo, J. Han, D. Herman, H. Hong, E. S. Kawasaki, Q. Z. Li, Y. Luo, Y. Ma, N. Mei, R. L. Peterson, R. K. Puri, R. Shippy, Z. Su, Y. A. Sun, H. Sun, B. Thorn, Y. Turpaz, C. Wang, S. J. Wang, J. A. Warrington, J. C. Willey, J. Wu, Q. Xie, L. Zhang, L. Zhang, S. Zhong, R. D. Wolfinger, and W. Tong. 2008. The balance of reproducibility, sensitivity, and specificity of lists of differentially expressed genes in microarray studies. BMC bioinformatics 9 Suppl 9:S10.

Stanley, P. 2014. O-Glycans in Mammalian Notch Signaling. Pages 1-8 in Glycoscience: Biology and Medicine. T. Endo, P. H. Seeberger, G. W. Hart, C.-H. Wong, and N. Taniguchi, ed. Springer Japan.

Tamminga, S., P. A. Luteijn, and R. G. M. Meijer. 1997. Changes in composition and energy content of liveweight loss in dairy cows with time after parturition. Livestock production science 52(1):31-38.

Thompson, G. R., R. P. Naoumova, and G. F. Watts. 1996. Role of cholesterol in regulating apolipoprotein B secretion by the liver. Journal of lipid research 37(3):439-447.

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

Valeri, F., F. Boess, A. Wolf, C. Goldlin, and U. A. Boelsterli. 1997. Fructose and tagatose protect against oxidative cell injury by iron chelation. Free radical biology and medicine 22(1-2):257-268.

Wallimann, T., M. Wyss, D. Brdiczka, K. Nicolay, and H. M. Eppenberger. 1992. Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: The 'phosphocreatine circuit' for cellular energy homeostasis. Biochemical journal 281 (Pt 1):21-40.

Ying, W. 2008. NAD+/NADH and NADP+/NADPH in cellular functions and cell death: Regulation and biological consequences. Antioxidants and redox signaling 10(2):179-206.

Zhao, H., M. Przybylska, I. H. Wu, J. Zhang, P. Maniatis, J. Pacheco, P. Piepenhagen, D. Copeland, C. Arbeeny, J. A. Shayman, J. M. Aerts, C. Jiang, S. H. Cheng, and N. S. Yew. 2009.

Inhibiting glycosphingolipid synthesis ameliorates hepatic steatosis in obese mice. Hepatology 50(1):85-93.

Zhou, Z., J. J. Loor, F. Piccioli-Cappelli, F. Librandi, G. E. Lobley, and E. Trevisi. 2016. Circulating amino acids during the peripartal period in cows with different liver functionality index. Journal of dairy science 99(3):2257-2267.

CHAPTER 6: FAR-OFF AND CLOSE-UP DRY MATTER INTAKE MODULATE INDICATORS OF IMMUNOMETABOLIC ADAPTATIONS TO LACTATION IN SUBCUTANEOUS ADIPOSE TISSUE OF PASTURE-BASED TRANSITION DAIRY COWS

Manuscript published.

Vailati Riboni, M., G. Farina, F. Batistel, A. Heiser, M.D. Mitchell, M.A. Crookenden, C.G. Walker, J.K. Kay, S. Meier, J.R. Roche, and J.J. Loor. 2017. Far-off and close-up dry matter intake modulate indicators of immunometabolic adaptations to lactation in subcutaneous adipose tissue of pasture-based transition dairy cows. Journal of Dairy Science, 100(3):2334-2350.

ABSTRACT

The common practice of increasing dietary energy density during the "close-up" dry period (last ~3 wk prepartum) has been recently associated with a higher incidence of metabolic disorders after calving. Despite these reports, over-feeding of metabolizable energy (ME) during the 'far-off', non-lactating period is a common management policy aimed at achieving optimum calving body condition score (BCS) in pasture-based systems, as cows are generally thinner than TMR cows at the end of lactation. Our hypothesis was that both far-off and close-up overfeeding influence the peripartum adipose tissue changes associated with energy balance and inflammatory state. Sixty mid-lactation, grazing dairy cows of mixed age and breed were randomly allocated to one of two groups that were managed through late lactation to achieve a low and high BCS (approximately 4.25 and 5.0 on a 10-point scale) at dry-off. The low BCS cows were then overfed ME to ensure that they achieved the same BCS as the higher BCS group by calving. Within each rate of BCS gain treatment, cows were offered either 65, 90, or 120% of their pre-calving ME requirements for three weeks pre-calving in a 2 x 3 factorial arrangement of treatments (i.e., 10 cows/treatment). Subcutaneous adipose tissue was collected via biopsy at -1,

1 and 4 wk relative to parturition. Quantitative PCR was used to measure mRNA and microRNA (miRNA) expression of targets related to adipogenesis and inflammation. Cows overfed in the far-off period had increased expression of miR-143 and miR-378 prepartum (-1 wk) indicating greater adipogenesis, consistent with their rapid gain in BCS following dry-off. Furthermore, the lower postpartum expression of *IL6*, *TNF*, *TLR4*, *TLR9*, and miR-145, and a higher abundance of miR-99a indicated lower body fat mobilization in early lactation in the same group. In the close-up period, feeding either 65 or 120% of ME requirements caused changes in *FASN*, *IL1B*, *IL6R*, *TLR9*, and the miRNA miR-143, miR-155, and miR-378. Their respective expression patterns indicate a tentative negative-feedback mechanism in metabolically compromised, feed-restricted cows, and a possible immune-related stimulation of lipolysis in apparently static adipocytes in overfed cows. Data from cows fed 90% of ME requirements indicate the existence of a balance between lipolytic (inflammatory-related) and anti-lipolytic signals, in order to prime the mobilization machinery in light of imminent lactation. Overall, results indicate that far-off dry cow nutrition influences peripartum adipose tissue metabolism, with neither strategy negatively affecting the physiological adaptation to lactation. Furthermore, to ensure a favorable transition, cows should be subjected to a small feed restriction in the close-up period, irrespective of far-off nutritional management.

INTRODUCTION

The body condition score (BCS) of a dairy cow is an assessment of the amount of body fat that it possesses. It is an important factor in dairy cattle management (Roche et al., 2009), due to its association with production and reproduction parameters and the chances for a successful lactation (Waltner et al., 1993, Roche et al., 2005, Pires et al., 2013, Randall et al., 2015). The

progression of BCS in a TMR-based system during the lactation cycle (e.g. intercalving) is inversely related with the lactation curve (i.e. it declines to a nadir 40 to 100 d after calving as milk production peaks, before increasing again as milk production declines; Roche et al., 2009). However, in seasonal spring-calving cows grazing fresh pasture, a second period of loss in mid-lactation (Roche et al., 2007) leads to thinner cows at the end of lactation, compared with counterparts fed a TMR (Roche et al., 2007).

To avoid the detrimental physiological and metabolic effects of calving with a low BCS (Pires et al., 2013, Akbar et al., 2015), cows in pasture-based systems have to consume metabolizable energy (ME) in excess of requirements during the "far-off" non-lactating period (>4 wk before calving) to achieve optimal calving BCS targets (Roche et al., 2009). However, Dann et al. (2006), working with TMR-fed cows, provided evidence that overfeeding in the far-off period might increase the risk of metabolic dysfunction during early lactation.

To further complicate peripartal nutritional management, cows are historically allowed ad libitum access to energy-dense feeds during the weeks before calving (Boutflour, 1928, i.e., during the so-called "close-up" dry period), thereby ensuring that cows do not lose condition pre-calving. Recent studies from different research groups have demonstrated, however, that this practice can lead to undesired outcomes including detrimental metabolic shifts such as increased postpartum blood FA concentration (Rukkwamsuk et al., 1999, Holtenius et al., 2003, Janovick et al., 2011, Ji et al., 2014, Khan et al., 2014) and poorer postpartum health indices (Dann et al., 2006, Soliman et al., 2007, Graugnard et al., 2013, Shahzad et al., 2014).

Adipose tissue plays an important role in the cow's adaptation to lactation and its metabolism is directly linked and responsive to DMI (McNamara, 1991, 1997). Furthermore, data from non-ruminants underscore that it plays an active role in its "self-regulation" [e.g.

through the production of adipokines, (Adamczak and Wiecek, 2013, McGown et al., 2014, Musi and Guardado-Mendoza, 2014)]. Among its self-regulating features, adipose has the ability to generate a local inflammatory response, also (in human and mice models) through the recruitment and regulation of the innate immune system (Grant and Dixit, 2015); leading scientists to hypothesize a homeorhetic role of inflammation as a physiological adaptation to lactation (Farney et al., 2013, Vailati Riboni et al., 2015, Vailati Riboni et al., 2016).
A recent study (Arner and Kulyte, 2015) investigated the involvement of microRNA (**miRNA**) in fat cell formation (adipogenesis) and regulation of metabolic and endocrine functions; the results demonstrated how adipocyte metabolic pathways are not only controlled by the well-established changes in mRNA expression, but that miRNA signaling through complex networks involving transcription factors plays an important role in the control of inflammation. Furthermore, miRNA expression patterns in humans have also been associated with levels of inflammatory molecules (e.g. cytokines) and the degree of immune cell infiltration (Kloting et al., 2009).

We previously demonstrated that prepartum BCS and level of nutrition in grazing cows can affect adipose tissue adaptation to lactation through complex immunometabolic pathways (Vailati-Riboni et al., 2016). Overfeeding optimally-conditioned cows during close-up primed adipose tissue for accretion of lipid and caused a robust localized inflammatory response, which upon parturition may increase the probability for metabolic disorders. We hypothesized that far-off overfeeding could impair the adipose tissue adaptation to lactation, with further detrimental effects, or mitigation of these, when combined with close-up overfeeding, or feed-restriction, respectively. In the present study, gene and miRNA expression profiling were used to further

understand the adipose responses to the physiological changes induced by the high metabolic demands of early lactation, and their interaction with far-off and close-up nutritional strategies.

MATERIALS AND METHODS

Animal Management

Complete details of the experimental design are reported elsewhere (Roche et al., 2017). Briefly, a group of 150 mid-lactation dairy cows (that passed a veterinary clinical examination, which included a full pathology health panel) of mixed age and breed (Holstein-Friesian, Jersey, Holstein-Friesian x Jersey) were allocated randomly to one of two treatment groups (75 cows per group) 18 wk before planned start of calving, and managed through late lactation to achieve a high and low BCS (approximately 4.75 and 4.25, on a 10-point scale, where 1 is emaciated and 10 obese; Roche et al. 2004). Consequently, to reach optimal calving BCS (5.00, Roche et al. 2004), the high BCS group had <0.25 BCS units to gain during the 5 wk far-off period (**Slow** BCS gain), while the low BCS cows were overfed to ensure a gain of 0.75-1.0 BCS units in the same period (**Fast** BCS gain). From approximately 3 wk before calving, cows within each BCS gain group were randomly assigned in a 2 x 3 factorial arrangement of treatments to one of three feeding level categories: 65, 90, and 120% of estimated ME requirements (**Feed65**, **Feed90**, and **Feed120**, respectively). Although cow allocation to treatment was random, groups were assessed to ensure they were balanced for age, breed, BCS at the time of enrolment, and expected calving date. For the current study, only a subset of 60 animals (10 cows per group) with adipose tissue biopsy samples available were considered.

RNA extraction and Quantitative PCR (qPCR)

Complete details of these procedures are included in the supplemental material. Briefly, subcutaneous adipose tissue was collected posterior to the shoulder blade and approximately 10 cm down the withers during wk −1, 1, and 4 relative to parturition as described previously (Grala et al., 2013). Average sampling date (mean ± SD) for wk -1, 1, and 4 was -10.4 ± 2.4, 6.4 ± 0.9, and 27.4 ± 0.9 days relative to parturition, respectively. RNA samples were extracted from the frozen tissue and used for cDNA synthesis using established protocols in our laboratory (Vailati Riboni et al., 2016). The Quanta qScript microRNA cDNA Synthesis Kit (Quanta BioSciences, Inc., Gaithersburg, MD) was used for miRNA following the manufacturer's protocols. The qPCR performed was SYBR Green-based, using a 7-point standard curve obtained from a diluted cDNA pool of all samples. Genes selected for transcript profiling are associated with fatty acid metabolism: fatty acid synthase (*FASN*), peroxisome proliferator-activated receptor gamma (*PPARG*); adipokines: adiponectin (*ADIPOQ*); and inflammation: chemokine (C-C motif) ligand 2 (*CCL2*), chemokine (C-C motif) ligand 5 (*CCL5*), haptoglobin (*HP*), interleukin-1β (*IL1B*), inteleukin-6 (*IL6*), inteleukin-6 receptor (*IL6R*), retinoid X receptor alpha (*RXRA*), serum amyloid A3 (*SAA3*), toll-like receptor 4 (*TLR4*), toll-like receptor 9 (*TLR9*), and tumor necrosis factor α (*TNF*). The miRNA selected for expression profiling are associated with immune cell infiltration (miR-26b, miR-126, miR-132, miR-155, miR-193b), inflammation and lipolysis (miR-99a, miR-145, miR-221), and positive regulation of adipogenesis (miR-103, miR-143, miR-378). The specific function of each target miRNA is reported in Table 6.1, including the model system in which the function was assessed. Primer sequences and qPCR performances are reported in the supplemental material.

Blood sampling and analysis

Blood was sampled by coccygeal venipuncture using evacuated blood tubes containing lithium heparin anticoagulant. Samples were placed immediately on ice and centrifuged within 30 min at 1,500 × *g* for 12 min at 4 °C. Following centrifugation, aspirated plasma was stored at −20 °C until assayed.

Blood free fatty acids (FA) and BHB were assayed using colorimetric techniques at 37 °C with a Hitachi Modular P800 analyzer (Roche Diagnostics, Indianapolis, IN). Plasma FA concentration (mmol/L) was measured using the Wako Chemicals (Osaka, Japan) kit NEFA HR2 measuring oxidative condensation of 3-methyl-N-ethyl-N-β hydroxyethyl aniline with 4-aminoantipyrine, while plasma BHB (mmol/L) concentration was assessed using Roche reagent kits measuring the reduction of NAD to NADH during oxidation of d-3-hydroxybutyrate to acetoacetate. Cholesterol was measured using a commercially-available fluorimetric kit (Cayman Chemical Company, East Ellsworth Road, Ann Arbor, U.S.A.).

Statistical Analysis

After normalization with the geometric mean of the internal control genes, qPCR data (mRNA and miRNA) were $\log_2$ transformed prior to statistical analysis to obtain a normal distribution. Statistical analysis was performed with SAS. Data were subjected to ANOVA and analyzed using repeated measures ANOVA with PROC MIXED. The statistical model included time (**T**; -1, 1 and 4 wk postpartum), far-off management (**FO**; Slow and Fast), close-up feeding (**CU**, 65, 90 and 120%) and their interactions (FO*T, CU*T and FO*CU*T) as fixed effects. Cow, nested within treatment, was 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. Data were considered significant at a $P \leq 0.05$ using the PDIFF statement

in SAS. For ease of interpretation, expression data reported in Tables 6.2 through 6.5 are the $\log_2$ back-transformed LSM that resulted from the statistical analysis. Standard errors were also adequately back-transformed. The three-way interaction LSM is not reported in the tables, and can be found in the supplemental material.

RESULTS

Gene expression

Infiltration of immune cells. There was no effect of feeding strategy or time on *CCL5* (T, FO, CU, and interactions, $P > 0.05$), while *CCL2* expression was affected by close-up feeding (CU, $P < 0.05$) (Table 6.2), time (T, $P < 0.05$), and their interaction (CU*T, $P < 0.05$) (Table 6.3). Expression of *CCL2* was greater prepartum in Feed120 cows, compared with Feed65 and Feed90. Early postpartum (1 wk), both Feed120 and Feed90 cows had a greater *CCL2* expression than Feed65 cows, but there was no detected effect of treatment later on (4 wk). This outcomes was due to the different progression in time; compared with prepartum, Feed90 cows experienced a strong upregulation ($P < 0.05$) of *CCL2* at both 1 and 4 wk postpartum time points, while the same increase ($P < 0.05$) in Feed65 cows did not materialize until the 4 wk postpartum. Expression of *CCL2* in Feed120 cows did not change ($P > 0.05$) during the peripartum period.

Inflammation and lipolysis-related proteins and receptors. Parturition affected expression of *HP* and *SAA3* (T, $P < 0.05$) due to an upregulation early postpartum independently from experimental groups ($P < 0.05$) (Table 6.3). *IL6, TLR4*, and *TLR9* were affected by far-off management (FO, $P < 0.05$), as SlowBCS cows had a greater expression ($P < 0.05$) compared with FastBCS cows (Table 6.2). For *TLR4*, however, a FO*T interaction ($P = 0.05$) indicated

that this effect was only present prepartum (Table 6.3). Close-up feeding also affected expression of *IL1B* (CU, $P < 0.05$), *IL6R*, *TLR4*, *TLR9* (CU, CU*T, $P < 0.05$), *IL6* and *TNF* (CU*T, $P < 0.05$) (Table 6.2 and 6.3). Compared with the other two groups, overfed cows (Feed120) had lower expression ($P < 0.05$) of *IL1B*, *TLR9* and *IL6R*. However, when time is taken into consideration, this effect was only present for *IL6R* and *TLR9* postpartum (wk 1 and 4), as prepartum (wk -1) both Feed90 and Feed120 had a lower expression ($P < 0.05$) compared with Feed65. Feed90 cows had increased the expression ($P < 0.05$) of *IL1B* over the entire period, *IL6* early postpartum (wk 1), *TLR4* overall postpartum (wk 1 and 4), and *TNF* late postpartum (wk 4). An interaction between FO and CU was detected for *IL1B* and *TNF* (FO*CU, $P < 0.01$) (Table 6.3). Overfeeding SlowBCS or feed-restricting FastBCS cows led to lower ($P < 0.05$) expression of these genes. Furthermore, *TNF* expression also was significant for the three-way interaction (FO*CU*T, $P < 0.05$) (Supplemental Figure 1). In SlowBCS cows, overfeeding decreased *TNF* expression at wk 4 postpartum; however, in FastBCS cows, feeding 90% of requirements increased expression at wk 1 postpartum (Supplemental Figure 1).

Adipogenesis and lipid metabolism. Far-off management did not affect the expression of any of the genes in this category (FO, $P > 0.05$); however, close-up feeding affected the expression of *FASN* (CU, CU*T, $P < 0.05$), *PPARG* (CU, CU*T, $P < 0.05$), and *ADIPOQ* (CU*T, $P = 0.05$), as feeding cows 90% of ME requirements during this period increased ($P < 0.05$) the expression of these genes prepartum (-1 wk) relative to feeding 60 or 120% of requirements (Table 6.2 and 6.3). A carry over-effect was also detected for *PPARG* and *ADIPOQ*, as their expression was still up-regulated 1 wk postpartum in Feed90 cows. Furthermore, parturition had a strong effect (T, $P < 0.01$), causing a significant down-regulation ($P < 0.05$) postpartum of all four genes (Table 6.3).

An interaction between FO and CU was detected for *FASN* and *RXRA* (FO*CU, $P < 0.01$). For both genes, the overall expression decreased ($P < 0.05$) when SlowBCS cows were overfeed (i.e. Feed120), while the same response ($P < 0.05$) was also detected for *FASN* when the FastBCS group were severely restricted (i.e. Feed65).

MicroRNA expression

Inflammation and lipolysis-related. Overfeeding cows during the far-off period (FastBCS) led to greater ($P < 0.05$) expression of miR-99a, mainly prepartum (FO*T, $P < 0.05$), while it decreased ($P < 0.05$) expression of miR-145 over the entire transition period (FO, $P < 0.05$) (Table 6.4 and 6.5). miR-221 expression was affected by time (T, $P < 0.05$) and close-up feeding (CU, $P < 0.05$), with greater ($P < 0.05$) overall expression postpartum and a greater ($P < 0.05$) expression in cows overfed close to parturition (Feed120).

All three miRNAs (miR-99a, miR-145, miR-221) had a significant two-way interaction between far-off management and close-up feeding (FO*CU, $P < 0.05$), with greater expression in either SlowBCS-Feed120 or FastBCS-Feed65 cows (Table 6.4).

Adipose infiltration of Immune Cells. Time had an opposite effect on expression of miR-155 and miR193b (T, $P < 0.05$), with an increase in expression ($P < 0.05$) postpartum for the former, and a decrease ($P < 0.05$) in expression after parturition with the latter (Table 6.5).

Far-off management had an overall effect on miR-132 (FO, $P < 0.05$) and a prepartum effect on miR-126 and miR-155 (FO*T, $P < 0.05$) (Table 6.4 and 6.5). Similar to prepartal expression of miR-155, the expression of miR-132 was greater ($P < 0.05$) in SlowBCS cows, while, in the same group, expression was lower ($P < 0.05$) prepartum for miR-126.

Close-up feeding had a significant effect on miRNA involved in immune cell infiltration (CU $P < 0.05$; miR-26b, miR-132, and miR-155; CU*T, $P < 0.05$; miR-126 and miR-193b). Feed120

cows had the greatest overall ($P < 0.05$) expression of miR-132 and miR155, with the lowest ($P < 0.05$) expression of miR-26b. miR-126 and miR-193b were only affected postpartum, with Feed90 cows having the greatest ($P < 0.05$) expression of miR-126 (wk 1) and the lowest ($P < 0.05$) expression of miR-193b (wk 1 and 4).

An interaction between far-off management and close-up feeding was detected for miR-126 and miR-155 (FO*CU, $P < 0.05$). In both cases, the greatest ($P < 0.05$) expression was detected in overfed (Feed120) SlowBCS and feed restricted (Feed65) FastBCS cows.

Proadipogenic miRNA. Far-off management affected expression of miR-143 and miR-378, with greater ($P < 0.05$) prepartal expression in FastBCS compared with SlowBCS cows (FO*T, $P < 0.05$). The same miRNA were also affected by close-up feeding (CU*T, $P = 0.01$) (Table 6.5). Expression of miR-143 was greater ($P < 0.05$) in Feed65 and Feed90 prepartum and early postpartum (wk 1), respectively. No differences ($P > 0.05$) were detected at 4 wk postpartum. Expression of miR-378 was greater ($P < 0.05$) in Feed90 cows prepartum and in Feed120 late postpartum (4 wk). No differences ($P > 0.05$) were detected early postpartum (wk 1) for this miRNA. Expression of miR-103 also was affected by close-up feeding (CU, $P < 0.05$), with increased expression in Feed90 compared with the other groups (Table 6.4). This was mainly due to an interaction with far-off management, such that SlowBCS cows experienced no change when fed differently in the close-up period, while FastBCS cows had the highest expression of miR-103 when feed-restricted (Feed65), and lowest in the Feed90 group (FO*CU, $P < 0.05$).

Blood metabolites

Fatty acids were the only metabolite affected by far-off management (FO, $P < 0.05$), with greater concentrations in SlowBCS cows (Table 6.6). Close-up feeding level also affected their

concentration (CU, CU*T, $P < 0.05$), mainly due to prepartum concentrations being inversely correlated with feeding level (Feed65 > Feed90 >Feed120). Similarly, BHB and cholesterol concentrations were greater (CU*T, $P < 0.05$) prepartum in underfed than overfed cows (CU*T, $P < 0.05$). However, for cholesterol, its concentrations changed at wk 4 postpartum, with higher ($P < 0.05$) concentrations in Feed90 compared with other feeding groups.

Time affected blood concentrations of fatty acids, BHB, and cholesterol (T, $P < 0.05$) (Table 6.7). Fatty acid and BHB concentrations were greatest ($P < 0.05$) early postpartum (wk 1). Compared with prepartum concentrations, postpartum concentrations of cholesterol decreased ($P < 0.05$).

DISCUSSION

The combination of mRNA and miRNA profiling has been used previously to understand the molecular "self-regulatory" mechanism within the adipose depot during the transition period in dairy cows in the context of the relationship between dry period BCS and close-up feeding (Vailati Riboni et al., 2016). Our present work demonstrates that part of the variation caused by BCS could be attributed to the nutritional strategies utilized to allow cows to reach optimal adiposity at calving. Furthermore, the level of nutrition from close-up to calving could interact with far-off management.

In our previous experiment (Vailati Riboni et al., 2016) we speculated that the infiltration of immune cells in the cow adipose tissue around parturition is part of the regulatory mechanisms in adipose tissue. Despite differences in cellularity, adipokine production and gene expression (e.g., abundance), and cell systems between omental and subcutaneous adipose tissue (Dodson et al., 2014), the latter was used in the present study to allow for multiple sampling

across time on the same animal, which is central for the mechanistic understanding during the transition period.

Recently Akter et al. (2012) concluded that the extent of fatness in early lactating dairy cows may not be high enough to stimulate significant infiltration of phagocytic cells and, therefore, these immune cells may have no major role in the immunologic and metabolic adaptations during early lactation. This was supported by the analysis of chemoattractant molecule *CCL2* mRNA and protein distribution in the adipose tissue of the same animals (Haussler et al., 2015). However both studies, based on the experiment of von Soosten et al. (2011), used Holstein heifers rather than multiparous mature cows as a model. As heifers are still growing and developing during their first lactation, adipose mobilization is generally less prominent than mature cows (e.g., lower fatty acids and BHB). The authors justified the choice of heifers as a way to avoid the influence of previous lactations on adaptations within the adipose tissue. However, the first lactation might be of substantial importance to develop the adaptive mechanisms that will help the animal support the greater production performance of the subsequent lactations.

When taking into consideration the work of Contreras et al. (2015) using multiparous cows with a displaced abomasum in early lactation compared to non-lactating healthy individuals, and flow cytometry rather than only immunostaining, a degree of immune infiltration was detected not only in both subcutaneous and omental fat, but also in healthy cows with no difference in cells markers (CD14, CD172a, CD11c, CD163, CD3) between depots. However, interpreting the immunostaining results, the authors described the macrophages count in subcutaneous adipose tissue of healthy cows as “sparse and randomly localized” in relation to the adipocytes number. Since those were multiparous non-lactating and non-gestating dairy cows

in an anabolic state, the possibilities that healthy cows might experience a "physiologically functional" degree of immune cell infiltration during the catabolic peripartal period is not to exclude.

We recognize the importance of direct measurements of adipose tissue immune cell infiltration (i.e., flow cytometry and/or immunohistochemistry), but despite their lack in the present study, miRNA and mRNA results support its existence. Despite the fact that data on miRNA function and correlations with immunity and inflammation come mainly from human models, they are highly conserved among species. For instance, the high similarity between miRNA sequences between *B. taurus* and *H. sapiens* obtained using blastn (NCBI, Bethesda, USA) indicate a similar function in bovine (Supplemental Table D.8). Furthermore, the degree of similarity between CCL2 mRNA and protein between bovine and human is high (blastn and blastp, Supplemental Table D.9). This is valid also for MMP12 (which activates CCL2), and the receptors CCR2 and CCR4. Thus, because CCL2 was detectable in the present study, we speculate a similarity in function in bovine compared with human. Overall, the blastn and the blastp results (Supplemental Table D.8 and D.9) support the existence of similar function in human and bovine of the many players involved in the immune cell infiltration of adipose tissue.

Far-off, non-lactating period nutrition

Far-off nutrition in TMR-based herds is normally designed to meet basic nutrition requirements (e.g., maintenance and gestation), while avoiding excessive storage of reserves that could impair the animal adaptation to the next lactation. However, in grazing systems, cows are generally dried-off at a BCS too low to ensure a proper transition into lactation; thus, leading to the need for fattening cows prior to parturition (Roche et al., 2007). Judging by the up-regulation of pro-adipogenic miRNA, also observed in previous research [miR-143, miR-378 (Jin et al.,

2009, Jin et al., 2010)], it can be surmised that overfeeding cows in the far-off period is a suitable management practice to meet adiposity requirements at calving, with FastBCS cows still exhibiting lipogenic traits at a wk prepartum (i.e., approximately 2 wk after they were switched to different close-up feeding management). Despite this, the lack of change in expression of their common target gene, *PPARG*, was suggestive that the pro-adipogenic effect of these miRNA might have been achieved through the regulation of other target genes (i.e. MPAK1, ERK5).

Although overfeeding thinner cows after dry-off (i.e., FastBCS) did not have long-term effects on expression of adipogenic genes, it seemed to prime the adipose tissue to retain rather than release fatty acid reserves in early lactation. This was surmised by the greater overall concentration of circulating fatty acids in SlowBCS cows compared with FastBCS cows (Roche et al., 2017, and Table 6.6). This hypothesis is also supported by the expression of miR-99a and miR-145 in FastBCS cow. In humans, miR-99a is negatively correlated with the concentrations of free FA within adipocytes (Kloting et al., 2009) and miR-145 regulates adipocyte lipolysis through different mechanisms (Lorente-Cebrian et al., 2014). Despite the lower BCS in FastBCS cows at 1 wk postpartum, the lower overall expression of miR-99a and the higher expression of miR-145 in SlowBCS cows support their greater lipolysis than in FastBCS cows.

It is possible that the greater degree of mobilization in SlowBCS cows was partly regulated by infiltration of immune cells within the adipose tissue. Despite both chemokines (*CCL2* and *CCL5*) not being affected by far-off management, signs of infiltration could be discerned in SlowBCS cows because of the greater expression of both miR-132 and miR-155, which are markers of macrophage infiltration in humans (Kloting et al., 2009) (i.e., an overall effect for the first and prepartum for the latter). In addition, the expression prepartum of miR-

126, a *CCL2* inhibitor that leads to reduced infiltration of immune cells (Arner et al., 2012), was lower in the same cows.

In the present study, the expression of both TLRs (*TLR4* and *TLR9*) was studied as a way to connect metabolism and inflammatory signals. Signaling through TLR4 in non-ruminants can induce insulin resistance and lipolysis in adipocytes (Shi et al., 2006, Song et al., 2006). Thus, the dual activation of *TLR4* in adipocytes by lipopolysaccharide and fatty acids represents a molecular gate that connects innate immunity with metabolism (Schaffler and Scholmerich, 2010). In the same fashion, TLR9, originally identified in non-ruminants as a sensor of exogenous DNA fragments (Scharfe-Nugent et al., 2012), can also be activated by fatty acids, leading to chronic adipose tissue inflammation and insulin resistance (Pallares et al., 2010, Nishimoto et al., 2016).

Because cows in the present experiment were clinically healthy and free from pathogen-related inflammatory events (e.g., cows passed a veterinary clinical examination), activation of *TLR4* and *TLR9* in SlowBCS cows likely was mediated by fatty acids; hence, they transmitted lipolytic signals to the tissue. The greater activation of both TLR could be surmised not only by their greater expression, but also by the greater expression of *IL6* in the same cows. In fact, TLR4 activation is known to induce *IL6* expression, both in adipocytes and macrophages (Shi et al., 2006). In non-ruminants, IL-6 is known to have lipolytic effects (Yang et al., 2008) at least, in part, due to its ability to enhance insulin resistance (Shoelson et al., 2007). Since miR-99a was negatively correlated with secretion of IL-6 (Kloting et al., 2009) in non-ruminants, we speculate that the greater overall expression of *IL6* in SlowBCS cows might partly be attributed to infiltrating macrophages rather than adipocytes themselves. This scenario has been demonstrated in models of human obesity, as release of interleukins and other inflammatory cytokines from

human adipose depots is enhanced in obesity, primarily due to the nonfat cells (Fain, 2006). However, further research is needed to localize the origin (e.g. immune cells, or nonfat cells) of *IL6* expression in the tissue.

At least in cows that reach the end of lactation at a greater BCS, and were not overfed during the far-off period, these relationships underscored the importance of immunological control of lipolysis. We further speculate the existence of a "positive-feedback" loop between FA and immune cell infiltration. Whether the observed effects were due to nutrition management only or BCS at drying-off requires further research.

Close-up feeding

A general outcome when overfeeding cows during the close-up period is a decrease in adipose mobilization and greater storage of surplus nutrients (Ji et al., 2012). The marked decrease in the overall concentration of circulating FA along the entire transition period, and BHB and cholesterol in the prepartum period, in cows fed 120% of ME requirements (Feed120) all indicate a decrease in lipid mobilization. This systemic effect was mirrored peripherally by the down-regulation of lipolytic and insulin resistance signaling genes (e.g., lower *IL1B*, *IL6R*, *TLR9*) (Lagathu et al., 2006, Yang et al., 2008, Pallares et al., 2010). Paradoxically, however, the down-regulation of *FASN* and the pro-adipogenic miR-378 and miR-143 (Jin et al., 2009, Jin et al., 2010), as well as *ADIPOQ*, in Feed120 cows were indicative of a reduction in the differentiation and proliferation of adipocytes in the overfeeding treatment.

The response in *ADIPOQ* expression (e.g., lower in Feed120) was particularly interesting because, in non-ruminants, the increase in its expression improves insulin sensitivity and exerts some regulation over fatty acid metabolism (Brochu-Gaudreau et al., 2010). Expression of *ADIPOQ* also is markedly increased during ruminant adipocyte differentiation (Roh et al., 2006).

