

TRANSITION COW METABOLISM IN RELATION TO PLANE OF ENERGY
PREPARTUM

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TRANSITION COW METABOLISM IN RELATION TO PLANE OF ENERGY

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Transition dairy cows face unique metabolic challenges with increased use of energetic substrates from mobilization of body tissue as a hallmark of this adaptation. This change is characterized by a dramatic drop in the circulating insulin concentrations with the onset of milk production and is accompanied by a significant reduction in circulating insulin concentration which may be exacerbated by insulin resistance on the receptor and post-receptor level.

Feeding strategies for dry cows have continuously evolved over the last decades and increasing evidence suggests that overfeeding of energy has detrimental effects on the ability of the dairy cow to successfully accomplish this transition, reflected in increased concentration of markers of negative energy balance postpartum. However, it is unclear if dry period plane of energy has direct effects on systemic or local insulin sensitivity in the peripartum period that hamper the metabolic adaptation of the transition cow.

The objectives therefore were to 1) evaluate the effect of three different dry period feeding strategies on markers of energy balance and colostrum and milk production 2) evaluate the effect of different feeding strategies on systemic glucose

tolerance and insulin response 3) evaluate the effect of different feeding strategies on adipose and muscle tissue accretion and loss as well as tissue-specific insulin signaling.

Cows overfed energy during the prepartum period were at higher risk of hyperketonemia and had elevated concentrations of serum nonesterified fatty acids postpartum, whereas milk production remained unchanged. Colostral IgG concentration was highest in cows fed a controlled energy diet prepartum. Whole body glucose tolerance was unaffected by plane of energy prepartum whereas resting concentrations of insulin and glucose remained more stable during the transition period in cows fed a controlled energy diet. Insulin signaling in muscle and adipose tissue was not affected by dry period feeding and overfeeding did not lead to overt inflammatory changes in adipose tissue.

Overall, feeding a controlled energy diet prepartum was associated with favorable metabolic parameters in the absence of changes in early lactation milk production. Changes in glucose tolerance or insulin signaling in peripheral tissues did not provide an explanation for the underlying metabolic mechanisms.

BIOGRAPHICAL SKETCH

Sabine Mann was born and raised in rural southwestern Germany. She earned her veterinary degree from the School of Veterinary Medicine in Hannover, Germany in 2007. After graduation, Sabine moved to Ithaca, NY, to start a mixed large animal internship in the Ambulatory and Production Medicine Clinic at Cornell University where she worked until 2009, fulfilling partial requirements for her residency. She completed her residency in the European College of Bovine Health Management (ECBHM) in the Clinic for Ruminants of the Ludwig-Maximilian-University (LMU) in Munich, Germany. Sabine started to work on research on the topic of antimicrobial resistance with Dr. Lorin Warnick during her residency, and in collaboration with Dr. Wolfgang Klee this work earned her the German doctorate (Dr. med. vet.) with a thesis defense in 2011. Upon completion of her residency, Sabine became a diplomate of the ECHBM in 2012 when she successfully passed her board exams. She subsequently started to work on her PhD in Dr. Daryl Nydam's group in the Comparative Biomedical Sciences Program at the Veterinary College at Cornell University. Her work focuses on the physiological and pathological, as well as epidemiological aspects of nutrition and peripartum energy metabolism of the dairy cow.

Für meinen Großvater

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CHAPTER 1

INTRODUCTION

Transition dairy cows face unique challenges in the adaptation to lactation that involve orchestrated changes in hormonal regulation, gluconeogenesis and glucose disposal, visceral organ size and function, liver metabolism, as well as adipose and lean tissue mobilization to support milk production during a state of negative energy balance (Bauman and Currie, 1980). The underlying physiological changes and effects on tissues in the transition period of dairy cows have received increasing attention in recent years and particular interest lies in the study of the potential association between metabolic adaptation during this high risk period and different dry cow feeding strategies currently used in the dairy industry.

Early nutritional strategies focused on feeding energy-dense diets to cows prepartum (steaming up) in an effort to prepare them for the energy demands of early lactation (Grummer, 1995; Vandehaar et al., 1999; Rabelo et al., 2001). However, in the last two decades this approach has been challenged repeatedly. In a report by Drehmann (2000) an approach of feeding cows a prepartum diet that more closely met their actual energy needs and avoided overfeeding was proposed. Since this time, research has intended to elucidate the effects of different dry cow planes of energy, not only on postpartum liver health and milk production, but also on the effects of whole body metabolism and risks that are associated with excessive negative energy balance in the immediate postpartum period.

Authors of a number of studies were able to show that the type of diet and how much of this diet fed to dry cows had direct effects on the success of the so called transition period, meaning the last three weeks before and the first three weeks after parturition (Grummer, 1995; Drackley, 1999). Despite an increase in the number of controlled feeding trials investigating the effects of different dietary strategies, we still lack a consensus on the best way to prepare cows metabolically for the ensuing lactation and energetic and nutritional demands of milk production. To this day, the exact mechanisms that are involved in making the transition period a metabolic success have not been fully elucidated. Drackley (1999) stated that “measurements during this time are fraught with a high degree of variability, reflecting differences among individual cows in the success of adaptation to lactation”. The variability in response is likely one of the reasons why the prevalence of disorders of energy metabolism has not declined greatly in the last decades, despite continued efforts of researchers to find methods of disease prevention.

The objective of the following literature review is to revisit the metabolic adaptations of transition cows and highlight what we have learned about the effects of different dry cow energy feeding strategies on these changes in the periparturient dairy cow until today. Because few research trials using total mixed rations (TMR) are available before 1995 on this topic (Grummer, 1995), this literature review will focus on the knowledge generated over the last two decades.

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CHAPTER 2

LITERATURE REVIEW

DRY COW FEEDING STRATEGIES AND NEGATIVE ENERGY BALANCE

Negative energy balance (NEB) of early lactation

Despite the physiological adaptations that are in place during the transition from pregnancy to lactation, metabolic disease incidence in this period is greatly increased and reflects inadequate nutrient supply, insufficient voluntary intake, failure of the physiological adaptive mechanisms, or a combination of these factors. Of particular importance is the fact that dry matter intake (DMI) can decrease substantially as cows approach parturition, particularly in animals that are entering second or later lactation (Bertics et al., 1992; Grummer et al., 2004). In fact, some of the detrimental consequences of negative energy balance (NEB) in early lactation could be prevented by force-feeding cows to avoid a drop in DMI prepartum (Bertics et al., 1992). Given the inadequate uptake of nutrients, all dairy cows are exposed to a certain degree of NEB during the transition to lactation (Goff, 2006; Ospina et al., 2013). Indicators or markers of NEB are increased concentrations of blood ketone bodies, specifically β -hydroxybutyrate (BHB), and an increase in the concentration of non-esterified fatty acids (NEFA) in serum or plasma from lipolysis (Ospina et al., 2013). Although an increase in these markers is part of the physiological adaptation during the transition period in dairy cattle, a rise above critical thresholds for BHB and NEFA concentrations has been associated repeatedly with negative health and

production outcomes in individual animals (Ospina et al., 2010a; Chapinal et al., 2011; Suthar et al., 2013) and at the herd level (Ospina et al., 2010b; Chapinal et al., 2012). Total cost per case of hyperketonemia (defined as a blood concentration equal to or greater than 1.2 mmol/L) was recently estimated at \$375 and \$256 for primiparous and multiparous animals, respectively (McArt et al., 2015). Comprehensive reviews summarizing the consequences of excessive NEB during the transition period (McArt et al., 2013) and the use of NEFA and BHB concentrations as markers for herd-level monitoring (Ospina et al., 2013) can be found elsewhere. The greatest degree of NEB occurs very early in lactation, with peak incidence for hyperketonemia and highest concentration of NEFA within the first week postpartum (McArt et al., 2012; McCarthy et al., 2015).

Based on these data, it becomes clear that a successful transition period comprises the adaptation to lactation without a large increase in these markers of negative energy balance, and that the prepartum period is of great importance to aid in the prevention of excessive negative energy balance in the immediate postpartum period.

Prepartum feeding strategies

The definition of plane of energy encompasses all the usable energy consumed that is not lost in faeces, urine, gas or heat production, and includes not only dietary carbohydrates, but also fat and protein (Berryman et al., 2013). Experimental evidence suggests that the total prepartum energy intake, regardless of the source, has effects on

postpartum lipid metabolism of dairy cattle (Douglas et al., 2006). To keep with the focus of this dissertation, this literature review will not include work on the supplementation of different fats or fatty acids in the prepartum diet, or research on different protein sources and amounts during this period, and instead focus on differences of plane of energy altered by the amount of starch and fiber, or carbohydrates in general, in the diet. The interested reader can find more information on fat supplementation prepartum in a number of recent studies (Grum et al., 1996; Komaragiri et al., 1998; Douglas et al., 2004; Douglas et al., 2006; Douglas et al., 2007; Castaneda-Gutierrez et al., 2009; Afzalzadeh et al., 2010; Damgaard et al., 2013; Karimian et al., 2015; Zapata et al., 2015), and more information on the effects of altering the amount of prepartum protein intake is available elsewhere (Curtis et al., 1985; van Saun et al., 1993; Putnam and Varga, 1998; Vandehaar et al., 1999; Bell et al., 2000; Santos et al., 2001; Doepel et al., 2002; Park et al., 2002; Phillips et al., 2003; Lean et al., 2013).

Transition dairy cows are considered to be in negative protein balance (NPB) for at least some part of the period of NEB since a decrease in DMI affects not only energy but also protein intakes while amino acid demands for the fetus in late pregnancy and for milk production steadily increase (Bell, 1995; Bell et al., 2000). The far-off and close-up period will generally be defined as they have been by Dann et al. (2006) as the beginning of the dry period until three weeks before expected calving, and the last three weeks before calving, respectively.

Traditionally it was thought that the additional energy provided to the prepartum cow would help offset the NEB of early lactation (Holcomb et al., 2001;

Rabelo et al., 2003) and improve production in the early postpartum period (McNamara et al., 2003). Grum et al. (1996) fed dry cows diets differing in energy and fat level in order to replenish energy reserves of cows before the onset of lactation. It was also seen as a way to reduce the degree of adipose tissue mobilization prepartum (Vandehaar et al., 1999; Rabelo et al., 2001). However, the effect on intake in the immediate postpartum period, when negative energy balance is most severe, has been inconclusive. A number of studies found a detrimental effect of feeding a higher energy diet prepartum on early postpartum intake (Douglas et al., 2006; Janovick and Drackley, 2010; Hayirli et al., 2011) whereas Doepel et al. (2002) found a less severe drop in peripartum intakes and higher postpartum intakes after feeding a high energy diet. Grummer et al. (2004) reviewed the existing literature and came to the conclusion that preventing a large drop in DMI in the last days before calving rather than maximizing DMI in the postpartum period should be the priority in considerations of transition cow feeding strategies. Management factors beyond feed composition and delivery such as grouping strategies, stocking density, heat abatement, and cow comfort clearly have a large influence on the success of this goal and may help explain the variability in findings (Van Saun and Sniffen, 2014) in addition to the cow-level variability discussed above.

Controlled energy diets in the prepartum period

In the early 2000s a number of research studies were conducted that showed a beneficial effect of restricting the energy intake prepartum to, or in some studies even

below requirements on liver health and prevention of fat accumulation in the liver (Douglas et al., 2006), which challenged the existing convention of the traditional close-up dry cow diets at this time (Dann et al., 2005). Energy intake in the prepartum period has been altered in studies by two different mechanisms, either by restricting the amount of feed, or by allowing *ad libitum* intake of a lower energy density diet with a higher inclusion rate of forage such as straw, or by a combination of both (Butler et al., 2011).

The distinction between controlling and restricting energy is important when regarding markers of negative energy balance, especially during the prepartum period, as energy intake has been limited to below requirements in some studies during this period, especially in those using feed restriction. In an experiment where researchers were attempting to isolate the effect of prepartum energy intake and fat supplementation, cows were either fed restricted to 80% of predicted requirements, or fed *ad libitum* (Douglas et al., 2006). The authors found that cows fed restricted amounts of TMR prepartum had higher DMI postpartum, as well as lower concentrations of both, NEFA and BHB in plasma after calving which was also observed previously by Holcomb et al. (2001).

Dann et al. (2006) conducted an experiment investigating the effect of dry period energy level during the far-off and close-up period separately. To achieve this, cows were fed 80, 100 or 150% of predicted requirements during far-off (60 to 22 d before expected calving) after which half of each group was assigned to be fed either restricted to approximately 80% of predicted requirements or fed the same diet *ad libitum* for the remainder of the dry period. Cows that were overfed energy during the

far-off period had the highest concentrations of BHB and the lowest energy balance postpartum, regardless of energy level during the close-up period, and also tended to have greater NEFA concentrations as well as lower DMI in the first week postpartum (Dann et al., 2006). The authors concluded that overfeeding energy during the far-off period had a significant carry-over effect into early lactation whereas close-up feeding strategy affected prepartum metabolism minimally.

In an extension of this work, Janovick and Drackley (2010) conducted an experiment in which cows were either fed a restricted to 80% of predicted requirements, or fed *ad libitum* to achieve approximately 100 and 150% of predicted energy requirements. The authors found that overfeeding energy prepartum resulted in large changes in periparturient energy balance, even in the absence of overt overconditioning (Janovick and Drackley, 2010) and that the inclusion of chopped wheat straw was successful in limiting energy intake to 100% of requirements at *ad libitum* intake. In addition, cows fed the controlled energy diet during the complete dry period had the lowest plasma NEFA and serum BHB concentrations postpartum among all groups (Janovick et al., 2011).

Hayirli et al. (2011) tested the hypothesis that prepartal feed restriction would help alleviate NEB by reducing the DMI depression observed in the peripartum period. Although no difference was found in postpartum NEFA and BHB concentrations, cows fed restricted prepartum did indeed show an increase by one kg in DMI postpartum (Hayirli et al., 2011). Restricted energy intake compared to an *ad libitum* fed group of the same diet (1.17 Mcal NE_L/kg) in the far-off period was investigated by Nowak et al. (2014); all cows were fed the same transition and

lactation diet *ad libitum* thereafter. Restricted feeding in the far-off period was associated with reduced BHB concentrations whereas NEFA concentrations were unchanged in early lactation (Nowak et al., 2014).

As has become clear with the above presented literature, feeding a limited amount of energy by restricting intakes has the potential to prevent excessive NEB. However, research situations where cows can be fed restricted on an individual basis are not translatable into modern free-stall housing systems where diets are fed to groups of animals.

More recent studies have thus investigated the effect of overfeeding energy in *ad libitum* feeding systems with varying results on the effect of postpartum concentration of markers of NEB. Diets that are fed *ad libitum* and are formulated to supply a controlled amount of energy are often bulky, high-fiber diets that include varying amounts of pre-chopped straw or hay. Potential differences when using chopped hay versus chopped straw to bulk up diets was the focus of two recent studies by Litherland et al. (2012; 2013). In the first study, wheat straw and orchard grass hay as a forage source were compared for *ad libitum* fed dry cow TMR compared with restricted fed TMR (Litherland et al., 2012). The authors found that cows fed *ad libitum* during the dry period had higher DMI in the first week postpartum compared with restricted fed cows, but that cows fed the straw based diet *ad libitum* had significantly higher concentrations of BHB in blood postpartum compared with the group fed orchard grass hay *ad libitum* (Litherland et al., 2012). In the second study, the authors found that, compared with grass hay, wheat straw forage addition prepartum resulted in lower postpartum NEFA concentrations, but no differences were

found for BHB concentrations. These studies were not conclusive in the optimal choice for the forage to be included in controlled energy *ad libitum* fed dry cow diets. However, higher energy, protein content, and NDF digestibility, as well as higher concentrations of potassium may limit the use of hay versus wheat straw as a forage source (Litherland et al., 2013).

A number of recent studies in North America have investigated the effect of diluting energy density of the dry cow TMR by inclusion of straw or hay. Richards (2011) conducted a study enrolling cows into three different *ad libitum* fed treatment groups, controlled or overfed energy during the whole dry period, and a third group that was fed a controlled energy TMR during far-off and a TMR supplying approximately 120% of predicted energy requirements during the close-up period. Both NEFA and BHB concentrations were highest postpartum in the overfed group and lowest in the one-group controlled energy group, whereas DMI did not differ between groups (Richards, 2011). In a Canadian study, researchers fed cows a high forage diet of different grass hay and rye grass straw during the far-off period (1.41 Mcal NE_L/kg, 13.6% starch) and continued to feed this diet to one group of cows until calving whereas the other group was fed a higher energy diet during the close-up period (1.46 Mcal NE_L/kg, 16.3% starch) (Vickers et al., 2013). Cows fed the higher energy diet in the close-up period had higher concentrations of blood BHB between day 1 to 10 postpartum (0.49 vs. 0.59 mmol/L).