Thus, lower expression of *ADIPOQ* is a marker of reduced pre-adipocyte differentiation (Soliman et al., 2007). In vivo data also revealed a tendency for a reduction of circulating ADIPOQ during overconditioning (similar to our mRNA data) (Locher et al., 2015), but the cows, opposite to the present study, also gained weight and BCS over time. In light of these seemingly-paradoxical responses, questions arise on the adipose's utilization of the excess energy. For example, because adipose did not seem to accrete additional triglycerides and BCS did not change in the wk before parturition, (Roche et al., 2017), the additional intake could have been entirely partitioned towards meeting the requirements for gestation. In fact, cows did not experience an increase in adiposity, but their BW increased before parturition (Roche et al., 2017).

Despite the apparent absence of new fat deposition, the adipose tissue of overfed cows seemed to respond similar to what is observed in fat depots from obese individuals in the sense that infiltration of immune cells appeared to be stimulated by the excess feeding. The overall upregulation of the chemokine gene, *CCL2,* coupled with upregulation of miR-132 and miR-155 led us to speculate an increase in infiltration of immune cells (Kloting et al., 2009). Such a response normally increases insulin resistance and lipid mobilization to avoid excess storage in adipocytes and associated detrimental effects (Olefsky and Glass, 2010).

As lipolytic and insulin resistance signals are suppressed (*IL1B*, *IL6R*, *TLR9*) in Feed120 cows, the tentative onset of an inflammatory cascade could be a response to the need for mobilization to meet lactation requirements postpartum; however, it could also be related to the need to establish new reserves. This idea is supported by recent data demonstrating that adipocyte inflammation is an important component for a healthy expansion and remodeling of the adipose tissue (Asterholm et al., 2014). In either scenario, the apparent immune-related

tendency to kick start an inflammatory response to modulate metabolism could be a reaction to what has been previously described as a "lazy" phenotype in cows overfed prepartum (Vailati Riboni et al., 2016). This concept shares strong similarities with the well-known calcium metabolism and nutrition of the dry cow, as overfeeding calcium before parturition will increase the risk of metabolic failures (e.g. milk fever) by dampening the physiological mechanisms behind calcium homeostasis (Horst et al., 1997).

Contrary to overfeeding, a strong feed restriction (Feed65) elicited a clear outcome. The phenotypic data from this study indicated that such severe restriction during the pre-calving period increases the risk of disease in early lactation and reduces milk production (Roche et al., 2017). These cows experienced an excessive degree of mobilization of tissue reserves prepartum (Table 6.6), together with a reduction in BCS, but without a loss of BW (Roche et al., 2017). The presented transcriptome data point to a negative-feedback salvage mechanism (i.e. due to the excess mobilization prepartum, cows experience an even greater loss of reserves in the postpartum). Despite the contention that evolutionary programming of animal physiology is pointed towards the offspring rather than the mother (Bauman and Currie, 1980), the downregulation of a possible immune infiltration signal (*CCL2*) and lipolytic signal (*IL6*) postpartum, and the upregulation of the pro-adipogenic miR-143 (at least prepartum) in Feed65 cows could represent an attempt by the fat depots of the cow to control and maintain its reserves, as under extreme circumstances (e.g. malnourishment) the physiological priority can come back to the mother (Bauman and Currie, 1980).

Considering the entire set of mRNA and miRNA analyzed, and contrary to expectations, a slight restriction (Feed90) during the transition period was the main driver of changes in expression of most target genes. These changes, however, did not create an extreme phenotype,

and rather seemed to be part of the natural physiological adaptation to lactation. The overall up-regulation of immune lipolytic signaling (e.g., *IL1B*, *TLR4*, *TLR9*), combined with the lowest expression of miR-155 and the absence of a clear change in chemokine expression (both *CCL2* and *CCL5*) underscored that the fat depots of these cows did not rely on the action of immune cells to regulate and induce changes in metabolism. Furthermore, the marked upregulation prepartum in Feed90 of pro-adipogenic gene (e.g. *PPARG*, *FASN*, and *ADIPOQ*) indicated an equilibrating mechanism to balance lipolytic and anti-lipolytic signals in order to prime the mobilization machinery in light of the imminent parturition. Once this balancing mechanism was complete (*PPARG*, *FASN*, and *ADIPOQ* expression decreased after parturition), the lipolytic signaling and insulin resistance mechanisms are already established and can fully act on adipocyte metabolism (i.e. *IL6*, *IL6R*, *TLR4*, *TLR9*, and *TNF* all had a higher expression postpartum in Feed90 cows).

Together, these changes led to a numerically greater, but non-significant, level of circulating FA in early lactation (Table 6.6). As indicated by the higher expression postpartum of miR-126, a direct inhibitor of chemokine CCL2 (Arner et al., 2012), these changes did not seem to encompass an infiltration of immune cells. We speculate that the involvement of the innate immune system in regulating adipocyte metabolism may only occur in extreme nutritional situations (e.g. Feed65, Feed120), similar to the obesity scenario in humans, or in clinical scenarios [e.g. displaced abomasum, Contreras et al. (2015)], in which most of the relationships among immunity and metabolism have already been well-studied.

Possible interaction of far-off and close-up nutritional strategies

No interactions among far-off management and close-up feeding level were detected for production and health outcomes as presented in the main manuscript concerning this experiment

(Roche et al., 2017). However, at a molecular level (adipocyte transcriptome), distinct and similar changes were caused by their interaction in SlowBCS-Feed120 and FastBCS-Feed65 cows. Both groups experienced a state of low lipolytic signaling and higher insulin sensitivity (low *IL1B*, *TNF*) during the entire transition period. Such a physiological state would have been supported by the higher expression of miR-99a, which in non-ruminants is inversely correlated with IL-6 secretion and FA acid concentration (Kloting et al., 2009), miR-221, which is inversely correlated with TNF-α secretion (Chou et al., 2013), and miR-126, which is a *CCL2* inhibitor (Arner et al., 2012).

Although adipose tissue of SlowBCS-Feed120 and FastBCS-Feed65 cows appears not to have been primed to mobilize its reserves, at least from the genes studied, it also did not seem to signal an increase in TAG storage because the expression of *FASN* and *RXRA* was markedly lower compared with the other experimental groups. To further complicate this scenario of an apparent metabolic "stasis", contradictory responses were detected. Both miR-155 and miR-145 were up-regulated in SlowBCS-Feed120 and FastBCS-Feed65, suggesting greater immune cell infiltration (Kloting et al., 2009) and lipolytic activity (Lorente-Cebrian et al., 2014), while miR-103 was also up-regulated, possibly increasing insulin sensitivity (Trajkovski et al., 2011) and stimulating pro-adipogenic signaling (Romao et al., 2011, Romao et al., 2014). Thus, the metabolic effect of the interactions detected remains unclear and are further complicated by the lack of interaction at the phenotype level. Further research is needed to better characterize the physiology of the relationships among far-off and close-up nutrition.

CONCLUSIONS

The current results support the hypothesis that bovine adipose tissue possesses a homeorhetic mechanism for the adaptation to lactation, driven, in part, by inflammatory changes and, we speculate, in a cross-talk with the innate immune system. This mechanism appears to be modulated by peripartal nutrition. Overfeeding animals in the far-off period to achieve optimal calving BCS (FastBCS) reduced the propensity to mobilize adipose depots after parturition; however, animals that were managed in late lactation to dried-off at optimal calving BCS (SlowBCS) seemed more primed to lose BCS in early lactation. Concerning close-up nutrition, what seems to be a natural progression of self-driven inflammatory events in slightly underfed cows (Feed90) can be modulated both by underfeeding (Feed65) or overfeeding (Feed120), which we speculate caused the recruitment of the innate immune system to help modulate adipocyte metabolism. In light of the present results, to obtain a favorable transition to lactation, at least in grazing systems, dairy cows should be managed to achieve an optimal calving BCS at close-up, either by overfeeding thinner cows or control-feeding those already dried-off at target BCS, as neither strategy (FastBCS, SlowBCS), despite their different outcomes, interfere in the physiological adaptation. Subsequently, in the close-up period, BCS should be managed by applying a slight feed restriction closer to calving.

ACKNOWLEDGEMENTS

The authors thank Bruce Sugar (Farm Manager, Lye Farm, DairyNZ) and his capable team, as well as Stu Morgan, Kate Watkins, Ben Fisher, Kelly Collier, and Chris Roach (DairyNZ) for technical support, and Barbara Kuhn-Sherlock (DairyNZ) for statistical analysis and advice. This research was supported by New Zealand dairy farmers through DairyNZ Inc.

(RD1403) and the New Zealand Ministry of Business, Innovation, and Employment (DRCX1201).

TABLES

Table 6.1. Details and functions of the miRNA targets analyzed in the current study, adapted from Vailati Riboni et al. (2016), Moisa et al. (2016), and Arner and Kulyte (2015)

miRNA	Function and/or expression pattern	Model System[1]	Reference
Infiltration of immune cells			
miR-26b	Expression is associated with the number of macrophages infiltrating the fat depot Affected by levels of circulating TNF, leptin and resistin	Hu	(Kloting et al., 2009, Xu et al., 2013)
miR-126	Directly inhibits *CCL2* expression	Hu	(Kloting et al., 2009, Arner et al., 2012)
miR-132	Expression levels are associated with the number of macrophages infiltrating fat depots Activates NF-κB signalling and the transcription of *IL8* and *CCL2* Lower expression is associated with increased secretion of IL-6	Hu	(Kloting et al., 2009, Strum et al., 2009, Estep et al., 2010)
miR-155	Expression levels are associated with the number of macrophages infiltrating fat depots	Hu	(Kloting et al., 2009)
miR-193	Indirectly inhibits *CCL2* expression through a network of transcription factors	Hu	(Arner et al., 2012)
Inflammation and lipolysis			
miR-99a	Negative correlation with secretion of IL-6 and level of free fatty acids	Hu	(Kloting et al., 2009)
miR-145	Affects secretion of TNFα, regulating lipolysis	Hu	(Lorente-Cebrian et al., 2014)
miR-221	Lower expression is associated with high levels of TNFα	Hu	(Chou et al., 2013)
Proadipogenic			
miR-103	Regulates expression of *PPARG*, *PANK1*, *CAV1*, *FASN*, *ADIPOQ* and *FABP4*	Bo, Ma, Mo	(Romao et al., 2011, Trajkovski et al., 2011, John et al., 2012, Romao et al., 2014)
miR-143	Regulates expression of *ERK5*, *SLC2A4*, *TFAP2A*, *LIPE*, *PPARG*, *CEBPA*, and *FABP4*	Bo, Hu, Ma, Mo	(Esau et al., 2004, Kajimoto et al., 2006, Xie et al., 2009, Jin et al., 2010, Li et al., 2011, Romao et al., 2011)
miR-378	Targets *PPARG* expression through the MAPK1 pathway	B, Mo	(Gerin et al., 2010, Jin et al., 2010, John et al., 2012, Sacco and Adeli, 2012, Liu et al., 2015)

[1]Bo = Bovine (*Bos taurus*); Hu = Human; Ma = Mammalian; Mo = Mouse

Table 6.2. Effect of far-off and close-up feeding management on subcutaneous adipose tissue expression (back-transformed LSM and SEM) in grazing dairy cows during the transition period.

						FO*CU									
	FO[1]		CU[2]			SlowBCS			FastBCS				*p*-value		
Target	Slow BCS	Fast BCS	Feed 65	Feed 90	Feed 120	Feed 65	Feed 90	Feed 120	Feed 65	Feed 90	Feed 120	SEM[3]	FO	CU	FO*CU
Infiltration of immune cells															
CCL2	0.80	0.64	0.58[a]	0.64[a]	0.99[b]	0.76	0.56	1.18	0.44	0.73	0.82	0.24	0.18	0.01	0.09
CCL5	0.89	0.86	1.00	0.87	0.78	1.03	1.05	0.66	0.97	0.72	0.93	0.22	0.86	0.52	0.22
Inflammation and lipolysis															
HP	0.11	0.11	0.15	0.07	0.12	0.23	0.06	0.08	0.10	0.07	0.19	0.09	0.86	0.09	0.10
IL1B	0.75	0.64	0.67[a]	0.99[b]	0.50[c]	1.09[ac]	1.20[a]	0.32[b]	0.41[b]	0.82[ac]	0.77[c]	0.18	0.21	< 0.01	< 0.01
IL6	0.46[a]	0.26[b]	0.25	0.40	0.42	0.47	0.38	0.55	0.13	0.41	0.32	0.16	0.01	0.12	0.06
IL6R	1.02	0.91	1[a]	1.09[a]	0.82[b]	1.13	1.23	0.78	0.89	0.97	0.87	0.12	0.15	0.02	0.13
SAA3	0.26	0.24	0.35	0.18	0.25	0.27	0.18	0.37	0.45	0.18	0.17	0.13	0.66	0.07	0.07
TLR4	1.03[a]	0.77[b]	0.74[a]	1.21[b]	0.79[a]	0.92	1.42	0.84	0.59	1.03	0.74	0.18	< 0.01	< 0.01	0.40
TLR9	0.68[a]	0.54[b]	0.63[a]	0.71[a]	0.50[b]	0.78	0.80	0.51	0.50	0.63	0.50	0.08	< 0.01	< 0.01	0.07
TNF	1.07	1.02	1.03	1.19	0.92	1.33[a]	1.21[a]	0.75[b]	0.80[b]	1.17[a]	1.12[a]	0.17	0.63	0.11	< 0.01
Adipogenesis and lipid metabolism															
ADIPOQ	0.75	0.63	0.57	0.87	0.66	0.59	1.02	0.70	0.54	0.74	0.63	0.20	0.28	0.09	0.82
FASN	0.13	0.14	0.09[a]	0.29[b]	0.09[a]	0.14[ac]	0.27[ac]	0.05[b]	0.05[b]	0.31[a]	0.16[c]	0.08	0.66	< 0.01	< 0.01
PPARG	0.99	0.83	0.75[a]	1.21[b]	0.83[a]	0.87	1.31	0.84	0.64	1.12	0.81	0.21	0.21	< 0.01	0.73
RXRA	0.96	1.01	1.02	1.02	0.92	1.05[a]	1.09[a]	0.77[b]	1.00[a]	0.95[a]	1.09[a]	0.09	0.37	0.28	< 0.01

[1] FO = Far-off feeding management. Cows that were dried off at a lower BCS than optimal (0.75-1.00 unit lower) were overfed to quickly reach optimal calving BCS at 5wk prepartum, hence FastBCS, While animals that were dried closer to optimal (<0.25 unit than optimal) were fed to maintenance to slowly reach calving BCS at 5wk prepartum, hence SlowBCS

[2] CU = close-up feeding management; Feed65, Feed90, and Feed120 were fed to reach 65, 90, and 120% of estimated ME requirements, respectively

[3] SEM = greatest standard error of the mean.

a,b,c = significant difference among groups ($P < 0.05$)

Table 6.3. Effect of far-off and close-up feeding management, and time on subcutaneous adipose tissue expression (back-transformed LSM and SEM) in grazing dairy cows during the transition period.

			FO*T[3]		CU*T[4]				*p*-values			
Target	**Wk[1]**	**T[2]**	**SlowBCS**	**FastBCS**	**Feed65**	**Feed90**	**Feed120**	**SEM[5]**	**T**	**FO*T**	**CU*T**	**FO*CU*T**
Infiltration of immune cells												
CCL2	-1	0.43^{x}	0.52	0.36	0.36^{a,x}	0.29^{a,x}	0.78^{b}	0.36	< 0.01	0.79	0.02	0.71
	1	0.77^{y}	0.81	0.74	0.40^{a,x}	0.91^{b,y}	1.27^{b}					
	4	1.10^{y}	1.20	1.00	1.34^{y}	1.01^{y}	0.97					
CCL5	-1	0.81	0.77	0.84	1.01	0.64	0.81	0.27	0.40	0.15	0.30	0.14
	1	1.00	1.23	0.82	1.20	0.94	0.90					
	4	0.84	0.75	0.94	0.82	1.09	0.66					
Inflammation and lipolysis												
HP	-1	0.07^{x}	0.07	0.07	0.08	0.02	0.18	0.17	0.01	0.87	0.08	0.55
	1	0.22^{y}	0.19	0.26	0.37	0.13	0.22					
	4	0.08^{x}	0.08	0.08	0.12	0.09	0.05					
IL1B	-1	0.69	0.80	0.59	0.59	0.73	0.75	0.33	0.22	0.71	0.07	0.45
	1	0.83	0.81	0.84	0.77	1.34	0.55					
	4	0.58	0.65	0.52	0.66	1.00	0.30					
IL6	-1	0.26^{x}	0.42	0.16	0.18^{x}	0.22^{x}	0.42	0.22	0.02	0.21	0.01	0.15
	1	0.30^{x}	0.33	0.28	0.12^{a,x}	0.62^{b,y}	0.36^{b}					
	4	0.53^{y}	0.71	0.40	0.66^{y}	0.46^{xy}	0.50					
IL6R	-1	0.84^{x}	0.96	0.74	1.03^{a}	0.73^{b,x}	0.80^{ab}	0.19	0.04	0.34	0.04	0.71
	1	1.10^{y}	1.19	1.03	1.05^{ab}	1.47^{a,y}	0.87^{b}					
	4	0.97^{xy}	0.95	0.99	0.93^{ab}	1.21^{a,y}	0.80^{b}					
SAA3	-1	0.23^{xy}	0.20	0.26	0.37	0.16	0.20	0.19	0.04	0.49	0.82	0.62
	1	0.37^{y}	0.40	0.33	0.57	0.24	0.36					
	4	0.18^{x}	0.22	0.15	0.19	0.15	0.22					
TLR4	-1	0.76	1.03^{a}	0.55^{b,x}	0.73	0.72^{x}	0.82	0.35	0.08	0.05	0.01	0.24
	1	0.96	0.96	0.96^{y}	0.64^{a}	1.89^{b,y}	0.74^{a}					
	4	0.96	1.10	0.85^{y}	0.85^{a}	1.30^{b,y}	0.80^{a}					
TLR9	-1	0.51^{x}	0.60	0.42	0.66^{a}	0.41^{b,x}	0.48^{b}	0.14	< 0.01	0.38	< 0.01	0.77
	1	0.73^{y}	0.79	0.67	0.61^{a}	1.20^{b,y}	0.54^{a}					
	4	0.61^{z}	0.66	0.56	0.62^{ab}	0.74^{a,z}	0.50^{b}					
TNF	-1	0.85^{x}	0.98	0.73	0.80	0.74^{x}	1.02^{x}	0.25	0.01	0.16	< 0.01	0.05
	1	1.28^{y}	1.33	1.23	1.23	1.38^{y}	1.22^{x}					
	4	1.05^{xy}	0.94	1.16	1.13^{a}	1.64^{b,y}	0.62^{b,y}					
Adipogenesis and lipid metabolism												
ADIPOQ	-1	1.02^{x}	1.07	0.98	0.95^{ab,x}	1.60^{a,x}	0.71^{b}	0.38	< 0.01	0.32	0.05	0.48
	1	0.46^{y}	0.58	0.36	0.29^{a,y}	0.70^{b,y}	0.47^{ab}					
	4	0.70^{z}	0.68	0.72	0.67^{x}	0.59^{y}	0.87					
FASN	-1	0.57^{x}	0.05	0.04	0.17^{a,x}	3.49^{b,x}	0.30^{a,x}	1.11	< 0.01	0.79	< 0.01	0.35
	1	0.05^{y}	0.52	0.62	0.03^{y}	0.08**y**	0.04^{y}					
	4	0.08^{z}	0.08	0.09	0.11^{x}	0.09**y**	0.06^{y}					
PPARG	-1	1.31^{x}	1.52	1.13	1.14^{a,x}	2.15^{b,x}	0.92^{a}	0.42	< 0.01	0.16	0.05	0.17
	1	0.62^{y}	0.75	0.51	0.39^{a,y}	0.91^{b,y}	0.65^{b}					
	4	0.92^{z}	0.84	1.01	0.92^{x}	0.90**y**	0.93					

Table 6.3 (cont)

RXRA	-1	1.40[x]	1.26	1.55	1.38	1.65	1.21	0.20	< 0.01	0.19	0.51	0.88
	1	0.84[y]	0.93	0.76	0.88	0.87	0.78					
	4	0.81[y]	0.75	0.88	0.88	0.75	0.81					

[1] Wk = week relative to parturition
[2] T = time
[3] FO = Far-off feeding management. Cows that were dried off at a lower BCS than optimal (0.75-1.00 unit lower) were overfed to quickly reach optimal calving BCS at 5wk prepartum, hence FastBCS, While animals that were dried closer to optimal (<0.25 unit than optimal) were fed to maintenance to slowly reach calving BCS at 5wk prepartum, hence SlowBCS
[4] CU = close-up feeding management; Feed65, Feed90, and Feed120 were fed to reach 65, 90, and 120% of estimated ME requirements, respectively
[5] SEM = greatest standard error of the mean.
a,b,c = significant difference among groups within the same week relative to parturition ($P < 0.05$)
x,y,z = significant difference among time points, within group ($P < 0.05$)

Table 6.4. Effect of far-off and close-up feeding management on subcutaneous adipose tissue microRNA expression (back-transformed LSM and SEM) in grazing dairy cows during the transition period.

						FO*CU									
	FO[1]		CU[2]			SlowBCS			FastBCS				*p*-value		
Target	Slow BCS	Fast BCS	Feed 65	Feed 90	Feed 120	Feed 65	Feed 90	Feed 120	Feed 65	Feed 90	Feed 120	SEM[3]	FO	CU	FO*CU
Infiltration of immune cells															
miR-26b	0.92	1.00	1.00^{a}	1.05^{a}	0.84^{b}	1.08	0.94	0.77	0.93	1.16	0.92	0.10	0.24	0.03	0.07
miR-126	0.81	0.92	0.85	0.92	0.82	0.67^{a}	0.87^{ab}	0.90^{ab}	1.09^{b}	0.97^{bc}	0.74^{ac}	0.14	0.22	0.70	0.04
miR-132	1.09^{a}	0.91^{b}	0.94^{a}	0.95^{a}	1.12^{b}	1.02	1.08	1.19	0.86	0.84	1.06	0.08	< 0.01	0.02	0.67
miR-155	1.18	1.05	1.11^{a}	0.88^{b}	1.42^{c}	1.02^{ab}	0.91^{bd}	1.76^{c}	1.20^{a}	0.84^{b}	1.14^{ad}	0.14	0.08	< 0.01	< 0.01
miR-193b	0.88	0.86	1.00	0.77	0.86	0.94	0.82	0.90	1.07	0.72	0.82	0.13	0.78	0.11	0.52
Inflammation and lipolysis															
miR-99a	0.78^{a}	0.94^{b}	0.87	0.84	0.85	0.67^{a}	0.75^{ab}	0.92^{bc}	1.14^{c}	0.94^{bc}	0.78^{ab}	0.13	0.04	0.94	0.01
miR-145	1.28^{a}	0.99^{b}	1.17	0.95	1.28	1.07^{ac}	1.09^{ac}	1.81^{b}	1.28^{c}	0.83^{a}	0.91^{a}	0.23	0.02	0.07	0.01
miR-221	0.94	0.92	0.93^{ab}	0.84^{a}	1.04^{b}	0.81^{a}	0.88^{ac}	1.17^{b}	1.06^{cb}	0.80^{a}	0.92^{ac}	0.09	0.72	0.03	0.01
Proadipogenic															
miR-103	0.99	0.95	1.05^{a}	0.89^{b}	0.98^{a}	1.00^{abc}	0.95^{ac}	1.02^{abc}	1.10^{b}	0.83^{d}	0.95^{c}	0.05	0.29	< 0.01	0.03
miR-143	0.93	0.89	0.91	1.07	0.79	0.85	1.04	0.92	0.97	1.09	0.68	0.15	0.72	0.09	0.23
miR-378	0.79	0.86	0.88	0.78	0.81	0.76	0.77	0.83	1.02	0.78	0.80	0.11	0.33	0.55	0.28

[1] FO = Far-off feeding management. Cows that were dried off at a lower BCS than optimal (0.75-1.00 unit lower) were overfed to quickly reach optimal calving BCS at 5wk prepartum, hence FastBCS, While animals that were dried closer to optimal (<0.25 unit than optimal) were fed to maintenance to slowly reach calving BCS at 5wk prepartum, hence SlowBCS

[2] CU = close-up feeding management; Feed65, Feed90, and Feed120 were fed to reach 65, 90, and 120% of estimated ME requirements, respectively

[3] SEM = greatest standard error of the mean.

a,b,c = significant difference among groups ($P < 0.05$)

Table 6.5. Effect of far-off and close-up feeding management, and time on subcutaneous adipose tissue microRNA expression (back-transformed LSM and SEM) in grazing dairy cows during the transition period.

			FO*T[3]		CU*T[4]				*p*-value			
Target	**Wk[1]**	**T[2]**	**SlowBCS**	**FastBCS**	**Feed65**	**Feed90**	**Feed120**	**SEM[5]**	**T**	**FO*T**	**CU*T**	**FO*CU*T**
Infiltration of immune cells												
miR-26b	**-1**	0.94	0.84	1.06	0.97	0.89	0.97	0.13	0.68	0.25	0.16	0.33
	1	1.00	0.98	1.03	1.12	1.16	0.78					
	4	0.93	0.96	0.91	0.93	1.11	0.79					
miR-126	**-1**	0.76	$0.57^{a,x}$	1.01^{b}	0.97	0.67^{x}	0.67	0.24	0.22	0.02	0.04	0.62
	1	0.96	0.97^{y}	0.96	0.78^{a}	$1.39^{b,y}$	0.82^{a}					
	4	0.88	0.96^{y}	0.81	0.82	0.82^{x}	1.01					
miR-132	**-1**	1.04	1.21	0.89	0.97	1.09	1.07	0.12	0.50	0.13	0.65	0.26
	1	0.94	1.05	0.85	0.90	0.86	1.10					
	4	1.02	1.02	1.01	0.95	0.93	1.21					
miR-155	**-1**	0.97^{x}	1.18^{a}	$0.79^{b,x}$	0.93	0.83	1.18	0.16	0.01	0.01	0.19	0.41
	1	1.14^{xy}	1.19	1.09^{y}	1.15	0.77	1.67					
	4	1.24^{y}	1.17	1.33^{y}	1.27	1.05	1.44					
miR-193b	**-1**	1.20^{x}	1.03	1.38	1.21	1.51^{x}	0.93	0.28	< 0.01	0.22	0.02	0.63
	1	0.82^{y}	0.96	0.70	1.11^{a}	$0.66^{b,y}$	0.75^{ab}					
	4	0.68^{y}	0.70	0.66	0.75^{ab}	$0.46^{a,y}$	0.91^{b}					
Inflammation and lipolysis												
miR-99a	**-1**	0.86	0.63^{a}	$1.17^{b,x}$	1.01	0.83	0.76	0.17	0.19	< 0.01	0.06	0.94
	1	0.95	0.87	1.04^{x}	0.93	1.12	0.82					
	4	0.76	0.85	0.69^{y}	0.71	0.64	0.98					
miR-145	**-1**	1.10	1.21	1.00	1.25	0.99	1.08	0.22	0.14	0.28	0.56	0.68
	1	1.29	1.64	1.01	1.40	1.01	1.51					
	4	1.01	1.07	0.95	0.92	0.86	1.30					
miR-221	**-1**	0.81^{x}	0.86	0.77	0.84	0.73	0.88	0.12	0.02	0.64	0.84	0.60
	1	0.98^{y}	0.95	1.00	0.96	0.83	1.16					
	4	1.02^{y}	1.02	1.01	0.98	0.97	1.10					
Proadipogenic												
miR-103	**-1**	0.88	0.83	0.94	0.94	0.78	0.94	0.09	0.07	0.14	0.51	0.14
	1	1.07	1.12	1.02	1.22	0.93	1.08					
	4	0.97	1.04	0.90	1.02	0.96	0.94					
miR-143	**-1**	0.80	$0.66^{a,x}$	0.97^{b}	1.12^{a}	$0.67^{b,x}$	0.69^{b}	0.32	0.08	0.04	0.01	0.83
	1	1.11	1.22^{y}	1.01	0.97^{a}	$1.77^{b,y}$	0.79^{a}					
	4	0.86	1.00^{y}	0.73	0.69	1.02^{x}	0.90					
miR-378	**-1**	1.10^{x}	0.86^{a}	$1.40^{b,x}$	$1.19^{ab,x}$	$1.36^{b,x}$	0.82^{a}	0.20	< 0.01	0.02	0.01	0.42
	1	0.72^{y}	0.76	0.68^{y}	0.78^{y}	0.69^{y}	0.71					
	4	0.70^{y}	0.74	0.66^{y}	$0.73^{ab,y}$	$0.50^{a,y}$	0.93^{b}					

[1] Wk = week relative to parturition

[2] T = time

[3] FO = Far-off feeding management. Cows that were dried off at a lower BCS than optimal (0.75-1.00 unit lower) were overfed to quickly reach optimal calving BCS at 5wk prepartum, hence FastBCS, While animals that were dried closer to optimal (<0.25 unit than optimal) were fed to maintenance to slowly reach calving BCS at 5wk prepartum, hence SlowBCS

[4] CU = close-up feeding management; Feed65, Feed90, and Feed120 were fed to reach 65, 90, and 120% of estimated ME requirements, respectively

[5] SEM = greatest standard error of the mean.

Table 6.5 (cont)

a,b,c = significant difference among groups within the same week relative to parturition ($P < 0.05$)
x,y,z = significant difference among time points, within group ($P < 0.05$)

Table 6.6. Effect of far-off and close-up feeding management on plasma concentrations of fatty acids (FA), β-hydroxybutyrate (BHB), and cholesterol in grazing dairy cows during the transition period.

	FO[1]		CU[2]			FO*CU						SEM[3]	*p*-value		
						SlowBCS			FastBCS						
	Slow BCS	Fast BCS	Feed 65	Feed 90	Feed 120	Feed 65	Feed 90	Feed 120	Feed 65	Feed 90	Feed 120		FO	CU	FO*CU
FA (mmol/L)	0.761[a]	0.671[b]	0.780[a]	0.747[a]	0.621[b]	0.840	0.771	0.673	0.720	0.723	0.568	0.054	0.04	0.01	0.77
BHB (mmol/L)	0.558	0.520	0.552	0.536	0.529	0.580	0.563	0.531	0.523	0.510	0.527	0.031	0.13	0.75	0.62
Cholesterol (mM)	2378	2285	2440	2377	2178	2467	2437	2230	2413	2316	2127	264	0.62	0.46	0.99

[1] FO = Far-off feeding management. Cows that were dried off at a lower BCS than optimal (0.75-1.00 unit lower) were overfed to quickly reach optimal calving BCS at 5wk prepartum, hence FastBCS, While animals that were dried closer to optimal (<0.25 unit than optimal) were fed to maintenance to slowly reach calving BCS at 5wk prepartum, hence SlowBCS

[2] CU = close-up feeding management; Feed65, Feed90, and Feed120 were fed to reach 65, 90, and 120% of estimated ME requirements, respectively

[3] SEM = greatest standard error of the mean.

a,b,c = significant difference among groups ($P < 0.05$)

Table 6.7. Effect of far-off and close-up feeding management, and time on plasma concentrations of fatty acids (FA), β-hydroxybutyrate (BHB), and cholesterol in grazing dairy cows during the transition period.

			FO*T[3]		CU*T[4]				*p*-value			
	Wk[1]	T[2]	SlowBCS	FastBCS	Feed65	Feed90	Feed120	SEM[5]	T	FO*T	CU*T	FO*CU*T
FA (mmol/L)	**-1**	0.653^{x}	0.705	0.602	0.893^{a,x}	0.690^{b,x}	0.377^{c,x}	0.064	< 0.01	0.89	< 0.01	0.55
	1	1.001^{y}	1.034	0.969	0.989^{x}	1.018^{y}	0.997^{y}					
	4	0.493^{z}	0.546	0.440	0.458^{y}	0.534^{z}	0.488^{x}					
BHB (mmol/L)	**-1**	0.455^{x}	0.494	0.416	0.557^{a,x}	0.459^{b,x}	0.350^{c,x}	0.036	< 0.01	0.23	< 0.01	0.41
	1	0.646^{y}	0.665	0.626	0.626^{x}	0.634^{y}	0.677^{y}					
	4	0.516^{z}	0.514	0.517	0.472^{y}	0.516^{x}	0.560^{z}					
Cholesterol (mM)	**-1**	2860^{x}	2992	2728	3286^{a,x}	2831^{ab,x}	2463^{b}	287	< 0.01	0.38	0.04	0.60
	1	2039^{y}	2172	1907	2351^{y}	1808^{y}	1959					
	4	2095^{y}	1969	2221	1682^{a,z}	2490^{b,xy}	2113^{ab}					

[1] Wk = week relative to parturition
[2] T = time
[3] FO = Far-off feeding management. Cows that were dried off at a lower BCS than optimal (0.75-1.00 unit lower) were overfed to quickly reach optimal calving BCS at 5wk prepartum, hence FastBCS, While animals that were dried closer to optimal (<0.25 unit than optimal) were fed to maintenance to slowly reach calving BCS at 5wk prepartum, hence SlowBCS
[4] CU = close-up feeding management; Feed65, Feed90, and Feed120 were fed to reach 65, 90, and 120% of estimated ME requirements, respectively
[5] SEM = greatest standard error of the mean.
a,b,c = significant difference among groups within the same week relative to parturition ($P < 0.05$)
x,y,z = significant difference among time points, within group ($P < 0.05$)

REFERENCES

Adamczak, M. and A. Wiecek. 2013. The adipose tissue as an endocrine organ. Seminars in nephrology 33(1):2-13.

Akbar, H., T. M. Grala, M. V. 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. Journal of dairy science 98(2):1019-1032.

Akter, S. H., S. Haussler, D. Germeroth, D. von Soosten, S. Danicke, K. H. Sudekum, and H. Sauerwein. 2012. Immunohistochemical characterization of phagocytic immune cell infiltration into different adipose tissue depots of dairy cows during early lactation. Journal of dairy science 95(6):3032-3044.

Arner, E., N. Mejhert, A. Kulyte, P. J. Balwierz, M. Pachkov, M. Cormont, S. Lorente-Cebrian, A. Ehrlund, J. Laurencikiene, P. Heden, K. Dahlman-Wright, J. F. Tanti, Y. Hayashizaki, M. Ryden, I. Dahlman, E. van Nimwegen, C. O. Daub, and P. Arner. 2012. Adipose tissue microRNAs as regulators of CCL2 production in human obesity. Diabetes 61(8):1986-1993.

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

Asterholm, I. W., C. Tao, T. S. Morley, Q. A. Wang, F. Delgado-Lopez, Z. V. Wang, and P. E. Scherer. 2014. Adipocyte inflammation is essential for healthy adipose tissue expansion and remodeling. Cell metabolism 20(1):103-118.

Bauman, D. E. and W. B. Currie. 1980. Partitioning of nutrients during pregnancy and lactation: A review of mechanisms involving homeostasis and homeorhesis. Journal of dairy science 63(9):1514-1529.

Brochu-Gaudreau, K., C. Rehfeldt, R. Blouin, V. Bordignon, B. D. Murphy, and M. F. Palin. 2010. Adiponectin action from head to toe. Endocrine 37(1):11-32.

Chou, W. W., Y. C. Liao, S. C. Chuang, S. N. Wang, and S. H. Juo. 2013. Decreased microRNA-221 is associated with high levels of TNF-alpha in human adipose tissue-derived mesenchymal stem cells from obese woman. Cellular physiology and biochemistry 32(1):127-137.

Contreras, G. A., E. Kabara, J. Brester, L. Neuder, and M. Kiupel. 2015. Macrophage infiltration in the omental and subcutaneous adipose tissues of dairy cows with displaced abomasum. Journal of dairy science 98(9):6176-6187.

Dann, H. M., N. B. Litherland, J. P. Underwood, M. Bionaz, A. D'Angelo, J. W. McFadden, and J. K. Drackley. 2006. Diets during far-off and close-up dry periods affect periparturient metabolism and lactation in multiparous cows. Journal of dairy science 89(9):3563-3577.

Dodson, M. V., M. Du, S. Wang, W. G. Bergen, M. Fernyhough-Culver, U. Basu, S. P. Poulos, and G. J. Hausman. 2014. Adipose depots differ in cellularity, adipokines produced, gene expression, and cell systems. Adipocyte 3(4):236-241.

Esau, C., X. Kang, E. Peralta, E. Hanson, E. G. Marcusson, L. V. Ravichandran, Y. Sun, S. Koo, R. J. Perera, R. Jain, N. M. Dean, S. M. Freier, C. F. Bennett, B. Lollo, and R. Griffey. 2004. MicroRNA-143 regulates adipocyte differentiation. Journal of biological chemistry 279(50):52361-52365.

Estep, M., D. Armistead, N. Hossain, H. Elarainy, Z. Goodman, A. Baranova, V. Chandhoke, and Z. M. Younossi. 2010. Differential expression of miRNAs in the visceral adipose tissue of patients with non-alcoholic fatty liver disease. Alimentary pharmacology and therapeutics 32(3):487-497.

Fain, J. N. 2006. Release of interleukins and other inflammatory cytokines by human adipose tissue is enhanced in obesity and primarily due to the nonfat cells. Vitamins and hormones 74:443-477.

Farney, J. K., L. K. Mamedova, J. F. Coetzee, B. KuKanich, L. M. Sordillo, S. K. Stoakes, J. E. Minton, L. C. Hollis, and B. J. Bradford. 2013. Anti-inflammatory salicylate treatment alters the metabolic adaptations to lactation in dairy cattle. American journal of physiology. Regulatory, integrative and comparative physiology 305(2):R110-117.

Gerin, I., G. T. Bommer, C. S. McCoin, K. M. Sousa, V. Krishnan, and O. A. MacDougald. 2010. Roles for miRNA-378/378* in adipocyte gene expression and lipogenesis. American journal of physiology. Endocrinology and metabolism 299(2):E198-206.

Grala, T. M., J. R. Roche, C. V. Phyn, A. G. Rius, R. H. Boyle, R. G. Snell, and J. K. Kay. 2013. Expression of key lipid metabolism genes in adipose tissue is not altered by once-daily milking during a feed restriction of grazing dairy cows. Journal of dairy science 96(12):7753-7764.

Grant, R. W. and V. D. Dixit. 2015. Adipose tissue as an immunological organ. Obesity 23(3):512-518.

Graugnard, D. E., K. M. Moyes, E. Trevisi, M. J. Khan, D. Keisler, J. K. Drackley, G. Bertoni, and J. J. Loor. 2013. Liver lipid content and inflammometabolic indices in peripartal dairy cows are altered in response to prepartal energy intake and postpartal intramammary inflammatory challenge. Journal of dairy science 96(2):918-935.

Haussler, S., C. Sacre, K. Friedauer, S. Danicke, and H. Sauerwein. 2015. Short communication: localization and expression of monocyte chemoattractant protein-1 in different subcutaneous and visceral adipose tissues of early-lactating dairy cows. Journal of dairy science 98(9):6278-6283.

Holtenius, K., S. Agenas, C. Delavaud, and Y. Chilliard. 2003. Effects of feeding intensity during the dry period. 2. Metabolic and hormonal responses. Journal of dairy science 86(3):883-891.

Horst, R. L., J. P. Goff, T. A. Reinhardt, and D. R. Buxton. 1997. Strategies for preventing milk fever in dairy cattle. Journal of dairy science 80(7):1269-1280.

Janovick, N. A., Y. R. Boisclair, and J. K. Drackley. 2011. Prepartum dietary energy intake affects metabolism and health during the periparturient period in primiparous and multiparous Holstein cows. Journal of dairy science 94(3):1385-1400.

Ji, P., J. K. Drackley, M. J. Khan, and J. J. Loor. 2014. Overfeeding energy upregulates peroxisome proliferator-activated receptor (PPAR)gamma-controlled adipogenic and lipolytic gene networks but does not affect proinflammatory markers in visceral and subcutaneous adipose depots of Holstein cows. Journal of dairy science 97(6):3431-3440.

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 molecular biology 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 molecular biology 10:90.

John, E., A. Wienecke-Baldacchino, M. Liivrand, M. Heinaniemi, C. Carlberg, and L. Sinkkonen. 2012. Dataset integration identifies transcriptional regulation of microRNA genes by PPARgamma in differentiating mouse 3T3-L1 adipocytes. Nucleic acids research 40(10):4446-4460.

Kajimoto, K., H. Naraba, and N. Iwai. 2006. MicroRNA and 3T3-L1 pre-adipocyte differentiation. RNA 12(9):1626-1632.

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 regulation and systems biology 8:97-111.

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.

Lagathu, C., L. Yvan-Charvet, J. P. Bastard, M. Maachi, A. Quignard-Boulange, J. Capeau, and M. Caron. 2006. Long-term treatment with interleukin-1beta induces insulin resistance in murine and human adipocytes. Diabetologia 49(9):2162-2173.

Li, H., Z. Zhang, X. Zhou, Z. Wang, G. Wang, and Z. Han. 2011. Effects of microRNA-143 in the differentiation and proliferation of bovine intramuscular preadipocytes. Molecular biology reports 38(7):4273-4280.

Liu, S. Y., Y. Y. Zhang, Y. Gao, L. J. Zhang, H. Y. Chen, Q. Zhou, M. L. Chai, Q. Y. Li, H. Jiang, B. Yuan, L. S. Dai, and J. B. Zhang. 2015. MiR-378 plays an important role in the differentiation of bovine preadipocytes. Cellular physiology and biochemistry 36(4):1552-1562.

Locher, L., S. Haussler, L. Laubenthal, S. P. Singh, J. Winkler, A. Kinoshita, A. Kenez, J. Rehage, K. Huber, H. Sauerwein, and S. Danicke. 2015. Effect of increasing body condition on key regulators of fat metabolism in subcutaneous adipose tissue depot and circulation of nonlactating dairy cows. Journal of dairy science 98(2):1057-1068.

Lorente-Cebrian, S., N. Mejhert, A. Kulyte, J. Laurencikiene, G. Astrom, P. Heden, M. Ryden, and P. Arner. 2014. MicroRNAs regulate human adipocyte lipolysis: Effects of miR-145 are linked to TNF-alpha. Plos one 9(1):e86800.

McGown, C., A. Birerdinc, and Z. M. Younossi. 2014. Adipose tissue as an endocrine organ. Clinics in liver disease 18(1):41-58.

McNamara, J. P. 1991. Regulation of adipose tissue metabolism in support of lactation. Journal of dairy science 74(2):706-719.

McNamara, J. P. 1997. Adipose tissue metabolism during lactation: Where do we go from here? Proceedings of the Nutrition Society 56(1A):149-167.

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.

Musi, N. and R. Guardado-Mendoza. 2014. Adipose tissue as an endocrine organ. Cellular endocrinology in health and disease:229-237.

Nishimoto, S., D. Fukuda, Y. Higashikuni, K. Tanaka, Y. Hirata, C. Murata, J. R. Kim-Kaneyama, F. Sato, M. Bando, S. Yagi, T. Soeki, T. Hayashi, I. Imoto, H. Sakaue, M. Shimabukuro, and M. Sata. 2016. Obesity-induced DNA released from adipocytes stimulates chronic adipose tissue inflammation and insulin resistance. Science advances 2(3):e1501332.

Olefsky, J. M. and C. K. Glass. 2010. Macrophages, inflammation, and insulin resistance. Annual review of physiology 72:219-246.

Pallares, V., A. Castell-Auvi, L. Cedo, M. Pinent, A. Ardevol, and M. Blay. 2010. A paracrine loop between adipocytes and macrophages in adipose tissue: An in vitro model to study obesity-induced inflammation. Journal of nutrigenetics and nutrigenomics 3(2-3):96-96.

Pires, J. A. A., C. Delavaud, Y. Faulconnier, D. Pomies, and Y. Chilliard. 2013. Effects of body condition score at calving on indicators of fat and protein mobilization of periparturient Holstein-Friesian cows. Journal of dairy science 96(10):6423-6439.

Randall, L. V., M. J. Green, M. G. Chagunda, C. Mason, S. C. Archer, L. E. Green, and J. N. Huxley. 2015. Low body condition predisposes cattle to lameness: An 8-year study of one dairy herd. Journal of dairy science 98(6):3766-3777.

Roche, J. R., D. P. Berry, J. M. Lee, K. A. Macdonald, and R. C. Boston. 2007. Describing the body condition score change between successive calvings: A novel strategy generalizable to diverse cohorts. Journal of dairy science 90(9):4378-4396.

Roche, J. R., P. G. Dillon, C. R. Stockdale, L. H. Baumgard, and M. J. VanBaale. 2004. Relationships among international body condition scoring systems. Journal of dairy science 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. Journal of dairy science 92(12):5769-5801.

Roche, J. R., A. Heiser, M. D. Mitchell, M. A. Crookenden, C. G. Walker, J. K. Kay, M. Vailati Riboni, J. J. Loor, and S. Meier. 2016. Strategies to gain body condition score in pasture-based dairy cows during late lactation and the far-off nonlactating period and their interaction with close-up dry matter intake. Journal of dairy science 100(3):1720-1738.

Roche, J. R., E. S. Kolver, and J. K. Kay. 2005. Influence of precalving feed allowance on periparturient metabolic and hormonal responses and milk production in grazing dairy cows. Journal of dairy science 88(2):677-689.

Roh, S.-G., D. Hishikawa, Y.-H. Hong, and S. Sasaki. 2006. Control of adipogenesis in ruminants. Animal science 77(5):472-477.

Romao, J. M., W. Jin, M. V. Dodson, G. J. Hausman, S. S. Moore, and L. L. Guan. 2011. MicroRNA regulation in mammalian adipogenesis. Experimental biology and medicine 236(9):997-1004.

Romao, J. M., W. Jin, M. He, T. McAllister, and L. Guan le. 2014. MicroRNAs in bovine adipogenesis: Genomic context, expression and function. BMC genomics 15:137.

Rukkwamsuk, T., T. Wensing, and M. J. Geelen. 1999. Effect of overfeeding during the dry period on the rate of esterification in adipose tissue of dairy cows during the periparturient period. Journal of dairy science 82(6):1164-1169.

Sacco, J. and K. Adeli. 2012. MicroRNAs: Emerging roles in lipid and lipoprotein metabolism. Current opinion in lipidology 23(3):220-225.

Schaffler, A. and J. Scholmerich. 2010. Innate immunity and adipose tissue biology. Trends in immunology 31(6):228-235.

Scharfe-Nugent, A., S. C. Corr, S. B. Carpenter, L. Keogh, B. Doyle, C. Martin, K. A. Fitzgerald, S. Daly, J. J. O'Leary, and L. A. J. O'Neill. 2012. TLR9 provokes inflammation in response to fetal DNA: Mechanism for fetal loss in preterm birth and preeclampsia. Journal of immunology 188(11):5706-5712.

Shahzad, K., M. Bionaz, E. Trevisi, G. Bertoni, S. L. Rodriguez-Zas, and J. J. Loor. 2014. Integrative analyses of hepatic differentially expressed genes and blood biomarkers during the peripartal period between dairy cows overfed or restricted-fed energy prepartum. Plos one 9(6):e99757.

Shi, H., M. V. Kokoeva, K. Inouye, I. Tzameli, H. Yin, and J. S. Flier. 2006. TLR4 links innate immunity and fatty acid-induced insulin resistance. Journal of clinical investigation 116(11):3015-3025.

Shoelson, S. E., L. Herrero, and A. Naaz. 2007. Obesity, inflammation, and insulin resistance. Gastroenterology 132(6):2169-2180.

Soliman, M., K. Kimura, M. Ahmed, D. Yamaji, Y. Matsushita, Y. Okamatsu-Ogura, K. Makondo, and M. Saito. 2007. Inverse regulation of leptin mRNA expression by short- and long-chain fatty acids in cultured bovine adipocytes. Domestic animal endocrinology 33(4):400-409.

Song, M. J., K. H. Kim, J. M. Yoon, and J. B. Kim. 2006. Activation of Toll-like receptor 4 is associated with insulin resistance in adipocytes. Biochemical and biophysical research communications 346(3):739-745.

Strum, J. C., J. H. Johnson, J. Ward, H. B. Xie, J. Feild, A. Hester, A. Alford, and K. M. Waters. 2009. MicroRNA 132 regulates nutritional stress-induced chemokine production through repression of SirT1. Molecular endocrinology 23(11):1876-1884.

Trajkovski, M., J. Hausser, J. Soutschek, B. Bhat, A. Akin, M. Zavolan, M. H. Heim, and M. Stoffel. 2011. MicroRNAs 103 and 107 regulate insulin sensitivity. Nature 474(7353):649-653.

Vailati Riboni, M., M. Kanwal, O. Bulgari, 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. 2016. Body condition score and plane of nutrition prepartum affect adipose tissue transcriptome regulators of metabolism and inflammation in grazing dairy cows during the transition period. Journal of dairy science 99(1):758-770.

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. Journal of dairy science 98(5):3079-3085.

von Soosten, D., U. Meyer, E. M. Weber, J. Rehage, G. Flachowsky, and S. Danicke. 2011. Effect of trans-10, cis-12 conjugated linoleic acid on performance, adipose depot weights, and liver weight in early-lactation dairy cows. Journal of dairy science 94(6):2859-2870.

Waltner, S. S., J. P. McNamara, and J. K. Hillers. 1993. Relationships of body condition score to production variables in high producing Holstein dairy cattle. Journal of dairy science 76(11):3410-3419.

Xie, H. M., B. Lim, and H. F. Lodish. 2009. MicroRNAs induced during adipogenesis that accelerate fat cell development are downregulated in obesity. Diabetes 58(5):1050-1057.

Xu, G., C. Ji, C. Shi, H. Fu, L. Zhu, L. Zhu, L. Xu, L. Chen, Y. Feng, Y. Zhao, and X. Guo. 2013. Modulation of hsa-miR-26b levels following adipokine stimulation. Molecular biology reports 40(5):3577-3582.

Yang, Y., D. Ju, M. Zhang, and G. Yang. 2008. Interleukin-6 stimulates lipolysis in porcine adipocytes. Endocrine 33(3):261-269.

CHAPTER 7: HEPATIC ONE-CARBON METABOLISM AND TRANSSULFURATION PATHWAY ENZYME ACTIVITY AND INTERMEDIATE METABOLITES ARE ALTERED TO DIFFERENT EXTENTS BY PREPARTUM BODY CONDITION SCORE AND PLANE OF NUTRITION IN GRAZING DAIRY COWS

ABSTRACT

Alterations in activity and intermediates of the hepatic one-carbon metabolism pathways were evaluated in 28 pregnant and non-lactating grazing dairy cows of mixed age and breed. They were randomly allocated to 1 of 4 treatment groups in a 2 × 2 factorial design: 2 prepartum body condition score (BCS) categories [4.0 (thin) and 5.0 (optimal); 10-point scale], and 2 levels of energy intake during the 3 wk preceding calving (75% or 125% of estimated requirements). Liver tissue was collected by biopsy at −7, 7, and 28 d relative to calving. Tissue samples were used for ^{14}C radio-labeling assays to measure betaine-homocysteine S-methyltransferase (BHMT), 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR), and cystathionine-β-synthase (CBS) activity. Liver metabolomics was undertaken using a targeted GC/MS-based profiling approach. Enzyme activity and metabolite area under the curve (AUC) were normalized with the homogenate protein concentration, and subjected to repeated measures ANOVA via PROC MIXED in SAS, with BCS, Feeding, and Time as fixed effects, and cow as random effect. All enzymes were affected by time, with BHMT activity peaking at 7 d, while CBS and MTR activity decreased post-partum. Overall, thin cows had greater MTR activity, while cows fed 125% requirements had greater CBS activity. An interaction was detected between BCS and feeding for CBS activity, as thin cows fed 125% of requirements had greater overall activity. Compared with liver from BCS 4 cows, BCS 5 cows had overall greater betaine, glycine,

butyrobetaine/acetylcholine, serine, and taurine concentrations. The same metabolites, plus choline, and N-N-dimethyl glycine, were overall greater in liver of cows fed 75% compared with those fed 125% of requirements. An interaction of BCS and feeding level was detected for the aforementioned metabolites plus methionine, cystathionine, cysteinesulfinate, and hypotaurine, due to greater overall concentrations in BCS 5 cows feed 75% of requirements compared with other groups. Overall, the present data highlight a potentially greater capacity in grazing cows to regenerate methionine from folate compared with cows in confinement systems, based on published data. The lower MTR and CBS activity postpartum could be associated with the greater abundance of antioxidant precursors in pasture. However, the differences in MTR and CBS activity and intermediate metabolites suggest that both BCS and feeding level can alter the internal antioxidant system (e.g., glutathione and taurine) throughout the periparturient period. The data support the need for differential management prepartum based on adiposity: feed restricting optimally conditioned cows while allowing a higher overall nutrient intake for thin cows via stocking rate manipulation.

INTRODUCTION

The advantage of a pasture-based dairy system is centered around the low cost of pasture compared with more controlled total mixed ration (**TMR**) systems, based on conserved forages and concentrates. For their profitability, however, grazing systems depend on high levels of pasture production, and on the efficiency with which cows are able to harvest it (Kolver, 2003). This is achieved by managing stocking rate (e.g., number of cows per pasture area), calving times in seasonal systems, and conservation and supplementation of feeds (Holmes et al., 1987, Roche et al., 2017).

Regarding periparturient or “transition” dairy cows, the research focus has been on energy nutrition (Roche et al., 2017), as intake of energy in pasture systems is a major limiting factor for milk production (Kolver, 2003). On the other hand, protein supply in pasture-based diets, both in terms of quantity and quality, has always been considered adequate. More than 30 years ago, Beever and Siddons (1986) suggested that milk production in grazing systems might also be limited by duodenal protein supply; however, when tested, this hypothesis yielded mixed results. Researchers looking at New Zealand pasture-based systems suggested that MP supply was adequate in grazing dairy cows, as their milk production did not respond to supplementation with undegraded dietary protein (Brookes, 1984, Penno and Carruthers, 1995). Similar earlier studies by Rogers et al. (1980) and Minson (1981) reported enhanced milk yield under a similar type of protein supplementation scheme, suggesting that MP supply from ryegrass pastures might be insufficient to maximize milk production.

Regarding specific amino acid supply, lysine and methionine supply is a well-known limiting factor for milk production in high-producing confinement systems, while in pasture-based systems, their supplementation has not proven effective (Pacheco-Rios et al., 1997, Rusdi and van Houtert, 1997). These amino acid limitations will, in fact, only affect production if there are no other first-limiting nutrients. Since energy intake is the first-limiting nutrient in milk production in grazing systems, supplementation of high-quality pasture would first need to correct the deficiency in energy rather than amino acid supply (Roche, 2017). This can also explain the inconsistent results from rumen undegradable protein supplementation studies.

In our recent work (Vailati-Riboni et al., 2016) we identified hepatic transcriptomic changes related to the methionine cycle (e.g., folate biosynthesis, one-carbon pool by folate, and vitamin B6 metabolism) when underconditioned cows were allowed a higher pasture area

prepartum, thus, increasing their overall energy supply. The changes detected in these three pathways seemed to indicate a greater flux through the one-carbon metabolism and the methionine cycle, with a potential increase in the availability of methionine and antioxidants. Together with the increase in activation of 'taurine and hypotaurine metabolism' during early lactation in those animals, these results suggested a link between amino acid metabolism and energy intake, together with a beneficial alteration of methionine metabolism via prepartal energy intake manipulation.

To better understand the activity and flux through the one-carbon metabolism, methionine cycle, and transsulfuration pathway in response to prepartum feed allowance and its interaction with body condition score (**BCS**), we measured the activity of 3 key enzymes: **(i)** betaine homocysteine methyltransferase (**BHMT**), which regulates the use of choline in the re-methylation of homocysteine, thus, regenerating methionine; **(ii)** 5-methyltetrahydrofolate-homocysteine methyltransferase (**MTR**), responsible for the methylation of homocysteine to regenerate methionine via the folate pathway; and **(iii)** cystathionine β-synthase (**CBS**), which channels homocysteine out of the cycle to generate glutathionine or taurine as important antioxidants. Furthermore, we also measured abundance of genes and concentration of metabolites involved in these pathways.

MATERIAL AND METHODS

Animal Management

Complete details of the experimental design are published elsewhere (Roche et al., 2015). Briefly, a group of 150 mid-lactation grazing dairy cows of mixed age and breed were enrolled in the experiment on 21st January 2013. Animals were allocated randomly to one of six treatment

groups (25 cows per group) in a 2 × 3 factorial arrangement: two pre-calving BCS categories (4.0 and 5.0, **BCS4** and **BCS5**; based on a 10-point scale, where 1 is emaciated and 10 obese; Roche et al. 2004) and three levels of energy intake during the three wk preceding calving (75, 100, and 125% of estimated requirements; (Roche et al., 2004)(Roche et al., 2004)(Roche et al., 2004)(Roche et al., 2004)(Roche et al., 2004)(Roche et al., 2004). The different levels of energy intake were obtained by daily manipulation of pasture allowance, adjusting area allocation (m^2/cow) in each group. Cows were randomly assigned to the six groups, balanced for age, breed (Holstein and Holstein × Jersey), BCS at the time of enrolment, and expected calving date. For the current study, only four groups, with a subset of 24 animals (6 cows per group), were considered. These were cows with prepartum BCS4 fed to meet 75 (**B4F75**) or 125 (**B4F125**) % of requirements, and cows with prepartum BCS5 fed to meet 75 (**B5F75**) or 125 (**B5F125**) % of requirements. The subset groups were still balanced for breed, age, and calving date. Average age and days in gestation ± SD at enrolment was 6.20 ± 2.18 years, and 259.32 ± 1.86 d, respectively.

The intermediate groups in both BCS classes (B4F100 and B5F100) were omitted from the present analysis as feeding to the exact requirements is rarely achievable in field conditions. In practice, multiple factors (e.g., pasture management and allocation, animal competition, and social interaction) result in cows being mostly overfed or underfed.

Sample Collection

Liver tissue was collected via percutaneous biopsy under local anesthesia at -7, 7, and 28 d relative to parturition. Tissue samples were immediately snap frozen in liquid nitrogen and stored at -80°C until further analysis.

Enzyme Activity Assays

Enzyme activity assays were performed as previously described (Zhou et al., 2017). Briefly, BHMT activity was measured as described by Garrow (1996). MTR activity was determined on the basis of Banerjee et al. (1997). CBS activity was determined on the basis of Lambert et al. (2002), except that the separation of 14C-cystathionine from 14C-Ser was done according to Taoka et al. (1998). Enzyme assay details are reported in Supplemental Methods.

Metabolomics

About 100 mg of frozen tissue was extracted by the two-step protocol described by Wu et al. (2008). Targeted metabolomics (LC-MS) was performed to quantify metabolites related to the one carbon metabolism and the transsulfuration pathway (Table 7.1). Samples were analyzed with the 5500 QTRAP LC/MS/MS system (Sciex, Framingham, MA). Software Analyst 1.6.2 was used for data acquisition and analysis. The 1200 series HPLC system (Agilent Technologies, Santa Clara, CA) includes a degasser, an autosampler, and a binary pump. The LC separation was performed on a Phenomenex C18 (2) column (4.6 × 150mm, 5 μm. Torrance, CA) with mobile phase A (10 mM ammonia formate) and mobile phase B (methanol). The flow rate was 0.4 mL/min. The linear gradient was as follows: 0-1min, 95% A; 8min, 50% A; 15-18min, 0% A; 18.1-26min, 95% A. The autosampler was set at 10°C. The injection volume was 10 μL. Mass spectra were acquired under both positive (ion spray voltage was +5500 V) and negative (ion spray voltage was -4500 V) electrospray ionization (**ESI**). The source temperature was 500°C. The curtain gas, ion source gas 1, and ion source gas 2 were 35, 65, and 55, respectively. Multiple reaction monitoring (**MRM**) was used for quantitation. Before statistical analysis, the area under the curve (**AUC**) was normalized by the protein concentration of the samples.

RNA isolation, cDNA Synthesis, and Quantitative PCR

Total RNA was isolated from 100 mg of tissue using the miRNeasy kit (Qiagen) following the manufacturer's protocols. Samples were treated on-column with DNaseI (Qiagen); quantification was accessed using the NanoDrop ND-1000 (NanoDrop Technologies), and RNA quality was measured using an Agilent 2100 Bioanalyzer (Agilent). The qPCR was performed as described previously (Vailati Riboni et al., 2016). Before statistical analysis, normalized (against internal control genes) gene relative abundance was $\log_2$ transformed to ensure a normal distribution.

Statistical Analysis

Enzyme activity (U · mg protein^{-1}, or nmol product · h^{-1} · mg protein^{-1}), mRNA abundance ($\log_2$ relative abundance), and metabolite relative concentrations (AUC · mg protein^{-1}) were subjected to repeated measures ANOVA in SAS using the PROC MIXED procedure. BCS (B, 4 or 5), feeding (F, 75 or 125%), time (T, -1, +1, +4 wk from parturition), and their interactions (B*F, B*T, F*T, B*F*T) were used as fixed effects. Cow nested in group (B*F) was the random effect. The Kenward Roger statement was used to compute the degrees of freedom, and spatial power was used as covariance structure for the repeated measurement. Normality of the residuals was checked via PROC UNIVARIATE. Data for fumarate, NAD^+, NADH, NAD^+:NADH, adenosine, and cysteinesulfinate were $\log_2$ transformed to obtain normality. All other data were normally distributed. Metabolite data reported in tables and graphs were properly back-transformed to aid in their interpretation. mRNA abundance data were back-transformed only in tables, and kept as $\log_2$ relative mRNA abundance for figures. Statistical significance was determine at $P \leq 0.05$, while tendencies were considered at $P \leq 0.10$.

RESULTS

One-Carbon Metabolism

Enzyme activity. There was no statistical effect of BCS, feeding, or their interaction on BHMT activity ($P > 0.05$), which was, however, affected by time, with greater activity (1 vs -1 wk, $P = 0.02$; 4 vs -1, $P = 0.07$) postpartum compared with prepartum (Table 7.2, Figure 7.1). The significant interaction (F*T, $P = 0.05$) between feeding level and time indicated that cows fed to 75% of requirements had stable BHMT enzyme activity throughout the transition period, while cows overfed to 125% of requirements experienced an increase in activity postpartum. Furthermore, compared with overfed, restricted cows tended ($P = 0.07$) to have greater activity prepartum. MTR activity was influence by time ($P < 0.0001$), but with an opposite trend. Activity was, in fact, highest prepartum and then decreased over time (-1 vs 1 wk, $P = 0.06$; -1 vs 4 wk, $P < 0.0001$). Feeding strategy did not statistically alter its activity, but thin animals (BCS 4) had a lower ($P = 0.001$) activity of MTR compared with optimally conditioned cows (BCS 5).

mRNA abundance. Abundance of *MAT2A* and *MTR* was stable over time (T, $P > 0.05$; Table 7.3), while *BHMT1* followed the enzyme activity trend, with greater ($P < 0.05$) abundance postpartum compared with prepartum (Table 7.3, Figure 7.2). Abundance of *MAT2A* was greater in optimally conditioned cows (B, $P = 0.004$), while no statistical effects of BCS ($P > 0.05$) were identified for *BHMT1* and *MTR*. Overfeeding animals prepartum induced an overall lower abundance of both *MAT2A* and *MTR* (F, $P < 0.05$), but not *BHMT1* ($P > 0.05$). No statistical interaction of BCS and feeding (B*F, $P > 0.05$) was recorded for these genes related to the one-carbon metabolism. Three-way interactions were detected for all three genes (B*F*T, $P < 0.05$).

Metabolomics. A time effect existed for all metabolites (T, $P < 0.05$; Table 7.4). Except for adenosine and S-5'-adenosyl-homocysteine, whose concentrations decreased ($P < 0.05$)

postpartum, all metabolites in this pathway increased ($P < 0.05$) in concentration postpartum compared with prepartum (Table 7.4, Figure 7.3). Compared with thin cows, optimally-conditioned cows had greater (B, $P < 0.05$) concentrations of betaine, carnitine, choline, glycine, and butyrobetaine/acetylcholine in liver tissue. Compared with feed restriction, overfeeding decreased (F, $P < 0.05$) the concentration of these metabolites, overall, including that of N,N-dimethylglycine (**N,N-DG**). Interactions of BCS and feeding (B*F, $P < 0.05$) were detected for betaine, choline, glycine, methionine, N,N-DG, and butyrobetaine/acetylcholine. Compared with all other groups, betaine, choline, carnitine, glycine, and N,N-DG concentrations were greatest (B*F, $P < 0.05$) in feed restricted, optimally-conditioned cows. The peak concentration of methionine was, instead, detected for B5F75 and B4F125 cows, while butyrobetaine/acetylcholine was greatest in B5F75 cows, followed by B5F125, and then both BCS 4 groups.

Interactions of BCS and feeding with time were detected for carnitine, glycine and butyrobetaine/acetylcholine (B*T, F*T, $P < 0.05$; Table 7.4, Figure 7.3). Overall, BCS5 compared with BCS4 or feed restricted compared with overfed cows had greater postpartal carnitine concentrations at 4 wk. Feed restricted cows also had greater ($P < 0.05$) levels of carnitine prepartum (-1 wk). Concentrations of glycine in BCS4 and BCS5 cows were similar at -1, and 1 wk relative to calving, but differed at 4 wk postpartum, with greater values in BCS5 cows. Similarly, cows feed-restricted and overfed had similar concentrations of glycine at -1 and 1 wk relative to parturition, but feed restricted animals had higher glycine 4 wk from calving. Regarding butyrobetaine/acetylcholine, compared with BCS4, concentration was greater in BCS5 cows both prepartum and at 4wk postpartum. Similar concentrations were detected at 1wk postpartum between the two BCS groups. Feeding level had no statistical effect at -1 and 1 wk

from parturition, but affected butyrobetaine/acetylcholine concentrations at 4 wk from calving, with restricted cows having greater concentrations compared with overfed cows. A three way interaction (B*F*T, $P < 0.05$) was identified for butyrobetaine/acetylcholine, carnitine, glycine, and N,N-DG.