Interest in feeding trials using different dry cow energy levels has increased also in Europe and other countries worldwide. In a Polish study, Nowak et al. (2013) fed diets that differed in energy density by different inclusion rates of wheat straw

(1.04 and 1.17 Mcal NE_L/kg) during the dry period until approximately one week prior to calving and did not observe differences in NEFA and BHB concentrations postpartum. However, both diets would be considered of low energy density even for far-off dry cows according to recent recommendations (Lean et al., 2013). The use of a high-fiber TMR with added wheat straw (1.17 Mcal NE_L/kg) was evaluated as an alternative to grass silage (1.40 Mcal NE_L/kg) by Butler et al. (2011) in the Irish pasture-based system; both diets were fed *ad libitum* during the whole dry period of approximately three months. Cows fed the controlled energy TMR showed no difference in plasma NEFA concentration, but had lower plasma BHB concentrations in the first four weeks postpartum (Butler et al., 2011).

In a recent Chinese study, Zhang et al. (2015) altered the energy density of prepartum diets during the close-up period by changing the ratio of inclusion of alfalfa hay (1.25, 1.41, 1.55 Mcal NE_L/kg DM, respectively) and found that those cows overfed energy had significantly higher postpartum NEFA concentrations, but BHB concentrations and DMI intake postpartum did not differ between groups.

In a study conducted in Finland, Selim et al. (2015) fed cows either grass silage (2.41 Mcal ME/kg DM) or grass silage with wheat straw (2.17 Mcal ME/kg DM) during the whole dry period and found no differences in NEFA and BHB concentrations either pre- or postpartum. Schultz et al. (2014) enrolled cows differing in body condition in a German study and overfed energy to those that were already overconditioned at the beginning of the dry period, whereas those with a normal body condition were fed a controlled energy diet. The authors found that the overconditioned and overfed dry cows went on to have over one and a half times the

concentration of BHB and NEFA in blood in the first two week postpartum. In an effort to isolate the effect of overconditioning and overfeeding of energy prepartum, Bjerre-Harpoth et al. (2014) set up a two by two factorial arrangement with two different dietary energy levels (1.19 vs 1.61 Mcal NE_L) and two different body condition groups (BCS 3.18 vs. 3.40) prepartum, achieved by altering the energy density of the diet in late lactation. The authors of this Danish study found that irrespective of BCS at the beginning of the dry period, cows fed a controlled energy diet had lower concentrations of plasma NEFA and BHB in early lactation (Bjerre-Harpoth et al., 2014).

Prepartum and postpartum energy interactions

Few studies have investigated the effect of prepartum energy levels in combination with postpartum plane of energy. Rabelo et al. (2005) fed cows a low and high energy prepartum diet (1.58 versus 1.70 Mcal NE_L/kg) and low and high postpartum energy diet (1.57 versus 1.63 Mcal NE_L/kg) in a 2 x 2 factorial design. The authors found that cows with the combination of low prepartum and low postpartum diet had highest concentrations of BHB postpartum whereas NEFA concentrations did not differ (Rabelo et al., 2005). However, the low and high prepartum diets had a nonfiber carbohydrate (NFC) content of 38.2 and 44.6%, respectively, and thus both diets were of comparatively high NFC content (Overton and Waldron, 2004). Urdl et al. (2015) enrolled 81 multiparous Holstein, Simmental, and Brown Swiss cows in a 3 x 3 factorial design to three different prepartum energy levels and three different

postpartum energy levels, in both phases supplying approximately 75, 100, and 125% of predicted requirements. Cows in this experiment were fed *ad libitum*, unless intake exceeded target values, when intake was restricted. The authors found that energy supply pre- and postpartum showed an interaction for postpartum energy balance and metabolic status. Cows fed restricted or to requirements during the dry period and fed a high energy diet postpartum had the highest energy balance and lowest BHB concentrations in the first 3 months postpartum and NEFA concentrations postpartum were highest in those animals overfed energy prepartum (Urdl et al., 2015). Dry matter intake postpartum also showed a strong interaction between pre- and postpartum energy level with highest postpartum intakes in cows fed a restricted or controlled energy diet prepartum and a high energy diet postpartum (Urdl et al., 2015).

These results show that dietary energy level prepartum cannot be seen independently of the postpartum diet. In addition to differences in management and cow-level response to different diets, this may be another factor that helps explain the variability of findings in studies on markers of NEB postpartum in relation to dry cow feeding strategies outlined above.

DRY COW FEEDING STRATEGIES AND MILK PRODUCTION

Milk production relative to prepartum plane of energy

Milk production is tightly linked to energy metabolism and intake of nutrients for the production of glucose for lactose synthesis. Immediately after parturition, liver glucose production nearly doubles within eleven days of calving as compared to

prepartum glucose output (Reynolds et al., 2003). The amount of insulin-independent glucose transporters in the mammary gland increases with lactation (Komatsu et al., 2005) and lactose yield increases with increasing milk production.

Besides the great interest in reducing negative energy balance because of the known detrimental effects on transition cow health, milk production is of great importance to the economic success of dairy operations. In fact, despite the increasing evidence that controlled energy dry cow strategies aid in a successful metabolic transition of cows into lactation, the adoption of such practices is met with some scepticism in the industry because of perceived differences in early lactation milk yield and of timing and magnitude of peak milk production (Drackley and Janovick Guretzky, 2007).

When reviewing the existing literature for the effect of different transition diets on milk production one has to be aware of potential sources of reduction of validity of the data, as many of the performed studies were restricted in cow numbers, leading to a large degree of variability in the measured response. Bias might stem from the relatively common practice of removal of those cows suffering from postpartum disorders from the dataset before reporting. Study animals are also under close supervision by the investigators so that disorders are treated (e.g. ketosis) and potential losses in milk production may therefore be prevented by early intervention (McArt et al., 2011).

Differences in milk production were observed in a number of studies over the last two decades. Minor et al. (1998) found a trend for a higher milk production in cows fed a higher energy diet prepartum. In a recent Irish study by Butler et al. (2011)

comparing a higher energy grass silage based diet with a diet containing wheat straw, data was available for early lactation and total lactation milk yields. Authors found a trend for higher milk yield and a higher solids-corrected yield in the first eight weeks of lactation for cows fed grass silage, as well as a numerically lower peak yield in the lower energy wheat straw group, whereas no differences were found over the whole course (average 294 days, no standardized 305-day lactation given) of the lactation (Butler et al., 2011). In a study comparing two one-group feeding strategies (controlled energy and overfed) with a two-stage strategy where cows were fed a controlled energy diet during the far-off period and a higher energy diet during close up, Richards (2011) found no differences in milk production, but lower lactose and fat concentration as well as lower fat-corrected milk yield in cows fed a controlled energy diet throughout the whole dry period compared with those overfed energy for the same period. Similar differences were found in the study by Janovick and Drackley (2010) where cows overfed energy during the dry period showed higher milk fat concentration and subsequently higher fat-corrected milk yield in the second and third week postpartum. Vickers et al. (2013) found milk production during the first month of lactation to be lower in cows fed a high forage, controlled energy diet throughout the whole dry period compared with cows receiving a moderately increased energy diet in the close-up period alone. A trend for a higher production in the step-up group was present over the first 22 weeks of lactation (Vickers et al., 2013). Another study found milk production in the first ten weeks of lactation to be increased in cows fed a controlled energy diet prepartum compared with cows overfed energy by approximately 50% of predicted requirements, whereas fat concentration was

decreased, resulting in no overall differences in 4% fat-corrected milk yield (Huang et al., 2014).

In contrast, others did not find an effect of prepartum dietary energy level on postpartum milk production (Grum et al., 1996; Mashek and Beede, 2000; Agenas et al., 2003; Rabelo et al., 2003; Dann et al., 2006; Douglas et al., 2006; Winkelman et al., 2008; Hayirli et al., 2011; Ji et al., 2012; Nowak et al., 2014).

It is apparent that there are only few studies investigating the persistence and whole lactation yield following different dry period treatments. In addition, effects of different energy densities prepartum may be confounded by the provision of adequate protein during this time, particularly in cows restricted in intake, since underfeeding of both protein and energy will limit the availability of metabolizable protein (Bell et al., 2000). Reduced availability of protein during the dry period may furthermore compromise labile protein reserves which could lead to an impairment of lactational performance and health in the subsequent lactation (Ji and Dann, 2013).

Colostrum quality and production relative to prepartum energy level

Whereas milk production in early lactation in relation to dry cow feeding strategies has been assessed in a multitude of studies, there is a dearth of data on the effect on the first milk, colostrum. Nowak et al. (2012) measured dry matter and concentrations of colostral immunoglobulins by ELISA and found no difference between colostrum of cows fed either one of two high-fiber diets differing in energy (1.04 Mcal NE_L/kg and 52.3% NDF vs. 1.17 Mcal NE_L/kg and 56.2% NDF) in the

prepartum period. Richards (2011) studied the effect of prepartum plane of energy in cows fed either a controlled energy diet (1.34 Mcal NE_L/kg, 53.9 % NDF) or a high energy diet (1.61 Mcal NE_L/kg, 37.2% NDF) for the whole dry period, or the controlled energy diet during the far-off period and the higher energy diet during the close up period. Colostrum weight did not differ, however this was only assessed in 3, 6, and 6 cows in the respective groups and colostral IgG concentration determined by colostrometer was also not different (Richards, 2011).

WHOLE BODY METABOLISM AND INSULIN SIGNALING IN THE PERIPARTUM PERIOD

Physiological insulin resistance and adaptations to metabolism in the prepartum period

Insulin resistance is defined as a state where a normal concentration of insulin elicits less than the biological response in insulin-sensitive tissues and is divided into reduced insulin sensitivity and insulin responsiveness, or a combination of both (Kahn, 1978). A decreased sensitivity is characterized by the ability to reach the maximal biological effect only at a higher than normal insulin concentration (i.e. shift of the dose-response curve to the right) whereas a decreased responsiveness is characterized by a decreased maximal effect (Kahn, 1978). A number of different methods including hyperinsulinemic euglycemic clamp test, intravenous glucose tolerance test, intravenous insulin tolerance test, and surrogate indices can be used to assess insulin sensitivity and have been described in detail for use in dairy cattle (De Koster and

Opsomer, 2013). Because of fundamental differences in ruminant glucose sources, ruminants have an inherently lower insulin responsiveness of peripheral tissues compared to non-ruminants (Sasaki, 2002). Unlike in non-ruminants, nutrients are subjected to ruminal microbial degradation and fermentation in ruminants. As a result only a small amount of glucose is absorbed directly and plasma glucose concentrations are therefore lower in ruminants than in non-ruminants (Hayirli, 2006).

Bauman and Currie (1980) described the coordinated changes of nutrient partitioning during pregnancy and lactation as homeorhesis. Despite fundamental differences in glucose metabolism between ruminants and humans, this situation seems to be part of a normal pregnancy in humans (Hodson et al., 2013) and cattle (Bell, 1995), when insulin sensitivity declines with advancing gestation and glucose concentrations are controlled by an increasing concentration of insulin (Baz et al., 2015), shifting the dose-response curve to the right. As in humans, fasting insulin and blood glucose are higher in cows prepartum (Janovick et al., 2011). The adaptations of energy metabolism in late pregnancy assure that the conceptus receives adequate nutrients in the form of glucose (or lactate from the placenta) and amino acids because fetal uptake of lipids is limited in ruminants (Bell, 1995). During the last two months of pregnancy, glucose demand of uterus and fetus is equal to the metabolic demands of about 3 to 6 kg of milk per day (Bauman and Currie, 1980).

Transfer of glucose to the fetus is an insulin-independent process, and reduced glucose use in the peripheral tissues together with high circulating glucose concentrations assures sufficient transfer of glucose from dam to fetus (Hayirli, 2006). During this time insulin also has a decreased ability to promote lipogenesis and to

oppose lipolysis during this time (Bell, 1995). The reduced ability of insulin to suppress adipose tissue mobilization is observed as a certain degree of increase in NEFA concentration, evident in most dairy cows in the days before calving. The increase in NEFA concentration is particularly evident when animals are restricted in energy intake before calving (Dann et al., 2006; Douglas et al., 2006) and is likely due to lower circulating glucose and insulin concentrations compared to animals fed above requirements as described before.

The increase in glucose and insulin concentrations prepartum is more pronounced in cows receiving higher energy diets compared with those fed restricted or controlled energy diets (Holtenius et al., 2003; Douglas et al., 2006; Zhang et al., 2015). This difference may be due to the greater availability of gluconeogenic substrates such as propionate (Janovick et al., 2011), but the exact mechanism has not been elucidated in detail.

A limited number of research studies have investigated if overfeeding energy during the dry period alters insulin sensitivity. Schoenberg et al. (2011, 2012) investigated how plane of nutrition affects the response of dry cows to a glucose challenge in two experiments. Cows were fed either approximately 90% or 160%, or 120% and 170% of predicted energy requirements during the dry period in the two experiments, respectively. Cows fed 90% of energy requirements tended to have higher glucose area under the curve following challenge and decreased glucose clearance and greater NEFA response compared with overfed cows. Diet had no effect on the insulin response in both studies. Holtenius et al. (2003) found a numerically higher insulin peak and higher glucose clearance following a glucose challenge in

cows overfed energy during the dry period compared with cows fed a lower energy diet.

Glucose sparing effect and alternative fuels in the peripartum period

Nutrient partitioning in late gestation leads to a glucose sparing effect of peripheral tissues and increased gluconeogenesis to fill the energy demands of the uterine tissues and growing fetus as described above (Bell, 1995). This increases the energetically costly process of gluconeogenesis, which accounts for almost all the ruminant animal's glucose supply (Bell, 1979).

With the onset of lactation glucose demands are vastly increased with mammary uptake increasing to approximately nine times the amount on the day after calving compared to the week prepartum (Bell, 1995). The expression of the major mammary gland glucose transporter GLUT1, which functions independently of insulin, is greatly increased in mammary tissue of peak lactating and late lactating cows compared to non-lactating animals, whereas the situation is reversed in adipose tissue (Komatsu et al., 2005; Sadri et al., 2010). In addition, the abundance of the insulin-dependent glucose transporter GLUT4 is decreased postpartum in adipose and muscle tissue (Sadri et al., 2010; Kuhla et al., 2011), likely in response to the decreased insulin concentrations at this time. The priority of glucose use lies in the mammary tissue with the onset of lactation, making it necessary for other tissues to use alternative fuels such as fatty acids (Bauman and Currie, 1980; Bell, 1995). Muscular fatty acid oxidation is increased early postpartum, and declines over the first

four weeks of lactation (Schaff et al., 2013), in addition lactate production from anaerobic glycolysis increases (Kuhla et al., 2011).

In addition to increased hepatic gluconeogenesis and reduced use of glucose in the periphery, adipose tissue lipolysis builds the hallmark of metabolic adaptation, allowing for increased mobilization of NEFA from adipose tissue in the days before parturition, as well as increased use of amino acids, partially from muscle mobilization (Bell, 1995). The liver takes up about a third of the circulating NEFA at first pass where it can be used for energy, repackaged or stored (Bell, 1979; Drackley et al., 2001). However, release of glycerol as a substrate for gluconeogenesis and NEFA as a source of ATP production from adipose tissue can lead to an elevated fat content in the liver, which in turn can lower the capacity for hepatic glucose production (Rukkwamsuk et al., 1999; Murondoti et al., 2004). The resulting lower blood glucose concentration is associated with further decreased insulin concentration, leading to even more adipose tissue mobilization (Adewuyi et al., 2005). In contrast to humans, VLDL secretory capacity and triglyceride concentration in blood are low (Pullen et al., 1990; Katoh, 2002) which limits the ability to export triglycerides from hepatic tissues. A higher concentration of circulating NEFA in the postpartum period has been linked to excess energy in the dry period (Kunz et al., 1985; Holtenius et al., 2003; Janovick et al., 2011; Cardoso et al., 2013; Khan et al., 2014) and fatty liver (Rukkwamsuk et al., 1999; Murondoti et al., 2004; Hammon et al., 2009) due to the slow rate at which the ruminant liver is able to secrete lipids (Bobe et al., 2003).