Transsulfuration pathway

Enzyme activity. Similar to MTR, CBS activity was lower postpartum (T, $P < 0.05$) compared with prepartum levels (Table 7.2, Figure 7.1). BCS did not statistically affect its activity overall (B, $P = 0.23$), but compared with feed restricted cows, overfed cows had greater overall CBS activity (F, $P < 0.0001$). The significant interaction between BCS and feeding (B*F, $P = 0.004$) indicated that overfeeding led to greater CBS activity, mostly in thin cows (BCS 4) and not in optimally conditioned cows (BCS 5).

mRNA abundance. Compared with prepartum levels, abundance of *CSB*, *GCLC*, and *GSS* was lower (T, $P < 0.05$) early postpartum (1 wk) (Table 7.3, Figure 7.2). Abundance for these 3 genes remained lower later postpartum (4 wk), but *GSS* returned to its prepartum level of abundance. No overall statistical effect of BCS, feeding, or their interaction was evident for genes involved in the transsulfuration pathway. Interaction of feeding and time was significant for *CSAD* and *GSS* abundance (F*T, $P < 0.05$). Compared with overfed cows, *CSAD* abundance was greater ($P = 0.04$) prepartum in feed restricted cows. Early postpartum its abundance was similar, but late postpartum feed restricted had lower ($P = 0.01$) *CSAD* abundance compared with overfed cows. An opposite trend was detected for *GSS* abundance, with feed restricted cows having greater abundance prepartum, followed by lower abundance late postpartum when compared with overfed cows. A three way interaction (B*F*T, $P < 0.05$) was identified for *CBS* and *GSS* expression.

Metabolites. Time affected (T, $P < 0.05$) concentrations of cystathionine, hypotaurine, and serine, with higher levels 4 wk postpartum compared with earlier times (Table 7.4, Figure 7.4). Overall effects of BCS and feeding were detected only for serine, whose concentration was greater in BCS5 (B, $P = 0.02$) or feed restricted (F, $P = 0.001$) cows. The interaction of BCS and feeding was significant for all metabolites measured (B*F, $P < 0.05$). Overall, compared with other groups, feed restricted optimally-conditioned cows had the greatest ($P < 0.05$) concentrations of cystathionine and serine. Concentration of cysteine sulfonate was greatest ($P < 0.05$) in overfed thin and feed restricted optimally-conditioned cows, while hypotaurine was greatest ($P < 0.05$) for B5F75 compared with B4F75 and B5F125 cows. Cows in B4F125 had an intermediate concentration of hypotaurine. An interaction of BCS and time was detected for cystathionine (B*T, $P = 0.05$), but no difference between BCS groups were detected at any time point. Only a tendency ($P = 0.07$) for greater concentration of cystathionine in BCS5 compared with BCS4 cows at 4 wk postpartum was detected. The interaction of feeding and time was significant for all metabolites measured in the transsulfuration pathway (F*T, $P < 0.05$). For all metabolites, there were no statistical differences between feeding levels at 1 wk postpartum ($P > 0.05$). In contrast, prepartum, overfeeding compared with feed restriction tended ($P < 0.10$) to increase concentrations of cystathionine, cysteinesulfinate, and hypotaurine, but not serine ($P > 0.10$). It is noteworthy that late postpartum (4 wk) overfeeding compared with feed restriction led to lower ($P < 0.05$) concentrations of all metabolites. A three way interaction (B*F*T, $P < 0.05$) was detected for serine.

Energy Metabolism

Differences due to time were detected for the concentrations of FAD, fumarate, glutamate, NAD^+, and NADH, and NAD^+:NADH ratio (T, $P < 0.05$; Table 7.4, Figure 7.5).

Concentrations of FAD increased linearly over the transition period, while fumarate concentrations increased to a stable level postpartum. Compared with prepartal concentrations, glutamate tended ($P = 0.09$) to be higher around parturition, and increased later in the transition period. In contrast, concentrations of NAD^+ and NADH decreased ($P = 0.001$) during the transition period, with the first being stable around calving and declining at 4 wk postpartum, and the latter decreasing after calving to stable levels throughout the postpartal period. In contrast, their ratio increased postpartum, with peak levels right after parturition. Overall, optimally conditioned cows had a greater concentration (B, $P < 0.05$) of FAD, and glutamate, and lower (B, $P = 0.002$) concentration of α-ketoglutarate compared with thin cows. Feed restriction caused an increase (F, $P < 0.05$) in concentrations of FAD, and glutamate. A BCS and feeding interaction was detected (B*F, $P < 0.05$) for FAD, glutamate, and glutamine, as feed restricted, optimally conditioned cows had greater concentrations of these metabolites compared with other cows.

Interactions between BCS and time were detected for FAD and glutamine (B*T, $P < 0.05$), with BCS5 cows having greater postpartal concentrations of these metabolites at 1 and 4 wk compared with BCS4 cows, and also at 1 wk prepartum (Table 7.4, Figure 7.5). FAD, glutamine, glutamine, and NAD^+:NADH ratio also had a significant interaction between feeding and time (F*T, $P < 0.05$). Compared with overfed cows, feed restricted cows had greater ($P < 0.05$) concentrations of glutamine and FAD postpartum (1 and 4 wk), and greater ($P < 0.05$) concentrations of glutamate late postpartum (4 wk). The NAD^+:NADH ratio was greater prepartum in feed restricted animals, but greater at 1 wk postpartum in overfed cows. A similar ratio between feeding groups was evident at the end of the transition period. A three way interaction was detected for FAD, and NAD^+:NADH ratio (B*F*T, $P < 0.05$).

DISCUSSION

The Transition Period

The one-carbon metabolism pathway comprises a network involving interactions between several B vitamins, homocysteine, and methionine. Its function is not limited to the regeneration of methionine via remethylation of homocysteine (e.g., BHMT, and MTR), as methionine is further converted to S-adenosyl-methionine (SAM). This step increases the functions of the one-carbon metabolism, as methyl groups in SAM can be donated to form a wide-range of vitally important methylated compounds including creatine, epinephrine, and sarcosine, as well as methylated DNA, RNA, and proteins (Ducker and Rabinowitz, 2017). The one-carbon metabolism further connects to the animal's antioxidant system via the transsulfuration pathway. Homocysteine together with serine are, in fact, used as substrates by CBS to generate cystathionine and then cysteine, leading to the synthesis of both glutathione and taurine, powerful cellular antioxidants.

The onset of lactation in dairy cows is characterized by high output of methylated compounds in milk when sources of methyl group are in short supply, even at the cost of depleting reserves of these compounds in liver tissue (Pinotti et al., 2002). Together with the well-known challenges of the transition period (e.g., negative energy and metabolizable protein balance), a negative methyl-donor balance could be an additional nutritional challenge of the peripartal dairy cow. At the same time, the overproduction of reactive oxygen species (**ROS**) leads to oxidative stress, which has been identified as an underlying factor of dysfunctional inflammatory responses (Abuelo et al., 2015), underlying the importance of the transsulfuration pathway in keeping an adequate supply of antioxidants. The work of Zhou et al. (2017) highlighted the importance of one-carbon metabolism in transition dairy cows reared in intense

TMR-based confinement systems. They reported a 130% and 26% increase of the hepatic activity of BHMT and MTR, respectively, around parturition, and a similar response (+72%) in *BHMT* hepatic abundance, but no changes in *MTR* abundance. Similarly, they reported a 20 and 13% increase in hepatic CBS activity and abundance, respectively, postpartum, when compared with prepartum.

Compared with their counterparts in confinement feeding systems, pasture-based systems utilize cows genetically selected with more emphasis on fertility and body condition score, and less on milk production. However, despite the lower milk production, the physiological and immunological dysfunction during the transition period in grazing dairy cows appears to be of similar magnitude to the higher producing TMR-fed counterparts (Kay et al., 2015). This said, our data appear to underscore fundamental differences in the one-carbon and transsulfuration pathway activity between the two systems. For instance, BHMT activity and mRNA abundance were the only parameters in pasture cows that resembled the trends reported in confinement systems. However, activity values, despite being similar prepartum, were half of those identified previously in TMR-fed cows (Zhou et al., 2017). The identified increase in BHMT activity was accompanied by an increase in concentrations of N,N-DG and choline, the products of the reaction, suggesting an increase in flux through the enzyme. The increase in concentrations of choline and butyrobetaine/acetylcholine, which feed betaine to BHMT, suggests they might have driven the increase in enzymatic activity. Despite the increase in concentrations of metabolites supplying substrates to MTR (e.g., folate, glycine, serine), its activity decreased postpartum; this is contrary to what was reported in TMR-fed cows (Zhou et al., 2017). Furthermore, the 2 to 3-fold greater activity prepartum and early postpartum in pasture fed cows suggested a more important role of this enzyme in grazing animals probably due to the high concentration of folate

in their diet, a precursor of 5-methyl-tetrahydrofolate and substrate of MTR, which is abundant in green leafy forage (Dong and Oace, 1975).

Concerning the transsulfuration pathway, the fact that homocysteine was undetectable, and that we detected a greater concentration of its metabolites (e.g., cystathionine, hypotaurine, serine) led us to speculate an increased flux postpartum. However, contrary to what was reported in TMR-based systems (Zhou et al., 2017), the activity and abundance of *CBS* decreased postpartum. Furthermore, in grazing cows activity values were close to half of those in higher-producing TMR-fed cows. Despite an extensive review of the literature concluding that grazing cows undergo a similar degree of peripartal physiological dysfunction than high-producing animals in TMR-systems (Roche et al., 2013), the substantial differences in BHMT, MTR, and CBS activity suggest a different load on the metabolic activity of the one-carbon metabolism and transsulfuration pathways. The unique trends during the transition period between systems might be explained by the difference in demand for lactation (e.g., lower milk production) and in nutrient intake.

Regarding energy status, the decrease in NAD^+ and NADH detected after calving was of particular interest. Our previous transcriptomic data indicated an increase in the activity of the "Nicotinate and nicotinamide metabolism" postpartum (responsible for the nicotinamide adenine dinucleotides family synthesis) in thin animals when overfed prepartum (Vailati-Riboni et al., 2016). The NAD^+ molecule plays a most-important role, as it is necessary to maintain hepatic fatty acid oxidative capacity, which is crucial after parturition when fat depot mobilization increases markedly (Schulz, 1991). The general decrease in concentrations of both NAD^+ and NADH postpartum suggested a lower energy status and possibly an impaired capacity to handle the flux of fatty acids arising from mobilization of fat depots. The fact that NAD^+:NADH ratio

increased postpartum, peaking right after calving, indicated that cows were able to maintain a higher level of reduced substrate to handle the increased demand for fatty acid β-oxidation. The rapid decrease of these molecules postpartum could be connected to the increased output of their precursors in milk. In fact, bovine milk is among the richest dietary sources of nicotinamide, tryptophan, and nicotinamide riboside (Trammell et al., 2016).

Prepartal Management

Manipulation of both prepartum cow adiposity and overall energy intake via pasture allocation (i.e., cow/m^2) had a large impact on activity and expression of enzymes involved in the one-carbon metabolism, transsulfuration pathway, and TCA cycle, including their metabolism. Overall, data support the idea of greater flux of nutrients through these pathways in optimally conditioned cows and in cows that were feed restricted prepartum. Few exceptions have to be noted: MTR activity and α-ketoglutarate concentration were greater in thin cows, and CBS activity was greater in overfed cows, despite all its downstream metabolites having greater concentrations in feed restricted cows. The latter suggested a negative feed-back effect. Since these metabolic pathways are integrated with energy status, and countless studies have demonstrated that BCS, DMI, and energy balance around parturition can be altered by management (reviewed elsewhere, (Roche et al., 2009, Drackley and Cardoso, 2014)), we speculate that the differences highlight a better energy and overall nutritional status in BCS5 or prepartum feed restricted cows. As the major inputs of the one-carbon metabolism (e.g., folate, methionine, choline) are naturally present in significant quantities in green pasture (NRC, 2001), it is logical that a better energy status (e.g., through greater DMI) may have led to greater concentrations of intermediates of this cycle and its related pathways.

Concerning the BCS effects detected in the present study, a direct relationship with calving adiposity and postpartum DMI and negative energy balance (NEB) has been observed in grazing conditions. Thinner cows often have greater DMI and more positive NEB postpartum compared with fat cows (Roche et al., 2009). However, compared with optimally conditioned cows, thin cows have similar energy balance, but slightly lower DMI and greater muscle mass mobilization after calving (Pires et al., 2013). Furthermore, thinner cows are at a greater risk of being afflicted by diseases and often demonstrate signs of poorer welfare compared with optimally conditioned cows (Roche et al., 2009). Thus, we speculate that the greater concentrations of metabolites in the one-carbon, transsulfuration, and TCA cycle pathways in BCS5 compared with BCS4 cows indicate a better metabolic and health status.

Despite lack of interactions of BCS and prepartal feeding regime for BHMT and MTR activity, and for mRNA abundance of all detected genes, the metabolites measured highlight a consistently greater flux throughout the one-carbon metabolism, methionine cycle and their related pathways in feed-restricted, optimally conditioned cows. These results confirm our previous hypothesis and recommendations regarding the use of separate prepartal nutritional strategies based on BCS at dry off. Not many interactions were detected for thin animals, who compared with optimally conditioned cows, displayed the greatest response at a transcription level (Vailati-Riboni et al., 2016). However, the greater CBS enzyme activity in overfed BCS4 cows, together with methionine, cysteinesulfinate, and hypotaurine concentrations comparable to those of feed restricted BCS5 cows, suggested a greater flux through the transsulfuration pathway. As such, optimally conditioned-feed restricted cows and thin-overfed cows were geared to better withstand the oxidative stress and inflammatory environment that characterizes the postpartum period. Furthermore, these results fit with our previous recommendation for

lower stocking density at pasture (e.g., increased feeding level) when managing thin cows prepartum (Vailati-Riboni et al., 2016).

CONCLUSIONS

The activity patterns over time of hepatic enzymes controlling one-carbon metabolism and transsulfuration pathway in grazing dairy cows highlight fundamental differences in the metabolic progression of these animals when compared with their counterparts reared in TMR-based, higher yield, confinement systems. Grazing cows, in fact, seem to have a greater capacity to regenerate methionine from folate. The lower CBS activity, and its decrease over time, indicate that grazing cows have a lower grade of oxidative stress or a lesser need to support the internal antioxidant system (e.g., glutathione and taurine), possibly thanks to the abundance of vitamin A and E precursors in pasture. Furthermore, metabolic data support our previous hypothesis that cows with different levels of adiposity at dry off need to be managed differently in the prepartum; this can be achieved by feed restricting optimally conditioned cows, while allowing a higher overall nutrient intake for thin cows by increasing feed allowance by either decreasing stocking density or providing supplementary feeds.

ACKNOWLEDGEMENTS

The authors thank Jason Philips, Farm Manager, Scott Farm, DairyNZ, and his capable team, as well as Stu Morgan, Kate Watkins, Hamish Hodgson, and Chris Roach for technical support, and Barbara Dow and Barbara Kuhn-Sherlock for statistical analysis and advice. This research was supported by New Zealand dairy farmers through DairyNZ Inc. (RD1403) and the Ministry of Business, Innovation, and Employment (DRCX1201).

TABLES AND FIGURES

Table 7.1. List of metabolites analyzed via targeted metabolomics in liver tissue of cows with different prepartum body condition score (BCS 4 or 5, on a 10-point scale), and different feed allowance prepartum (75 or 125% of estimated energy requirements).

Metabolites	Detected
Folic acid and methionine cycle	
5-methyltetradydrofolic acid	No
Adenosine	Yes
Betaine	Yes
Betaine aldehyde	No
Butyrobetaine/acetylcholine	Yes
Choline	Yes
Carnitine	Yes
Folic acid	Yes
Glycine	Yes
Homocysteine	
Methionine	Yes
N,N-dimethylglycine (N,N-DG)	Yes
N-methylglycine	No
S-5'-adenosyl-homocysteine (S-5-AH)	Yes
S-5'-adenosyl-methionine	No
Tetrahydrofolic acid	No
Transsulfuration pathway	
Cystathionine	Yes
Cysteine	No
Cysteinesulfinic acid	Yes
Glutathione	No
Hypotaurine	Yes
Serine	Yes
Taurine	No
Vitamin B_{12}	No
Vitamin B_6	No
γ-Glutamylcysteine	No
TCA cycle	
Acetyl-CoA	No
Adenosine monophosphate (AMP)	No
Adenosine triphosphate (ATP)	No
α-ketoglutaric acid (α-KG)	Yes
Cyclic adenosine monophosphate (cAMP)	No
Citrate	No
Flavin adenine dinucleotide (FAD)	Yes
Fumarate	Yes
Glutamate	Yes
Glutamine	Yes
Guanosine-5'-triphosphate (GTP)	No

Table 7.1 (cont)

Isocitric acid	No
Malate	Yes
NAD^+:NADH	Yes
Nicotinamide adenine dinucleotide – oxidized form (NAD^+)	Yes
Nicotinamide adenine dinucleotide – reduced form (NADH)	Yes
Nicotinamide adenine dinucleotide phosphate – oxidized form ($NADP^+$)	No
Nicotinamide adenine dinucleotide phosphate – reduced form (NADPH)	No
Nicotinic acid	No
Pyruvate	No
Succinyl-CoA	No
Tri-methyl-lysine	No
Uridine diphosphate (UDP)	No

Table 7.2. Activity (nmol product/h/mg protein) of betaine-homocysteine S-methyltransferase (BHMT), 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR), and cystathionine-β-synthase (CBS) at -1, 1, and 4 weeks around parturition (T) in liver tissue of cows with different prepartum body condition score (BCS 4 or 5, on a 10-point scale), and different feed allowance prepartum (F 75 or 125% of estimated energy requirements).

	BCS		F		BCS*F				Time (T)				*P*-value						
					4		5												
	4	5	75	125	75	125	75	125	-1	1	4	SEM[1]	BCS	F	BCS*F	T	BCS*T	F*T	BCS*F*T
Folic acid and methionine cycle																			
BHMT	15.83	14.76	15.10	15.49	15.37	16.30	14.84	14.68	13.69^{a}	16.47^{b}	15.73^{ab}	1.13	0.33	0.72	0.62	0.06	0.99	0.05	0.69
MTR	72.75^{a}	39.15^{b}	51.60	60.30	64.11	81.38	39.09	39.22	78.46^{a}	57.14^{a}	32.25^{b}	9.30	<0.01	0.34	0.35	<0.0001	0.44	0.16	0.22
Transsulfuration pathway																			
CBS	22.61	20.27	16.02^{b}	26.86^{a}	14.15^{a}	31.08^{c}	17.89^{ab}	22.65^{b}	27.63^{a}	20.52^{b}	16.17^{b}	2.01	0.23	<0.0001	<0.01	<0.0001	0.46	0.22	0.16

[1]Largest standard error of the mean

[a,b,c] Different superscripts indicate that means differ ($P \leq 0.05$)

Table 7.3. Relative mRNA abundance of genes associated with the folic acid cycle, methionine cycle, and transsulfuration pathway at -1, 1, and 4 weeks around parturition (T) in liver tissue of cows with different prepartum body condition score (BCS 4 or 5, on a 10-point scale), and different feed allowance prepartum (F 75 or 125% of estimated energy requirements).

	BCS		F		BCS*F				Time (T)				*P*-value[1]						
					4		5												
	4	5	75	125	75	125	75	125	-1	1	4	SEM[1]	BCS	F	BCS*F	T	BCS*T	F*T	BCS*F*T
Folic acid and methionine cycle																			
BHMT1	0.95	0.98	1.01	0.92	1.01	0.89	1.01	0.95	0.77[a]	1.10[b]	1.05[b]	0.09	0.72	0.26	0.72	0.003	0.43	0.93	0.02
MAT2A	0.78[a]	0.92[b]	0.91[a]	0.79[b]	0.86	0.70	0.96	0.88	0.84	0.92	0.79	0.07	0.004	0.01	0.31	0.22	0.91	0.12	0.04
MTR	1.16	1.12	1.26[a]	1.03[b]	1.29	1.04	1.23	1.02	1.12	1.13	1.16	0.05	0.37	<0.0001	0.59	0.78	0.96	0.16	0.02
Transsulfuration pathway																			
CBS	0.95	0.91	0.93	0.93	0.98	0.92	0.88	0.94	1.07[a]	0.86[b]	0.88[b]	0.06	0.39	0.93	0.23	0.02	0.99	0.17	0.02
CDO1	0.75	0.72	0.74	0.72	0.76	0.74	0.72	0.71	0.75	0.74	0.71	0.04	0.45	0.69	0.86	0.53	0.73	0.09	0.45
CSAD	0.97	0.88	0.99	0.87	1.16	0.81	0.84	0.93	0.82	0.96	1.02	0.18	0.55	0.41	0.15	0.24	0.90	0.002	0.23
GCLC	0.80	0.66	0.78	0.68	0.91	0.70	0.66	0.66	1.06[a]	0.64[b]	0.56[b]	0.13	0.20	0.35	0.40	<0.0001	0.85	0.14	0.12
GSS	0.94	0.98	0.93	0.98	0.97	0.91	0.90	1.06	1.00[a]	0.88[b]	1.01[a]	0.06	0.47	0.36	0.06	0.07	0.87	0.004	0.05

[1]Largest standard error of the mean

[a,b,c] Different superscripts indicate that means differ ($P \leq 0.05$)

Table 7.4. Relative concentration [area under the curve (AUC) · mg protein^{-1}] of metabolites in the folic acid and methionine cycle, transsulfuration, and energy metabolism pathways at -1, 1, and 4 weeks around parturition (T) in liver tissue of cows with different prepartum body condition score (BCS 4 or 5, on a 10-point scale), and different feed allowance prepartum (F 75 or 125% of estimated energy requirements).

	BCS		F		BCS*F				Time (T)				*P*-value						
					4		5												
Metabolite	4	5	75	125	75	125	75	125	-1	1	4	SEM[1]	BCS	F	BCS*F	T	BCS*T	F*T	BCS*F*T
Folic acid and methionine cycle																			
Butyrobetaine/acetylcholine	1528^{a}	2231^{b}	2099^{a}	1659^{b}	1523^{a}	1532^{a}	2676^{c}	1785^{b}	1460^{a}	1758^{a}	2419^{b}	149	<.0001	<.0001	<.0001	<.0001	0.01	0.02	0.005
Adenosine	676	690	695	671	713	642	678	703	1521^{a}	671^{b}	313^{c}	307	0.93	0.89	0.78	<.0001	0.06	0.63	0.09
Betaine	3082^{a}	3973^{b}	4089^{a}	2966^{b}	3305^{a}	2859^{a}	4873^{b}	3072^{a}	2815^{a}	3612^{ab}	4155^{a}	340	0.01	0.003	0.05	0.01	0.46	0.72	0.59
Carnitine	967^{a}	1307^{b}	1351^{a}	923^{b}	1020^{a}	913^{a}	1682^{b}	933^{a}	884^{c}	1081^{b}	1446^{a}	73	<.0001	<.0001	0.0001	<.0001	<.0001	0.04	0.0002
Choline	574482^{a}	702855^{b}	723915^{a}	553422^{b}	535837^{a}	613128^{a}	911993^{b}	493716^{a}	371518^{a}	575745^{a}	968742^{b}	80123	0.10	0.03	0.003	<.0001	0.24	0.13	0.54
Folate	344	381	376	350	318	370	433	329	178^{a}	386^{b}	524^{b}	62	0.53	0.66	0.19	0.0003	0.99	0.92	0.83
Glycine	35897^{a}	45147^{b}	47554^{a}	33490^{b}	34568^{a}	37226^{a}	60540^{b}	29755^{a}	20166^{a}	35470^{b}	65930^{c}	3057	0.003	<.0001	<.0001	<.0001	0.002	<.0001	<.0001
Methionine	41842	48255	48015	42082	30645^{a}	53039^{b}	65385^{b}	31125^{a}	26103^{a}	38237^{a}	70804^{b}	7124	0.36	0.39	0.0003	<.0001	0.50	0.17	0.09
N,N-dimethylglycine	6407	7287	7574^{a}	6120^{b}	6592^{a}	6221^{a}	8556^{b}	6019^{a}	5369^{a}	6941^{b}	8231^{b}	568	0.11	0.01	0.05	0.0004	0.08	0.14	0.0003
S-5'-adenosyl-homocysteine	21567	22993	24973	19587	23133	20001	26813	19173	29572^{a}	21988^{ab}	15281^{b}	4998	0.77	0.26	0.64	0.06	0.61	0.41	0.70
Transsulfuration pathway																			
Cystathionine	9122	11161	11226	9057	8653^{a}	9592^{a}	13799^{b}	8522^{a}	6491^{a}	7156^{a}	16778^{b}	1455	0.16	0.13	0.04	<.0001	0.09	0.001	0.15
Cysteinesulfinate	265	310	275	298	161^{a}	436^{b}	471^{b}	204^{a}	305	255	302	102	0.50	0.73	0.001	0.67	0.05	0.001	0.17
Hypotaurine	2260	2570	2624	2206	1800^{a}	2720^{ab}	3448^{b}	1692^{a}	1855^{a}	1830^{a}	3561^{b}	457	0.47	0.34	0.01	0.001	0.96	0.002	0.70
Serine	20965^{a}	24872^{b}	25793^{a}	20044^{b}	20205^{a}	21726^{a}	31382^{b}	18363^{a}	16165^{a}	20043^{a}	32548^{b}	1913	0.02	0.001	<.0001	<.0001	0.12	0.01	0.01
TCA cycle																			
α-ketoglutarate	3859^{a}	2572^{b}	3451	2979	4100	3617	2802	2341	2695	3185	3765	398	0.002	0.21	0.98	0.08	0.39	0.07	0.08
FAD	1234^{a}	1510^{b}	1604^{a}	1140^{b}	1300^{a}	1169^{a}	1908^{b}	1111^{a}	1131^{c}	1317^{b}	1668^{a}	83	0.002	<.0001	0.0004	<.0001	0.04	<.0001	0.01
Fumarate	5948	5300	5992	5261	5838	6060	6151	4566	4553^{b}	6188^{a}	6282^{a}	684	0.30	0.24	0.14	0.04	0.70	0.58	0.40
Glutamate	474799^{a}	564473^{b}	600389^{a}	438883^{b}	449666^{bc}	499933^{b}	751113^{a}	377833^{c}	369451^{b}	463805^{b}	725651^{a}	39531	0.03	0.0002	<.0001	<.0001	0.13	0.004	0.15
Glutamine	238	313	319	232	231^{a}	245^{a}	407^{b}	220^{a}	258	256	313	52	0.12	0.08	0.05	0.07	0.02	0.0004	0.06
Malate	587237	472793	577446	482584	583124	591351	571769	373818	435528	562923	591595	61258	0.06	0.11	0.09	0.09	0.76	0.78	0.64
NAD	1234	1465	1453	1244	1215	1253	1738	1235	2557^{a}	1429^{a}	665^{b}	540	0.53	0.57	0.49	0.001	0.12	0.10	0.25
NADH	679	1048	956	744	700	658	1305	842	1883^{a}	754^{b}	422^{b}	440	0.15	0.41	0.53	0.001	0.10	0.32	0.77
NAD:NADH	1.68	1.45	1.57	1.55	1.71	1.66	1.44	1.45	1.28^{c}	1.93^{a}	1.54^{b}	0.31	0.27	0.94	0.91	0.004	0.44	0.002	0.01

[1]Largest standard error of the mean

[a,b,c] Different superscripts indicate that means differ ($P \leq 0.05$)

Figure 7.1. Activity (nmol product/h/mg protein) of betaine-homocysteine S-methyltransferase (BHMT), 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR), and cystathionine-β-synthase (CBS) at -1, 1, and 4 weeks around parturition (T) in liver tissue of cows with different prepartum body condition score (BCS 4 or 5, on a 10-point scale), and different feed allowance prepartum (F 75 or 125% of estimated energy requirements).

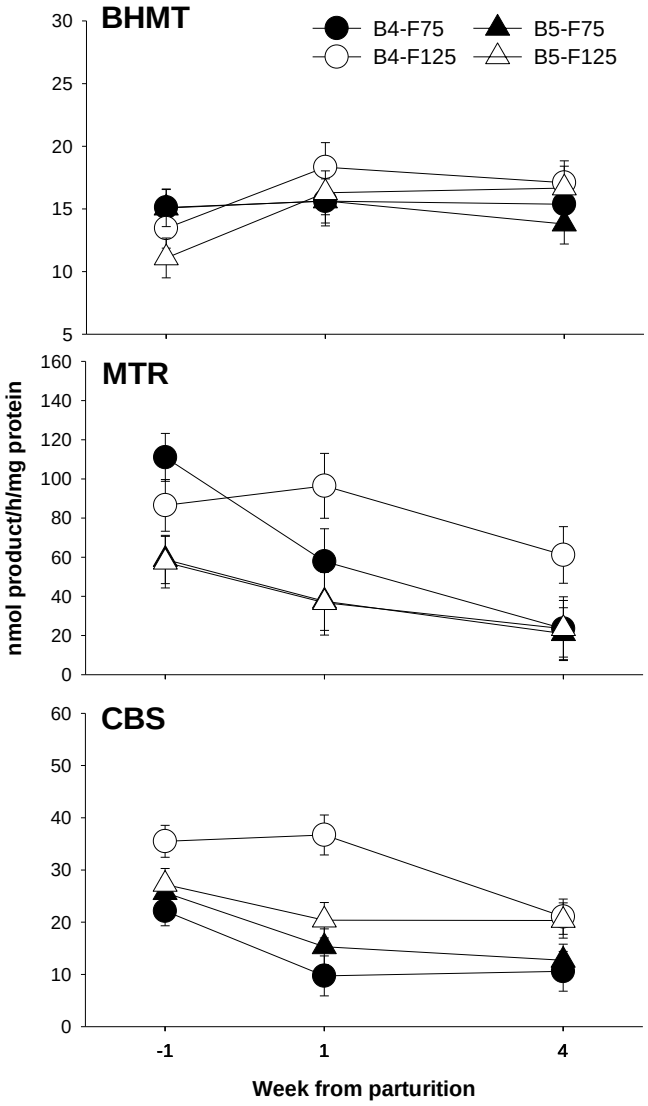


Figure 7.2. mRNA abundance of genes in the folic acid cycle, methionine cycle, and transsulfuration pathway at -1, 1, and 4 weeks around parturition (T) in liver tissue of cows with different prepartum body condition score (BCS 4 or 5, on a 10-point scale), and different feed allowance prepartum (F 75 or 125% of estimated energy requirements). [a-d]Different letters indicate that means differ ($P \leq 0.05$).

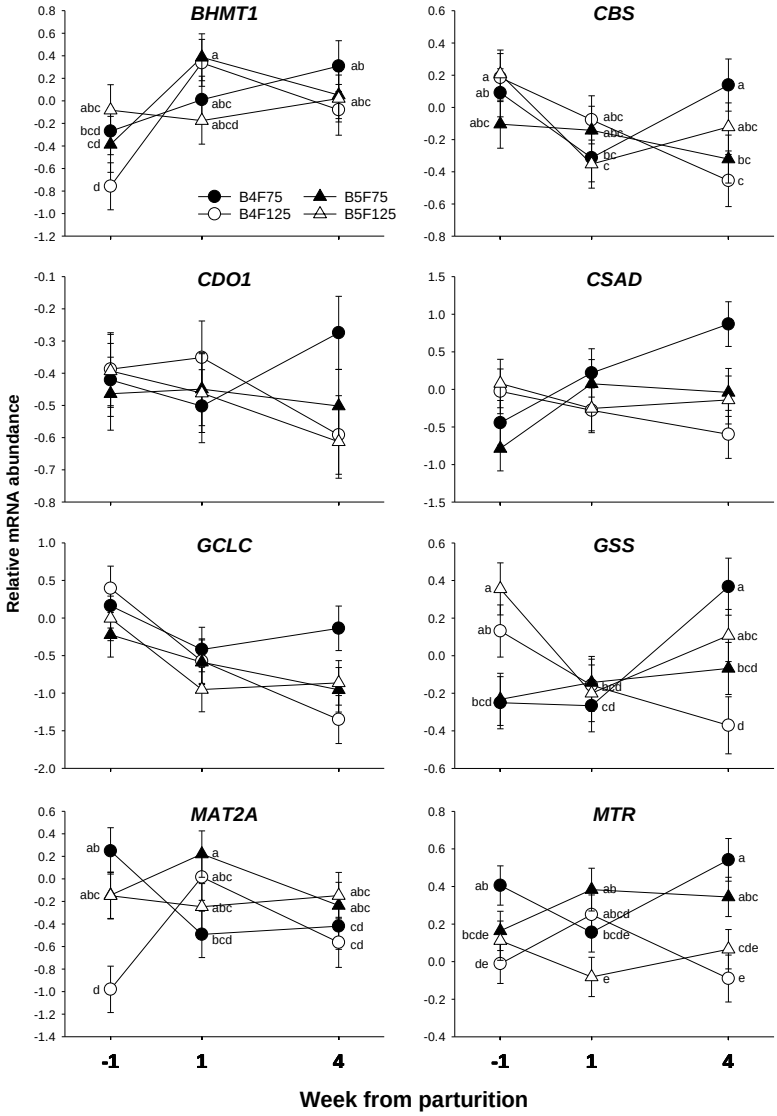


Figure 7.3. Concentration [area under the curve (AUC)/mg protein] of metabolites in the folic acid and methionine cycle at -1, 1, and 4 weeks around parturition (T) in liver tissue of cows with different prepartum body condition score (BCS 4 or 5, on a 10-point scale), and different feed allowance prepartum (F 75 or 125% of estimated energy requirements). [a-g]Means differ ($P \leq 0.05$).

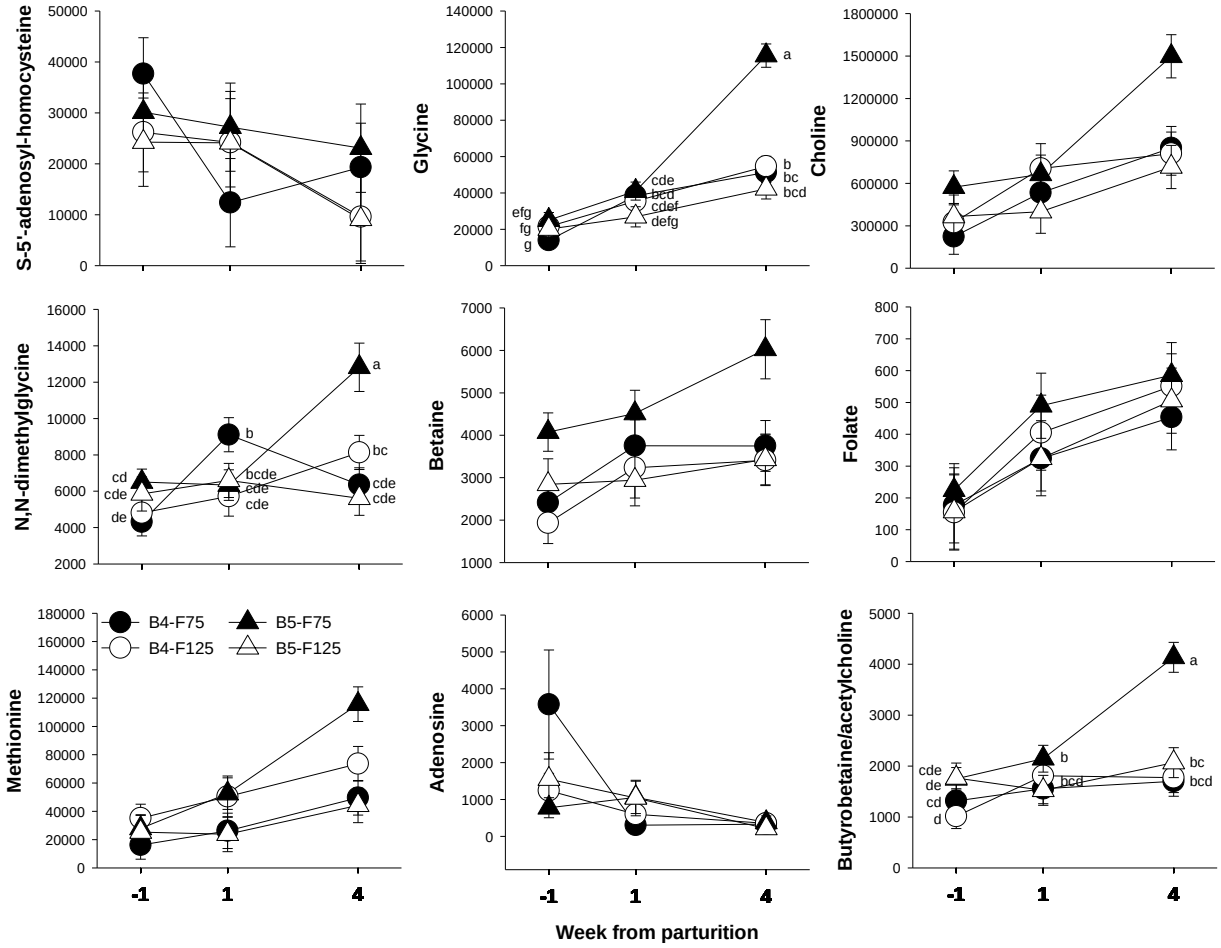


Figure 7.4. Relative concentration [area under the curve (AUC)/mg protein] of metabolites in the hepatic transsulfuration pathway at -1, 1, and 4 weeks around parturition (T) in liver tissue of cows with different prepartum body condition score (BCS 4 or 5, on a 10-point scale), and different feed allowance prepartum (F 75 or 125% of estimated energy requirements). [a-d]Means differ ($P \leq 0.05$).

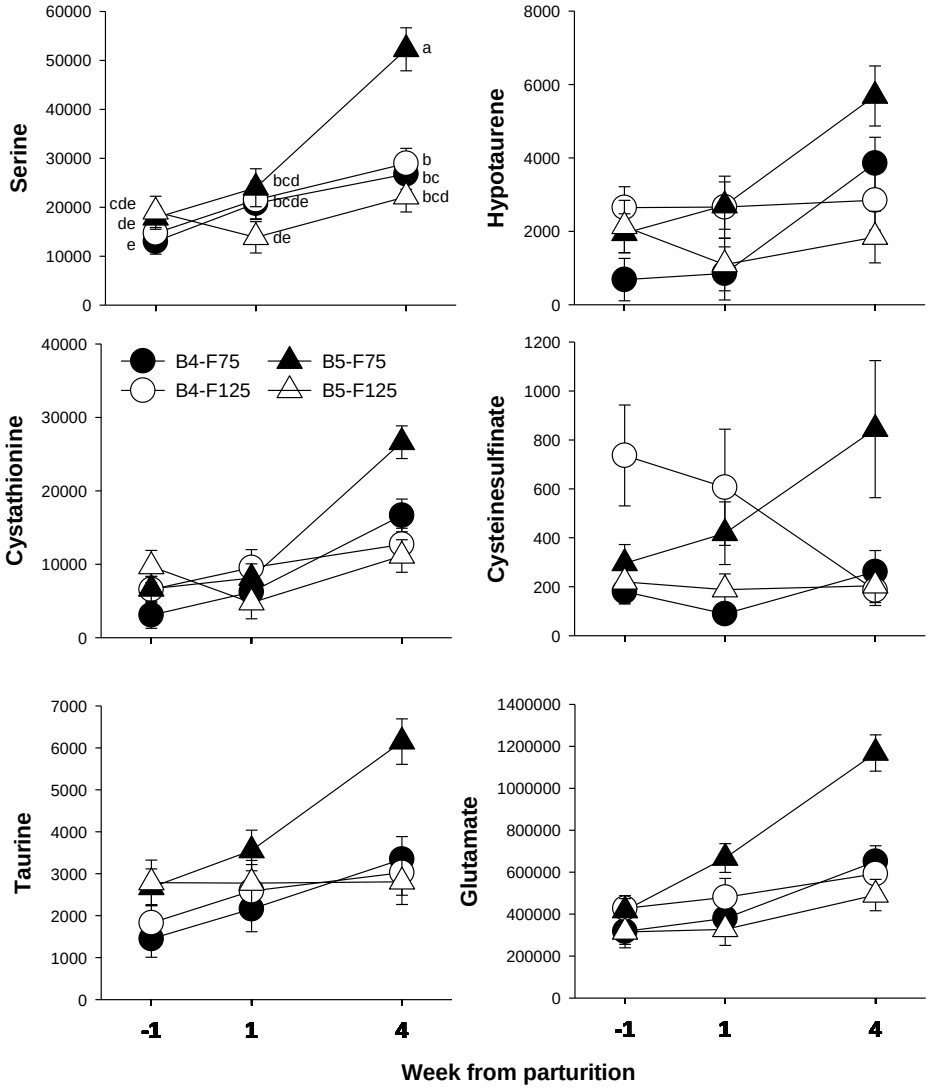
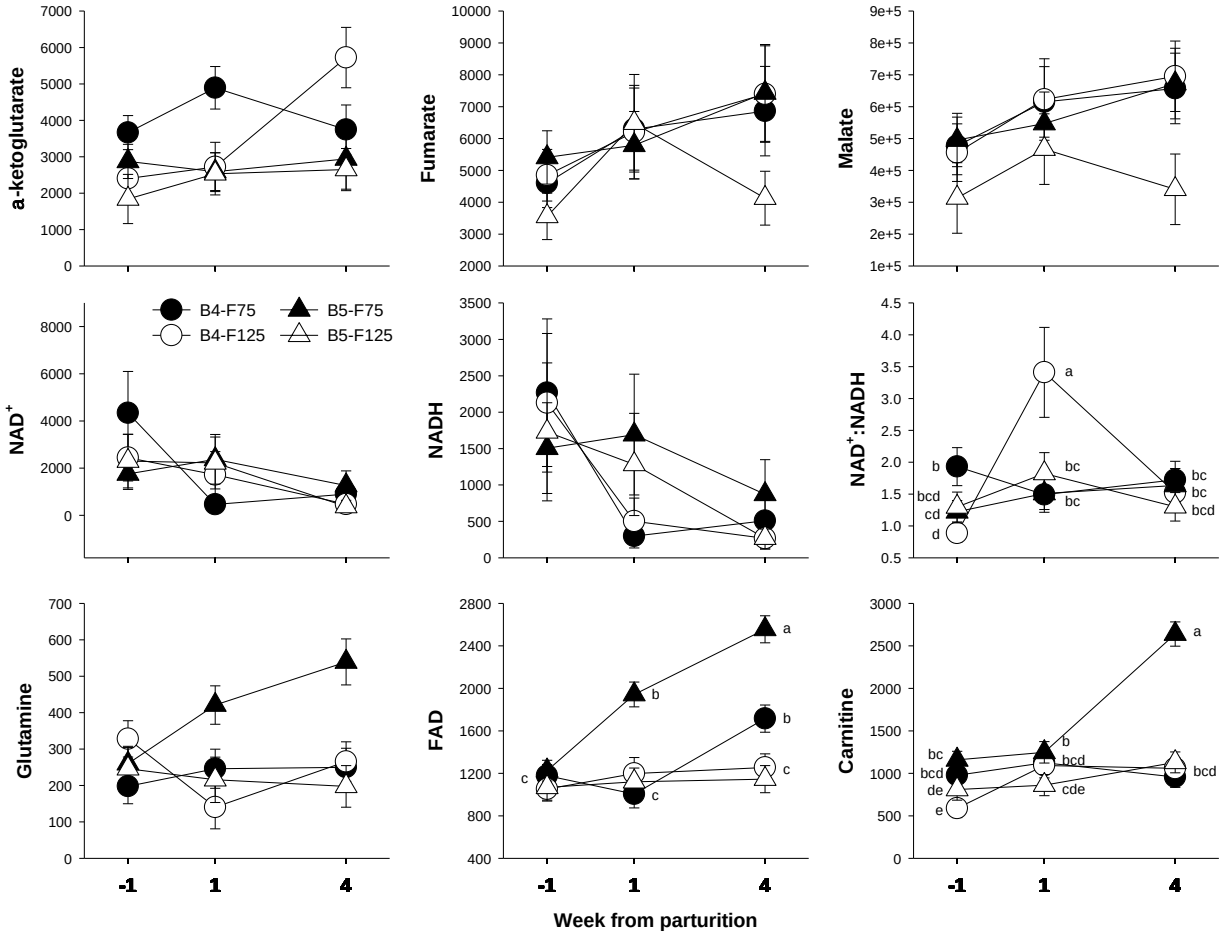


Figure 7.5. Relative concentration [area under the curve (AUC)/mg protein] of metabolites associated with hepatic energy metabolism at -1, 1, and 4 weeks around parturition (T) in liver tissue of cows with different prepartum body condition score (BCS 4 or 5, on a 10-point scale), and different feed allowance prepartum (F 75 or 125% of estimated energy requirements). [a-d]Means differ ($P \leq 0.05$).



REFERENCES

Abuelo, A., J. Hernandez, J. L. Benedito, and C. Castillo. 2015. The importance of the oxidative status of dairy cattle in the periparturient period: revisiting antioxidant supplementation. Journal of animal physiology and animal nutrition 99(6):1003-1016.

Banerjee, R., Z. Chen, and S. Gulati. 1997. Methionine synthase from pig liver. Methods in enzymology 281:189-196.

Beever, D. E. and R. C. Siddons. 1986. Digestion and metabolism in the grazing ruminant. Pages 479-497 in Control of digestion and metabolism in ruminants. L.P. Milligan, W.L. Grovum, and A. Dobson, eds. Prentice-Hall, Englewood Cliffs, NJ.

Brookes, I. M. 1984. Effects of formaldehyde-treated and untreated casein supplements on performance of dairy-cows offered ryegrass clover pasture. New Zealand journal of agricultural research 27(4):491-493.

Dong, F. M. and S. M. Oace. 1975. Folate concentration and pattern in bovine milk. Journal of agricultural and food chemistry 23(3):534-538.

Drackley, J. K. and F. C. Cardoso. 2014. Prepartum and postpartum nutritional management to optimize fertility in high-yielding dairy cows in confined TMR systems. Animal 8 Suppl 1:5-14.

Ducker, G. S. and J. D. Rabinowitz. 2017. One-carbon metabolism in health and disease. Cell metabolism 25(1):27-42.

Garrow, T. A. 1996. Purification, kinetic properties, and cDNA cloning of mammalian betaine-homocysteine methyltransferase. Journal of biological chemistry 271(37):22831-22838.

Holmes, C. W., G. F. Wilson, D. D. S. Mackenzie, D. S. Flux, I. M. Brookes, and A. W. F. Davey. 1987. Nutrition: Quantitative requirements of dairy cattle. Pages 107-130 in Milk production from pasture. C.W. Holmes, and G. F. Wilson, eds. Wellington: Butterworth's Agricultural Books, Wellington, New Zealand.

Kay, J. K., J. J. Loor, A. Heiser, J. McGowan, and J. R. Roche. 2015. Managing the grazing dairy cow through the transition period: A review. Animal production science 55(7):936-942.

Kolver, E. S. 2003. Nutritional limitations to increased production on pasture-based systems. Proceedings of the Nutrition Society 62(2):291-300.

Lambert, B. D., E. C. Titgemeyer, G. L. Stokka, B. M. DeBey, and C. A. Loest. 2002. Methionine supply to growing steers affects hepatic activities of methionine synthase and betaine-homocysteine methyltransferase, but not cystathionine synthase. Journal of nutrition 132(7):2004-2009.

Minson, D. J. 1981. The effects of feeding protected and unprotected casein on the milk production of cows grazing ryegrass. Journal of agricultural science 96(Feb):239-241.

NRC. 2001. Nutrient requirements of dairy cattle. 7th revised edition. National Academy Press, Washington, D.C.

Pacheco-Rios, D., W. C. McNabb, J. P. Hill, T. N. Barry, and D. D. S. Mackenzie. 1997. The effects of methionine supplementation on milk production of dairy cows in mid-lactation. Proceedings of the New Zealand Society of Animal Production 57:147–150.

Penno, J. W. and V. R. Carruthers. 1995. Energy and protein supplementation in spring: Experimental results. Massey dairyfarming annual 47:57–61.

Pinotti, L., A. Baldi, and V. Dell'Orto. 2002. Comparative mammalian choline metabolism with emphasis on the high-yielding dairy cow. Nutrition research reviews 15(2):315-332.

Pires, J. A. A., C. Delavaud, Y. Faulconnier, D. Pomies, and Y. Chilliard. 2013. Effects of body condition score at calving on indicators of fat and protein mobilization of periparturient Holstein-Friesian cows. Journal of dairy science 96(10):6423-6439.

Roche, J. R., D. P. Berry, A. M. Bryant, C. R. Burke, S. T. Butler, P. G. Dillon, D. J. Donaghy, B. Horan, K. A. Macdonald, and K. L. Macmillan. 2017. A 100-Year Review: A century of change in temperate grazing dairy systems. Journal of dairy science 100(12):10189-10233.

Roche, J. R., P. G. Dillon, C. R. Stockdale, L. H. Baumgard, and M. J. VanBaale. 2004. Relationships among international body condition scoring systems. Journal of dairy science 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. Journal of dairy science 92(12):5769-5801.

Roche, J. R., E. S. Kolver, and J. K. Kay. 2005. Influence of precalving feed allowance on periparturient metabolic and hormonal responses and milk production in grazing dairy cows. Journal of dairy science 88(2):677-689.

Roche, J. R., K. A. Macdonald, K. E. Schutz, L. R. Matthews, G. A. Verkerk, S. Meier, J. J. Loor, A. R. Rogers, J. McGowan, S. R. Morgan, S. Taukiri, and J. R. Webster. 2013. Calving body condition score affects indicators of health in grazing dairy cows. Journal of dairy science 96(9):5811-5825.

Roche, J. R., S. Meier, A. Heiser, M. D. Mitchell, C. G. Walker, M. A. Crookenden, M. Vailati Riboni, J. J. Loor, and J. K. Kay. 2015. Effects of pre-calving body condition score and prepartum feeding level on production, reproduction, and health parameters in pasture-based transition dairy cows. Journal of dairy science 98(10):7164-7182.

Rogers, G. L., R. H. D. Porter, T. Clarke, and J. A. Stewart. 1980. Effect of protected casein supplements on pasture intake, milk-yield and composition of cows in early lactation. Australian journal of agricultural research 31(6):1147-1152.

Rusdi and M. F. J. van Houtert. 1997. Responses to protected amino acids or protected protein in dairy cows grazing ryegrass pastures in early lactation. Proceedings of the New Zealand Society of Animal Production 57:120-125.

Schulz, H. 1991. Beta oxidation of fatty acids. Biochimica et biophysica acta 1081(2):109-120.

Taoka, S., S. Ohja, X. Shan, W. D. Kruger, and R. Banerjee. 1998. Evidence for heme-mediated redox regulation of human cystathionine beta-synthase activity. Journal of biological chemistry 273(39):25179-25184.

Trammell, S. A. J., L. P. Yu, P. Redpath, M. E. Migaud, and C. Brenner. 2016. Nicotinamide riboside is a major NAD(+) precursor vitamin in cow milk. Journal of nutrition 146(5):957-963.

Vailati-Riboni, M., S. Meier, C. R. Burke, J. K. Kay, M. D. Mitchell, C. G. Walker, M. A. Crookenden, A. Heiser, S. L. Rodriguez-Zas, J. R. Roche, and J. J. Loor. 2016. Prepartum body condition score and plane of nutrition affect the hepatic transcriptome during the transition period in grazing dairy cows. BMC genomics 17(1):854.

Vailati Riboni, M., M. Kanwal, O. Bulgari, 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. 2016. Body condition score and plane of nutrition prepartum affect adipose tissue transcriptome regulators of metabolism and inflammation in grazing dairy cows during the transition period. Journal of dairy science 99(1):758-770.

Wu, H., A. D. Southam, A. Hines, and M. R. Viant. 2008. High-throughput tissue extraction protocol for NMR- and MS-based metabolomics. Analytical biochemistry 372(2):204-212.

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. Journal of nutrition 147(1):11-19.

CHAPTER 8: CONCLUSIONS AND FUTURE PERSPECTIVES

Proper management of the dairy cows during its peripartal period is of paramount importance for the productivity of the dairy industry and the welfare of the operating herds. This transitional period from the non-lactating to the lactating status, together with its own neonatal days, is in fact the highest risk stages in a dairy cow life cycle, capable of determining the outcome of its future lactation, reproduction potential, and health. The use of nonsteroidal anti-inflammatory drugs (e.g., carprofen) injection postcalving to alleviate the well-documented underlying inflammatory status post calving did not produced positive effect on the animal health, fertility, and production. The possibility that the cows in our study (grazing Holstein*Jersey mix on a seasonal calving system) did not undergo a pronounced inflammatory state in early lactation (e.g., due to differences in production system and genetics) could have masked any positive effects of the treatment.

Focus was then moved from pharmacological intervention postpartum to nutritional intervention prepartum tailored to the animal adiposity level measured through the assessment of body conditioned score (BCS), a procedure commonly practice in commercial herds. Feed restriction or increased feed allowance (75 vs 125 % of calculated metabolizable energy, respectively) managed through animal density (cow/m^2 of pasture) was tested in thin or optimally conditioned animals. As hypothesized, the effect of prepartum plane of nutrition on hepatic function and adipose tissue metabolism was dependent on the BCS of the cow, underscoring how these management tools need to be evaluated together to optimize the biological adaptations of the cow during the peripartum period. Omics analysis of the liver transcriptome highlighted how thin animals were more susceptible to prepartal nutritional

management. Furthermore, pathways expression patterns, and metabolomics analysis suggested how overfeeding in late-pregnancy should be limited to thin cows, while cows with optimal adiposity should be maintained on an energy-restricted diet. Adipose tissue mRNA and microRNA expression further confirmed this hypothesis, and indicated a relationship between the immune and metabolic response of the adipose tissue underscoring the existence of a "self-regulatory" mechanism, which can be modulated via nutritional strategies. Further supported by performance and blood profile data, New Zealand farmers, thought DairyNZ (the industry organization that represents all New Zealand dairy producers), are now instructed that optimally conditioned cows will benefit from a short-term (2–3 wk) controlled feed restriction (75–90% of requirements), whereas cows in less than optimal condition should be fed to requirements before calving. This is an easily implementable strategy based on pasture allocation capable of benefitting the farmer with a minimum cost.

In a seasonal pasture based system, due to season fluctuation of pasture mass availability, cows are at greater risk to reach the end of lactation at a suboptimal level of adiposity; overfeeding thin animal at the end of their lactation is thus standard practice of the New Zealand production system. Our results suggested that to obtain a favorable transition to lactation, at least in grazing systems, dairy cows should be managed to achieve an optimal calving adiposity at close-up, providing confidence that over-feeding in the far-off nonlactating cow, as is customary in grazing systems, is not detrimental to the subsequent health of the cow. Afterwards animals should be maintained on a slight feed restriction until calving, following on the previously determined strategy of feed-restricting optimally condition animals.

Future perspective

Across all three experiment undertaken to understand the effect of different peripartal strategies in grazing dairy cows one results was consistently observed: the existence of a mechanism linking the animal immune system to the metabolic homeorhetic adaptation during the cow transition to the new lactation. This is an arising concept observed in recent literature, with the recognition that a certain degree of inflammation is likely necessary to support the intricate adaptation in the transition period. Most notably, its role in the facilitation of parturition, through (i) influx of innate immune cells into the uterus lining which promotes its contraction, cervix dilation, rupture of the fetal membranes, and expulsion of the placenta. Similarly, our results support the role of innate cells (e.g., macrophages) infiltration in the cow adipose tissue around parturition to induce a localize inflammatory status aiming at the impairment of the tissue insulin sensitivity, allowing for the release of stored energy with the goal of covering the animal energy deficit typical of the postpartal period. This resembles mechanisms already observed in nature, including human adipose response in obese patient.

The interface of immunity and tissue/organ metabolism is an emerging field of study. Relatively recent immunity and metabolism were considered separate entity, with the only link being metabolism as the simple source of energy for the immune system cellular components and function. Nowadays instead, the field of immunometabolism considers the role of immune cells in metabolism of organs and their effect on the whole organism metabolism, and the effect of specific metabolic pathways on immune cells functionality and the broader organism immunity. These concepts will have to be applied also to livestock physiological sciences, and specifically to dairy science to (i) better understand the immune system role during the transition period, and

(ii) redefine the maintenance requirements for a more complete nutritional management of the dairy cow. To the end future research goal will have to aim at:

1. ***The understanding of the molecular mechanism that ignite the inflammatory processes around calving, and the identification of the cellular players involved in the signaling cascade***. After either blocking (e.g., NSAID) or enhancing (e.g., pro-inflammatory molecules) the inflammatory scenario, scientist could combine targeted traditional approaches with more holistic technologies, such as transcriptomic, proteomic, and metabolomics to identify molecules, mediators, and targets involved in the immunometabolic adaptation to lactation.
2. ***The effect that immune cells and inflammatory mediators have on specific organs at the center of peripartal metabolic adaptation***. Response to similar immunological stimuli is different depending on the receiving system; if influx of immune cells in the uterus is aimed at the facilitating parturition and subsequent expulsion of placental and fetal lining tissues, influx in the adipose tissue appears to be linked to the organism energy metabolism. Furthermore, the role of liver residing immune cells (e.g., Kupffer cells) in the hepatic adaptation to lactation is still to be explored. Similarly, the role of immune cells (e.g., brain microglia) in the central nervous system regulation of feed intake in dairy animals is a potential aspect to consider in the complexity of ruminant physiology. As important part of animal protein metabolism, the involvement of muscle tissue and its possible link with immunity has also to be further investigated.
3. ***The system network that link organ/tissue crosstalk as part of the physiological changes throughout the peripartal period.*** As organ/tissues secreted molecules (e.g., myokines, adipokines, hepatokines) are involved in the fundamental molecular mechanism that allows

communication amongst metabolic organs in the endocrine and metabolic system, immune cells can act as mediators of organs crosstalk.

The pursuit of these lines of research will yield a greater understanding of the complex network of adaptation during the transition period, allowing future scientist to design nutritional and non-nutritional strategies to target specific node of this network aiming at the optimization of the dairy cow transition. As consumer scrutiny of the goods and feeds marketed by the dairy industry, including the strategies applied to produce such foods, has increased in today's society, I believe that nutritional intervention (e.g., macro and micronutrients, bioactive compounds, pre and probiotics, phytonutrients) will become the norm as future preventative and prophylactic strategies to ensure food safety and security, while supporting animal productivity, health, and welfare.

APPENDIX A

CHAPTER 3 - SUPPLEMENTAL MATERIAL

RNA extraction and Real-Time Quantitative PCR (qPCR)

Liver RNA extraction. Liver tissue (30-50 mg) was homogenized in a TissueLyser II (Qiagen GmbH) for 2 x 2 min bursts at 30 Htz. Total RNA and DNA were extracted using a Qiagen AllPrep DNA/RNA mini kit (Qiagen GmbH) as per manufacturer's instructions and RNA was treated with DNase (Ambion DNA-free kit; Ambion Inc., Austin, TX).

Adipose RNA extraction. Total RNA was extracted using Trizol® reagent (Life Technologies, New Zealand). Adipose tissue (30-80 mg) was placed in 1 mL of Trizol reagent and tissue was homogenized with a PRO200 homogeniser (Pro Scientific, CT, USA) using two 20 sec bursts of the homogeniser at full speed (30,000 RPM) and placed on ice after any homogenisation. The homogeniser tool was rinsed between each sample twice with 75% molecular grade ethanol (Merk, Millipore, Darmstadt, Germany), and UltraPure™ RNAse/DNase-free distilled water (Life Technologies, CA, USA) and once with RNAseZap® (Life Technologies, USA). Choloroform was added to homogenised sample, centrifuged, and aqueous phase carefully removed. Precipitation of RNA was achieved with the addition of ispopropanol (Merk Millipore, Germany), and the subsequent RNA pellet was washed (with 75% ethanol), dried, resuspended in UltraPure™ RNAse/DNase-free distilled water and DNAse treated (Ambion DNA-free kit; Ambion Inc., Austin, TX).

RNA quality evaluation. The RNA quantity, purity, and integrity were determined using a NanoDrop ND-1000 (NanoDrop Technologies Inc., Wilmington, DE) and the Agilent 2100

Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA). RNA samples were stored at -80 °C awaiting analysis. From the original set of samples, a subset of 10 animals per treatment was chosen based on RIN scores >7 for liver tissue and >6 for adipose tissue. After screening, average RIN scores for liver and adipose tissues were 7.50 ± 0.39 and 6.59 ± 0.58 respectively.

cDNA synthesis and qPCR performance. Genes selected for transcript profiling in liver tissue are associated with fatty acid oxidation (*PPARA, CPT1A, ACOX1*), growth hormone/IGF-1 axis (*GHR, IGF1, IGFALS, IGFBP1*), liver-secreted hepatokines (*FGF21, ANGPTL4*), ketogenesis (*HMGCS2*), lipoprotein metabolism (*MTTP, APOB*), gluconeogenesis (*PC, PCK1, PDK4*), inflammation mediators (*NFKB1, STAT3, XBP1*), and acute-phase proteins (*ALB, HP, SAA2, SAA3*). In adipose tissue, the selected genes are associated with fatty acid metabolism (*ADIPOQ*, *FASN*, *LEP*, *PPARA*, *PPARG*, *RXRA*), acute-phase proteins (*HP*, *SAA3*), and inflammation (*CCL2*, *CCL5*, *TLR4*, *IL6R*, *IL1B*, *IL6*, *TNFA*). Complete information about primer sequences and qPCR performance are reported in Suppl. Table A.1-4.

A portion of the RNA was diluted to 100 ng/μL with DNase/RNase-free water for cDNA synthesis through RT-PCR. To accommodate the analysis of all genes, per each sample cDNA was synthesized using 6 μL of diluted RNA, 6 μL of Random Primers (3 μg/μL; cat#11034731001, Roche) and 54 μL of DNase/RNase-free water. The mixture was incubated at 65°C for 5 min and kept on ice for 3 min. A total of 54 μL of master mix, composed of 24 μL of 5X First-Strand Buffer (cat#EP0441, Thermo Scientific), 6 μL of Oligo dT18 (Custom DNA Oligo Tubes, Integrated DNA Technologies), 12 μL of 10mM dNTP mix (cat#18427-088, Invitrogen), 1.5 μL of Reverse Transcriptase (200 U/μL as final concentration, cat#EP0441, Thermo Scientific), 0.75 μL of Rnase inhibitor (200 U/μL as final concentration, cat#EO0381, Thermo Scientific), and 9.75 μL of DNase/RNase-free water, was added. The reaction was

performed in an Eppendorf Mastercycler Gradient following the appropriate temperature program: 25°C for 5 min, 42°C for 60 min, and 70°C for 5 min. The cDNA was then diluted 1:4 (to a final volume of 480 μL) with DNase/RNase-free water, prior to Quantitative Polymerase Chain Reaction Analysis.

Quantitative PCR was performed using 4 μL diluted cDNA combined with 6 μL of a mixture composed of 5 μL SYBR Green master mix (cat#95073-05K, Quanta BioSciences), 0.4 μL each of 10 μM forward and reverse primers, and 0.2 μL DNase/RNase free water in a MicroAmp™ Optical 384-Well Reaction Plate (Applied Biosystems). Each sample was run in triplicate and a 7 point relative standard curve plus the non-template control were used. The reactions were performed in an ABI Prism 7900 HT SDS instrument using the following conditions: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C (denaturation), and 1 min at 60 °C (annealing + extension). The presence of a single PCR product was verified by the dissociation protocol using incremental temperatures to 95 °C for 15 s plus 65 °C for 15 s. Data were calculated with the 7900 HT Sequence Detection Systems Software. The final data were normalized using the geometric mean of three internal control genes (ICG): ACTB, GAPDH and RPS9 for adipose tissue (Ji et al., 2012; Khan et al., 2013), and GAPDH, RPS9 and UXT for liver tissue (Khan et al., 2014).

Suppl. Table A.1. Accession number, gene symbol, and forward and reverse primer sequences of genes analyzed in liver tissue.