Insulin sensitivity postpartum

Although the presence of insulin resistance in late gestation in dairy cows has been proposed (Hayirli, 2006; De Koster and Opsomer, 2013) and is said to correspond to a physiological adaptation to pregnancy also seen in other ruminants and humans (Pettersson et al., 1993; Barbour et al., 2007) as described above, it is of particular interest to know if such a state of insulin resistance is carried over into early lactation (Komatsu et al., 2005), and may be an underlying factor in the failure of successful adaptation to lactation.

When considering this question, one has to keep in mind that the prominent change in metabolic adaptation to lactation leads to low insulin and high glucagon concentrations in circulation. This situation promotes lipolysis and gluconeogenesis as well as ketogenesis, preserving glucose supply during NEB (Hue and Taegtmeier, 2009). Glucagon is essential for maintenance of blood glucose levels through increasing hepatic glucose output (Torres et al., 2009). Insulin action in turn is tightly linked to the circulating glucose concentrations (Aronoff et al., 2004) via GLUT2 in the pancreas and subsequent production of ATP, however additional nutrients can influence insulin secretion to a certain extent (Torres et al., 2009).

Insulin concentrations decline continuously from about a month before calving to the lowest concentrations in the first week after calving, parallel to the sharp drop in glucose concentrations after calving (Vallimont et al., 2001). The decrease in insulin in the days before calving and after parturition has been documented in a number of studies (Vallimont et al., 2001; Janovick et al., 2011) and is more pronounced in animals undergoing a prolonged period of negative energy balance (Hayirli, 2006).

Since insulin has a strong inhibitory effect on lipolysis, this drop in the circulating levels and low insulin status allows for a greater degree of adipose tissue mobilization and an increase in NEFA in the blood stream as well as for a reduced inhibiting effect of insulin on gluconeogenesis (Zachut et al., 2013).

Insulin is predominantly metabolized in the liver (Ferrannini and Cobelli, 1987), and liver blood flow is greatly increased with the onset of lactation (Lomax and Baird, 1983), which may add to the decrease in blood concentrations found in early lactation. This complicates any comparison to prepartum physiology together with vastly different glucose disposal rates due to the large insulin-independent drain to the udder. Moreover, earlier studies using sheep as the animal model have not reached conclusive results (Ji et al., 2012).

Researchers have attempted to address this fundamental question of insulin resistance both on a whole-body basis, as well as by examining the insulin sensitivity of certain tissues independently from the rest of the body and mammary gland. A few early studies suggested an impaired insulin-stimulated uptake of glucose postpartum in several ruminant models (Debras et al., 1989; Faulkner and Pollock, 1990; Vernon et al., 1990; Petterson et al., 1993). In the study by Debras and coauthors (1989) on goats, postpartum insulin resistance (inhibition of insulin-stimulated radioactively labelled glucose utilization in a hyperinsulinemic euglycemic clamp test) could only be caused at higher than physiological plasma insulin concentrations. In the same study an improved ability of insulin to decrease hepatic glucose production during early lactation was also found. Using a clamp test in the hind-limb in sheep, Vernon and coauthors (1990) found that insulin-stimulated glucose uptake in lactating animals

could not be increased to the same degree as in non-lactating non-pregnant sheep. In a previous *in vitro* study by Vernon and Taylor (1988) on lactating sheep adipocytes, glucose utilization was decreased compared to non-lactating sheep adipocytes. Yet the insulin signal transduction could be restored at longer exposure (48 h) to insulin (Vernon and Taylor, 1988) reflecting an adaptability of the cells to changing insulin concentrations over a course of several days. The differences observed in lactating animals highlight the importance of taking the lower resting blood glucose and insulin concentrations preceding the experiment into consideration since insulin release as a response to a glucose induced signal is dependent on the current physiological state (Hove, 1978). In fact, insulin concentrations to reach the half maximal effect in hyperinsulinemic euglycemic clamp tests are correlated with resting insulin concentrations (Kraeft, 2004). In addition, prolonged negative energy balance leads to decrease in pancreatic islet cell number and size, lower insulin secretion, lower basal insulin and glucose as well as lower glucose clearance rates (Hayirli, 2006). Moreover, fasting reduces resting insulin concentrations and insulin response to a glucose stimulus (Oikawa and Oetzel, 2006). There is recent evidence that resting insulin concentrations postpartum are affected by prepartum plane of energy (Hayirli et al., 2011; Janovick et al., 2011; Zhang et al., 2015) with cows fed a restricted or controlled energy in the dry period showing higher resting insulin concentrations postpartum.

Ketogenesis and types of ketosis

The liver and ruminal epithelium are the major sites of production of the three ketone bodies acetoacetate (AcAc), β -hydroxybutyrate (BHB), and acetone in ruminants (Bergman, 1971). During times of positive energy balance in fed ruminants, ketone bodies are mainly synthesized from butyrate in the ruminal wall, whereas hepatic partial oxidation is a secondary source of ketone bodies (Bell, 1979; Chilliard et al., 1998). Postpartum ketogenesis is initiated in light of high rates of gluconeogenesis and a relative lack of oxaloacetate as a major causal factor. Ketones are used as alternative energetic substrates, especially during the precarious glucose supply in early lactation. Skeletal muscle has a particularly high capacity of uptake and use of ketone bodies (Bell, 1979). Acetoacetate can be reduced to BHB in a reversible reaction, but AcAc is an unstable compound, forming acetone and CO₂, the former giving the characteristic sweet breath (Bergman, 1971). BHB has to be converted back to AcAc to be metabolized by tissues by an enzyme located at the mitochondrial membrane (Bergman, 1971). It is worth noting that BHB is more abundant than AcAc, but the ratio decreases as the animal becomes increasingly hyperketonemic and is usually greater in the liver (Bergman, 1971). Insulin and glucose concentrations in animals with postpartum hyperketonemia are frequently lower compared with normal animals (Hove, 1974; 1978).

Historically ketosis has been separated in type I and type II, with type I occurring later in lactation (3-6 weeks) after calving in cows with high milk production unable to take up sufficient nutrients for the energetic demands of their production level and is associated with hypoglycemia and hypoinsulinemia (Holtenius

and Holtenius, 1996). In contrast, type II ketosis has been linked to overfeeding in the dry period, which is thought to lead to disturbances in hormonal adaptation of metabolism and lead to a hyperglycemia and hyperinsulinemic form of ketosis earlier in lactation in association with increased adipose tissue lipolysis and fatty liver development (Holtenius and Holtenius, 1996). Because of the apparent similarities of this metabolic state it has been compared to type II diabetes in humans (Holtenius and Holtenius, 1996), however recent data showing this clear distinction are lacking.

It is therefore of particular interest to investigate the effect of overfeeding not only on resting glucose and insulin concentrations and whole body insulin response, but also on adipose tissue insulin signaling to ascertain if the periparturient cow overfed energy during the dry period is indeed in a similar endocrine state as humans with type II diabetes. Knowledge on the particular metabolic changes that are associated with overfeeding can help provide an explanation to the increased concentration of NEFA and ketone bodies in cows overfed energy before calving as hypothesized (Beever, 2006; De Koster and Opsomer, 2013).

PERIPARTUM CHANGES IN ADIPOSE TISSUE

Regulation of adipose tissue accretion and mobilization

Accretion – The liver of ruminants, unlike in humans, is not a primary site of fatty acid synthesis and hepatic fatty acid synthesis is negligible (Bell, 1979; Vernon, 1981). Lipogenesis is dependent on the availability of insulin in ruminants (Hayirli, 2006) and is modulated by leptin and other factors regulating energy metabolism (Wronska and Kmiec, 2012). In ruminants acetate is the major precursor for lipogenesis in adipose tissue, and glucose only the second largest contributor, which is different from non-ruminants where glucose is the major substrate for lipogenesis (Vernon, 1981; Hayirli, 2006). The first step in fatty acid synthesis is catalysed by acetyl-CoA carboxylase (ACC) to form malonyl-CoA with elongation of the fatty acid being enzymatically regulated by fatty acid synthase (FASN) (Moore and Christie, 1981).

In addition to adipose tissue *de novo* lipogenesis, lipoprotein lipase attached to the endothelium of blood vessels can hydrolyse preformed triacylglycerides from circulating chylomicrons and very low-density lipoproteins, leading to fatty acid uptake into the adipocyte (Wronska and Kmiec, 2012). This process is necessary since triacylglycerides are unable to pass through biological membranes (Fruhbeck et al., 2014). Alternatively, NEFA can be taken up directly by fat cells and be re-esterified into triacylglycerides. These same processes are taking place in the mammary gland

for the synthesis of milk fat with the difference that almost no glucose carbon is used for fatty acid synthesis in this organ (Moore and Christie, 1981).

Reflecting the need for positive energy balance and presence of insulin for fatty acid synthesis, the abundance of the enzymes critically involved in synthesis of fatty acids (ACC and the multi-enzyme protein FASN) was greatly reduced postpartum in subcutaneous adipose tissue of cows fed high and low energy diets in the close-up period (Doepel et al., 2002) and in cows fed the same diet prepartum (Sadri et al., 2011). Cows overfed energy during the far-off period gained more adipose tissue mass prepartum compared to cows fed a controlled energy diet (Kokkonen et al., 2005). Upregulation of genes involved in lipogenesis in adipose tissue of cows overfed energy during the dry period has also been described by Ji et al. (2014a) in a recent study. In another experiment by the same author, cows fed a moderate energy diet during the close-up period had higher expression of both FASN and ACC compared with cows fed a controlled energy diet, but both groups showed a decrease to the same low expression postpartum (Ji et al., 2012).

Lipolysis – Adipose tissue represents the major energy reserve in mammals during fasting and exercise (Nielsen et al., 2014) and accounts for over 90% of the change in total body energy in early lactation (Komaragiri and Erdman, 1997). Adipose tissue mobilization in early lactation is estimated to have a mean of 50 kg, but can be up to 90 kg (Komaragiri et al., 1998). The mobilization of adipose tissue and an increase in circulating NEFA in dairy cattle is considered the hallmark of the transition to lactation (Bell, 1995). The low insulin to glucagon ratio that has its nadir in the first

week postpartum is considered as an important trigger of postpartum lipolysis and NEFA production (Adewuyi et al., 2005) since insulin suppresses lipolysis (Hayirli, 2006) and cows with higher concentrations of plasma NEFA postpartum showed lower plasma insulin concentrations compared with cows having lower NEFA concentrations (van Dorland et al., 2012).

In addition, the ability of catecholamines to stimulate lipolysis through β -adrenergic receptors (Nielsen et al., 2014) is already increased in late pregnancy (Bell, 1995) and release of glycerol in response to epinephrine in vitro peaks postpartum (Jaster and Wegner, 1981; Kokkonen et al., 2005). Kokkonen et al. (2005) showed that different degrees of body fatness following overfeeding in the far-off period were associated with increased NEFA concentrations, but no differences in baseline lipolytic rates of adipose tissue were observed. However, regarding the glycerol release following a norepinephrine stimulus, cows with increased body condition that were overfed in the far-off period had a higher response of adipose tissue biopsies *ex vivo* (Kokkonen et al., 2005).

The process of lipolysis involves the orchestrated activation of several cellular components, including perilipin, hormone-sensitive lipase (HSL), and adipose triglyceride lipase (ATGL) (Koltes and Spurlock, 2011). Activation of HSL is through phosphorylation, and is directly linked to β -adrenergic signaling via protein kinase A (PKA) activation (Koltes and Spurlock, 2011) and is inhibited by insulin by direct and indirect pathways within minutes upon binding of the hormone to its receptor (Fruhbeck et al., 2014; Nielsen et al., 2014). Insulin inhibits lipolysis via a pathway that reduces the amount of cyclic adenosine monophosphate (cAMP) in the cell which

in turn reduces the stimulation of HSL, and by activating protein phosphatase-1 which subsequently dephosphorylates HSL, rendering it inactive (Fruhbeck et al., 2014). Perilipin is located around the lipid droplet in the adipocyte as a coating protein and its phosphorylation leads to conformational changes that expose the droplet to HSL (Wronska and Kmiec, 2012). Phosphorylation of HSL and perilipin increases with the increased rate of lipolysis in early lactation (Koltes and Spurlock, 2011; Locher et al., 2011) and differences exist in the sensitivity of different adipose tissue depots to catecholamine-driven lipolysis signals (Locher et al., 2011).

The gene expression of HSL and perilipin was found unchanged in the peripartum period in one study (Sadri et al., 2011) whereas Sumner and McNamara (2007) described an increase in both HSL and perilipin expression postpartum. Locher et al. (2011) showed a decrease in the protein abundance of this enzyme in adipose tissue after calving. Cows overfed energy in the close-up period had a higher mRNA expression of ATGL and perilipin on ten days prepartum and seven days postpartum compared with cows fed a controlled energy diet whereas no difference was observed for HSL expression (Ji et al., 2012). Interestingly, postpartum ketotic cows had lower subcutaneous HSL mRNA abundance despite a higher rate of lipolysis as expressed by higher NEFA concentrations in plasma compared with non-ketotic cows (Xia et al., 2012).

Studies in dairy cows are mostly limited to subcutaneous fat because it is readily biopsied (Saremi et al., 2014), yet lipolytic response of the visceral adipose depot might differ. Given the findings in human medicine where visceral fat depots is described as having a more important role in development of insulin resistance, the

current lack of data on visceral adipose tissue characteristics in transition dairy cows is disconcerting. A recent study addressed this lack of knowledge by comparing gene expression of key regulators of lipogenesis, lipolysis, transcription regulation, and adipose tissue inflammation in animals overfed or fed a controlled energy diet (Ji et al., 2014a). After a period of eight weeks of overfeeding or feeding a controlled energy diet, subcutaneous, omental, mesenteric, and perirenal adipose tissue were harvested at the time of euthanasia (Ji et al., 2014a). Expression of FASN and ACC was greater in subcutaneous adipose tissue whereas that of lipoprotein lipase, leptin and adiponectin was smaller, and expression of PPAR γ was unchanged compared with visceral adipose tissue (Ji et al., 2014b). The authors used non-pregnant, non-lactating Holstein cows as a model which limits the generalizability to transition dairy cows.

A smaller mRNA abundance of adiponectin in sternal and mesenteric subcutaneous adipose tissue compared with retroperitoneal fat was recently also shown, however, there were no significant differences to other depots tested (tail head and withers subcutaneous, omental visceral locations) (Saremi et al., 2014). The results of a study by Saremi et al. (2014) suggests that differences in gene expression for a number of genes might exist within different visceral, but also within different subcutaneous adipose tissues. Therefore care should be exerted when comparing data between studies using different sources of visceral and subcutaneous adipose tissue.

Insulin signaling in adipose tissue of peripartum dairy cows

As described previously, excessive adipose tissue mobilization is associated with negative health outcomes. Since insulin is a potent inhibitor of lipolysis, specific interest lies in assessing insulin signaling and insulin sensitivity in adipose tissue in the peripartum period, and in relation to overfeeding during the prepartum period. Alterations in insulin signaling might help explain the increased NEFA concentrations often observed in early postpartum dairy cows overfed energy prepartum. In addition, high circulating concentrations of NEFA in humans have been implicated in the development of impaired insulin responsiveness (Wronska and Kmiec, 2012).

Insulin signaling occurs with a complex, integrated network that activates intracellular pathways controlling cell metabolism, growth and differentiation and can be simplified in the mainly metabolic PI3 kinase/AKT, and the mainly mitogenic Ras/MAPK/ERK pathway (Vigneri et al., 2010). After binding of insulin to its receptor, insulin receptor substrate (IRS) proteins activate those two pathways, (Taniguchi et al., 2006). Insulin can also bind to the insulin-growth factor-1 (IGF-1) receptor, as well as to the hybrid receptor formed by the insulin as well as insulin-growth factor dimeric structure, albeit with lower affinity (Velloso, 2008). Both receptor types are tyrosine kinase receptors with the capacity to phosphorylate the tyrosine residues in the effector proteins. Insulin signal transduction at the cellular level occurs in three stages: 1) phosphorylation of insulin receptors, 2) signaling to the intracellular secondary messengers, and 3) translocation of glucose transporters and other effects of insulin signaling following second messenger activation (Hayirli, 2006).