Accession #	Symbol	Forward sequence	Reverse sequence
NM_001035289.3	*ACOX1*	ACCCAGACTTCCAGCATGAGA	TTCCTCATCTTCTGCACCATGA
BC151546.1	*ALB*	AGTGCTGCACAGAGTCATTGGT	GGCTTTGGGTACATATGTTTCATCA
DQ355521.1	*ANGPTL4*	AGGAAGAGGCTGCCCAAGAT	CCCTCTCTCCCTCTTCAAACAG
XM_005895759.1	*APOB*	CAAGGCTTTGTACTGGGTTAACG	ACCATGTCCTGCTCATGTTTATCA
FJ415874.1	*CPT1A*	TCGCGATGGACTTGCTGTATA	CGGTCCAGTTTGCGTCTGTA
XM_001789587.2	*FGF21*	CAGAGCCCCGAAAGTCTCTTG	AAAGTGCAGCGATCCGTACAG
NM_001034034.2	*GAPDH*	TGGAAAGGCCATCACCATCT	CCCACTTGATGTTGGCAG
JQ711177.1	*GHR*	TCCAGCCTCTGTTTCAGGAG	GCTGCCAGAGATCCATACCT
NM_001045883.1	*HMGCS2*	TTACGGGCCCTGGACAAAT	GCACATCATCGAGAGTGAAAGG
NM_001040470.2	*HP*	GGTTCGGAAAACCATCGCTA	CACTCGTGTCCCCTCCCTC
HQ324241.1	*IGF1*	CCAATTCATTTCCAGACTTTGCA	CACCTGCTTCAAGAAATCACAAAA
NM_001075963.1	*IGFALS*	GCTGGTCCTGGCAGGTAACA	TAGAGTTTCTGGAGCTTGGGCA
NM_174554.3	*IGFBP1*	CCTGCCAGCGAGAACTCTACA	TTGTTGCAGTTTGGCAGATAAAA
NM_001101834.1	*MTTP*	ACCAGGCTCATCAAGACAAAGTG	GTGACACCCAAGACCTGATGTG
BC153232.1	*NFKB1*	TTCAACCGGAGATGCCACTAC	ACACACGTAACGGAAACGAAATC
NM_177946.4	*PC*	GCAAGGTCCACGTGACTAAGG	GGCAGCACAGTGTCCTGAAG
JQ733516.1	*PCK1*	AAGATTGGCATCGAGCTGACA	GTGGAGGCACTTGACGAACTC
NM_001101883.1	*PDK4*	CAGGTGGACAGGGCAGTCTAG	TCCTCCTTTTCCATCTTTCTTCTTT
NM_001034036.1	*PPARA*	CATAACGCGATTCGTTTTGGA	CGCGGTTTCGGAATCTTCT
NM_001101152.2	*RPS9*	CCTCGACCAAGAGCTGAAG	CCTCCAGACCTCACGTTTGTTC
NM_001075260.2	*SAA2*	GACCAGTGCTCTCCTCCACTG	CCCCTTCATAAGCCTCACCAA
DQ298246.1	*SAA3*	GGGCATCATTTTCTGCTTCCT	TTGGTAAGCTCTCCACATGTCTTTAG
NM_001012671.2	*STAT3*	GGTAGCATGTGGGATGGTCTCT	GCATCCCTAGAAACTCTGGTCAA
BC108205.1	*UXT*	TGTGGCCCTTGGATATGGTT	GGTTGTCGCTGAGCTCTGTG
NM_001271737.1	*XBP1*	GAGAGCGAAGCCAATGTGGTA	ACTGTGAATTCAGGGTGATCTTTCT

Suppl. Table A.2. Accession number, gene symbol, and forward and reverse primer sequences of genes analyzed in adipose tissue.

Accession#	Symbol	Forward sequence	Reverse sequence
XM_005225005.1	*ACTB*	ACCAACTGGGACGACATGGA	GTCTCGAACATGATCTGGGTCAT
BC140488.1	*ADIPOQ*	GATCCAGGTCTTGTTGGTCCTAA	GAGCGGTATACATAGGCACTTTCTC
BC142093.1	*CCL2*	TCAACAGTAAGAAGATCTCCATGCA	CAGGACGGTCTTGAAAATCACA
BC102064.1	*CCL5*	CCCTGCTGCTTTGCCTATATCT	ATAAAGACAACTGCTGCCATGGA
AF285607.2	*FASN*	ACCTCGTGAAGGCTGTGACTCA	TGAGTCGAGGCCAAGGTCTGAA
NM_001040470.2	*HP*	GGTTCGGAAAACCATCGCTA	CACTCGTGTCCCCTCCCTC
EU276067.1	*IL1B*	TCCACCTCCTCTCACAGGAAA	TACCCAAGGCCACAGGAATCT
EU276071.1	*IL6*	CCAGAGAAAACCGAAGCTCTCAT	CCTTGCTGCTTTCACACTCATC
NM_001110785.1	*IL6R*	GCTCTTTCTACGTATTGTCCCTGTGT	GGGTCGGGCTGTAGGAGTTT
NM_173928.2	*LEP*	GGCTTTGGCCCTATCTGTCTTA	GAGACGGACTGCGTGTGTGA
NM_001034036.1	*PPARA*	CATAACGCGATTCGTTTTGGA	CGCGGTTTCGGAATCTTCT
NM_181024.2	*PPARG*	CCAAATATCGGTGGGAGTCG	ACAGCGAAGGGCTCACTCTC
XM_005213506.1	*RXRA*	ACGTCCTTGCTGTGTTTGAAAA	TGAACCAGATTGGTCTACTGAAGGT
DQ298246.1	*SAA3*	GGGCATCATTTTCTGCTTCCT	TTGGTAAGCTCTCCACATGTCTTTAG
DQ839567.1	*TLR4*	GCTGTTTGACCAGTCTGATTGC	GGGCTGAAGTAACAACAAGAGGAA
NM_173966.3	*TNF*	CCAGAGGGAAGAGCAGTCCC	TCGGCTACAACGTGGGCTAC

Suppl. Table A.3. qPCR performance of genes measured in adipose tissue.

Gene	Median Ct[1]	Median ΔCt[2]	Slope[3]	(R^2)[4]	Efficiency[5]
ADIPOQ	19.041	-2.884	-3.158	0.996	2.073
CCL2	24.127	2.202	-2.950	0.990	2.183
CCL5	28.851	6.926	-3.258	0.988	2.027
FASN	25.073	3.148	-3.246	0.998	2.033
HP	23.851	1.922	-3.248	0.993	2.032
IL1B	26.699	4.774	-2.960	0.984	2.176
IL6	28.168	6.245	-2.975	0.980	2.168
IL6R	23.240	1.316	-3.256	0.991	2.028
LEP	29.284	7.360	-3.383	0.980	1.975
PPARA	26.544	4.619	-2.980	0.974	2.165
PPARG	22.121	0.189	-2.980	0.994	2.165
RXRA	21.610	-0.315	-2.942	0.990	2.187
SAA3	26.765	4.840	-2.982	0.992	2.164
TLR4	24.466	2.543	-3.057	0.991	2.124
TNF	29.777	7.861	-2.953	0.982	2.180

[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 [$10^{(-1 / \text{Slope})}$].

Suppl. Table A.4. qPCR performance of genes measured in liver.

Gene	Median Ct[1]	Median ΔCt[2]	Slope[3]	(R^2)[4]	Efficiency[5]
ACOX1	19.392	-1.593	-3.407	0.996	1.966
ALB	13.056	-7.929	-3.330	0.997	1.997
ANGPTL4	25.280	4.295	-3.286	0.991	2.015
APOB	20.215	-0.770	-3.731	0.990	1.854
CPT1A	22.335	1.350	-3.388	0.993	1.973
FGF21	23.763	2.778	-3.173	0.992	2.066
GHR	21.726	0.741	-3.509	0.993	1.927
HMGSC2	19.260	-1.725	-3.549	0.992	1.913
HP	21.985	1.000	-3.700	0.991	1.863
IGF1	24.938	3.953	-2.911	0.920	2.206
IGFALS	23.302	2.317	-3.695	0.991	1.865
IGFBP1	18.343	-2.642	-3.695	0.984	1.865
MTTP	21.590	0.605	-3.197	0.991	2.055
NFKB1	24.243	3.258	-3.240	0.994	2.035
PC	22.532	1.547	-3.452	0.981	1.948
PCK1	19.177	-1.808	-3.663	0.992	1.875
PDK4	24.765	3.780	-3.474	0.987	1.940
PPARA	23.943	2.958	-3.073	0.986	2.116
SAA2	19.921	-1.064	-3.422	0.993	1.960
SAA3	23.894	2.909	-3.148	0.994	2.078
STAT3	23.087	2.102	-3.412	0.991	1.964
XBP1	19.422	-1.563	-3.616	0.992	1.890

[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 [$10^{(-1 / \text{Slope})}$].

REFERENCES

Ji, P., JS Osorio, J. K. Drackley, and J. J. Loor. 2012. Overfeeding a moderate energy diet prepartum does not impair bovine subcutaneous adipose tissue insulin signal transduction and induces marked changes in peripartal gene network expression. Journal of dairy science 95:4333-4351.

Khan, M. J., C. B. Jacometo, D. E. Graugnard, M. N. Corrêa, E. Schmitt, F. Cardoso, and J. J. Loor. 2014. Overfeeding dairy cattle during late-pregnancy alters hepatic PPARα-regulated pathways including hepatokines: Impact on metabolism and peripheral insulin sensitivity. Gene regulation and systems biology. 2014 Apr 3;8:97-111.

APPENDIX B

CHAPTER 4 - SUPPLEMENTAL MATERIAL

RNA Extraction. Adipose tissue was weighted (~0.2 g), immediately placed in 1.2 mL of QIAzol Lysis Reagent (cat#79306, Qiagen) and homogenized using a Mini-Beadbeater-24 (cat#112011, Biospec Products Inc.) with two 30 s cycles, and a 1 min incubation on ice in between the cycles. Samples were then centrifuged for 10 min at 12,000 g and 4 °C, and the supernatant was transfer in a separate tube and mix with 240 µL of Chloroform (cat#C298, Fisher Chemical). After centrifugation for 15 min at 12,000 g and 4 °C, the aqueous phase was transferred to a new tube, mixed with 900 µL of 100% Ethanol (cat#2701, Decon Labs), and total RNA was cleaned using miRNeasy mini kit columns (cat# 217004, Qiagen) following manufacture protocol. During purification, genomic DNA was removed using the RNase-Free DNase Set (cat# 79254, Qiagen).

RNA Quality Evaluation. The RNA quantity, purity, and integrity were determined using a NanoDrop ND-1000 (cat#E112352, NanoDrop Technologies Inc.) and the Agilent 2100 Bioanalyzer (cat#G2939AA, Agilent Technologies Inc.). Samples had an average RIN score of 6.5 ± 0.8. RNA samples were stored at -80 °C until analysis.

mRNA cDNA Synthesis and qPCR Performance. Genes selected for transcript profiling are associated with fatty acid metabolism: fatty acid synthase (*FASN*), peroxisome proliferator-activated receptor gamma (*PPARG*); adipokines: adiponectin (*ADIPOQ*), leptin (*LEP*); and inflammation: chemokine (C-C motif) ligand 2 (*CCL2*), chemokine (C-C motif) ligand 5 (*CCL5*), haptoglobin (*HP*), interleukin-1β (*IL1B*), inteleukin-6 (*IL6*), inteleukin-6 receptor

(*IL6R*), retinoid X receptor alpha (*RXRA*), serum amyloid A3 (*SAA3*), toll-like receptor 4 (*TLR4*), and tumor necrosis factor α (*TNF*). Complete information about primer sequences and qPCR performance are reported in the supplemental file (Suppl. Tab. B.1 and 3). A portion of the RNA was diluted to 100 ng/μL with DNase/RNase-free water for cDNA synthesis through RT-PCR. Per each sample, 4 μL of diluted RNA, 5 μL of Random Primers (3 μg/μL; cat#11034731001, Roche) and 45 μL of DNase/RNase-free water. The mixture was incubated at 65°C for 5 min and kept on ice for 3 min. A total of 36 μL of master mix, composed of 20 μL of 5X First-Strand Buffer (cat#EP0441, Thermo Scientific), 5 μL of Oligo dT18 (Custom DNA Oligo Tubes, Integrated DNA Technologies), 10 μL of 10mM dNTP mix (cat#18427-088, Invitrogen), 1.25 μL of Reverse Transcriptase (200 U/μL as final concentration, cat#EP0441, Thermo Scientific), 0.625 μL of Rnase inhibitor (200 U/μL as final concentration, cat#EO0381, Thermo Scientific), and 8.125 μL of DNase/RNase-free water, was added. The reaction was performed in an Eppendorf Mastercycler Gradient following the appropriate temperature program: 25°C for 5 min, 42°C for 60 min, and 70°C for 5 min. The cDNA was then diluted 1:4 with DNase/RNase-free water, prior to quantitative PCR analysis.

Quantitative PCR was performed using 4 μL diluted cDNA combined with 6 μL of a mixture composed of 5 μL SYBR Green master mix (cat#95073-05K, Quanta BioSciences), 0.4 μL each of 10 μM forward and reverse primers, and 0.2 μL DNase/RNase free water in a MicroAmp™ Optical 384-Well Reaction Plate (Applied Biosystems). Each sample was run in triplicate and a 7 point relative standard curve plus the non-template control were used. The reactions were performed in an ABI Prism 7900 HT SDS instrument using the following conditions: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C (denaturation), and 1 min at 60 °C (annealing + extension). The presence of a single PCR product was verified by the dissociation protocol using

incremental temperatures to 95 °C for 15 s plus 65 °C for 15 s. Data were calculated with the 7900 HT Sequence Detection Systems Software. The final data were normalized using the geometric mean of three internal control genes (ICG): *ACTB*, *GAPDH* and *RPS9* (Khan et al., 2014, Vailati Riboni et al., 2015).

microRNA cDNA Synthesis and qPCR Performance. miRNA selected for expression profiling are associated with immune cell infiltration (miR-26b, miR-126, miR-132, miR-155, miR-193), inflammation and lipolysis (miR-99a, miR-145, miR-221), and positive regulation of adipogenesis (miR-103, miR-143, miR-378). Complete information about primer sequences and qPCR performance are reported in the supplemental file (Suppl. Tab. 2 and 4). microRNA-specific cDNA synthesis was performed starting from 5 μL of 100 ng/ μL diluted RNA, using the Quanta qScript microRNA cDNA Synthesis Kit (cat# 95107, Quanta BioSciences, Inc.), following the manufacturer's protocols. The cDNA was diluted 1:4 with DNase/RNase-free water, and quantitative PCR analysis was performed according to the Quanta qScript kit protocol using SybrGreen, the specific primers and a universal primer provided with the kit. Each sample was run in triplicate and a 7 point relative standard curve plus the non-template control were used. The reactions were performed in an ABI Prism 7900 HT SDS instrument. Data were calculated with the 7900 HT Sequence Detection Systems Software. The final data were normalized using the geometric mean of, miR-let-7b, miR-16b and miR-181a, which served as the internal controls.

Suppl. Table B.1. Accession number, gene symbol, and forward and reverse primer sequences of genes analyzed in adipose tissue.

Accession#	Symbol	Forward sequence	Reverse sequence
BC140488.1	*ADIPOQ*	GATCCAGGTCTTGTTGGTCCTAA	GAGCGGTATACATAGGCACTTTCTC
NM_174006.2	*CCL2*	TCAACAGTAAGAAGATCTCCATGCA	CAGGACGGTCTTGAAAATCACA
NM_175827.2	*CCL5*	CCCTGCTGCTTTGCCTATATCT	ATAAAGACAACTGCTGCCATGGA
NM_001012669.1	*FASN*	ACCTCGTGAAGGCTGTGACTCA	TGAGTCGAGGCCAAGGTCTGAA
NM_001040470.2	*HP*	GGTTCGGAAAACCATCGCTA	CACTCGTGTCCCCTCCCTC
NM_174093.1	*IL1B*	TCCACCTCCTCTCACAGGAAA	TACCCAAGGCCACAGGAATCT
NM_173923	*IL6*	CCAGAGAAAACCGAAGCTCTCAT	CCTTGCTGCTTTCACACTCATC
NM_001110785.1	*IL6R*	GCTCTTTCTACGTATTGTCCCTGTGT	GGGTCGGGCTGTAGGAGTTT
NM_173928.2	*LEP*	GGCTTTGGCCCTATCTGTCTTA	GAGACGGACTGCGTGTGTGA
NM_181024.2	*PPARG*	CCAAATATCGGTGGGAGTCG	ACAGCGAAGGGCTCACTCTC
NM_001304343.1	*RXRA*	ACGTCCTTGCTGTGTTTGAAAA	TGAACCAGATTGGTCTACTGAAGGT
NM_181016.3	*SAA3*	GGGCATCATTTTCTGCTTCCT	TTGGTAAGCTCTCCACATGTCTTTAG
NM_174198.6	*TLR4*	GCTGTTTGACCAGTCTGATTGC	GGGCTGAAGTAACAACAAGAGGAA
NM_173966.3	*TNF*	CCAGAGGGAAGAGCAGTCCC	TCGGCTACAACGTGGGCTAC

Suppl. 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-26b	TTCAAGTAATTCAGGATAGGTT
miR-99a	AACCCGTAGATCCGATCTTGT
miR-103	AGCAGCATTGTACAGGGCTATGA
miR-126	CGTACCGTGAGTAATAATGCG
miR-132	TAACAGTCTACAGCCATGGTCG
miR-143	TGAGATGAAGCACTGTAGCTCG
miR-145	GTCCAGTTTTCCCAGGAATCCCT
miR-155	TTAATGCTAATCGTGATAGGGGT
miR-193	AACTGGCCCACAAAGTCCCGCTTT
miR-221	AGCTACATTGTCTGCTGGGTTT
miR-378	ACTGGACTTGGAGTCAGAAGGC

Suppl. Table B.3. qPCR performance of measured genes.

Gene	Median Ct[1]	Median ΔCt[2]	Slope[3]	(R^2)[4]	Efficiency[5]
ADIPOQ	18.11	-0.75	-3.404	0.997	1.967
CCL2	24.27	5.44	-3.127	0.997	2.088
CCL5	26.76	8.10	-3.321	0.995	2.001
FASN	21.31	2.26	-3.334	0.994	1.995
HP	23.87	4.69	-3.398	0.996	1.969
IL1B	27.07	8.28	-3.297	0.988	2.011
IL6	28.64	9.86	-3.330	0.964	1.997
IL6R	22.40	3.66	-3.490	0.993	1.934
LEP	24.44	5.47	-3.027	0.996	2.140
PPARG	21.48	2.55	-3.489	0.993	1.935
RXRA	21.58	2.73	-3.402	0.996	1.968
SAA3	26.37	7.76	-3.080	0.992	2.112
TLR4	24.88	5.86	-3.168	0.994	2.069
TNF	29.42	10.62	-3.293	0.974	2.012

[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 $[10^{(-1 / \text{Slope})}]$.

Suppl. Table B.4. qPCR performance of measured miRNAs.

Gene	Median Ct[1]	Median ΔCt[2]	Slope[3]	(R^2)[4]	Efficiency[5]
miR-26b	18.37	1.37	-3.08	0.992	2.113
miR-99a	14.34	-2.43	-3.28	0.998	2.019
miR-103	21.19	4.43	-3.31	0.994	2.007
miR-126	18.37	1.92	-3.29	0.996	2.015
miR-132	17.47	1.17	-3.17	0.998	2.069
miR-143	16.92	0.22	-3.26	0.998	2.028
miR-145	19.16	2.45	-3.20	0.998	2.053
miR-155	21.61	4.82	-3.22	0.995	2.044
miR-193	18.14	1.62	-3.39	0.999	1.972
miR-221	17.86	1.13	-3.28	0.996	2.018
miR-378	21.25	4.34	-3.25	0.999	2.030

[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 $[10^{(-1 / \text{Slope})}]$.

REFERENCES

Khan, M. J., C. B. Jacometo, D. E. Graugnard, M. N. Corrêa, E. Schmitt, F. Cardoso, and J. J. Loor. 2014. Overfeeding dairy cattle during late-pregnancy alters hepatic PPARα-regulated pathways including hepatokines: Impact on metabolism and peripheral insulin sensitivity. Gene regulation and systems biology. 2014 Apr 3;8:97-111

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. Journal of dairy science 98(5):3079-3085.

APPENDIX C

CHAPTER 5 – SUPPLEMENTAL MATERIAL

Supplemental file 1. Provides detailed and complete information about the protocol used during the microarray and qPCR procedures, and blood collection and analysis. Moreover, it provides additional discussion points, omitted from the main body of the manuscript.

SUPPLEMENTAL MATERIAL AND METHODS

RNA extraction

Liver tissue (30-50 mg) was homogenized in a TissueLyser II (Qiagen GmbH) for 2 × 2 min bursts at 30 Htz. Total RNA and DNA were extracted using a Qiagen AllPrep DNA/RNA mini kit (Qiagen GmbH) as per manufacturer's instructions and RNA was treated with DNase (Ambion DNA-free kit; Ambion Inc., Austin, TX). The RNA quantity, purity, and integrity were determined using a NanoDrop ND-1000 (NanoDrop Technologies Inc., Wilmington, DE) and the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA). Average RNA Integrity Number (**RIN**) for samples at -7, 7, and 28 d relative to parturition were 8.18 (± 0.39), 7.77 (± 0.43), and 8.17 (± 0.45), respectively. RNA samples were stored at -80 °C until analysis.

Microarrays

cRNA Synthesis, Labeling, and Purification. The Agilent platform was chosen to conduct the microarray experiment, using the 44K Bovine (V2) gene expression microarray chip (Agilent Technologies Inc.), following the manufacturer's protocols. Briefly, a total of 200 ng of RNA per sample were used to generate first-strand cDNA, which was reverse transcribed to cRNA using the low-input quick amp labeling kit (Agilent Technologies Inc). The resulting cRNA was labeled with either Cy3 or Cy5 fluorescent dye, purified using

RNeasy mini spin columns (Qiagen), and subsequently eluted in 30 μL of DNase-RNase-free water. The NanoDrop ND-1000 (Thermo Fisher Scientific Inc., Waltham, MA) was used to confirm the manufacturer's recommended criteria for yield and specific activity of at least 0.825 μg and ≥6.

Hybridization and Scanning. The labeled cRNA was fragmented and then hybridized to the microarray slide according to manufacturer's protocol. Briefly, 825 ng of Cy3 and Cy5 labeled cRNA sample were combined, mixed with 11 μL of 10× Blocking Agent (Agilent Technologies Inc.), 2.2 μL of 25× Fragmentation Buffer (Agilent Technologies Inc.), and nuclease-free water (to a final volume of 55 μL); and then fragmented at 60°C for 30 s. The reaction was then stopped by adding 55 μL of 2× GEx Hybridization Buffer (Agilent Technologies Inc.), and the samples were loaded onto the slide. These were hybridized in a rotating hybridization oven (Agilent Technologies Inc.) at 65°C for 17 h. The slides were washed according to the manufacturer's recommended procedures and scanned using a GenePix 4000B scanner (Axon Instruments Inc., Sunnyvale, CA) and GenePix Pro v.6.1 software. Resulting spots where features were substandard were flagged as bad and excluded from subsequent analysis.

qPCR Validation

cDNA synthesis. A portion of the RNA was diluted to 100 ng/μL with DNase/RNase-free water for cDNA synthesis through RT-PCR. Per each sample, 4 μL of diluted RNA, 5 μL of Random Primers (3 μg/μL; cat#11034731001, Roche) and 45 μL of DNase/RNase-free water. The mixture was incubated at 65°C for 5 min and kept on ice for 3 min. A total of 36 μL of master mix, composed of 20 μL of 5X First-Strand Buffer (cat#EP0441, Thermo Scientific), 5 μL of Oligo dT18 (Custom DNA Oligo Tubes, Integrated DNA Technologies), 10 μL of

10mM dNTP mix (cat#18427-088, Invitrogen), 1.25 μL of Reverse Transcriptase (200 U/μL as final concentration, cat#EP0441, Thermo Scientific), 0.625 μL of Rnase inhibitor (200 U/μL as final concentration, cat#EO0381, Thermo Scientific), and 8.125 μL of DNase/RNase-free water, was added. The reaction was performed in an Eppendorf Mastercycler Gradient following the appropriate temperature program: 25°C for 5 min, 42°C for 60 min, and 70°C for 5 min. The cDNA was then diluted 1:4 with DNase/RNase-free water, prior to quantitative PCR analysis.

Real Time qPCR. Primer couples were designed using NCBI Primer-BLAST toll, and tested through normal PCR, using the same thermo cycle as the final qPCR analysis, and gel electrophoresis to certify the presence of a single PCR product of the expected size. The product was then purified and send for sequencing at the UIUC Core Sequencing Facility, to asses amplification of the correct target. Complete details on primer couples and amplification products sequences can be found in Supplemental Table C.8. Quantitative PCR was performed using 4 μL diluted cDNA combined with 6 μL of a mixture composed of 5 μL SYBR Green master mix (cat#95073-05K, Quanta BioSciences), 0.4 μL each of 10 μM forward and reverse primers, and 0.2 μL DNase/RNase free water in a MicroAmp™ Optical 384-Well Reaction Plate (Applied Biosystems). Each sample was run in triplicate and a 7 point relative standard curve plus the non-template control were used. The reactions were performed in an ABI Prism 7900 HT SDS instrument using the following conditions: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C (denaturation), and 1 min at 60 °C (annealing + extension). The presence of a single PCR product was verified by the dissociation protocol using incremental temperatures to 95 °C for 15 s plus 65 °C for 15 s. Data were calculated with the 7900 HT Sequence Detection Systems Software. The final data were normalized using the geometric

mean of three internal control genes (ICG): *GAPDH*, *RPS9*, and *UXT*. Details on qPCR performances can be found in Supplemental Table C.9.

Blood Collection and analysis

Blood was sampled by coccygeal venipuncture using evacuated blood tubes containing a lithium heparin anticoagulant. Samples were placed immediately on ice and centrifuged within 30 min at 1,500 × *g* for 12 min at 4°C. Following centrifugation, aspirated plasma was stored at −20°C until assayed.

Blood NEFA and BHBA analysis was performed by Gribbles Veterinary Pathology Ltd. Blood metabolites were assayed using colorimetric techniques at 37°C with a Hitachi Modular P800 analyzer (Roche Diagnostics, Indianapolis, IN). Plasma NEFA concentration (mmol/L) was measured using Wako Chemicals (Osaka, Japan) kit NEFA HR2 measuring oxidative condensation of 3-methyl-*N*-ethyl-*N*-β hydroxyethyl aniline with 4-aminoantipyrine, while plasma BHBA (mmol/L) concentrations was assessed using Roche reagent kits measuring the reduction of NAD to NADH during oxidation of d-3-hydroxybutyrate to acetoacetate.

SUPPLEMENTAL DISCUSSION

B4F125 vs B4F75

Lipid metabolism. Lipid metabolism in thinner cows was much more impacted by prepartum feeding management, with a distinct postpartum activation of every pathway among the top 25 impacted when animals were fed 125% compared with 75% of requirements. Biosynthetic pathways rather than catabolic ones were impacted around parturition. In fact, out of the five most-impacted pathways in this category, three are concerned with the biosynthesis

of fatty acids, unsaturated fatty acids, and their elongation in the mitochondria. Despite the lack of change in blood NEFA (Table 5.2), this response could be taken as an indication of lower postpartum mobilization of body reserves in BCS4 animals fed 125% than 75%. This idea agrees with the fact that overfeeding energy during the dry period also increases the concentration of plasma insulin, hence, preventing excessive adipose tissue lipolysis (Ji et al., 2012). We further speculate that a possible greater postpartum mobilization in B4F75 cows could increase the hepatic uptake of fatty acids, consequently inhibiting the biosynthetic pathways, which is a well-established effect in non-ruminant liver. Additionally, NEFA values are often quite variable, especially as a function of meal patterns (Allen et al., 2005, Nakamura et al., 2014). Hepatic transcriptome alterations, in comparison, reflect longer-term regulatory responses to changes in BCS and nutrition.

Among the non-biosynthetic pathways, 'Glycerolipid metabolism' was impacted and activated at 7 d postpartum in cows fed 125% of requirements. Because this pathway is involved in the synthesis of triacylglycerol (**TAG**) from free fatty acids, its activation could be a consequence of an increased flux of NEFA or could suggest a better 'handling' of the hepatic NEFA flux. Among the top DEG, the expression of *DGAT1* (diacylglycerol O-acyltransferase 1) had a fold change of + 4.5 in B4F125 cows. As the protein product of this gene catalyzes the final step of TAG synthesis, and the fact that BCS4 cows had an overall lower plasma concentration of NEFA (Table 5.2), the greater *DGAT1* expression indicates a faster handling of mobilized fatty acids, avoiding accumulation in the liver that could lead to partial oxidation and ketogenesis.

Also of great interest, is the activation of the 'α-Linolenic acid metabolism' in the first week after parturition combined with activation of the 'Biosynthesis of unsaturated fatty acids'

in lean animals fed 125%. The α-linolenic fatty acid (C18:3) is part of both pathways, being partially oxidized for energy in the first or converted to eicosapentaenoic acid (**EPA**) and docosahexaenoic acid (**DHA**) in the second. These two ω-3 fatty acids elicit numerous effects on functional responses of cells involved in inflammation and immunity (Calder, 2013), all of which have been interpreted in the context of reducing inflammation, i.e. they are beneficial during inflammatory conditions (Calder, 2006). The concurrent increase in activity of the two pathways leads us to hypothesize a greater availability of C18:3 at the hepatic level (because its metabolism was increased), for synthesis of EPA or DHA and, hence, an improvement of the immunological status of the cow. This scenario would be beneficial as cows always experience an inflammatory state after parturition that can impair production and reproduction, and predispose to disease (Bertoni et al., 2008).

B5F125 vs B5F75

Lipid metabolism. The pathways of 'Lipid metabolism' impacted in optimally-conditioned cows (BCS5) mainly concerned pathways of utilization rather than synthesis (as detected in the BCS4 group), and they were mostly impacted in early lactation (7 d). 'Sphingolipid metabolism' and 'Arachidonic acid metabolism' were both impacted, being activated in B5F125 compared with B5F75 cows. However, because flux in 'sphingolipid metabolism' was close to zero (-0.58), we considered the pathway impacted and induced in both groups (B5F75 and B5F125). This pathway is associated with the production of ceramides, a lipid species that exerts biological effects through cellular proliferation, differentiation, and cell death, and interacts with several pathways involved in insulin resistance, oxidative stress, inflammation, and apoptosis, all of which are linked to non-alcoholic fatty liver (Pagadala et al., 2012).

The fact that ‘sphingolipid metabolism’ was unequivocally activated early postpartum in BCS5 cows compared with BCS4 cows, regardless of feeding management, highlights how underconditioned animals should be less susceptible to liver TAG accumulation. In a recent study, it was reported that plasma concentrations of total ceramide and monohexosylceramide increase as lactation approaches, with greater concentrations in cows exhibiting higher adiposity a month prior to parturition relative to those with a lean phenotype (Rico et al., 2015). Furthermore, changes in plasma ceramide concentration occurred concomitantly with changes in plasma NEFA, insulin, and insulin sensitivity, leading the authors to speculate that circulating ceramides may be fundamentally involved in the homeorhetic adaptation to early lactation (Rico et al., 2015).

The activation of ‘Arachidonic acid metabolism’ in BCS5 animals fed 75% compared with 125% of requirements indicated some degree of inflammation in the former. Arachidonic acid metabolites can have both pro- and anti-inflammatory effects (Samuelsson, 1991). However, among the top DEG at 7 d postpartum with FC ≥ |3| (Supplemental Table C.5), the pro-inflammatory pathways seem to prevail, e.g. *ALOX12* was upregulated in B5F75 cows. This enzyme catalyzes the conversion of arachidonic acid to 12-hydroxyeicosatetraenoic acid (12-HETE), a chemotactic compound in human neutrophils (Goetzl and Pickett, 1980). If biologically-active, this compound could initiate a local inflammatory response that might impair liver function. Activation of inflammatory-related hepatic functions also have been detected in feed-restricted compared with overfed cows (Shahzad et al., 2014); however, despite blood biomarkers revealing greater distress in liver and a low-grade inflammatory response in feed-restricted cows prepartum, overfed cows postpartum had greater bilirubinemia and hepatic lipid accumulation leading authors to conclude that feed restriction served to

"prime" the liver to better handle the postpartal metabolic and inflammatory challenges (Shahzad et al., 2014).

The 'Glycerophospholipid metabolism' pathway was impacted and activated prepartum in B5F125 compared with B5F75. One of the central metabolites of the pathway is phosphatidylcholine, the main building block of cellular membranes that are components of lipoproteins. Lecithin also is secreted in bile to increase emulsification of micelles during intestinal lipid absorption. Even if in low amounts, the greater pasture allowance prepartum in B5F125 could have led to a greater amount of lipid ingestion and the induction of phosphatidylcholine production through the 'glycerophospholipid metabolism' to aid in absorption and repackaging of fatty acids in the liver (Bauchart, 1993).

Suppl. Table C.1. Differentially expressed genes at -7 days from parturition with fold change (FC) ≤ −3 or ≥ +3 in liver of animals with BCS 4 fed either 125 (B4F125) compared with 75 (B4F75) % of requirement for the last 3 weeks before parturition.