Recent studies in transition dairy cows have focused on insulin signaling in particular tissues, especially adipose tissue. The mRNA expression of insulin receptors in liver and adipose tissue was found to be unchanged in the transition period (Sadri et al., 2010; Ji et al., 2012) or increased postpartum (Gross et al., 2011; Zachut et al., 2013). The abundance of insulin receptor mRNA and insulin receptor substrate 1 and 2 was unchanged in subcutaneous adipose tissue of transition dairy cows (Sadri et al., 2010) and overfeeding energy in the dry period had no measurable effect on gene expression of adipose tissue IRS-1 in a recent Finnish study (Selim et al., 2014). Phosphorylation of insulin receptor substrate 1 (IRS-1) was decreased in samples postpartum, which could be due to a state of insulin resistance, but also be caused by the concurrent hypoinsulinemia of early lactation (Ji et al., 2012). In the same study, the expression of *GLUT4* was downregulated after calving, indicating a reduced insulin state as well (Ji et al., 2012). The authors found no difference in the level of phosphorylation of IRS-1 in subcutaneous adipose tissue of cows either fed a moderate or controlled energy diet (1.47 vs. 1.24 Mcal NE_L/kg) during the close-up period (Ji et al., 2012).

Phosphorylation of the kinase AKT in adipose tissue biopsies, which is increased through insulin signaling, was increased only in four out of eight cows in a recent study after glucose infusion leading to an endogenous insulin stimulus (Zachut et al., 2013). The animals that had an absence of increase in insulin signaling as defined by AKT phosphorylation were different from those that did respond in the respect that they had greater body weight loss in early lactation. The authors

hypothesized that adipose tissue specific insulin resistance occurs in cows prone to high weight loss (Zachut et al., 2013).

The ability of the adipose tissue in prepartum dairy cows to respond to an insulin stimulus was tested recently using the hyperinsulinemic euglycemic clamp test method in cows of varying degrees of body fatness (De Koster et al., 2015). No difference was found in insulin responsiveness between five overconditioned and four normally conditioned animals in respect to the ability to decrease lipolysis; however, cows were euthanized before parturition and no measurements taken after calving (De Koster et al., 2015).

Adipokines

Increasing evidence in the literature ascertains that adipose tissue is not merely an organ of fuel storage, but functions as a regulator of metabolic homeostasis through adipokines such as leptin and adiponectin (Wronska and Kmiec, 2012). It also influences other biological functions such as blood pressure, blood clotting, and immunity by secreting hormones and cytokines, referred to as adipokines which may act locally or systemically (Wronska and Kmiec, 2012). Leptin and adiponectin are among the best characterized adipokines in dairy cattle and will be presented herein.

Leptin – Leptin is produced mainly in adipose tissue and has the effect of lowering lipid levels in tissues by stimulating lipolysis, inhibiting lipogenesis, increasing muscle glucose utilization, and fatty acid oxidation in muscle (Chilliard et al., 2005). Leptin concentration increases with increased concentration of plasma

insulin (Leury et al., 2003). Plasma leptin, insulin, and glucose, as well as subcutaneous adipose tissue leptin mRNA abundance were significantly decreased in cows with ketosis (BHB concentration on average 3.5 mmol/L) compared with non-ketotic cows (BHB concentration on average 0.6 mmol/L) (Xia et al., 2012). Leptin gene expression did not change drastically from pre- to postpartum values in dairy cows two studies (Sadri et al., 2011; Selim et al., 2014) but declined dramatically from prepartum values in another study (Saremi et al., 2014). Plasma concentrations of leptin were consistently shown to decline in the immediate peripartum period when energy balance is negative (Block et al., 2003; Liefers et al., 2003; Meikle et al., 2004; Sadri et al., 2011; Saremi et al., 2014). This is seen as part of the metabolic adaptation to coordinate energy metabolism during this time of undernutrition and hypoinsulinemia (Chilliard et al., 1998; Block et al., 2003). Holtenius et al. (2003) studied the plasma concentration of leptin in cows fed diets with different levels of energy prepartum. Cows receiving the highest energy diet had higher concentrations of leptin and insulin during the prepartum period, but no difference in leptin concentration postpartum was noted (Holtenius et al., 2003). Leptin was found to be positively associated with body condition, energy balance, body weight and insulin and showed seasonal variation with the highest concentrations in spring (Reist et al., 2003). Xia et al. (2012) investigated the relationship of ketosis with leptin mRNA abundance in adipose tissue and leptin plasma concentrations and found that both were decreased in animals with BHB concentrations greater than 1.4 mmol/L compared with those with concentrations below 1.0 mmol/L.

Adiponectin – Adiponectin is considered an upregulator of insulin sensitivity and lipogenesis and its abundance in adipose tissue decreases around parturition (Saremi et al., 2014). The amount of adipose tissue is positively correlated with mesenteric adiponectin expression (Mielenz et al., 2013b). In humans, adipose tissue expression as well as circulating concentrations of adiponectin are decreased in obesity (Fruhbeck et al., 2014). Reduced serum concentrations might enhance gluconeogenesis (Saremi et al., 2014) and increased adipose tissue mass could therefore have a detrimental effect on gluconeogenesis through increasing circulating adiponectin. Adiponectin monomers have a size of approximately 30 kD, but the adipokine circulates in different molecular weight forms ranging from trimeric to multimeric exceeding 250 kD. The high molecular weight (HMW) adiponectin plays the most important role in mediating the insulin sensitizing effect of the adipokine in humans where a decrease in this weight form was associated with obesity-induced insulin resistance (Yamauchi and Kadowaki, 2008). In fact, the HMW to total adiponectin ratio was proposed as more useful than total adiponectin in the diagnosis of metabolic syndrome (Yamauchi and Kadowaki, 2008). Circulating adiponectin in dairy cows was found to be mainly of the HMW form and unaffected by stage of lactation (Giesy et al., 2012). Adiponectin also has potent anti-inflammatory effects and might play a role in the inflammatory processes around parturition when its expression is decreased (Kabara et al., 2014). In fact, adiponectin treatment of bovine macrophages in vitro was associated with a decrease in TNF α expression after LPS challenge (Kabara et al., 2014).

Peripartal adipose tissue adiponectin expression has been shown to increase due to overfeeding in the dry period (Ji et al., 2012). Circulating adiponectin concentrations drop in the week before calving, reach a nadir in the first days after parturition and then start increasing again (Giesy et al., 2012; Mielenz et al., 2013a). Concentrations of this protein are higher one month after calving compared with the dry period and transition period time-points (Kabara et al., 2014). Gene expression in subcutaneous adipose tissue reached a nadir during the immediate postpartum period and was highest in the dry period (Saremi et al., 2014). This reduction in adiponectin around the time of most severe NEB might facilitate lipolysis from adipose depots (Kabara et al., 2014). The abundance of adiponectin receptors 1 and 2 mRNA was decreased postpartum in comparison with prepartum expression in one study (Lemor et al., 2009) whereas no change in receptor gene expression was seen in another study (Sadri et al., 2011).

Peroxisomes and PPAR

Peroxisome proliferator-activated receptors (PPAR) are a class of ligand-activated transcription factors involved in lipid metabolism (Astapova and Leff, 2012). PPAR α expression in the liver of transition cows plays a key role in hepatic long chain fatty acid uptake, intracellular activation, oxidation and ketogenesis (Khan et al., 2014). During times of increased NEFA supply to the liver, the peroxisomal β -oxidation pathway can be induced and can account for almost 50 percent of β -oxidation capacity from palmitate (Grum et al., 1996). The expression of this

transcription factor increases after calving (Loor et al., 2005) and at a higher rate in cows overfed energy during late gestation, possibly due to the greater NEFA flux to the liver (Khan et al., 2014).

PPAR γ is expressed mainly in adipose tissue, increases fatty acid uptake, adipocyte differentiation and adipogenesis, increases expression of ACC, and improves insulin sensitivity (Vidal-Puig et al., 1996; Hue and Taegtmeyer, 2009; Astapova and Leff, 2012). Expression reduced from prepartum values in the postpartum period (Gupta et al., 2005; Schmitt et al., 2011; Saremi et al., 2014) and expression was higher in the immediate peripartum period in cows overfed energy during the dry period (Ji et al., 2012). The nadir of PPAR γ expression has been reported to be around three weeks postpartum (van Dorland et al., 2012; Saremi et al., 2014) when PPAR α is upregulated (van Dorland et al., 2012). Cows with high NEFA concentrations postpartum showed a decrease in PPAR γ expression from the day after calving to the third week postpartum whereas cows with low NEFA concentrations showed a stable expression of this gene (van Dorland et al., 2012).

In two studies by Smith et al. (Smith et al., 2007; Smith et al., 2009), the intravenous administration of a PPAR γ ligand (thiazolidinedione, TZD) in prepartum dairy cows was tested. In the first study, prepartum cows were treated daily from 25 days before expected parturition until the day of calving (Smith et al., 2007). Concentrations of NEFA prepartum and in the week postpartum were decreased in the treatment group, but plasma glucose, as well as liver lipid and glycogen concentrations remained unchanged (Smith et al., 2007). Upon daily treatment of dry cows for three weeks with one of two doses of TZD (2 or 4 mg/kg BW) in a second study, plasma

NEFA concentration as well as liver triglyceride in the postpartum period were decreased and plasma glucose and liver glycogen concentration increased and body condition was maintained through the transition period in TZD treated cows (Smith et al., 2009). The results of these two studies highlight the potential for PPAR agonists to alter and potentially improve metabolic health in the periparturient period.

Studies in human medicine suggest that dual activation of PPAR γ and PPAR α is able to increase total and high-molecular weight adiponectin as well as adiponectin receptors (Yamauchi and Kadowaki, 2008). Recently it was proposed that adiponectin is a significant mediator of PPAR γ activation and a PPAR-response element that regulates adiponectin was identified in humans (Astapova and Leff, 2012). An increased activity of PPAR γ led to an increase in adiponectin and the authors hypothesized that a part of the insulin sensitizing effect of PPAR γ was mediated by an adiponectin-dependent pathway (Yamauchi and Kadowaki, 2008).

Data on the coordinated regulation of PPARs and adiponectin in transition dairy cows are sparse. Cows fed a moderate energy diet had higher gene expression of PPAR γ and adiponectin compared with cows fed a controlled energy diet, but expression of both declined in the postpartum period (Ji et al., 2012). Gene expression of PPAR γ in subcutaneous adipose tissue was markedly reduced by the addition of fish oil to the close-up diet and in the first week postpartum which was associated with a decreased expression of adiponectin as well (Schmitt et al., 2011).

Inflammatory properties of bovine adipose tissue

Periparturient dairy cows have a high risk of exposure to inflammatory diseases, including mastitis and metritis (Kabara et al., 2014) which may be exacerbated by a pro-inflammatory state induced by excessive lipolysis (Sordillo and Raphael, 2013). The magnitude and persistence of this inflammation are linked to disease risk during the postpartum period (Bradford et al., 2015). The immunologically active role of adipose tissue has been established in humans and rodent models (Weisberg et al., 2003), and has recently become the focus of research effort in peripartum dairy cows as well (Akter et al., 2012; Ji et al., 2014b). Although this is considered as an emerging aspect of transition cow biology, evidence is lacking that the dairy cow undergoes similar changes as those seen in human obesity adipose tissue inflammation (Bradford et al., 2015).

Adipose tissue immune cell infiltration shows mainly increases of the monocyte/macrophage lineage, especially during increased TNF α production (Sordillo and Streicher, 2002). Immune cells are found within the stromal vascular fraction of adipose tissue which is about 6% of the omental adipose tissue in bovine (Ampem et al., 2015) and comprised an inhomogeneous population of endothelial cells, adipocyte precursors, fibroblasts, and leukocytes (Bai and Sun, 2015).

In a recent study on peripartum dairy cows by Akter et al (2012), immune cell infiltration was rarely observed (positive staining for macrophage markers was only observed in only 7 out of 25 cows), and the authors concluded that immunohistochemical evidence for an appreciable infiltration into bovine subcutaneous or visceral adipose depots was lacking. The study used healthy animals and no samples

were obtained between 2 and 41 days after calving which led the authors to speculate that a potential influx of immune cells could have been missed by the choice of sampling time-points (Akter et al., 2012).

Since visceral adipocytes produce more pro-inflammatory cytokines (Wronska and Kmiec, 2012), it is particularly interesting to see the absence of a difference between those two depots. A recent study by Ji et al. (2014a) investigated the effect of both, overfeeding versus a controlled energy diet fed for a period of eight weeks, and of adipose tissue sample site on inflammatory gene expression of non-pregnant, non-lactating Holstein cows. Whereas the authors “did not find strong evidence to support the hypothesis that overfeeding energy would stimulate an inflammatory response in bovine adipose tissue as observed in nonruminants” (Ji et al., 2014a), markers of inflammatory genes in adipose tissue samples from omental and mesenterial locations were increased compared with subcutaneous adipose tissue samples, particularly for mesenterial adipose tissue (Ji et al., 2014b). A limitation of the study was that the possibility of a greater influx of immune cells versus an inherent inflammatory state of the adipose tissue could not be differentiated with the use of mRNA expression profiles (Ji et al., 2014b).

The cytokine tumor necrosis factor alpha (TNF α) and the chemokine monocyte chemoattractant protein 1 (MCP-1) are discussed as potential mediators in bovine adipose tissue inflammation in more detail.

TNF α - TNF α of adipose tissue origin can act locally or systemically with evidence from human medicine that it acts predominantly as a local factor (Fruhbeck et al., 2014). Macrophages of the M1 type that are infiltrating adipose tissue are the

main contributor to the secreted cytokine in humans (Wronska and Kmiec, 2012). TNF α blocks insulin signaling through several pathways. It inactivates IRS-1 both by decreasing tyrosine phosphorylation which is the phosphorylation that activates IRS-1, and by increasing serine phosphorylation which inhibits IRS-1 (Fruhbeck et al., 2014). In addition, it reduces the amount of IRS-1 protein in adipocytes (Fruhbeck et al., 2014). TNF α also activates perilipin, the adipose droplet coating protein that protects the droplet from hydrolysis (Fruhbeck et al., 2014). And lastly, TNF α was shown to decrease adiponectin expression, reducing the metabolically advantageous effects of this adipokine (Astapova and Leff, 2012; Bai and Sun, 2015).

Evidence for a detrimental effect of TNF α in transition dairy cows has been largely experimental. Daily injections of recombinant TNF α subcutaneously to late-lactation dairy cows led to decreased feed intake and a higher liver fat content (Bradford et al., 2009). Daily subcutaneous injection of the cytokine in the first seven days after calving also decreased DMI and increased the risk for health disorders in a separate study (Yuan et al., 2013). The most recent investigation included continuous infusion into the subcutaneous adipose tissue over a seven day period (Martel et al., 2014), but no metabolic effects were observed. The authors hypothesized that a sound anti-inflammatory response may have protected the metabolic homeostasis of the treated animals (Martel et al., 2014).

The expression of TNF α in subcutaneous adipose tissue of dairy cows was found to be upregulated postpartum (Sadri et al., 2010) but this was dependent on parity in another study (Saremi et al., 2014) with upregulation demonstrated in primiparous, but not pluriparous cows. Overfeeding of non-pregnant, non-lactating

Holstein cows did not lead to an inflammatory response in adipose tissue based on expression levels of chemokines and cytokine receptors (Ji et al., 2014a). Overfeeding prepartum in transition dairy cows did not lead to expression differences of TNF α in subcutaneous adipose tissue in a recent Finnish study (Selim et al., 2014).

MCP-1 – Adipose tissue macrophages play a critical role in the establishment of a chronic inflammatory state that contributes to metabolic dysfunctions in humans and are predominantly of the pro-inflammatory M1 and less of the anti-inflammatory M2 type in obese phenotypes (Bai and Sun, 2015). Expression of PPAR γ favours the induction of the M2 type in humans, contributing to the balance of inflammatory stimuli in adipose tissue (Lackey and Olefsky, 2015). This phenomenon may have importance in the transition dairy cow because of the demonstrated changes in PPAR γ expression around the time of calving and in early lactation. Adipocytes and M2 macrophages secrete the pro-inflammatory chemokine MCP-1 to attract more monocytes from the circulation into the adipose tissue, aggravating the inflammatory state. This essentially represents a feed-forward mechanism where the number of pro-inflammatory macrophages in the tissue will remain elevated (Lackey and Olefsky, 2015). Microscopically observed crown-like structures are accumulations of pro-inflammatory macrophages in adipose tissue surrounding dead adipocytes, and have been shown in human obesity to be involved in the chronic adipose tissue inflammation leading to insulin resistance (Wronska and Kmiec, 2012). It is thought that death of adipocytes is greatly increased by increasing size of the adipose cells and that this death is sufficient to attract macrophages into the tissue (Bai and Sun, 2015).

However, it still remains to be clarified if macrophage infiltration may contribute to cell death.

While the level of knowledge in human medicine is increasing steadily, elucidating the role of macrophages and MCP-1 production in adipose tissue inflammation, few studies are available on the role of MCP-1 in dairy cows. Häussler et al (2015) studied the mRNA abundance and presence of MCP-1 in three different subcutaneous and three different visceral adipose tissue depots of high-yielding dairy cows with or without supplementation of a fatty acid supplement (conjugated linoleic acid), and demonstrated the presence of both in the tissue from different locations, yet the protein signal from immunostaining was very low. The abundance of mRNA was increased in visceral adipose tissue compared with the subcutaneous localization, which may have been due to the slightly higher number of resident immune cells found in this location, and no effect of fatty acid supplementation was observed (Häussler et al., 2015). The baseline mRNA expression of MCP-1 and response of adipose tissue *ex vivo* to an immunological challenge were studied in subcutaneous and mesenteric adipose tissue samples from non-pregnant cows (Mukesh et al., 2010). The authors found that mesenteric adipose tissue had a greater abundance of MCP-1 mRNA and that adipose tissue was capable of mounting an inflammatory response reflected in the increased expression of MCP-1 and other inflammatory markers such as TNF α and interleukin 6 (Mukesh et al., 2010).

PERIPARTUM CHANGES IN MUSCLE TISSUE

Regulation of muscle mass

Muscle hypertrophy - There are two main mechanisms by which muscle mass can increase, hypertrophy (increase in myofiber size), and hyperplasia (increase in myofiber number). In adult animals, and most relevant to transition dairy cows, an increase in muscle mass is due to hypertrophy (Velloso, 2008) because it is thought that the muscle fiber number is set at the time of birth (Sharples and Stewart, 2011). The adaptation of growth and repair is achieved by satellite cells, resident adult stem cells that can be activated to form myoblasts and fuse with the damaged muscle fiber (Sharples and Stewart, 2011). Recent literature in human medicine has led to challenging of the idea that satellite cells are necessary for muscle mass maintenance (Bonaldo and Sandri, 2013).

Muscle synthesis and degradation are simultaneous processes influenced by mechanical stress, activity, nutrient availability, and growth factors (Sandri, 2008). A delicate dynamic balance regulates the muscle mass as a function of both, production and degradation of existing proteins (Stitt et al., 2004). Protein synthesis is an energetically costly process with one peptide bond estimated to cost 5 ATP equivalents (Wright et al., 2005), and hence is tightly regulated by nutritional and hormonal factors. An increase in muscle mass is associated with increased circulatory concentrations or increased autocrine or paracrine activity of the muscle growth promoting factor, insulin-like growth factor 1 (IGF-1). This growth factor in the

presence of normal concentration of insulin (Glass, 2003; Sandri, 2008) induces muscle hypertrophy and satellite cell proliferation and differentiation via the phosphatidylinositol 3 kinase (PI3K)/AKT pathway (Sandri et al., 2004; Solomon and Bouloux, 2006). IGF-1 expression and production is normally regulated by growth hormone (GH), but in transition cows, circulating concentrations of IGF-1 decrease from prepartum values to reach lowest concentrations within the first two weeks postpartum (Meikle et al., 2004). During this time, an effective uncoupling of the GH-IGF-1 axis occurs, also expressed as GH resistance. This situation in early lactation is characterized by decreased circulating concentrations of IGF-1 despite elevated concentrations of GH leading to increased peripheral lipolysis and nutrient partitioning towards milk production (Kim, 2014) in the absence of anabolic effects in the liver that are typically induced by high concentrations of GH (Bauman and Vernon, 1993). These effects include the anabolic effects of GH on lean body mass (Solomon and Bouloux, 2006) and are likely mediated by regulation of IGF-binding proteins since IGF-1 mRNA expression returns to prepartum levels while plasma IGF-1 concentrations remain depressed (Kim, 2014). In particular, IGF-binding protein 3 and the acid labile subunit (ALS) control IGF-1 activity and may remain low until approximately eight weeks after calving (Velloso, 2008; Kim, 2014).

As discussed, circulating concentrations of IGF-1 (largely from hepatic origin), as well as autocrine and paracrine generation of IGF-1 in muscle tissue play a role in determination of myofiber size and function (Solomon and Bouloux, 2006; Velloso, 2008). Increased concentrations of GH in a bovine muscle cell *in vitro* experiment did not lead to an increase of muscular IGF-1 mRNA expression (Ge et al., 2012).

However, IGF-1 addition to the cell culture medium did increase proliferation and protein synthesis (Ge et al., 2012). The authors hypothesized that this may point to an inability of GH to induce local production of IGF-1 in bovine muscle.

Following activation by IGF-1, two major downstream effectors of AKT are important for muscle hypertrophy: the mammalian target of rapamycin (mTOR) kinase, which is activated by AKT, and the glycogen synthase kinase (GSK3 β), which is blocked by AKT (Sandri, 2008). The protein synthesis is increased through two mechanisms downstream of mTOR activation: inactivation of the repressor protein 4E-binding protein (4EBP1) that inhibits mRNA translation, and the activation of the 70 kDa ribosomal protein S6 kinase (S6K1) (Carbone et al., 2012).

Myostatin, a member of the TGF superfamily, acts as a negative regulator of muscle growth (Solomon and Bouloux, 2006). It acts both to inhibit myoblast differentiation and to block the AKT pathway, however IGF-1 signaling is able to rescue the activation of the AKT pathway that is blunted by myostatin (Glass, 2010; Sharples and Stewart, 2011). Mutations of the myostatin gene lead to excessive hypertrophy of muscle (Bonaldo and Sandri, 2013) such as found in high frequency in Belgian Blue and Piedmontese cattle.

Recently, a role of myostatin in mediating insulin resistance in skeletal muscle during times of high calorie intake was identified following the discovery that myostatin in plasma and skeletal muscle of obese and diabetic humans is increased (Bonala et al., 2014). The authors proposed that increased expression of myostatin in mouse muscle and liver following exposure to high fat and glucose in an *in vivo* and *in vitro* model leads to enhanced ubiquitin proteasome-mediated degradation of IRS-1 in

muscle tissue by upregulation of a specific E3-ligase. These data provide further evidence for the emerging role of muscle tissue as a regulator of metabolism (Bonala et al., 2014).

Muscle atrophy - Atrophy occurs when proteolysis exceeds protein synthesis and can be caused by a multitude of stimuli, including inflammation, oxidative stress, disuse, aging, as well as periods of starvation (Sharples and Stewart, 2011). Muscle protein is a crucial component of the energy balance in periods of undernutrition (Pasiakos et al., 2010) and is therefore of importance to the periparturient dairy cow, in particular since muscle lean mass has the potential to offset the increased demand for metabolic substrates in addition to the energy from adipose tissue mobilization. However, loss of skeletal muscle mass may negatively affect metabolic processes (Carbone et al., 2012). Characterization of the mechanism and regulation of protein utilization in transition dairy cows has been the focus of more recent research efforts (Phillips et al., 2003; Chibisa et al., 2008; Kuhla et al., 2011).

Transition dairy cows in NEB have been shown to mobilize over 20 kg of body protein in the transition period (Komaragiri and Erdman, 1997; Kuhla et al., 2011) because of an amino acid deficit and negative nitrogen balance (Plaizier et al., 2000; Phillips et al., 2003). Although muscle tissue mobilization is responsible for less than 10% of the total body energy change, mobilization of lean tissue is regulated by the hormonal changes in the transition period and supports the availability of amino acids for milk protein synthesis (Komaragiri and Erdman, 1997). As in other mammals during a catabolic state, muscle amino acid profiles in transition dairy cows reflect the breakdown of tissue (Meijer et al., 1995) which is most pronounced in the first two

weeks of lactation (Bruckmaier et al., 1998) and shows a maximum on day 7 after calving (Bell et al., 2000; Doepel et al., 2002). This is consistent with the greatest extent of muscle breakdown expressed as urinary 3-methylhistidine:creatinine (3-MH) ratio (Phillips et al., 2003). Urinary 3-MH is commonly used as an indicator of whole body myofibrillar protein degradation (Seashore et al., 1981) and increases in the postpartum period in dairy cows, indicating breakdown of muscle mass (Chibisa et al., 2008).

Free essential amino acids (except histidine and methionine) were shown to reach the lowest concentration on the first day after calving which could be either due to the decreased DM intake, the removal of free amino acids from the circulation for gluconeogenesis, or a combination of both (Doepel et al., 2002; Kuhla et al., 2011). The concentration of free essential amino acids shows a decrease in the immediate peripartum period (Meijer et al., 1995; Kuhla et al., 2011). Amino acids can contribute up to 30 percent of the liver glucogenic substrate during early lactation and are important in meeting the postpartum glucose needs of the cow (Reynolds et al., 2003). The capacity of the liver to convert amino acids into glucose improves, as was shown by the increase in conversion of alanine to glucose on the day after calving compared to prepartum values (Overton et al., 1998). Subsequent observations confirm that only alanine contributes substantially to gluconeogenesis in postpartum transition cows (Larsen and Kristensen, 2012).

The time-point of initiation of muscle mobilization is less well studied compared to the end-point of muscle mobilization during the transition period. In a few studies, the onset was observed within the last four weeks (Schaff et al., 2013) or

two weeks (Doepel et al., 2002) before calving, or within the last 7 days prepartum and the day after calving (Kokkonen et al., 2005). The endpoint of protein mobilization was determined to be until approximately the fourth or fifth week postpartum while body fat mobilization continues for an extended period (Blum et al., 1985; Komaragiri and Erdman, 1997; Komaragiri et al., 1998; Kokkonen et al., 2005; Schaff et al., 2013).

In an experiment where transition cows were supplemented with a gluconeogenic precursor (propylene glycol in the form of a top-dress of 300 mL twice per day), authors were interested in the effects that added energy substrates during this period had on muscle breakdown (Chibisa et al., 2008). Propylene glycol supplementation as a top-dress led to a numerical reduction in urinary 3-MH excretion in this experiment on 16 cows, however, differences were not statistically significant (Chibisa et al., 2008). Van der Drift et al. (2012) showed that higher plasma 3-MH concentrations were associated with lower serum BHB concentrations postpartum. In addition, cows with higher muscle thickness had larger loss in muscle mass with the greatest loss around parturition, indicating that this period was that of most severe protein mobilization (van der Drift et al., 2012).

There is limited data available on the effects of dry period energy level and postpartum mobilization of muscle mass. Cows overfed energy during the dry period had a higher urinary 3-MH:creatinine ratio postpartum compared with those cows fed a controlled energy diet despite higher DMI (Doepel et al., 2002). Postpartum, cows overfed energy during the far-off period lost a greater amount of diameter of the

longissimus lumborum muscle compared with cows fed a controlled energy diet (Kokkonen et al., 2005).

Mechanisms of muscle breakdown

There are four primary pathways contributing to muscle atrophy: the ubiquitin-proteasome system, the caspase-mediated, the Ca²⁺/calpain-dependent system, and the autophagy/lysosomal degradation pathway, of which the caspase-mediated and proteasome system are considered major contributors to atrophy during periods of NEB (Carbone et al., 2012) whereas the contribution of the two other pathways remains to be clarified. In animal models of starvation, the ATP-ubiquitin-proteasome pathway of muscle degradation is highly upregulated (Medina et al., 1995) following the initial cleavage of actomyosin by caspase-3 (Lee et al., 2004). In a state of NEB, this is linked to the reduced concentration of insulin since the ubiquitin-proteasome proteolytic pathway is inhibited by insulin through increased activity of phosphatidylinositol-3 kinase (PI3K) (Chilliard et al., 1998) and with a coordinated upregulation of the E3-ubiquitin ligases atrogen-1 and muscle RING finger 1 (mURF-1) in catabolic states (Lee et al., 2004). The formation of ubiquitin-protein conjugate is a necessary step for the degradation of proteins by the proteasome and is facilitated by the muscle-specific E3-ligases atrogen-1 and mURF1 which are indispensable for ubiquitin-tagging of proteins for degradation by the proteasome (Glass, 2010). Supplementation of transition dairy cows with propylene glycol as a top-dress reduced

the mRNA expression of ubiquitin postpartum, as well as the expression of the 26S subunit of the proteasome (Chibisa et al., 2008).

While mURF1 acts specifically in the degradation of several muscle structural proteins, few proteins have been identified as specific targets for atrogin-1 to date and include proteins related to cellular growth or survival pathways such as MyoD, a key muscle transcription factor, and eIF3f, an activator of protein synthesis (Bonaldo and Sandri, 2013). Other E3-ligases such as Trim32 and TRAF6 are involved in muscle atrophy, but their role has only recently begun to be elucidated (Bonaldo and Sandri, 2013).

The activation of the PI3K/AKT pathway inhibits the expression of both atrogin-1 and mURF1 through the phosphorylation and inactivation of forkhead box (FOXO) class of transcription factors (Stitt et al., 2004), in particular FOXO3 (Velloso, 2008), and leads to activation of the kinase mTOR in the presence of adequate concentration of amino acids and insulin (Sandri et al., 2004). A decrease in essential amino acid concentrations was shown to decrease mTOR activity independent of insulin concentrations in bovine mammary cells (Appuhamy et al., 2011). It becomes clear that the AKT pathway has a central role not only in stimulating muscle hypertrophy following IGF-1 signaling, but also in repressing atrophy signaling by reducing the expression of muscle-specific E3-ligases (Glass, 2010; Bonaldo and Sandri, 2013).

Activation of the caspase-mediated pathway of atrophy is associated with an increase in proteasome degradation since caspase cleavage leads to the initial steps of myofibrillar degradation that is completed by the proteasome once smaller cleavage

products are tagged by ubiquitin (Carbone et al., 2012). Caspase activity, like proteasome activity, is regulated through PI3K and hence increased during hypoinsulinemia (Carbone et al., 2012).

Upregulation of the Ca^{2+} - dependent protease system has previously been demonstrated in transition dairy cows and propylene glycol could partially suppress the upregulation of μ -calpain, one of the central components of this pathway (Chibisa et al., 2008).

Autophagy, the process by which a cell degrades its own components in lysosomes, is another mechanism in muscle atrophy (Sandri, 2008). Although first described many years ago, this process has not been recognized as a contributor of muscle atrophy until more recently (Bonaldo and Sandri, 2013). In response to starvation, NEB or other types of cellular stress, the cell forms a phagophore which subsequently engulfs portions of the cytosol and organelles in a double-layered vesicle called the autophagosome (Stipanuk, 2009). The rate of autophagy is regulated through the mTOR pathway (Stipanuk, 2009) thereby linking autophagy regulation to this nutrient sensing kinase. Abundance of insulin, amino acids, and leucine in particular decreased macroautophagy in neonatal pigs (Suryawan and Davis, 2014). Excess autophagy during prolonged times of a catabolic metabolic state causes excessive removal of cellular components which may lead to impairment of normal cellular activity with structural and functional impairment causing muscle weakness (Bonaldo and Sandri, 2013). The autophagy activity can be approximated by the ratio of protein 1 light chain 3 (LC3)-I to the conjugated form LC3-II (Stipanuk, 2009).

SUMMARY AND OBJECTIVE

The transition to lactation is accompanied by homeorhetic changes of metabolism which are complicated by a transient but significant drop in dry matter intake. These adaptations include an increase in lipolysis and utilization of fatty acids as alternate fuel, sparing of glucose in favor of its use by the conceptus or mammary gland, decreased lipogenesis in adipose tissue, increased gluconeogenesis, and increased use of protein from muscle tissue as a source of amino acids for milk protein synthesis and support of gluconeogenesis. The review of the existing literature shows that dry period nutritional energy level is intricately linked with the metabolism in the peripartum period and leads to alterations in the concentrations of NEFA and BHB, predisposing cows to negative health events and loss of production.