Gene	Description	FC at -7 d
Upregulated		
LOC788413	olfactory receptor, family 10, subfamily C, member 1-like	11,16
PTPRU	protein tyrosine phosphatase, receptor type, U	4,81
KIAA1045	KIAA1045 ortholog	4,66
OBSCN	obscurin, cytoskeletal calmodulin and titin-interacting RhoGEF	4,45
ARHGAP21	Rho GTPase activating protein 21	4,35
AGR2	anterior gradient homolog 2	4,25
ZNF445	zinc finger protein 445	4,21
LOC100137795	uncharacterized LOC100137795	4,20
SPATA2	spermatogenesis associated 2	4,00
KIAA0319L	KIAA0319-like ortholog	3,87
DCP1A	DCP1 decapping enzyme homolog A	3,84
GTF2A1	general transcription factor IIA, 1, 19/37kDa	3,81
ANKRD34B	ankyrin repeat domain 34B	3,78
PTPN4	protein tyrosine phosphatase, non-receptor type 4	3,77
RNPEP	arginyl aminopeptidase	3,39
PLCL2	phospholipase C-like 2	3,34
BDA20	major allergen BDA20	3,25
C25H16orf45	chromosome 25 open reading frame, human C16orf45	3,08
MBD1	methyl-CpG binding domain protein 1	3,02
Downregulated		
MAU2	MAU2 chromatid cohesion factor homolog	-7,61
ZNF236	zinc finger protein 236	-7,49
LRRC49	leucine rich repeat containing 49	-5,34
AQP6	aquaporin 6	-4,85
CACNB2	calcium channel, voltage-dependent, beta 2 subunit	-4,56
LOC789904	ribosomal protein L7a-like	-4,30
FGF11	fibroblast growth factor 11	-3,97
SSPO	SCO-spondin homolog	-3,95
LRRTM3	leucine rich repeat transmembrane neuronal 3	-3,59
CDH7	cadherin 7, type 2	-3,59
LOC524560	olfactory receptor, family 10, subfamily H, member 1-like	-3,50
SASH3	SAM and SH3 domain containing 3	-3,49
EZH2	enhancer of zeste homolog 2	-3,39
C2CD4D	C2 calcium-dependent domain containing 4D	-3,36
KCNG3	potassium voltage-gated channel, subfamily G, member 3	-3,31
PTH2R	parathyroid hormone 2 receptor	-3,19
KCTD1	potassium channel tetramerization domain containing 1	-3,11

Suppl. Table C.1 (cont)

HIATL1	hippocampus abundant transcript-like 1	-3,11
RHOF	ras homolog gene family, member F	-3,05
PDLIM7	PDZ and LIM domain 7	-3,03
MS4A18	membrane-spanning 4-domains, subfamily A, member 18	-3,03
WDR87	WD repeat domain 87	-3,01

Suppl. Table C.2. Differentially expressed genes at +7 days from parturition with fold change (FC) ≤ −3 or ≥ +3 in liver of animals with BCS 4 fed either 125 (B4F125) compared with 75 (B4F75) % of requirement for the three weeks before parturition.

Gene	Description	FC at +7 d
Upregulated		
SOSTDC1	sclerostin domain containing 1	12,07
MYOC	myocilin, trabecular meshwork inducible glucocorticoid response	7,45
MBOAT2	membrane bound O-acyltransferase domain containing 2	6,94
SYT15	synaptotagmin XV	6,71
HSFY2	heat shock transcription factor, Y linked 2	6,23
GABRA6	gamma-aminobutyric acid (GABA) A receptor, alpha 6	5,69
GAD2	glutamate decarboxylase 2	5,69
LOC530773	germinal histone H4-like	5,54
GALNT2	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 2 (GalNAc-T2)	5,41
FSCN3	fascin homolog 3, actin-bundling protein, testicular	5,16
SLC24A6	solute carrier family 24 (sodium/potassium/calcium exchanger), member 6	4,86
ANAPC15	anaphase promoting complex subunit 15	4,83
SLC16A14	solute carrier family 16, member 14 (monocarboxylic acid transporter 14)	4,61
DGAT1	diacylglycerol O-acyltransferase 1	4,50
HS3ST2	heparan sulfate (glucosamine) 3-O-sulfotransferase 2	4,26
EIF5A	eukaryotic translation initiation factor 5A	4,24
AARS	alanyl-tRNA synthetase	4,19
GRM1	glutamate receptor, metabotropic 1	4,06
CRLF2	cytokine receptor-like factor 2	4,04
GOLGA1	golgin A1	3,98
PALM2	paralemmin 2	3,94
TRIM63	tripartite motif containing 63	3,89
TCRA	T cell receptor, alpha	3,88
GJD4	gap junction protein, delta 4, 40.1kDa	3,87
HRH4	histamine receptor H4	3,84
MGAT5	mannosyl (alpha-1,6-)-glycoprotein beta-1,6-N-acetyl-glucosaminyltransferase	3,84
MUC13	mucin 13, cell surface associated	3,83
NFE2L1	nuclear factor (erythroid-derived 2)-like 1	3,79
MAK	male germ cell-associated kinase	3,74
LOC783998	olfactory receptor, family 11, subfamily G, member 2-like	3,69
TTLL6	tubulin tyrosine ligase-like family, member 6	3,69
HERC2	hect domain and RLD 2	3,68
ATG16L1	ATG16 autophagy related 16-like 1	3,64
CACNA1A	calcium channel, voltage-dependent, P/Q type, alpha 1A subunit	3,62
TMEM160	transmembrane protein 160	3,57
FAM84B	family with sequence similarity 84, member B	3,48

Suppl. Table C.2. (cont)

ASXL3	additional sex combs like 3	3,48
CELF4	CUGBP, Elav-like family member 4	3,46
LRRC73	leucine rich repeat containing 73	3,43
LBH	limb bud and heart development	3,42
OR52W1	olfactory receptor, family 52, subfamily W, member 1	3,42
TLR7	toll-like receptor 7	3,41
RPRD2	regulation of nuclear pre-mRNA domain containing 2	3,41
KCNIP1	Kv channel interacting protein 1	3,39
SP4	Sp4 transcription factor	3,35
LOC615782	PWWP domain-containing protein MUM1L1	3,34
TTLL8	tubulin tyrosine ligase-like family, member 8	3,32
MAST1	microtubule associated serine/threonine kinase 1	3,32
NKIRAS2	NFKB inhibitor interacting Ras-like 2	3,31
MARCH10	membrane-associated ring finger (C3HC4) 10, E3 ubiquitin protein ligase	3,31
SLC14A1	solute carrier family 14 (urea transporter), member 1	3,31
THBS2	thrombospondin 2	3,29
ZFYVE20	zinc finger, FYVE domain containing 20	3,29
PPP1R9A	protein phosphatase 1, regulatory subunit 9A	3,29
ENTPD6	ectonucleoside triphosphate diphosphohydrolase 6	3,27
LOC614895	olfactory receptor, family 2, subfamily T, member 27-like	3,26
DDX4	DEAD (Asp-Glu-Ala-Asp) box polypeptide 4	3,22
SLC8A1	solute carrier family 8 (sodium/calcium exchanger), member 1	3,21
SLC18A2	solute carrier family 18 (vesicular monoamine), member 2	3,20
WDR16	WD repeat domain 16	3,19
SLC6A9	solute carrier family 6 (neurotransmitter transporter, glycine), member 9	3,16
IPO4	importin 4	3,15
SLC22A17	solute carrier family 22, member 17	3,15
KLK4	kallikrein-related peptidase 4	3,14
VWA2	von Willebrand factor A domain containing 2	3,14
KCNT1	potassium channel, subfamily T, member 1	3,13
ABCA13	ATP-binding cassette, sub-family A (ABC1), member 13	3,13
ACTR3B	ARP3 actin-related protein 3 homolog B	3,12
SSTR2	somatostatin receptor 2	3,10
COL4A6	collagen, type IV, alpha 6	3,09
TXNDC8	thioredoxin domain containing 8	3,09
MARCH4	membrane-associated ring finger (C3HC4) 4, E3 ubiquitin protein ligase	3,08
WC1.3	WC1.3 molecule	3,07
MYH9	myosin, heavy chain 9, non-muscle	3,06
NLN	neurolysin (metallopeptidase M3 family)	3,06
APLP2	amyloid beta (A4) precursor-like protein 2	3,05
LOC100847827	transcription elongation regulator 1-like protein-like	3,05

Suppl. Table C.2. (cont)

DOPEY1	dopey family member 1	3,02
ATP5SL	ATP5S-like	3,02
NDP	Norrie disease	3,01
BTN1A1	butyrophilin, subfamily 1, member A1	3,01
CALM	calmodulin-like	3,01
TNR	tenascin R	3,00
LOC100125776	olfactory receptor	3,00
Downregulated		
ASPM	asp (abnormal spindle) homolog, microcephaly associated	-9,75
BUB1	budding uninhibited by benzimidazoles 1 homolog	-8,29
O3FAR1	omega-3 fatty acid receptor 1	-7,98
HIP1	huntingtin interacting protein 1	-6,12
GLRA4	glycine receptor, alpha 4	-5,88
LGSN	lengsin, lens protein with glutamine synthetase domain	-5,60
TMEM169	transmembrane protein 169	-5,50
CCK	cholecystokinin	-5,36
GPX3	glutathione peroxidase 3	-5,25
INS	insulin	-5,24
CAMKK1	calcium/calmodulin-dependent protein kinase kinase 1, alpha	-5,18
LOC787081	UPF0632 protein A	-5,13
KIF4A	kinesin family member 4A	-4,88
DAB2IP	DAB2 interacting protein	-4,69
CKAP2	cytoskeleton associated protein 2	-4,69
CHRDL1	chordin-like 1	-4,61
RNASEH2A	ribonuclease H2, subunit A	-4,34
RRM2	ribonucleotide reductase M2	-4,34
GRASP	GRP1 (general receptor for phosphoinositides 1)-associated scaffold protein	-4,22
LOC526064	histone cluster 1, H2bl-like	-4,13
PLIN4	perilipin 4	-4,07
RNF168	ring finger protein 168, E3 ubiquitin protein ligase	-4,01
LOC539789	stathmin-like pseudogene	-4,01
MC4R	melanocortin 4 receptor	-3,75
LOC524985	olfactory receptor 1038-like	-3,71
ABCC9	ATP-binding cassette, sub-family C (CFTR/MRP), member 9	-3,71
OVOL2	ovo-like 2 (Drosophila)	-3,66
AGR2	anterior gradient homolog 2	-3,56
MEIS2	Meis homeobox 2	-3,53
E2F1	E2F transcription factor 1	-3,52
NKG2C	NKG2C protein	-3,49
UBE2C	ubiquitin-conjugating enzyme E2C	-3,48
MYBL2	v-myb avian myeloblastosis viral oncogene homolog-like 2	-3,47

Suppl. Table C.2. (cont)

ME1	malic enzyme 1, NADP(+)-dependent, cytosolic	-3,45
GPNMB	glycoprotein (transmembrane) nmb	-3,34
BAALC	brain and acute leukemia, cytoplasmic	-3,33
MEF2C	myocyte enhancer factor 2C	-3,33
LOC787642	Olfactory receptor-like protein OLF2-like	-3,28
LOC510902	olfactory receptor, family 4, subfamily B, member 1-like	-3,27
CCDC162P	coiled-coil domain containing 162, pseudogene	-3,26
VLDLR	very low density lipoprotein receptor	-3,26
MED13L	mediator complex subunit 13-like	-3,25
MAPRE3	microtubule-associated protein, RP/EB family, member 3	-3,25
COL8A1	collagen, type VIII, alpha 1	-3,21
LRRC16A	leucine rich repeat containing 16A	-3,20
WDR93	WD repeat domain 93	-3,18
VILL	villin-like	-3,17
TMEM38A	transmembrane protein 38A	-3,16
IKZF1	IKAROS family zinc finger 1 (Ikaros)	-3,15
C2H1orf64	chromosome 2 open reading frame, human C1orf64	-3,12
METTL19	methyltransferase like 19	-3,09
FAM13C	family with sequence similarity 13, member C	-3,08
RFX5	regulatory factor X, 5 (influences HLA class II expression)	-3,08
RGS17	regulator of G-protein signaling 17	-3,07
KCNMB1	potassium large conductance calcium-activated channel, subfamily M, beta member 1	-3,07
MARS	methionyl-tRNA synthetase	-3,05
SPC24	SPC24, NDC80 kinetochore complex component, homolog	-3,05
WT1	Wilms tumor 1	-3,04
LOC789904	ribosomal protein L7a-like	-3,04
BOLA-DMB	major histocompatibility complex, class II, DMB	-3,02
LOC618591	killer cell lectin-like receptor-like	-3,02

Suppl. Table C.3. Differentially expressed genes at +28 days from parturition with fold change (FC) ≤ −3 or ≥ +3 in liver of animals with BCS 4 fed either 125 (B4F125) compared with 75 (B4F75) % of requirement for the three weeks before parturition.

Gene	Description	FC at +28 d
Upregulated		
HSPA1A	heat shock 70kDa protein 1A	4,27
MYO7B	myosin VIIB	3,82
MMP9	matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	3,69
PPP1R21	protein phosphatase 1, regulatory subunit 21	3,41
CDHR1	cadherin-related family member 1	3,31
ALPK1	alpha-kinase 1	3,26
APOA4	apolipoprotein A-IV	3,25
FGF6	fibroblast growth factor 6	3,19
Downregulated		
CD46	CD46 molecule, complement regulatory protein	-9,87
KRT9	keratin 9	-9,84
PPP1R14C	protein phosphatase 1, regulatory (inhibitor) subunit 14C	-8,70
AKAP6	A kinase (PRKA) anchor protein 6	-8,29
KIR2DL5A	killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 5A	-7,86
FGF8	fibroblast growth factor 8 (androgen-induced)	-7,40
LOC100139191	uncharacterized LOC100139191	-7,36
FGD3	FYVE, RhoGEF and PH domain containing 3	-7,33
C7H5orf46	chromosome 7 open reading frame, human C5orf46	-7,22
SGIP1	SH3-domain GRB2-like (endophilin) interacting protein 1	-7,21
TCHHL1	trichohyalin-like 1	-6,97
PLEKHG7	pleckstrin homology domain containing, family G (with RhoGef domain) member 7	-6,85
MYPN	myopalladin	-6,18
OPN1SW	opsin 1 (cone pigments), short-wave-sensitive	-6,05
LOC789829	sorting nexin-5-like	-6,01
RYR1	ryanodine receptor 1	-5,84
KIAA1456	putative methyltransferase KIAA1456 homolog	-5,79
OVCH2	ovochymase 2	-5,78
AGR2	anterior gradient homolog 2	-5,34
LALBA	lactalbumin, alpha-	-5,14
SLC35D3	solute carrier family 35, member D3	-5,10
C26H10orf90	chromosome 26 open reading frame, human C10orf90	-5,08
ALPK3	alpha-kinase 3	-5,06
KRT6B	keratin 6B	-5,04
CCDC178	coiled-coil domain containing 178	-5,01
METTL24	methyltransferase like 24	-4,95

Suppl. Table C.3. (cont)

LOC786089	establishment of cohesion 1 homolog 2-like	-4,93
TGM7	transglutaminase 7	-4,91
PPP1R3A	protein phosphatase 1, regulatory subunit 3A	-4,90
NEUROD6	neurogenic differentiation 6	-4,87
SVOP	SV2 related protein homolog (rat)	-4,85
CHRDL1	chordin-like 1	-4,78
NIM1	serine/threonine-protein kinase NIM1	-4,73
MMP13	matrix metallopeptidase 13 (collagenase 3)	-4,64
LOC532848	centrosomal protein KIAA1731-like	-4,61
RYR2	ryanodine receptor 2 (cardiac)	-4,58
HDAC9	histone deacetylase 9	-4,53
ACHE	acetylcholinesterase	-4,52
LAT2	linker for activation of T cells family, member 2	-4,47
CTLA4	cytotoxic T-lymphocyte-associated protein 4	-4,45
GABRA1	gamma-aminobutyric acid (GABA) A receptor, alpha 1	-4,44
TAF1C	TATA box binding protein (TBP)-associated factor, RNA polymerase I, C, 110kDa	-4,35
MCF2	MCF.2 cell line derived transforming sequence	-4,34
GLYCAM1	glycosylation-dependent cell adhesion molecule 1	-4,33
GHRL	ghrelin/obestatin prepropeptide	-4,33
MTMR12	myotubularin related protein 12	-4,29
CD4	CD4 molecule	-4,28
TEX13B	testis expressed 13B	-4,27
DLGAP5	discs, large (Drosophila) homolog-associated protein 5	-4,24
LOC618010	olfactory receptor, family 51, subfamily F, member 2-like	-4,22
GPR77	G protein-coupled receptor 77	-4,20
LOC508589	olfactory receptor, family 8, subfamily A, member 1-like	-4,20
PI3	peptidase inhibitor 3, skin-derived (SKALP)	-4,19
IKZF1	IKAROS family zinc finger 1 (Ikaros)	-4,11
ATP2B3	ATPase, Ca++ transporting, plasma membrane 3	-4,09
ITGA4	integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	-4,08
UNC5B	unc-5 homolog B	-4,07
NKX3-1	NK3 homeobox 1	-4,04
DNAI2	dynein, axonemal, intermediate chain 2	-4,02
PLAU	plasminogen activator, urokinase	-4,02
KRT20	keratin 20	-4,02
ATP8A2	ATPase, aminophospholipid transporter, class I, type 8A, member 2	-4,02
DYNLRB1	dynein, light chain, roadblock-type 1	-4,02
GPR88	G protein-coupled receptor 88	-4,00
SLX4	SLX4 structure-specific endonuclease subunit homolog	-3,98
GALNT13	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 13 (GalNAc-T13)	-3,97

Suppl. Table C.3. (cont)

SUV39H1	suppressor of variegation 3-9 homolog 1	-3,96
TUBB1	tubulin, beta 1 class VI	-3,95
VTCN1	V-set domain containing T cell activation inhibitor 1	-3,92
C1H3orf52	chromosome 1 open reading frame, human C3orf52	-3,91
PSMF1	proteasome (prosome, macropain) inhibitor subunit 1 (PI31)	-3,90
SMIM24	small integral membrane protein 24	-3,87
GPNMB	glycoprotein (transmembrane) nmb	-3,85
PCDH10	protocadherin 10	-3,82
SYMPK	symplekin	-3,77
LOC785406	olfactory receptor 5AL1	-3,74
KIF5C	kinesin family member 5C	-3,74
RNF183	ring finger protein 183	-3,72
LOC508124	homer protein homolog 3-like	-3,71
LOC506891	olfactory receptor, family 9, subfamily G, member 4-like	-3,71
PTPN22	protein tyrosine phosphatase, non-receptor type 22 (lymphoid)	-3,67
NR4A2	nuclear receptor subfamily 4, group A, member 2	-3,67
CNNM1	cyclin M1	-3,66
GINS2	GINS complex subunit 2 (Psf2 homolog)	-3,65
RAX2	retina and anterior neural fold homeobox 2	-3,64
NXF3	nuclear RNA export factor 3	-3,64
IQGAP3	IQ motif containing GTPase activating protein 3	-3,62
SPATS1	spermatogenesis associated, serine-rich 1	-3,61
FGF18	fibroblast growth factor 18	-3,60
PTX3	pentraxin 3, long	-3,58
SCN1A	sodium channel, voltage-gated, type I, alpha subunit	-3,55
RBM28	RNA binding motif protein 28	-3,54
CDH8	cadherin 8, type 2	-3,54
SCRN1	secernin 1	-3,51
LOC523060	olfactory receptor, family 4, subfamily A, member 15-like	-3,49
FAM70A	family with sequence similarity 70, member A	-3,46
PRKCD	protein kinase C, delta	-3,45
PREX1	phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 1	-3,44
SLC46A2	solute carrier family 46, member 2	-3,44
SAA3	serum amyloid A 3	-3,42
DPYSL4	dihydropyrimidinase-like 4	-3,40
LOC618422	huntingtin-associated protein 1-like	-3,38
RPS29	ribosomal protein S29	-3,38
OLFM4	olfactomedin 4	-3,36
F2RL1	coagulation factor II (thrombin) receptor-like 1	-3,35
PADI4	peptidyl arginine deiminase, type IV	-3,32
CHRAC1	chromatin accessibility complex 1	-3,31

Suppl. Table C.3. (cont)

CLEC4E	C-type lectin domain family 4, member E	-3,30
SHH	sonic hedgehog	-3,29
TNFRSF9	tumor necrosis factor receptor superfamily, member 9	-3,26
TAP	tracheal antimicrobial peptide	-3,25
RPRD2	regulation of nuclear pre-mRNA domain containing 2	-3,24
CCDC60	coiled-coil domain containing 60	-3,23
ARMC3	armadillo repeat containing 3	-3,23
ARF1	ADP-ribosylation factor 1	-3,23
FLOT2	flotillin 2	-3,22
TKDP2	trophoblast Kunitz domain protein 2	-3,22
LOC510902	olfactory receptor, family 4, subfamily B, member 1-like	-3,21
CLSPN	claspin	-3,18
LYZ1	ysozyme 1	-3,17
GDPD3	glycerophosphodiester phosphodiesterase domain containing 3	-3,15
DLX4	distal-less homeobox 4	-3,15
C14H8orf34	chromosome 14 open reading frame, human C8orf34	-3,15
PTPRU	protein tyrosine phosphatase, receptor type, U	-3,14
FRY	furry homolog	-3,14
BOSTAUV1R404	vomeronasal 1 receptor bosTauV1R404	-3,13
CCDC136	coiled-coil domain containing 136	-3,12
INTU	inturned planar cell polarity effector homolog	-3,12
SNRPC	small nuclear ribonucleoprotein polypeptide C	-3,11
ZZEF1	zinc finger, ZZ-type with EF-hand domain 1	-3,10
VAV1	vav 1 guanine nucleotide exchange factor	-3,10
YBX2	Y box binding protein 2	-3,09
CLOCK	clock circadian regulator	-3,09
OR6C75	olfactory receptor, family 6, subfamily C, member 75	-3,08
ZNF383	zinc finger protein 383	-3,06
TK1	thymidine kinase 1, soluble	-3,06
LOC532330	insulin receptor substrate 3-like	-3,04
RCOR1	REST corepressor 1	-3,04
RASAL2	RAS protein activator like 2	-3,03
SNX29	sorting nexin 29	-3,03
DYTN	dystrotelin	-3,01
NTS	neurotensin	-3,00

Suppl. Table C.4. Differentially expressed genes at -7 days from parturition with fold change (FC) ≤ −3 or ≥ +3 in liver of animals with BCS 5 fed either 125 (B5F125) compared with 75 (B5F75) % of requirement for the three weeks before parturition.

Gene	Description	FC at -7 d
Upregulated		
ESYT2	extended synaptotagmin-like protein 2	5,17
CRYGD	crystallin, gamma D	4,98
RSPH1	radial spoke head 1 homolog	4,54
ANGPTL7	angiopoietin-like 7	4,39
PLD5	phospholipase D family, member 5	3,64
RHBDL2	rhomboid, veinlet-like 2	3,54
OBSCN	obscurin, cytoskeletal calmodulin and titin-interacting RhoGEF	3,18
LOC787878	putative olfactory receptor 56B2-like	3,10
Downregulated		
C1QL3	complement component 1, q subcomponent-like 3	-6,66
CADPS	Ca^{++}-dependent secretion activator	-6,15
TRIM36	tripartite motif containing 36	-5,52
MAEL	maelstrom homolog	-5,45
NRCAM	neuronal cell adhesion molecule	-4,13
FEZF1	FEZ family zinc finger 1	-4,03
SLC26A8	solute carrier family 26, member 8	-3,80
CKMT1B	creatine kinase, mitochondrial 1B	-3,71
LOC782400	uncharacterized LOC782400	-3,71
ELN	elastin	-3,25
RPRD2	regulation of nuclear pre-mRNA domain containing 2	-3,11
FLNA	filamin A, alpha	-3,09
RPS28	ribosomal protein S28	-3,08
FGFR2	fibroblast growth factor receptor 2	-3,03

Suppl. Table C.5. Differentially expressed genes at +7 days from parturition with fold change (FC) ≤ −3 or ≥ +3 in liver of animals with BCS 5 fed either 125 (B5F125) compared with 75 (B5F75) % of requirement for the three weeks before parturition.

Gene	Description	FC at +7 d
Upregulated		
CRB1	crumbs homolog 1	7,77
ALPK1	alpha-kinase 1	5,87
TMEM163	transmembrane protein 163	5,18
ARL14	ADP-ribosylation factor-like 14	4,76
DAB2IP	DAB2 interacting protein	4,66
IPPK	inositol 1,3,4,5,6-pentakisphosphate 2-kinase	4,65
CALML5	calmodulin-like 5	4,41
LOC616722	immunity-related GTPase family, cinema-like	4,30
LOC618985	interferon, omega 1-like	4,16
XPO4	exportin 4	4,15
PGPEP1L	pyroglutamyl-peptidase I-like	4,00
LOC539818	ribophorin I-like	3,97
MCART6	mitochondrial carrier triple repeat 6	3,93
RASD2	RASD family, member 2	3,79
MAN2C1	mannosidase, alpha, class 2C, member 1	3,73
LOC788761	uncharacterized LOC788761	3,72
C10H14orf39	chromosome 10 open reading frame, human C14orf39	3,69
CCK	cholecystokinin	3,66
MAP3K12	mitogen-activated protein kinase kinase kinase 12	3,66
NEIL3	nei endonuclease VIII-like 3	3,57
LOC509124	olfactory receptor, family 9, subfamily G, member 4-like	3,51
BMP7	bone morphogenetic protein 7	3,49
CCDC113	coiled-coil domain containing 113	3,47
LOC100140382	olfactory receptor, family 4, subfamily F, member 21-like	3,46
DBIL5	endozepine-like peptide 2	3,41
LOC618124	olfactory receptor, family 1, subfamily E, member 2-like	3,40
CCBP2	chemokine binding protein 2	3,39
LOC785309	T-cell receptor alpha chain V region PY14-like	3,31
TRPC4	transient receptor potential cation channel, subfamily C, member 4	3,31
DNAL4	dynein, axonemal, light chain 4	3,30
KCNMB2	potassium large conductance calcium-activated channel, subfamily M, beta member 2	3,30
CRY1	cryptochrome 1 (photolyase-like)	3,29
CLDN22	claudin 22	3,25
LYG2	lysozyme G-like 2	3,23
STAT5A	signal transducer and activator of transcription 5A	3,15
PDE10A	phosphodiesterase 10A	3,13

Suppl. Table C.5. (cont)

LOC532330	insulin receptor substrate 3-like	3,13
FCGBP	Fc fragment of IgG binding protein	3,11
SLC6A6	solute carrier family 6 (neurotransmitter transporter, taurine), member 6	3,09
LOC506670	solute carrier family 7 (anionic amino acid transporter), member 13-like	3,09
LOC510100	olfactory receptor, family 10, subfamily A, member 5-like	3,08
NKD1	naked cuticle homolog 1	3,04
Downregulated		
FIGLA	folliculogenesis specific basic helix-loop-helix	-7,19
KCNH6	potassium voltage-gated channel, subfamily H (eag-related), member 6	-6,74
NDNF	neuron-derived neurotrophic factor	-6,71
ELOVL7	ELOVL fatty acid elongase 7	-6,39
TTF2	transcription termination factor, RNA polymerase II	-5,68
RTP3	receptor (chemosensory) transporter protein 3	-5,42
PLEKHB2	pleckstrin homology domain containing, family B (evectins) member 2	-5,20
PLD5	phospholipase D family, member 5	-4,79
IRG1	immunoresponsive 1 homolog	-4,70
SLIT3	slit homolog 3	-4,50
CAPN3	calpain 3, (p94)	-4,49
SUN3	Sad1 and UNC84 domain containing 3	-4,49
FGF8	fibroblast growth factor 8 (androgen-induced)	-4,29
RS1	retinoschisin 1	-4,28
OR10K1	olfactory receptor, family 10, subfamily K, member 1	-4,18
GTPBP4	GTP binding protein 4	-4,10
ANXA8L1	annexin A8-like 1	-4,09
TRAF5	TNF receptor-associated factor 5	-4,02
IFI47	interferon gamma inducible protein 47	-3,91
UHRF1	ubiquitin-like with PHD and ring finger domains 1	-3,79
KRT73	keratin 73	-3,71
MTMR12	myotubularin related protein 12	-3,69
NXPH3	neurexophilin 3	-3,63
EFHB	EF-hand domain family, member B	-3,61
AGAP2	ArfGAP with GTPase domain, ankyrin repeat and PH domain 2	-3,51
IFNA16	interferon, alpha 16	-3,47
TAP1	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	-3,46
KCNS1	potassium voltage-gated channel, delayed-rectifier, subfamily S, member 1	-3,32
AMOTL2	angiomotin like 2	-3,30
TRPA1	transient receptor potential cation channel, subfamily A, member 1	-3,29
ALOX12	arachidonate 12-lipoxygenase	-3,26
FLT1	fms-related tyrosine kinase 1	-3,24
KCTD6	potassium channel tetramerisation domain containing 6	-3,21
RNF168	ring finger protein 168, E3 ubiquitin protein ligase	-3,21

Suppl. Table C.5. (cont)

MIS18BP1	MIS18 binding protein 1	-3,19
NEU4	sialidase 4	-3,13
ADAMTS4	ADAM metallopeptidase with thrombospondin type 1 motif, 4	-3,13
DAB2	disabled homolog 2, mitogen-responsive phosphoprotein	-3,12
RNF145	ring finger protein 145	-3,11
LRRC3B	leucine rich repeat containing 3B	-3,10
ICOS	inducible T-cell co-stimulator	-3,08
ME1	malic enzyme 1, NADP(+)-dependent, cytosolic	-3,04
TMEM196	transmembrane protein 196	-3,01
BCAT1	branched chain amino-acid transaminase 1, cytosolic	-3,01

Suppl. Table C.6. Differentially expressed genes at +28 days from parturition with fold change (FC) ≤ −3 or ≥ +3 in liver of animals with BCS 5 fed either 125 (B5F125) compared with 75 (B5F75) % of requirement for the three weeks before parturition.

Gene	Description	FC at +28 d
Upregulated		
OPA3	optic atrophy 3	6,18
LRRC49	leucine rich repeat containing 49	5,45
FOXJ1	forkhead box J1	3,63
ZNF19	zinc finger protein 19	3,61
OTOL1	otolin 1	3,59
CXCL11	chemokine (C-X-C motif) ligand 11	3,49
UCN	urocortin	3,39
ZBTB46	zinc finger and BTB domain containing 46	3,03
TNFAIP8L3	tumor necrosis factor, alpha-induced protein 8-like 3	3,01
Downregulated		
LOC785406	olfactory receptor 5AL1	-6,05
TMEM196	transmembrane protein 196	-4,22
IFIT1	interferon-induced protein with tetratricopeptide repeats 1	-4,12
TMEM54	transmembrane protein 54	-3,70
SAA3	serum amyloid A 3	-3,43
APOBEC3A	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3A	-3,42
M-SAA3.2	mammary serum amyloid A3.2	-3,34
MVK	mevalonate kinase	-3,07
ATAD1	ATPase family, AAA domain containing 1	-3,03

Suppl. Table C.7. Complete result datasets generated by the DIA analysis. The KEGG pathways are sorted by category and subcategory. Blue bars represent the Impact along the transition period, while red and green bars show the Direction of the Impact (red = upregulation, green = downregulation). [available for download from publisher at https://doi.org/10.1186/s12864-016-3191-3]

Suppl. Table C.8. Accession number, gene symbol and EntrezID, forward and reverse primer sequences and product length of target genes for microarray validation.

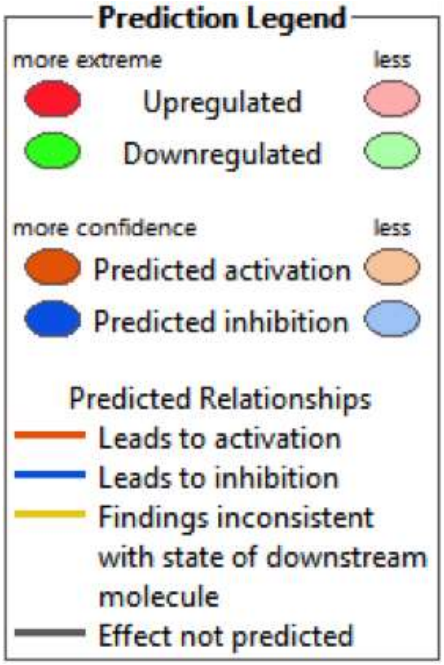
Accession #	EntrezID	Symbol	Forward sequence	Reverse Sequence	Lenght
NM_001081581.1	280969	*ABAT*	TCATGTGGTACCGGAGCAAG	GAATGGCTTTGGAGTGCGTG	174
NM_001205333.1	515338	*ACACB*	CACCCGCACCGGATGAC	CCTCGAATCTCTGGAAGGCG	139
NM_001192336.1	407169	*ALOX12*	AGGGCCAGCTGGACTGGTAT	CGGACATCAGGTAGTGAGCC	121
NM_001075321.1	507559	*B4GALT7*	GCCCTTTCGAGAACGCTTTG	TGCCCGGTTGAACCTGAAAT	130
NM_001083644.1	505926	*BCAT1*	GGCCCCACGATGAAGGATT	TAAAACGGTGGCTCGTGTGA	123
NM_001113232.2	514028	*DSE*	TGGAGTACCTCCCTCCTTGG	TCACCAACCAACTAGGCTGAG	177
NM_001100337.1	511060	*EXTL1*	GCACAGGAAGGCCAGTGAC	TGTGAGAAAGCCGACCATCC	157
NM_174077.4	281210	*GPX3*	CCCTGCAACCAATTTGGAAA	ACATACTTGAGGGTGGCTAGGATCT	78
NM_001076814.1	507817	*ME2*	GCTCGAGTCCAGCAGAGC	TTTCTCACCCCGCTTCTTGC	179
NM_001206826.1	535975	*UST*	AGAACAAACGTTCCCACAGGT	TGTACCTGGGAAGGACCCAA	113
NM_001008667.1	493989	*XYLT2*	GGCCGAGTCCTTCTTTCACA	CAGCTTGCGGTTCCAGTTG	145

Suppl. Table C.9. qPCR performance of measured genes for microarray validation.