Insulin has a central role in the adaptation to lactation, both on a whole body as well as tissue-specific level as shown by the multitude of cellular processes under regulation of this hormone in adipose and muscle tissue. Overfeeding energy may affect insulin signaling and thus allow for increased rates of lipolysis and ketogenesis in the postpartum period and affect the ability of insulin to regulate lipolysis and lean muscle loss.

Therefore the objective of this dissertation is to create a sound model of nutritional regulation of transition cow metabolism through dry period feeding strategies commonly used in the dairy industry, assess the effects on productivity and markers of NEB to confirm previous findings, evaluate the potential effect on colostrum quality, and to use this model system to investigate changes in insulin

signaling. Particular attention is given to assessing insulin sensitivity on the cow-level and by using molecular techniques on the level of the adipose and muscle tissue.

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CHAPTER 3

DRY PERIOD PLANE OF ENERGY: EFFECTS ON FEED INTAKE, ENERGY BALANCE, MILK PRODUCTION AND COMPOSITION IN TRANSITION DAIRY COWS

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ABSTRACT

The objective was to investigate the effect of different dry cow feeding strategies on the degree of ketonemia postpartum. Epidemiologic studies provide evidence of an association between elevated β -hydroxybutyrate (BHBA) concentrations in postpartum dairy cows and a decreased risk for reproductive success as well as increased risk for several diseases in early lactation, such as displacement of the abomasum and metritis. The plane of energy fed to cows in the prepartum period has been shown to influence ketogenesis and the degree of negative energy balance postpartum. Our hypothesis was that a high fiber, controlled energy diet (C) fed during the dry period would lead to a lower degree of hyperketonemia in the first weeks postpartum compared to either a high energy diet (H), or a diet where an intermediate level of energy would only be fed in the close-up period (starting at 28 d before expected parturition), following the same controlled energy diet in the far-off period (I). Hyperketonemia in this study was defined as a blood BHBA concentration of ≥ 1.2 mmol/L. Holstein cows (n=84) entering parity 2 or greater were enrolled using a randomized block design and housed in individual tie-stalls. All treatment diets were fed for ad libitum intake and contained monensin. Cows received the same fresh cow ration after calving. Blood samples were obtained three times weekly before and after calving and analyzed for BHBA and nonesterified fatty acids (NEFA). Milk components, production and dry matter intake (DMI) were recorded and energy balance was calculated. Repeated measures ANOVA was conducted for the outcomes DMI, energy balance, BHBA and NEFA concentrations, milk and energy-corrected

milk yield, as well as milk composition. Predicted energy balance tended to be less negative postpartum in group C and cows in this group had fewer episodes of hyperketonemia compared to both groups I and H in the first three weeks after calving. Postpartum BHBA and NEFA concentrations over time were highest in group H and lowest in group C whereas milk production was not affected by prepartum plane of energy. Analysis of milk fatty acid composition showed a higher yield of preformed fatty acids in group H compared to group C, suggesting higher lipid mobilization for cows fed H. In this study, a one group controlled energy dry period approach decreased the degree of negative energy balance as well as the number of episodes and degree of hyperketonemia postpartum.

Key Words: dairy cow, transition period, energy, ketosis

INTRODUCTION

As dairy cows transition from the dry period into early lactation, the risk for metabolic and infectious disease is particularly high (Goff and Horst, 1997; Hammon et al., 2006; McArt et al., 2013b). After parturition, dairy cattle are faced with a sudden and marked increase of nutrient requirements to support milk production (Drackley, 1999). Improving energy balance in the postpartum period can reduce the incidence of periparturient diseases (Duffield et al., 2009) and decrease the mobilization of body reserves (Busato et al., 2002). To achieve this, it has been recommended to either maximize DMI prepartum, mitigate the drop in DMI around the time of calving, or both. Because a certain degree of decrease in DMI seems

unavoidable, some authors have proposed to increase the energy content of the prepartum ration in order to improve production and health in early lactation (Grummer, 1995; McNamara et al., 2003; Rabelo et al., 2003). Cows fed a dry period ration with a higher energy level had a higher DMI prepartum compared with those being fed a lower energy density (Janovick and Drackley, 2010). However, research has shown that higher energy levels fed in the far-off dry period have a negative impact on peripartal metabolism (Dann et al., 2006) and lead to a greater decline in DMI prepartum (Minor et al., 1998; Olsson et al., 1998). Feeding a higher energy diet prepartum can lead to an increase in body condition with subsequently more severe negative energy balance postpartum (Rukkwamsuk et al., 1999). Recent research has shown that cows with a higher prepartum body condition are at a higher risk for developing hyperketonemia (McArt et al., 2013a).

Several researchers have investigated the potential benefits of feeding a controlled energy diet during the dry period (Grum et al., 1996; Drackley, 1999; Dann et al., 2005) and a significant improvement of fresh cow health after feeding a high fiber, low starch diet during the dry period has been reported in practice (Drehmann, 2000). More recent research has demonstrated that feeding a high-forage, low-energy diet prepartum improved metabolic status postpartum and reduced rates of subclinical ketosis (Janovick et al., 2011; Vickers et al., 2013). Exacerbated periparturient negative energy balance has been linked to downstream negative health events (Duffield et al., 2009; Ospina et al., 2010a; McArt et al., 2012), reduced reproductive success and decreased production (Duffield et al., 2009; Ospina et al., 2010b; c) in a number of recent studies.

Current recommendations for feeding a controlled energy diet have to address the questions of how to feed dry cows to meet their requirements in an ad libitum system and how much energy should be restricted. A majority of the available data is based on studies where intakes were restricted to control energy, a strategy that is not advisable on commercial dairy farms because it can lead to increased competition at the feed bunk in a free stall management system. This potentially decreased DMI in at least a part of the feed restricted group leading to some cows energy intake to be well below what was planned. Some uncertainty also exists about whether cows should be fed the same dry cow ration for the whole dry period or if it is preferable to change them to a higher energy plane of nutrition in the immediate prepartum period. Because we specifically wanted to isolate the effect of different energy levels and avoid differences introduced in protein availability, which might alter the plane of nutrition, diets were formulated for similar predicted amounts of MP.

Because different dry period feeding strategies have been shown to affect the level of milk production and milk composition in the immediate postpartum period, our study also aimed to describe the effects of different dry period dietary strategies on production in early lactation. Although some authors did not find a difference in ECM yield (Kunz et al., 1985; Mashek and Beede, 2001; Agenas et al., 2003) when feeding a lower energy diet throughout the dry period, trends for a lower fat percentage (Holter et al., 1990; McNamara et al., 2003; Dann et al., 2006) and decreased ECM production (Olsson et al., 1998) were observed and differences were shown to be parity-dependent (Rabelo et al., 2003). Several authors have shown that milk FA composition is related to the extent of energy deficit (Rukkwamsuk et al., 2000).

The objectives of this study were therefore to compare the effect of three different ad libitum dietary strategies differing in energy, starch and fiber content, representing a range of diets that are currently used on dairy farms, including monensin, on peripartum energy balance in order to identify which approach would allow for a better transition of dairy cows into early lactation without affecting milk yield.

MATERIALS AND METHODS

Animals, Feeding and Management

All procedures were evaluated and approved by the Cornell University Institutional Animal Care and Use Committee (protocol no. 2011-0016). Holstein cows (n=84) entering second or greater lactation from the herd at the Cornell Teaching and Research Center (Harford, NY) were enrolled between September 2012 and April 2013. Exclusion criteria for enrollment were: known twin pregnancy and chronic lameness, as well as having a record of a milk sample cultured positive for *Staphylococcus aureus* because these animals would be managed separately. All animals were housed in individual sawdust-bedded tie-stalls with feed bins and were exercised three times per week during the dry period. After calving, cows were milked twice daily at 0900 and 2100 h. Cows were allocated to one of three dry period dietary treatment groups using a randomized block design with 3 treatments in 28 blocks on the day of dry off (approximately 57 d before expected parturition) and blocked by expected calving date. Animals were enrolled in one of three feeding groups: those that were fed a TMR formulated to meet but not greatly exceed energy requirements at

predicted ad libitum intake (**C**), those that received a TMR formulated to supply 150% of energy requirements (**H**), and an intermediate group that received the same TMR as group C for the first 28 d after dry off and a TMR formulated to supply 125% of energy requirements from d 28 before expected parturition until calving (**I**, representing a 50:50 blend of both C and H diets). Predicted DMI was anticipated to be different across the three treatment groups and rations were formulated using the Cornell Net Carbohydrate and Protein System software (CNCPS, Cornell University, version 6.1) accordingly, so that they were balanced for predicted MP, Lys and Met requirements as well as for a Lys: Met ratio of 2.9:1 All cows received the same fresh cow TMR (**F**) from the onset of lactation until the end of the study period (42 DIM). Diet composition is summarized in Table 3.1. For all dry cow rations, predicted MP was formulated to be 1,280 g/d and monensin was included at a target intake of 300 mg/d in all dry cow rations and at 400 mg/d in the fresh period. The straw included in all dry cow rations had a target length of no more than 5 cm before inclusion in the TMR mix. Dry matter content of all feed components including the grain mix and TMR was determined on a weekly basis by drying the sample at 60°C for 48 h. Rations were adjusted each week for changes in DM content of the component. Weekly TMR samples were combined into monthly composite samples, dried and ground in a Thomas Wiley Mill to pass through a 2-mm screen. All composite samples were sent for wet chemistry analysis to a commercial laboratory (Dairy One Cooperative Inc, Ithaca, NY). Samples were analyzed for DM (method 930.15; (AOAC, 2012), CP (method 990.03; (AOAC, 2012), starch (YSI Biochemistry Analyzer, Dairy One Coop., (2014), fat (method 2003.05; AOAC, 2012), ADF

(method 973.18; AOAC 2012), NDF (Van Soest et al., 1991) and macro-and microminerals (Sirois et al., 1994). Throughout the study, four composites of the commercial grain mix were analyzed accordingly in order to monitor the composition compared with the initial formulation. For all forages a single composite of all monthly samples was sent for analysis. All diets were offered ad libitum, fed once daily at 0900 h and amounts fed were adjusted to allow for a minimum of 5% refusals.

Individual animal sampling

Individual feed intakes were recorded daily and calculated on a DM basis using the weekly information as described above. Blood samples (10 ml) were taken before feeding (between 0600 and 0730 h) from the coccygeal vein or artery using evacuated tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) with 158 USP units of sodium heparin for plasma separation and a 20 gauge x 2.54 cm blood collection needle, and in tubes without additive for the separation of serum. Sampling was done on the day of enrollment (57 d before expected parturition) and weekly thereafter until 21 d before expected parturition when sampling frequency was increased to three times weekly until d 21 postpartum in order to capture the greater variability in blood metabolites expected during the transition period (Dann et al., 2006). After d 21 postpartum, blood was again collected at weekly intervals until 42 DIM. The concentration of BHBA was determined using a handheld device (Precision Xtra meter, Abbott Diabetes Care Inc., Alameda, CA) as previously described (Iwersen et al., 2009). The calibration of all Precision Xtra meters used in the study was tested at regular intervals using a standardized solution (Medisense Glucose&

Ketone Control Solutions, Abbott Diabetes Care Inc., Alameda, CA). All blood samples were immediately placed on ice and serum and plasma were separated within one h at 2,800 x g for 20 min at 4°C, snap-frozen in liquid nitrogen and stored at -20°C until analysis. Serum concentrations of NEFA were analyzed by colorimetric measurement of an enzymatic reaction (HR Series NEFA-HR (2); Wako Life Sciences, Mountain View, CA) with a microplate spectrophotometer (Epoch, Biotek, Winooski, VT). In-house quality control samples were run on every plate.

Animals were weighed weekly on a commercial large animal scale (Fairbanks 2200, Fairbanks Scales, Inc., Kansas City, MO) and BCS according to a 5-point scale with 0.25 point increments according to Edmonson et. al. (1989) was determined by two trained investigators at dry off. Calving ease on a scale from one to five (1 = normal/no assistance, 2 = moderate assistance, provided by farm staff, 3= moderate assistance, but vet called as a precaution, 4 = difficult calving, with extraction done by skilled farm staff, 5 = very difficult calving, with maximum veterinary assistance) was recorded by the herd personnel attending each calving. Milk yield was recorded for each milking. Milk samples were obtained from two consecutive milkings each week. One aliquot was mixed with bronopol preservative, refrigerated at 4°C, and analyzed for fat, true protein, TS, lactose, MUN using infrared analysis on an automated Fossomatic (Foss, Eden Prairie, MN) FT+ (method 972.160; AOAC, 2012) and for somatic cell counts (SCC) using optical fluorescence on Fossomatic FC (method 972.160; AOAC, 2012) (Dairy One Cooperative Inc, Ithaca, NY) within 24 h of collection. The second aliquot was stored at -20°C without the addition of a

preservative until FA composition analysis. Linear scores (LS) were calculated as follows: $LS = \{\log_2[(SCC/100,000)] + 3\}$.

Hyperketonemia was defined as BHBA ≥ 1.2 mmol/L (McArt et al., 2013a). Cows were considered to have clinical ketosis if BHBA ≥ 2.5 mmol/L and were treated with 250 ml of a 50% dextrose solution (Dextrose 50% inj, VetOne, Boise, ID, i.v.) on two consecutive days as well as 300 ml (approximately 310 g) of propylene glycol orally for five consecutive days starting on the day of diagnosis. Subsequent episodes of clinical ketosis after this initial treatment were treated with another 5-d course of oral propylene glycol alone. This was repeated until BHBA concentrations were determined to be < 2.5 mmol/L. Health events such as displacement of the abomasum, mastitis and ketosis treatments were recorded daily for all animals throughout the study period. Retained placenta was defined as the failure to pass fetal membranes by 24 h after calving.

On d -28 ± 1 and -10 ± 1 relative to expected calving as well as on d 4 ± 1 and 21 ± 1 in milk, all cows on study had s.c. fat and muscle biopsies taken and a glucose tolerance test was performed (data not presented) with 0.25 g/kg of dextrose (Dextrose 50% inj., VetOne) administered i.v. Additionally, animals received 2.2 mg/kg flunixin meglumine (Prevail, VetOne) i.v. for pain mitigation following biopsy.

Calculations and Estimations

Rations were formulated using CNCPS. The chemical composition of all forages, as determined by Dairy One, and the composition of the grain as formulated were entered into CNCPS to estimate the energy content of all the diets. The energy

density of the diets was also estimated separately according to the NRC software (NRC, 2012). Energy balance was estimated in CNCPS based on weekly averages for each cow's BW and DMI and either a dry period or milk yield as well as percent milk fat, protein and lactose for the postpartum period. Energy balance was expressed as percentage of requirements. The results of energy density estimates obtained from the NRC software also were used to calculate energy balance for the dry period, as well as for early lactation according to NRC (2001) as previously described by Dann and coauthors (2006). Predicted MP amount for average intakes in all prepartum treatment groups was estimated with CNCPS. Weight gain comparison among the groups was calculated as the weight at 45 d after dry off minus the weight at dry off.

ECM yield was calculated for 3.5% fat and 3.0% protein as follows: $\text{kg ECM} = \{[(0.0929 \times \text{fat } \%) + (0.0563 \times \text{true protein } \%) + 0.192]\} \times \text{milk kg} / 0.68605$. Fat and protein concentrations for each of the two consecutive milkings within a sample day were summed and the average entered into the formula. Milk samples from the second and fourth week after calving were analyzed for FA composition as previously described (Lock et al., 2013). In brief, morning and afternoon milk samples from each sampling period were composited based on milk fat yield. Following extraction and methylation of lipids, GLC was used to determine the composition of FA methyl esters. Using the molecular weight of each FA, milk fat yield and FA concentration, yields of individual FA (g/d) were calculated (Piantoni et al., 2013).

Analytical approach

The primary outcome of interest in the study was the difference in BHBA blood concentrations among the treatment groups. The sample size of 28 animals per group was based on identifying a biological difference in BHBA concentration between the C and H groups of 0.4 mmol/L with a SD of 0.4, confidence level of 95% and power of 95%. To minimize potentially unmeasured bias associated with environmental factors on a dairy farm that change over time, animals were blocked in groups of three. Allocation within the block was performed following the sequence of a random number generator (Research Randomizer v.4.0, Urbaniak, 2012). Secondary outcomes included differences in DMI, NEFA concentrations, BW, milk production and composition.

Chi-squared tests were generated using PROC FREQ of SAS (SAS 9.3, SAS Institute Inc., Cary, NC) for differences in calving scores, parity, calf gender, episodes of BHBA ≥ 1.2 mmol/L as well as treatment episodes for clinical ketosis. One-way ANOVA were carried out with PROC ANOVA for differences in days dry, BCS, and BW measurements and fatty acid composition. Repeated measures ANOVA was performed for the outcomes DMI, energy balance, BHBA and NEFA concentrations, milk and ECM yield, percentage of milk fat, protein, lactose and total solids as well as MUN concentration and linear scores using PROC MIXED in SAS. Five covariance structures were tested for each variable analyzed (simple, compound symmetry, autoregressive order 1, Toeplitz and unstructured). The covariance structure with the smallest Akaike's information criterion was chosen. Fixed effects were treatment group and parity with the REPEATED statement for the time variable. Interaction

terms were removed from the model unless the p-value was ≤ 0.05 ; the interaction of treatment group and time was forced into every model. Data were analyzed separately for pre- and postpartum. For every model, the effect of enrollment block was tested. When results of the ANOVA analysis yielded a p-value of ≤ 0.05 , Tukey's posthoc test was used for comparison of means across all groups to control experimentwise error rate. Normality of residuals was tested after each model fit.

RESULTS

Description of study population

Two cows had to be replaced two weeks after enrollment because one animal aborted and one was severely lame. 28 cows in each group completed the dry period. One cow in group C had to be euthanized in the third wk postpartum due to severe toxic mastitis following a teat laceration, two animals in group I were sold, one in the fourth and one in the fifth wk postpartum because of a presumed perforating abomasal ulcer and severe mastitis, respectively.

Descriptive statistics are provided in Table 3.2. Cows were dry for an average of 55.5 d (C: 56.5, I: 55.0, H: 55.2; $P=0.4$), One cow in the C group was pregnant to a later breeding date and was dry for 99 d, however, she was still included in the analysis unless indicated. Weight gain in the dry period was 72.1 ± 18.7 , 71.0 ± 19.2 and 90.4 ± 23.5 kg in the C, I and H groups ($P = 0.0008$). Weight loss from d 4 to 21 in milk was 24.8 ± 22.1 , 40.6 ± 32.9 , 37.8 ± 31.8 kg in the C, I and H group ($P=0.11$).

Weight loss from d 4 to 42 in milk was 30.7 ± 26.5 , 45.5 ± 34.8 , and 40.3 ± 32.2 kg in the C, I and H group ($P = 0.22$).

Calving scores in the C, I and H groups were distributed as follows: 20/28 (71.4%), 16/28 (57.1%) and 22/28 (78.6%) animals Score 1; 6/28 (21.4%), 8/28 (28.6%) and 5/28 (17.9%) animals score 2; 2/28 (7.1%), 3/28 (10.7%) and 1/28 (3.6%) animals Score 3, and one animal in group I 1/28 (3.6%) had Score 4 ($P = 0.60$). One animal in group I had twins.

Description of diets and intakes

The analyzed composition of pre- and postpartum diets is presented in Table 3.3. Prepartum DMI was different between groups with the lowest intakes (14.2 ± 0.3 kg) in group C and the highest intake in group H (16.4 ± 0.3 kg) (Figure 3.1, Table 3.4). Postpartum DMI was not different among groups (22.3 ± 0.6 , 22.38 ± 0.6 and 22.4 ± 0.6 kg for the C, I and H groups respectively ($P = 0.99$) (Figure 3.1, Table 3.4). Energy balance predictions using the CNCPS model are listed in Table 3.4. Predictions obtained from the NRC model are shown in Figure 3.2 in order to contrast estimates from the CNCPS model for both prepartum and postpartum timepoints. The NRC model yielded the highest estimates of prepartum energy balance (C: 140.7 ± 3.1 , I: 156.6 ± 3.1 and H: 176.8 ± 3.1 % respectively), whereas the CNCPS calculations yielded overall lower results (C: 111.9 ± 2.2 , I: 125.8 ± 2.2 and H: 152.5 ± 2.3 %). The predicted energy density of the diets in the CNCPS model were 1.98, 2.12, 2.23 and 2.42 Mcal ME/kg DM for the C, I, H and F diets whereas the NRC model predicted higher energy densities across all groups (2.31, 2.38, 2.45 and 2.55

Mcal ME/kg DM). Based on average DMI, predicted MP was on average 1,490, 1,520 and 1,520 g/d in the C, I and H group, thus supplying 124, 123 and 118% of the MP requirements in the treatment groups as estimated by CNCPS.

Milk production and composition

Milk production and ECM are presented in Table 3.5. Milk and ECM production was not different in the three treatment groups. Results for milk components are summarized in Table 3.5. Milk fat was the component with the most notable difference among the three groups and was 3.96 ± 0.09 , 4.12 ± 0.09 and $4.24 \pm 0.09\%$ in the C, I and H groups, respectively ($P = 0.10$). Milk FA yield and concentration for wk 2 and 4 postpartum are summarized in Table 3.6. The yield of preformed FA in the high group was 117 g higher in wk 2 and 76 g higher in wk 4 compared to the controlled energy group, while no difference was found in the yield of de novo FA (Table 3.6). The difference in preformed FA was driven by a higher yield of both C18:0 and *cis*-9 C18:1. Cows fed a high energy diet during the dry period also had higher concentrations of C18:0 in milk fat (Table 3.6). Neither yield nor concentration of mixed source fatty acids (C16:0 and *cis*-9 C16:1) were different among treatment groups.

Energy metabolites and health events

BHBA concentrations in the three groups were different over time (Figure 3.3). Compared with group H, cows in group C had overall lower BHBA levels in the last 2 weeks prepartum (0.29 ± 0.01 vs. 0.34 ± 0.01 mmol/L), as well as in the first three weeks postpartum (0.61 ± 0.06 vs. 0.84 ± 0.07 mmol/L) (Table 3.7). Of all BHBA

tests within the first 21 DIM, there were 13, 32 and 31 episodes of hyperketonemia in groups C, I and H ($P = 0.007$). The median time to first positive test was 7 d. No cows were treated for clinical ketosis in group C while four cows in group I and five cows in group H received treatment ($P = 0.07$).

Intra- and interassay coefficients of variation for NEFA measurements determined from 15 assays were 3.1 and 8.2%, respectively. Concentrations of NEFA were also different among the dietary treatment groups over time (Figure 3.3). In the prepartum period, animals in group C had higher concentrations compared with both group I and H (C: 237 ± 12 , I: 180 ± 13 , H: 175 ± 12 $\mu\text{Eq/L}$). Postpartum NEFA concentrations were lower in group C and I compared with group H (C: 659 ± 36 , I: 665 ± 37 , H: 796 ± 40 $\mu\text{Eq/L}$) (Table 3.7).

One animal in group C and one animal in group I, as well as two animals in group H suffered from left displacement of the abomasum. All cases were corrected via the roll and toggle method. One, three and four cows in groups C, I and H developed clinical mastitis, respectively. Six, four and two cows in group C, I and H had retention of the placenta, respectively. Sample size was not sufficient to analyze differences in the number of health events.

Parity effect

Compared with parity 2 animals, DMI of older animals was higher prepartum (15.88 ± 0.03 vs. 14.73 ± 0.2 kg, $P = 0.001$), but energy balance postpartum tended to be lower (Table 3.4). Parity had an effect on postpartum BHBA and NEFA concentrations with lower levels in parity 2 compared with parity ≥ 3 (BHB: $0.66 \pm$

0.05 vs. 0.81 ± 0.06 mmol/L, $P = 0.03$; NEFA: 655 ± 26 vs. 757 ± 32 μ Eq/L, $P = 0.01$) (Table 3.5). The percentage of milk fat was lower in parity 2 animals (3.98 ± 0.07 vs. $4.24 \pm 0.08\%$, $P = 0.02$), on the contrary, the percent of lactose was higher in this parity group ($4.77 \pm .03$ vs. $4.67 \pm 0.03 \%$, $P = 0.004$) (Table 3.5).

Table 3.1. Ingredient composition of diets (% of DM)

Ingredient	Treatment ¹			Fresh
	Controlled	Intermediate	High	
Corn silage	28.5	42.2	55.9	44.2
Wheat straw	35.6	24.0	12.4	–
Hay crop silage	–	–	–	10.7
Grass Hay	–	–	–	2.7
Amino Plus ²	10.5	6.0	1.5	4.5
Canola meal, solvent extracted	6.8	7.9	8.9	9.8
Distillers grain, ethanol	4.5	3.0	1.6	7.2
Citrus pulp	3.7	4.4	5.1	–
Corn grain, finely ground	2.6	3.6	4.6	4.7
Soybean hulls	2.4	3.7	5.0	6.0
Corn germ meal	–	–	–	3.8
Chocolate dairy mix	–	–	–	1.0
Blood meal	0.53	0.45	0.38	0.84
Energy booster ³	–	–	–	0.78
Dextrose	–	–	–	0.73
Molasses	–	–	–	0.93
Soy Chlor ⁴	1.71	1.26	0.82	–
Calcium Carbonate	1.05	1.28	1.53	0.93
Calcium Sulfate	0.64	0.45	0.25	0.00
Magnesium sulfate 9.9%	0.54	0.79	1.05	–
Salt	0.31	0.29	0.27	0.50
Mono-dicalcium phosphate	0.21	0.27	0.34	–
Magnesium oxide 56%	0.17	0.17	0.15	0.15
Alimet ⁵	0.07	0.07	0.06	0.04
Dairy ADE ⁶	0.05	0.05	0.05	0.03
Selenium 0.06%	0.05	0.05	0.05	0.05
Urea	–	–	–	0.06
1100 Dairy TM ⁷	0.03	0.03	0.03	0.03
Rumensin 90 ⁸	0.01	0.01	0.01	0.01
Sodium bicarbonate	–	–	–	0.01
Vitamin E premix ⁹	0.005	0.004	0.003	–
Zinc sulfate	0.003	0.002	0.002	–
Copper sulfate	0.0007	0.0008	0.0009	0.0001

Table 3.1. Footnote

¹Treatments: Controlled energy prepartum diet, fed for ad libitum intake to control intake to 100% of energy requirements; intermediate energy prepartum diet, fed for ad libitum intake to control intake to 100% of energy requirements from 57 to 29 d before expected parturition and approximately 125% of energy requirements from 28 d before expected parturition until calving; high energy prepartum diet, fed for ad libitum intake to achieve energy intake at approximately 150% of requirements.

²Soybean product, Ag Processing Inc, Omaha, NE.

³Prilled FA, Milk Specialities Global, Eden Prairie, MN.

⁴Anionic feed supplement, West Central, Ralston, IA.

⁵2-hydroxy-4-methyl-thio-butanoic acid, Novus International, St. Charles, MO

⁶Contained 30,464 IU/kg of Vitamin A, 5,862 IU/kg of Vitamin D, and 93,784 IU/kg of Vitamin E (Cargill Animal Nutrition, Minneapolis, MN)

⁷Contained 30,317 mg/kg of Cu, 136,466 mg/kg of Mn, 3,393 mg/kg of Co, 3,040 mg/kg of I, and 153,916 mg/kg of Zn (Cargill Animal Nutrition).

⁸Contained 200 g monensin/kg, Elanco Animal Health, Greenfield, IN.

⁹Contained 499,400 IU/kg of vitamin E.

Table 3.2. Descriptive statistics of all animals by treatment group; results presented as mean value \pm SD

Measurement		Treatment ¹			Overall	<i>P</i>
		Controlled	Intermediate	High		
Entering parity	2	15	16	20	51 (60.71%)	0.34
	3	11	11	5	27 (32.14%)	
	4	1	0	3	4 (4.76%)	
	5	1	1	0	2 (2.38%)	
Body weight (kg) \pm SD		673 \pm 73	684 \pm 64	660 \pm 65	672 \pm 67	0.43
BCS \pm SD		3.1 \pm 0.3	3.2 \pm 0.3	3.1 \pm 0.2	3.1 \pm 0.3	0.24
Dry period (d) \pm SD ²		56.5 \pm 3.1	55.0 \pm 5.4	55.2 \pm 4.3	56.1 \pm 6.4	0.40

¹Treatments: Controlled energy prepartum diet, fed for ad libitum intake to control

intake to 100% of energy requirements; intermediate energy prepartum diet, fed for ad libitum intake to control intake to 100% of energy requirements from 57 to 29 d before expected parturition and approximately 125% of energy requirements from 28 d before expected parturition until calving; high energy prepartum diet, fed for ad libitum intake to achieve energy intake at approximately 150% of requirements.

²Excluding one cow in controlled energy group with 99 d dry period.

Table 3.3. Analyzed composition of diets

Component ²	Treatment ¹			
	Controlled	Intermediate	High	Fresh
DM, % ³	56.4 ± 3.8	49.8 ± 3.1	44.7 ± 3.9	46.8 ± 3.1
MP, g/d ⁴	1490 (1272-1738)	1520 (1241-1808)	1520 (1223-1774)	2650 (1673-3009)
CP, % of DM	14.2 ± 1.6	13.9 ± 0.7	12.5 ± 0.9	17.8 ± 0.9
NDF, % of DM	48.4 ± 5.0	42.2 ± 4.5	41.0 ± 4.2	35.4 ± 2.3
ADF, % of DM	30.1 ± 4.2	28.5 ± 3.2	26.55 ± 3.3	21.3 ± 4.4
Starch, % of DM	15.0 ± 2.5	20.1 ± 3.6	23.7 ± 2.6	21.2 ± 2.3
Fat, % of DM	2.7 ± 0.2	2.9 ± 0.2	3.1 ± 0.1	4.1 ± 0.3
Ca, % of DM	0.90 ± 0.08	0.96 ± 0.11	0.91 ± 0.14	0.83 ± 0.07
P, % of DM	0.36 ± 0.03	0.39 ± 0.03	0.37 ± 0.04	0.45 ± 0.02
Mg, % of DM	0.34 ± 0.02	0.38 ± 0.02	0.37 ± 0.04	0.34 ± 0.03
K, % of DM	1.07 ± 0.10	1.06 ± 0.08	0.99 ± 0.11	1.29 ± 0.14
S, % of DM	0.38 ± 0.03	0.39 ± 0.02	0.37 ± 0.02	–
Cl, % of DM	0.44 ± 0.03	0.43 ± 0.06	0.38 ± 0.04	–
Zinc, ppm	79.6 ± 23.3	79.2 ± 3.4	76.0 ± 10.2	85.5 ± 6.9
DCAD, meq/100g ⁵	-0.76 ± 1.65	-0.39 ± 3.32	-0.53 ± 2.24	–

¹Treatments: Controlled energy prepartum diet, fed for ad libitum intake to control intake to 100% of energy requirements; intermediate energy prepartum diet, fed for ad libitum intake to control intake to 100% of energy requirements from 57 to 29 d before expected parturition and approximately 125% of energy requirements from 28 d before expected parturition until calving; high energy prepartum diet, fed for ad libitum intake to achieve energy intake at approximately 150% of requirements.

Table 3.3 Footnote continued

²Chemical composition is presented as average of eleven monthly composites \pm SD (except DM).

³Average \pm SD of 36 weekly DM measurements.

⁴Prediction of MP supply for average DMI (range given for \pm SD in DMI) in each treatment group for the last seven weeks before calving and for overall average intakes postpartum (CNCPS v.6.1).

⁵Calculated as $[(\text{Na} + \text{K}) - (\text{Cl} + \text{S})]$. Percent sodium, potassium, chloride and sulfur were multiplied by 434.98, 255.74, 282.06 and 623.75 to convert to mEq/kg, respectively.