Gene	Median Ct[1]	Median ΔCt[2]	Slope[3]	(R^2)[4]	Efficiency[5]
ABAT	21.81	0.69	-3.183	0.996	2.061
ACACB	32.28	11.37	-3.194	0.975	2.056
ALOX12	30.47	9.41	-3.014	0.998	2.147
B4GALT7	25.72	4.72	-3.089	0.998	2.108
BCAT1	29.53	8.58	-3.453	0.976	1.948
DSE	28.15	7.20	-3.043	0.994	2.131
EXTL1	27.25	6.20	-3.036	0.994	2.135
GPX3	20.506	-0.521	-3.225	0.999	2.042
ME2	26.935	5.995	-3.280	0.992	2.018
UST	24.763	3.700	-3.183	0.990	2.061
XYLT2	25.935	4.936	-3.380	0.994	1.976

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

Suppl. Figure C.1. Ingenuity pathway analysis legend for network analysis.



REFERENCES

Allen, M. S., B. J. Bradford, and K. J. Harvatine. 2005. The cow as a model to study food intake regulation. Annual review of nutrition 25:523-547.

Bauchart, D. 1993. Lipid absorption and transport in ruminants. Journal of dairy science 76(12):3864-3881.

Bertoni, G., E. Trevisi, X. Han, and M. Bionaz. 2008. Effects of inflammatory conditions on liver activity in puerperium period and consequences for performance in dairy cows. Journal of dairy science 91(9):3300-3310.

Calder, P. C. 2006. n-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. American journal of clinical nutrition 83(6 Suppl):1505S-1519S.

Calder, P. C. 2013. N-3 fatty acids, inflammation and immunity: New mechanisms to explain old actions. Proceedings of the Nutrition Society 72(3):326-336.

Goetzl, E. J. and W. C. Pickett. 1980. The human PMN leukocyte chemotactic activity of complex hydroxy-eicosatetraenoic acids (HETEs). Journal of immunology 125(4):1789-1791.

Ji, P., J. S. Osorio, J. K. Drackley, and J. J. Loor. 2012. Overfeeding a moderate energy diet prepartum does not impair bovine subcutaneous adipose tissue insulin signal transduction and induces marked changes in peripartal gene network expression. Journal of dairy science 95(8):4333-4351.

Nakamura, M. T., B. E. Yudell, and J. J. Loor. 2014. Regulation of energy metabolism by long-chain fatty acids. Progress in lipid research 53:124-144.

Pagadala, M., T. Kasumov, A. J. McCullough, N. N. Zein, and J. P. Kirwan. 2012. Role of ceramides in nonalcoholic fatty liver disease. Trends in endocrinology and metabolism 23(8):365-371.

Rico, J. E., V. V. Bandaru, J. M. Dorskind, N. J. Haughey, and J. W. McFadden. 2015. Plasma ceramides are elevated in overweight Holstein dairy cows experiencing greater lipolysis and insulin resistance during the transition from late pregnancy to early lactation. Journal of dairy science.

Samuelsson, B. 1991. Arachidonic acid metabolism: Role in inflammation. Zeitschrift fur rheumatologie 50 Suppl 1:3-6.

Shahzad, K., M. Bionaz, E. Trevisi, G. Bertoni, S. L. Rodriguez-Zas, and J. J. Loor. 2014. Integrative analyses of hepatic differentially expressed genes and blood biomarkers during the peripartal period between dairy cows overfed or restricted-fed energy prepartum. Plos one 9(6):e99757.

APPENDIX D

CHAPTER 6 - SUPPLEMENTAL MATERIAL

RNA Extraction. Adipose tissue was weighted (~0.2 g), immediately placed in 1.2 mL of QIAzol Lysis Reagent (cat#79306, Qiagen) and homogenized using a Mini-Beadbeater-24 (cat#112011, Biospec Products Inc.) with two 30 s cycles, and 1 min incubation on ice in between the cycles. Samples were then centrifuged for 10 min at 12,000 g and 4 °C, and the supernatant was transfer in a separate tube and mix with 240 µL of Chloroform (cat#C298, Fisher Chemical). After centrifugation for 15 min at 12,000 g and 4 °C, the aqueous phase was transferred to a new tube, mixed with 900 µL of 100% Ethanol (cat#2701, Decon Labs), and total RNA was cleaned using miRNeasy mini kit columns (cat# 217004, Qiagen) following manufacture protocol. During purification, genomic DNA was removed using the RNase-Free DNase Set (cat# 79254, Qiagen).

RNA Quality Evaluation. The RNA quantity, purity, and integrity were determined using a NanoDrop ND-1000 (cat#E112352, NanoDrop Technologies Inc.) and the Agilent 2100 Bioanalyzer (cat#G2939AA, Agilent Technologies Inc.). Samples had an average RIN score of 6.5 ± 0.8. RNA samples were stored at -80 °C until analysis.

mRNA cDNA Synthesis and qPCR Performance. Genes selected for transcript profiling are associated with fatty acid metabolism: fatty acid synthase (*FASN*), peroxisome proliferator-activated receptor gamma (*PPARG*); adipokines: adiponectin (*ADIPOQ*); and inflammation: chemokine (C-C motif) ligand 2 (*CCL2*), chemokine (C-C motif) ligand 5 (*CCL5*), haptoglobin (*HP*), interleukin-1β (*IL1B*), inteleukin-6 (*IL6*), inteleukin-6 receptor (*IL6R*), retinoid X receptor alpha (*RXRA*), serum amyloid A3 (*SAA3*), toll-like receptor 4 (*TLR4*), toll-like receptor 9 (*TLR9*)

and tumor necrosis factor α (*TNF*). Complete information about primer sequences and qPCR performance are reported in the supplemental file (Suppl. Tab.D. 1 and 3). A portion of the RNA was diluted to 100 ng/μL with DNase/RNase-free water for cDNA synthesis through RT-PCR. Per each sample, 4 μL of diluted RNA, 5 μL of Random Primers (3 μg/μL; cat#11034731001, Roche) and 45 μL of DNase/RNase-free water. The mixture was incubated at 65°C for 5 min and kept on ice for 3 min. A total of 36 μL of master mix, composed of 20 μL of 5X First-Strand Buffer (cat#EP0441, Thermo Scientific), 5 μL of Oligo dT18 (Custom DNA Oligo Tubes, Integrated DNA Technologies), 10 μL of 10mM dNTP mix (cat#18427-088, Invitrogen), 1.25 μL of Reverse Transcriptase (200 U/μL as final concentration, cat#EP0441, Thermo Scientific), 0.625 μL of Rnase inhibitor (200 U/μL as final concentration, cat#EO0381, Thermo Scientific), and 8.125 μL of DNase/RNase-free water, was added. The reaction was performed in an Eppendorf Mastercycler Gradient following the appropriate temperature program: 25°C for 5 min, 42°C for 60 min, and 70°C for 5 min. The cDNA was then diluted 1:4 with DNase/RNase-free water, prior to quantitative PCR analysis.

Quantitative PCR was performed using 4 μL diluted cDNA combined with 6 μL of a mixture composed of 5 μL SYBR Green master mix (cat#95073-05K, Quanta BioSciences), 0.4 μL each of 10 μM forward and reverse primers, and 0.2 μL DNase/RNase free water in a MicroAmp™ Optical 384-Well Reaction Plate (Applied Biosystems). Each sample was run in triplicate and a 7 point relative standard curve plus the non-template control were used. Standard curve was created using a pool of RNA from all samples. The pool was then diluted 1:2 to obtain the first point of the curve, and then a serial dilution (1:4) was performed to obtain the remaining points. The reactions were performed in an ABI Prism 7900 HT SDS instrument using the following conditions: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C (denaturation), and 1 min

at 60 °C (annealing + extension). The presence of a single PCR product was verified by the dissociation protocol using incremental temperatures to 95 °C for 15 s plus 65 °C for 15 s. Data were calculated with the 7900 HT Sequence Detection Systems Software. The final data were normalized using the geometric mean of three internal control genes (ICG): *ACTB*, *GAPDH* and *RPS9* (Khan et al., 2014, Vailati Riboni et al., 2015, Vailati Riboni et al., 2016). Stability of the ICG was confirmed by the Genorm algorithm (stability value < 1.5) (Vandesompele et al., 2002).

microRNA cDNA Synthesis and qPCR Performance. MicroRNA (**miRNA**) selected for expression profiling are associated with immune cell infiltration (miR-26b, miR-126, miR-132, miR-155, miR-193b), inflammation and lipolysis (miR-99a, miR-145, miR-221), and positive regulation of adipogenesis (miR-103, miR-143, miR-378). Complete information about primer sequences and qPCR performance are reported in the supplemental file (Suppl. Tab. 2 and 4). miRNA-specific cDNA synthesis was performed starting from 5 μL of 100 ng/ μL diluted RNA, using the Quanta qScript microRNA cDNA Synthesis Kit (cat# 95107, Quanta BioSciences, Inc.), following the manufacturer's protocols. The cDNA was diluted 1:4 with DNase/RNase-free water, and quantitative PCR analysis was performed according to the Quanta qScript kit protocol using SybrGreen, the specific primers and a universal primer provided with the kit. Each sample was run in triplicate and a 7 point relative standard curve plus the non-template control were used. The reactions were performed in an ABI Prism 7900 HT SDS instrument. Data were calculated with the 7900 HT Sequence Detection Systems Software. The final data were normalized using the geometric mean of, miR-let-7b, miR-16b and miR-181a, which served as the internal controls. As for mRNA expression analysis, stability of the ICG was confirmed by the Genorm algorithm (stability value < 1.5) (Vandesompele et al., 2002).

Suppl. Table D.1. Accession number, gene symbol, and forward and reverse primer sequences of genes analyzed in adipose tissue.

Accession#	Symbol	Forward sequence	Reverse sequence
BC140488.1	*ADIPOQ*	GATCCAGGTCTTGTTGGTCCTAA	GAGCGGTATACATAGGCACTTTCTC
NM_174006.2	*CCL2*	TCAACAGTAAGAAGATCTCCATGCA	CAGGACGGTCTTGAAAATCACA
NM_175827.2	*CCL5*	CCCTGCTGCTTTGCCTATATCT	ATAAAGACAACTGCTGCCATGGA
NM_001012669.1	*FASN*	ACCTCGTGAAGGCTGTGACTCA	TGAGTCGAGGCCAAGGTCTGAA
NM_001040470.2	*HP*	GGTTCGGAAAACCATCGCTA	CACTCGTGTCCCCTCCCTC
NM_174093.1	*IL1B*	TCCACCTCCTCTCACAGGAAA	TACCCAAGGCCACAGGAATCT
NM_173923	*IL6*	CCAGAGAAAACCGAAGCTCTCAT	CCTTGCTGCTTTCACACTCATC
NM_001110785.1	*IL6R*	GCTCTTTCTACGTATTGTCCCTGTGT	GGGTCGGGCTGTAGGAGTTT
NM_181024.2	*PPARG*	CCAAATATCGGTGGGAGTCG	ACAGCGAAGGGCTCACTCTC
NM_001304343.1	*RXRA*	ACGTCCTTGCTGTGTTTGAAAA	TGAACCAGATTGGTCTACTGAAGGT
NM_181016.3	*SAA3*	GGGCATCATTTTCTGCTTCCT	TTGGTAAGCTCTCCACATGTCTTTAG
NM_174198.6	*TLR4*	GCTGTTTGACCAGTCTGATTGC	GGGCTGAAGTAACAACAAGAGGAA
NM_183081	*TLR9*	CTGACACCTTCAGTCACCTGAG	TGGTGGTCTTGGTGATGTAGTC
NM_173966.3	*TNF*	CCAGAGGGAAGAGCAGTCCC	TCGGCTACAACGTGGGCTAC

Suppl. Table D.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-26b	TTCAAGTAATTCAGGATAGGTT
miR-99a	AACCCGTAGATCCGATCTTGT
miR-103	AGCAGCATTGTACAGGGCTATGA
miR-126	CGTACCGTGAGTAATAATGCG
miR-132	TAACAGTCTACAGCCATGGTCG
miR-143	TGAGATGAAGCACTGTAGCTCG
miR-145	GTCCAGTTTTCCCAGGAATCCCT
miR-155	TTAATGCTAATCGTGATAGGGGT
miR-193b	AACTGGCCCACAAAGTCCCGCTTT
miR-221	AGCTACATTGTCTGCTGGGTTT
miR-378	ACTGGACTTGGAGTCAGAAGGC

Suppl. Table D.3. qPCR performance of measured genes.

Gene	Median Ct[1]	Median ΔCt[2]	Slope[3]	(R^2)[4]	Efficiency[5]
ADIPOQ	19.61	0.52	-3.279	0.997	2.018
CCL2	23.82	4.51	-3.073	0.980	2.116
CCL5	24.51	5.51	-3.095	0.991	2.104
FASN	23.95	4.92	-3.369	0.997	1.981
HP	21.65	2.40	-3.357	0.990	1.986
IL1B	27.40	8.48	-3.398	0.984	1.969
IL6	28.70	9.80	-3.011	0.992	2.148
IL6R	21.93	3.13	-3.204	0.995	2.052
PPARG	23.71	4.69	-3.238	0.991	2.036
RXRA	23.61	4.70	-3.249	0.994	2.031
SAA3	25.46	6.33	-3.318	0.981	2.002
TLR4	29.43	10.60	-3.101	0.990	2.101
TLR9	29.71	10.90	-3.803	0.999	1.832
TNF	27.77	8.81	-3.184	0.991	2.061

[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 $[10^{(-1 / \text{Slope})}]$.

Suppl. Table D.4. qPCR performance of measured miRNAs.

Gene	Median Ct[1]	Median ΔCt[2]	Slope[3]	(R^2)[4]	Efficiency[5]
miR-26b	18.57	0.87	-3.43	0.993	1.956
miR-99a	18.51	0.86	-3.32	0.995	2.003
miR-103	19.52	2.00	-3.21	0.996	2.050
miR-126	17.22	-0.64	-3.30	0.996	2.007
miR-132	23.72	6.24	-3.28	0.992	2.019
miR-143	20.82	3.24	-3.26	0.993	2.025
miR-145	17.21	-0.30	-3.50	0.991	1.931
miR-155	21.95	4.37	-3.29	0.991	2.015
miR-193b	19.35	1.44	-3.16	0.994	2.074
miR-221	21.16	3.76	-3.34	0.999	1.991
miR-378	22.90	5.30	-3.20	0.999	2.051

[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 $[10^{(-1 / \text{Slope})}]$.

Suppl. Table D.5. Interaction between far-off and close-up feeding management, and time (FO*CU*T) on subcutaneous adipose tissue expression (back-transformed LSM and SEM) in grazing dairy cows during the transition period

		FO*CU*T							
		SlowBCS			FastBCS				*p*-value
Target	Wk[1]	Feed60	Feed90	Feed120	Feed60	Feed90	Feed120	SEM[2]	FO*CU*T
Infiltration of immune cells									
CCL2	**-1**	0.59	0.26	0.92	0.22	0.32	0.66	0.59	0.71
	1	0.52	0.78	1.28	0.31	1.05	1.26		
	4	1.39	0.87	1.41	1.30	1.16	0.67		
CCL5	**-1**	0.83	0.67	0.83	1.24	0.61	0.78	0.50	0.14
	1	1.65	1.20	0.95	0.87	0.74	0.85		
	4	0.81	1.44	0.36	0.83	0.82	1.22		
Inflammation and lipolysis									
HP	**-1**	0.08	0.02	0.22	0.08	0.03	0.15	0.46	0.55
	1	0.69	0.10	0.10	0.20	0.18	0.48		
	4	0.20	0.10	0.03	0.07	0.08	0.09		
IL1B	**-1**	0.98	0.94	0.56	0.35	0.57	1.01	0.58	0.45
	1	1.34	1.14	0.35	0.44	1.56	0.86		
	4	1	1.63	0.16	0.44	0.62	0.53		
IL6	**-1**	0.71	0.19	0.57	0.05	0.26	0.31	0.46	0.15
	1	0.18	0.57	0.35	0.09	0.68	0.37		
	4	0.82	0.52	0.86	0.54	0.40	0.29		
IL6R	**-1**	1.26	0.81	0.86	0.85	0.66	0.74	0.29	0.71
	1	1.22	1.63	0.84	0.91	1.32	0.90		
	4	0.93	1.40	0.65	0.93	1.04	1		
SAA3	**-1**	0.22	0.18	0.20	0.64	0.14	0.19	0.31	0.62
	1	0.50	0.19	0.69	0.65	0.31	0.19		
	4	0.17	0.17	0.36	0.21	0.13	0.13		
TLR4	**-1**	1.29	0.42	0.82	0.63	1.04	0.64	0.52	0.24
	1	0.63	0.64	1.95	1.84	0.72	0.76		
	4	0.94	0.77	1.80	0.95	0.78	0.83		
TLR9	**-1**	0.91	0.47	0.52	0.48	0.35	0.45	0.20	0.77
	1	0.73	1.25	0.55	0.51	1.15	0.52		
	4	0.72	0.89	0.46	0.53	0.62	0.54		
TNF	**-1**	1.16	0.74	1.09	0.55	0.75	0.96	0.42	0.05
	1	1.78	1.21	1.08	0.85	1.58	1.39		
	4	1.15	1.98	0.37	1.10	1.35	1.06		
Adipogenesis and Lipid metabolism									
ADIPOQ	**-1**	1.20	1.38	0.74	0.74	1.85	0.68	0.62	0.48
	1	0.33	1.04	0.58	0.25	0.47	0.39		
	4	0.52	0.73	0.81	0.85	0.47	0.94		
FASN	**-1**	0.22	2.83	0.22	0.14	4.31	0.40	1.94	0.35
	1	0.06	0.08	0.03	0.02	0.07	0.06		
	4	0.23	0.09	0.02	0.05	0.09	0.16		
PPARG	**-1**	1.78	1.81	1.09	0.72	2.56	0.78	0.71	0.17
	1	0.46	1.26	0.73	0.34	0.65	0.58		
	4	0.80	0.98	0.75	1.06	0.83	1.16		
RXRA	**-1**	1.23	1.58	1.03	1.54	1.73	1.41	0.33	0.88
	1	1.09	1.09	0.68	0.71	0.69	0.89		
	4	0.85	0.76	0.64	0.91	0.73	1.04		

[1] Wk = week relative to parturition; T = time FO = Far-off feeding management; CU = close-up feeding management

[2] SEM = greatest standard error of the mean.

Suppl. Table D.6. Interaction between far-off and close-up feeding management, and time (FO*CU*T) on subcutaneous adipose tissue microRNA expression (back-transformed LSM and SEM) in grazing dairy cows during the transition period

		FO*CU*T							
		SlowBCS			FastBCS				*p*-value
Target	**Wk[1]**	**Feed60**	**Feed90**	**Feed120**	**Feed60**	**Feed90**	**Feed120**	**SEM[2]**	**FO*CU*T**
Infiltration of immune cells									
miR-26b	**-1**	0.99	0.65	0.91	0.95	1.23	1.03	0.19	0.33
	1	1.16	1.12	0.71	1.08	1.19	0.86		
	4	1.09	1.15	0.70	0.79	1.07	0.89		
miR-126	**-1**	0.58	0.48	0.64	1.61	0.93	0.70	0.38	0.62
	1	0.76	1.30	0.92	0.80	1.49	0.73		
	4	0.68	1.03	1.25	0.99	0.66	0.81		
miR-132	**-1**	1.18	1.31	1.16	0.80	0.90	0.99	0.18	0.26
	1	0.99	1.08	1.10	0.81	0.68	1.10		
	4	0.91	0.88	1.34	0.99	0.98	1.08		
miR-155	**-1**	0.96	1.07	1.60	0.89	0.64	0.87	0.29	0.41
	1	0.98	0.83	2.07	1.34	0.71	1.35		
	4	1.13	0.85	1.66	1.44	1.30	1.25		
miR-193	**-1**	0.98	1.44	0.79	1.51	1.59	1.10	0.41	0.63
	1	1.37	0.80	0.81	0.89	0.55	0.69		
	4	0.62	0.49	1.14	0.91	0.44	0.72		
Inflammation and lipolysis									
miR-99a	**-1**	0.59	0.62	0.68	1.73	1.10	0.85	0.35	0.94
	1	0.71	1.03	0.89	1.21	1.22	0.75		
	4	0.72	0.66	1.30	0.70	0.63	0.73		
miR-145	**-1**	1	1.25	1.40	1.56	0.78	0.83	0.52	0.68
	1	1.54	1.15	2.49	1.27	0.89	0.91		
	4	0.79	0.90	1.70	1.06	0.83	0.99		
miR-221	**-1**	0.78	0.86	0.95	0.91	0.62	0.81	0.21	0.6
	1	0.85	0.82	1.24	1.08	0.85	1.08		
	4	0.80	0.98	1.35	1.20	0.97	0.90		
Proadipogenic									
miR-103	**-1**	0.79	0.82	0.90	1.12	0.75	0.97	0.15	0.14
	1	1.38	0.95	1.06	1.07	0.90	1.10		
	4	0.93	1.10	1.11	1.12	0.84	0.79		
miR-143	**-1**	0.77	0.54	0.69	1.61	0.83	0.69	0.49	0.83
	1	1.12	1.68	0.95	0.84	1.86	0.65		
	4	0.71	1.22	1.17	0.67	0.85	0.69		
miR-378	**-1**	0.82	1.10	0.71	1.75	1.67	0.95	0.36	0.42
	1	0.90	0.70	0.71	0.67	0.67	0.71		
	4	0.60	0.59	1.15	0.89	0.43	0.75		

[1] Wk = week relative to parturition; T = time FO = Far-off feeding management; CU = close-up feeding management

[2] SEM = greatest standard error of the mean.

Suppl. Table D.7. Interaction between far-off and close-up feeding management, and time (FO*CU*T) on plasma concentration of fatty acids, BHB, and cholesterol grazing dairy cows during the transition period

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		FO*CU*T							
		SlowBCS			FastBCS				*p*-value
	Wk[1]	Feed60	Feed90	Feed120	Feed60	Feed90	Feed120	SEM[2]	FO*CU*T
Fatty Acids (mmol/L)	**-1**	1.011	0.697	0.406	0.776	0.682	0.348	0.090	0.55
	1	1.005	1.073	1.023	0.974	0.964	0.970		
	4	0.505	0.543	0.589	0.410	0.524	0.387		
BHB (mmol/L)	**-1**	0.613	0.480	0.390	0.501	0.437	0.311	0.053	0.41
	1	0.668	0.650	0.678	0.583	0.618	0.676		
	4	0.460	0.558	0.525	0.484	0.474	0.594		
Cholesterol (mM)	**-1**	3425	3036	2515	3146	2627	2412	424	0.60
	1	2241	2095	2180	2462	1521	1737		
	4	1734	2180	1994	1630	2800	2231		

[1] Wk = week relative to parturition; T = time FO = Far-off feeding management; CU = close-up feeding management

[2] SEM = greatest standard error of the mean.

Suppl. Table D.8. Blast results for the comparison of the analyzed *Bos taurus* miRNA, and some common target mRNA sequences, with the respective *Homo sapiens* sequences

Subject	# *B. taurus*	# *H. sapiens*	Cover[1]	E-score[2]	Identity[3]
miRNA					
miR-26b	NR_030883.1	NR_029500.1	90%	2.00e-37	99%
miR-99a	NR_030927.1	NR_029514.1	100%	2.00e-41	100%
miR-103	NR_031387.1	NR_029519.1	100%	1.00e-38	100%
miR-126	NR_030923.1	NR_029695.1	97%	3.00e-34	99%
miR-132	NR_031374.1	NR_029674.1	100%	2.00e-50	99%
miR-143	NR_030868.1	NR_029684.1	100%	1.00e-47	97%
miR-145	NR_030906.1	NR_029686.1	100%	4.00e-45	100%
miR-155	NR_031030.1	NR_030784.1	100%	2.00e-26	94%
miR-193	NR_031097.1	NR_030177.1	100%	1.00e-39	98%
miR-221	NR_030881.1	NR_029635.1	97%	4.00e-48	95%
miR-378	NR_030898.1	NR_029870.1	100%	9.00e-32	98%
mRNA[4]					
CCL2[5]	NM_174006.2	NM_002982.3	95%	2.00e-141	74%
FABP	NM_174314.2	NM_001442.2	100%	0.00	86%
PPARG[5]	NM_181024.2	NM_015869.4	94%	0.00	89%

[1] percent of the *B. taurus* sequence that overlaps the *H. sapiens* sequence

[2] describes the random background noise. The lower the E-value, or the closer it is to zero, the more 'significant' the match is.

[3] percent similarity between the *B. taurus* and *H. sapiens* sequences over the length of the coverage area

[4] as miRNA act on the transcribed mRNA, rather than the DNA sequence, the blast was conducted on the first

[4] human isoform A was chosen for the blast

[5] human transcription variant 2 was chosen for the blast

Suppl. Table D.9. Blastn (nucleotide) and Blastp (protein) results for the comparison of *Bos taurus* with the respective *Homo sapiens* mRNA and protein sequences of the players involved in CCL2 immune cells infiltration signaling.

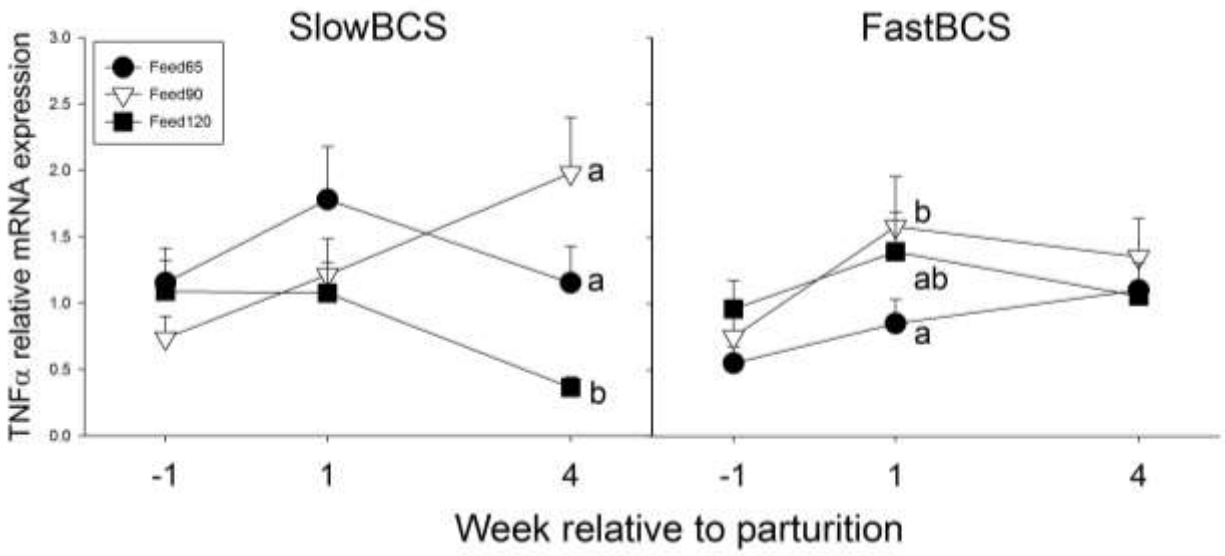
Subject	# *B. taurus*	# *H. sapiens*	Cover[1]	E-score[2]	Identity[3]
mRNA sequence					
CCL2	NM_174006.2	NM_002982.3	95%	2.00e-141	74%
MMP12	NM_001206640.1	NM_002426.5	79%	0.00	78%
CCR2, isoform A	NM_001194959.1	NM_001123041.2	85%	0.00	85%
CCR2, isoform B	NM_001194959.1	NM_001123396.1	97%	0.00	85%
CCR4	NM_001100293.2	NM_005508.4	59%	0.00	83%
Protein sequence					
CCL2	NP_776431.1	NP_002973.1	98%	1.00e-47	72%
MMP12	NP_001193569.1	NP_002417.2	99%	0.00	68%
CCR2 isoform A	NP_001181888.1	NP_001116513.2	84%	0.00	81%
CCR2 isoform B	NP_001181888.1	NP_001116868.1	97%	0.00	80%
CCR4	NP_001093763.1	NP_005499.1	100%	0.00	90%

[1] percent of the *B. taurus* sequence that overlaps the *H. sapiens* sequence

[2] describes the random background noise. The lower the E-value, or the closer it is to zero, the more 'significant' the match is.

[3] percent similarity between the *B. taurus* and *H. sapiens* sequences over the length of the coverage area

Suppl. Figure D.1. Effect of far-off and close-up feeding management on TNF (tumor necrosis factor α) subcutaneous adipose tissue expression (back-transformed LSM and SEM) in grazing dairy cows during the transition period



APPENDIX E

CHAPTER 7 – SUPPLEMENTARY MATERIAL

BHMT activity assay. All liver samples were weighed and homogenized immediately in 4 vol of 50 mM potassium phosphate buffer (pH 7.4) containing 2 mM EDTA and 1 mM mercaptoethanol. The homogenates was centrifuged at 4 °C for 45 min at 18,000 × g. The supernatant fraction was used for enzyme assay and protein concentration determination via the Bradford assay. DL-homocysteine was prepared fresh daily from homocysteine thiolactone hydrochloride (15.4 mg) in 400 µL 2 N sodium hydroxide and 600 µL saturated mono potassium phosphate. The standard assay contained 5 mM DL-Hcy, 2 mM betaine (0.05-0.1 µCi), and 50 mM Tris-HCl (pH = 7.5). The final reaction volume was 0.5 mL. Reaction tubes were capped with rubber stoppers and kept in ice water until the assay was started by transferring the tubes into a 37 °C water bath. After 1 h incubation, the reaction was terminated by adding 2.5 mL of ice-cold water into each tube. The samples were applied to a Dowex 1-X4 (OH-; 200-400 mesh) column (0.6 × 3.75 cm), and the unreacted betaine was washed off the column with 15 mL water. Dimethylglycine and methionine were eluted into scintillation vials with 3 mL of 1.5 N HCl. Seventeen milliliters of scintillation mixture (Ecolume, ICN) were added to each vial and counted. Blanks contained all of the reaction components except enzyme, and their values were subtracted from the sample values. All samples were assayed in duplicate.

MTR activity assay. All liver samples were weighed and homogenized immediately in 4 vol of 50 mM potassium phosphate buffer (pH 7.5) containing 2 mM EDTA. The homogenates was centrifuged at 4 °C for 45 min at 18,000 × g. The supernatant fraction was used for enzyme assay and protein concentration determination by the Bradford assay. The standard assay contained 1

M potassium phosphate buffer (pH 7.2), 0.5 M DTT, 3.8 mM S-adenosylmethionine, 100 mM DL-Hcy, 5 mM hydroxocobalamin, and 5 mM methyl-tetrahydrofolate (0.1 μCi). The final reaction vol was 0.5 mL. Reaction tubes were capped with rubber stoppers and kept in ice water until the assay was started by transferring the tubes into a 37 °C water bath. After 0.5 h incubation, the reaction was terminated by adding 0.5 mL of ice-cold water into each tube. The samples were applied to an AG 1X8 column, and elute was collected also from additional 2 mL of ice-cold water applied to the column. Seventeen milliliters of scintillation mixture (Ecolume, ICN) were added to each vial and counted. Blanks contained all of the reaction components except enzyme, and their values were subtracted from the sample values. All samples were assayed in duplicate.

CBS activity assay. All liver samples were weighed and homogenized immediately in 4 vol of 50 mM potassium phosphate buffer (pH 7.4) containing 2 mM EDTA and 1 mM mercaptoethanol. The homogenates was centrifuged at 4 °C for 45 min at 18,000 × g. The supernatant fraction was used for enzyme assay and protein concentration determination by the Bradford assay. The standard assay mixture (400 μL) contained 0.38 $mmol \cdot L^{-1}$ S-adenosylmethionine, 7.5 mM L-Hcy, 10 mM ^{14}C-Ser (0.07 μCi), and 50 μL cow liver extract. Reaction tubes were capped with rubber stoppers and kept in ice water until the assay was started by transferring the tubes into a 37 °C water bath. After 1 h incubation, the reaction was terminated by adding 0.4 mL 10% trichloroacetic acid and 2.2 mL ice-cold water into each tube. The samples were applied to an AG 50 W X8 column, 8 mL of water was applied to the column, and ^{14}C-Ser was eluted by applying 24 mL of 0.6M hydrochloric acid to the column. Following an additional wash with 16 mL water, ^{14}C-cystathionine was eluted by applying 5 mL of 3M ammonium hydroxide to the column and collected in scintillation vials. Seventeen milliliters of scintillation mixture

(Ecolume, ICN) were added to each vial and counted. Blanks contained all of the reaction components except enzyme, and their values were subtracted from the sample values. All samples were assayed in duplicate.