Table 3.4. Repeated measures LSM for DMI as well as energy balance estimates obtained with the Cornell Net Carbohydrate and Protein model (CNCPS version 6.1). Fixed effects treatment (T), parity (P), and time as well as their interactions

Variable	Treatment ¹ (\pm SE)			Parity (\pm SE)		<i>P</i> -value for fixed effects					
	C	I	H	2	≥ 3	T	Time	P	T*Time _e	T*P	Time*P
EBAL ² (%) prepartum											
wk -7 to -1	112 \pm 2 ^a	126 \pm 2 ^b	153 \pm 2 ^c	130 \pm 2	130 \pm 2	<0.001	<0.001	0.85	<0.001	–	0.005
wk -7	126 \pm 3	127 \pm 3	171 \pm 3	139 \pm 2	144 \pm 3						
wk -4	115 \pm 4	129 \pm 4	157 \pm 4	132 \pm 2	135 \pm 3						
wk -1	91 \pm 3	113 \pm 3	121 \pm 3	113 \pm 2	103 \pm 3						
EBAL ² (%) postpartum											
wk 1 to 6	77 \pm 1.5	74 \pm 1.6	72 \pm 1.5	77 \pm 1.1	72 \pm 1.4	0.09	<0.001	0.02	0.75	–	–
wk 2	69 \pm 2.5	65 \pm 2.5	64 \pm 2.6								
wk 4	78 \pm 1.8	76 \pm 1.8	74 \pm 1.8								
wk 6	87 \pm 1.6	85 \pm 1.6	82 \pm 1.6								

Table 3.4. Continued

DMI (kg) prepartum											
wk -7 to -1	14.2 ± 0.3 ^a	15.3 ± 0.3 ^b	16.4 ± 0.3 ^c	14.7 ± 0.2	15.9 ± 0.3	<0.001	<0.001	< 0.001	0.03	–	<0.001
wk -7	14.9 ± 0.4	15.3 ± 0.4	17.3 ± 0.4	14.9 ± 0.3	16.7 ± 0.4						
wk -4	13.9 ± 0.4	15.2 ± 0.4	16.5 ± 0.4	14.3 ± 0.3	16.1 ± 0.4						
wk -1	12.5 ± 0.4	14.3 ± 0.4	13.9 ± 0.4	14.0 ± 0.3	13.1 ± 0.4						
DMI (kg) postpartum											
wk 1 to 6	22.3 ± 0.6	22.4 ± 0.6	22.4 ± 0.6	22.1 ± 0.4	22.6 ± 0.5	0.99	<0.001	0.37	0.75	0.04	–
wk 2	19.7 ± 0.9	19.5 ± 0.9	20.7 ± 0.9								
wk 4	22.7 ± 0.7	23.1 ± 0.7	23.5 ± 0.8								
wk 6	25.0 ± 0.6	25.7 ± 0.6	25.3 ± 0.7								
C				22.0 ± 0.8	21.7 ± 0.8						
I				22.7 ± 0.7	21.5 ± 0.8						
H				22.0 ± 0.8	23.8 ± 1.0						

Table. 3.4. Footnotes

^{a,b,c}Main effects of treatment in the same row with different superscripts differ ($P \leq 0.05$).

¹Treatments: C= controlled energy prepartum diet, fed for ad libitum intake to control intake to 100% of energy requirements, I= intermediate energy prepartum diet, fed for ad libitum intake to control intake to 100% of energy requirements from 57 to 29 d before expected parturition and approximately 125% of energy requirements from 28 d before expected parturition until calving, H= high energy prepartum diet, fed for ad libitum intake to achieve energy intake at approximately 150% of requirements.

²EBAL= energy balance in % of requirements (CNCPS v. 6.1).

Table 3.5. Repeated measures ANOVA LSM for milk and ECM yield as well as milk composition

Variable	Treatment ¹ (\pm SE)			Parity (\pm SE)		<i>P</i> -value for fixed effects			
	C	I	H	2	≥ 3	T	Time	P	T*Time
Milk, kg									
wk 1 to 6	43.8 \pm 1.2	43.6 \pm 1.2	43.9 \pm 1.2	43.5 \pm 0.9	44.0 \pm 1.1	0.98	<0.01	0.73	0.31
wk 2	40.9 \pm 1.4	40.9 \pm 1.4	41.9 \pm 1.4						
wk 4	48.1 \pm 1.4	46.5 \pm 1.4	46.7 \pm 1.4						
wk 6	49.4 \pm 1.3	48.5 \pm 1.3	49.0 \pm 1.3						
ECM, kg									
wk 1 to 6	46.1 \pm 1.2	47.0 \pm 1.2	48.2 \pm 1.2	46.0 \pm 0.9	48.2 \pm 1.1	0.49	<0.01	0.13	0.92
wk 2	47.0 \pm 1.5	47.1 \pm 1.5	49.8 \pm 1.5						
wk 4	47.9 \pm 1.5	48.3 \pm 1.5	49.6 \pm 1.5						
wk 6	46.4 \pm 1.5	47.4 \pm 1.5	48.1 \pm 1.5						
Fat, %									
wk 1 to 6	3.96 \pm 0.09	4.12 \pm 0.09	4.24 \pm 0.09	3.98 \pm 0.07	4.24 \pm 0.08	0.10	<0.01	0.02	0.97
wk 2	4.44 \pm 0.16	4.63 \pm 0.15	4.76 \pm 0.15						
wk 4	3.65 \pm 0.12	3.93 \pm 0.12	4.11 \pm 0.12						
wk 6	3.30 \pm 0.12	3.47 \pm 0.12	3.57 \pm 0.12						

Table 3.5. Continued

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Protein, %									
wk 1 to 6	3.29 ± 0.08	3.27 ± 0.08	3.18 ± 0.08	3.24 ± 0.05	3.24 ± 0.06	0.59	<0.01	0.94	0.73
wk 2	3.39 ± 0.34	3.38 ± 0.34	3.26 ± 0.34						
wk 4	2.89 ± 0.07	2.81 ± 0.07	2.79 ± 0.07						
wk 6	2.77 ± 0.06	2.74 ± 0.06	2.70 ± 0.06						
Lactose %									
wk 1 to 6	4.68 ± 0.04	4.72 ± 0.04	4.75 ± 0.04	4.77 ± 0.03	4.67 ± 0.03	0.53	<0.01	0.004	0.88
wk 2	4.63 ± 0.08	4.74 ± 0.08	4.76 ± 0.08						
wk 4	4.72 ± 0.05	4.79 ± 0.05	4.83 ± 0.05						
wk 6	4.77 ± 0.04	4.81 ± 0.04	4.82 ± 0.05						
Linear score ²									
wk 1 to 6	2.64 ± 0.36	2.61 ± 0.35	2.66 ± 0.37	2.36 ± 0.26	2.91 ± 0.32	0.99	<0.01	0.18	0.28
wk 2	2.92 ± 0.37	2.58 ± 0.36	2.59 ± 0.38						
wk 4	2.31 ± 0.42	2.20 ± 0.42	2.58 ± 0.43						
wk 6	1.52 ± 0.45	2.26 ± 0.45	2.15 ± 0.46						
MUN, mg/dl									
wk 1 to 6	12.7 ± 0.3	13.5 ± 0.3	13.4 ± 0.4	13.1 ± 0.3	13.3 ± 0.3	0.22	0.04	0.60	0.33
wk 2	11.7 ± 0.5	13.1 ± 0.5	13.9 ± 0.5						
wk 4	12.6 ± 0.4	13.5 ± 0.4	13.1 ± 0.4						
wk 6	13.6 ± 0.5	14.6 ± 0.5	13.6 ± 0.5						
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Table 3.5. Footnotes

¹Treatments: C= controlled energy prepartum diet, fed for ad libitum intake to control intake to 100% of energy requirements, I= intermediate energy prepartum diet, fed for ad libitum intake to control intake to 100% of energy requirements from 57 to 29 d before expected parturition and approximately 125% of energy requirements from 28 d before expected parturition until calving, H= high energy prepartum diet, fed for ad libitum intake to achieve energy intake at approximately 150% of requirements.

²Linear score calculated $\{\log_2[(\text{SCC}/100,000)]+ 3\}$.

Table 3.6. Milk FA yield (g/d) and concentrations (g/100g) of cows for week 2 and 4 postpartum fed different dry period treatment diets

	Wk 2 postpartum					Wk 4 postpartum				
	Treatment ¹			SEM	P	Treatment ¹			SEM	P
	C (n=26)	I (n=28)	H (n=28)			C (n=26)	I (n=26)	H (n=28)		
FA yield (g/ d)										
Summation by source										
<i>De novo</i> ²	324	313	320	9.65	0.90	344	369	342	8.2	0.32
Mixed ²	488	490	524	11.7	0.36	474	528	513	11.0	0.13
Preformed ²	838	860	955	24.4	0.12	774	864	850	22.3	0.21
Selected individual FA ³										
4:0	60.4	61.1	64.8	1.76	0.55	56.4	62.9	58.5	1.48	0.19
6:0	32.4	32.2	33.0	1.02	0.95	33.2	36.7	33.6	0.83	0.17
8:0	16.7	16.2	16.3	0.58	0.94	18.0	19.5	17.8	0.49	0.30
10:0	35.2	32.8	32.4	1.40	0.68	39.2	41.3	37.5	1.22	0.43
12:0	37.3	34.1	33.7	1.46	0.56	42.1	43.5	39.7	1.28	0.47
14:0	136	131	133	4.07	0.90	147	157	147	3.66	0.43
14:1 <i>cis</i> -9	6.47	5.75	6.14	0.22	0.42	8.03	8.16	8.12	0.21	0.97
16:0	459	461	492	10.9	0.37	448	497	483	10.2	0.13
16:1 <i>cis</i> -9	29.4	29.8	32.5	1.04	0.40	26.8	31.0	29.9	1.06	0.26

Table 3.6. Continued

18:0	212 ^a	227 ^{ab}	252 ^b	6.99	0.05	191	217	207	5.98	0.22
18:1 <i>trans</i> -9	3.45	3.33	3.82	0.10	0.13	3.71	4.06	3.67	0.10	0.25
18:1 <i>cis</i> -9	435	445	507	14.6	0.09	391	444	451	14.0	0.17
18:2 <i>cis</i> -9, <i>cis</i> -12	35.1	36.1	37.1	0.91	0.68	33.2	37.3	36.0	0.92	0.19
18:2 <i>cis</i> -9, <i>trans</i> -11	6.22	5.91	5.76	0.19	0.60	6.53	6.92	6.43	0.20	0.55
FA concentration (g/ 100 g)										
Summation by source										
<i>De novo</i> ²	12.0	18.6	17.8	0.46	0.14	21.8	21.3	20.0	0.40	0.14
Mixed ²	29.6	29.6	29.3	0.18	0.78	29.8	30.0	30.2	0.22	0.81
Preformed ²	50.4	51.9	52.9	0.54	0.17	48.3	48.7	49.8	0.51	0.45
Selected individual FA ³										
4:0	3.66	3.62	3.58	0.04	0.77	3.53	3.57	3.42	0.04	0.27
6:0	1.98	1.89	1.82	0.04	0.29	2.09	2.10	1.96	0.03	0.12
8:0	1.03	0.96	0.90	0.03	0.22	1.14	1.13	1.04	0.03	0.19
10:0	2.19	1.94	1.80	0.08	0.14	2.50	2.42	2.18	0.07	0.18
12:0	2.33	2.02	1.88	0.09	0.11	2.69	2.55	2.32	0.08	0.16
14:0	8.36	7.80	7.43	0.21	0.19	9.38	9.10	8.60	0.19	0.25
14:1 <i>cis</i> -9	0.39	0.34	0.35	0.01	0.08	0.52	0.47	0.47	0.01	0.25
16:0	27.9	27.8	27.5	0.19	0.73	28.2	28.2	28.4	0.23	0.89
16:1 <i>cis</i> -9	1.75	1.81	1.82	0.04	0.78	1.66	1.73	1.75	0.04	0.63
18:0	12.7 ^a	13.6 ^{ab}	13.9 ^b	0.19	0.02	11.8	12.2	12.1	0.16	0.61

Table 3.6. Continued

18:1 <i>trans</i> -9	0.21	0.20	0.21	0.004	0.33	0.24	0.23	0.22	0.005	0.21
18:1 <i>cis</i> -9	26.0	27.0	28.1	0.48	0.21	24.3	24.9	26.4	0.45	0.15
18:2 <i>cis</i> -9, <i>cis</i> -12	2.14	2.20	2.06	0.03	0.16	2.08	2.11	2.12	0.03	0.81
18:3 <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15	5.05	5.00	5.45	0.14	0.33	4.87	5.37	5.26	0.13	0.28

^{a,b}Main effects of treatment in the same row with different superscripts differ ($P \leq 0.05$).

Table 3.6. Footnotes

¹Treatments: C= controlled energy prepartum diet, fed for ad libitum intake to control intake to 100% of energy requirements, I= intermediate energy prepartum diet, fed for ad libitum intake to control intake to 100% of energy requirements from 57 to 29 d before expected parturition and approximately 125% of energy requirements from 28 d before expected parturition until calving, H= high energy prepartum diet, fed for ad libitum intake to achieve energy intake at approximately 150% of requirements.

²De novo FA originate from de novo synthesis in the mammary gland (<16 carbons), preformed FA originate from extraction from circulating plasma FA (> 16 carbons), and mixed FA originate from both sources (C16:0 + *cis*-9 C16:1).

³A total of approximately 70 individual FA were quantified and used for calculations (summation by yield). Only select FA are reported.

Table 3.7. Repeated measures ANOVA LSM for blood concentration of BHBA and serum concentration of NEFA

Metabolite	Treatment ¹ (\pm SE)			Parity (\pm SE)		<i>P</i> -value for fixed effects					
	C	I	H	2	≥ 3	T	Time	P	T* Time	T* P	Time* P
BHBA (mmol/L), prepartum											
d -14 to -1	0.29 \pm 0.01 ^a	0.30 \pm 0.01 ^{ab}	0.34 \pm 0.01 ^b	0.30 \pm 0.01	0.32 \pm 0.01	0.04	< 0.001	0.34	0.03	–	0.04
d-14	0.27 \pm 0.02	0.26 \pm 0.02	0.31 \pm 0.02	0.27 \pm 0.01	0.28 \pm 0.02						
d -8	0.27 \pm 0.02	0.30 \pm 0.02	0.33 \pm 0.02	0.30 \pm 0.01	0.30 \pm 0.02						
d -2	0.36 \pm 0.02	0.33 \pm 0.02	0.39 \pm 0.02	0.34 \pm 0.02	0.38 \pm 0.02						
BHBA (mmol/L), postpartum											
d 1 to 21	0.61 \pm 0.06 ^a	0.76 \pm 0.06 ^{ab}	0.84 \pm 0.07 ^b	0.66 \pm 0.05	0.81 \pm 0.06	0.04	< 0.001	0.03	0.36	–	–
d 1	0.54 \pm 0.10	0.53 \pm 0.10	0.58 \pm 0.10								
d 7	0.67 \pm 0.10	0.89 \pm 0.10	0.89 \pm 0.10								
d 10	0.65 \pm 0.10	0.89 \pm 0.10	1.15 \pm 0.10								
d 13	0.63 \pm 0.10	0.87 \pm 0.10	0.85 \pm 0.10								
d 19	0.54 \pm 0.10	0.61 \pm 0.10	0.74 \pm 0.10								
NEFA (μ Eq/L), prepartum											
d -14 to -1	237 \pm 12 ^a	180 \pm 13 ^b	175 \pm 12 ^b	186 \pm 9	209 \pm 10	0.001	< 0.001	0.07	0.03	–	–
d-14	149 \pm 11	130 \pm 11	118 \pm 11								
d -8	205 \pm 13	156 \pm 13	168 \pm 13								
d -2	397 \pm 26	275 \pm 27	260 \pm 26								

Table 3.7. continued

NEFA ($\mu\text{Eq/L}$), postpartum											
d 1 to 21	659 \pm 36 ^a	665 \pm 37 ^a	796 \pm 40 ^b	655 \pm 26	757 \pm 32	0.02	< 0.001	0.01	0.37	0.004	–
d 1	638 \pm 50	583 \pm 50	729 \pm 52								
d 7	835 \pm 67	780 \pm 67	955 \pm 69								
d 10	734 \pm 56	745 \pm 56	864 \pm 58								
d 13	616 \pm 49	693 \pm 49	689 \pm 51								
d 19	518 \pm 43	550 \pm 43	702 \pm 46								
C				676 \pm 47	643 \pm 50						
I				642 \pm 46	687 \pm 52						
H				649 \pm 42	943 \pm 63						

^{a,b,c}Main effects of treatment in the same row with different superscripts differ ($P \leq 0.05$).

¹Treatments: C= controlled energy prepartum diet, fed for ad libitum intake to control intake to 100% of energy requirements, I= intermediate energy prepartum diet, fed for ad libitum intake to control intake to 100% of energy requirements from 57 to 28 d before expected parturition and approximately 125% of energy requirements from 28 d before expected parturition until calving, H= high energy prepartum diet, fed for ad libitum intake to achieve energy intake at approximately 150% of requirements.

Figure 3.1. DMI (kg) wk 7 to 1 prepartum (A) and wk 1 to 6 postpartum (B) for the three treatment groups

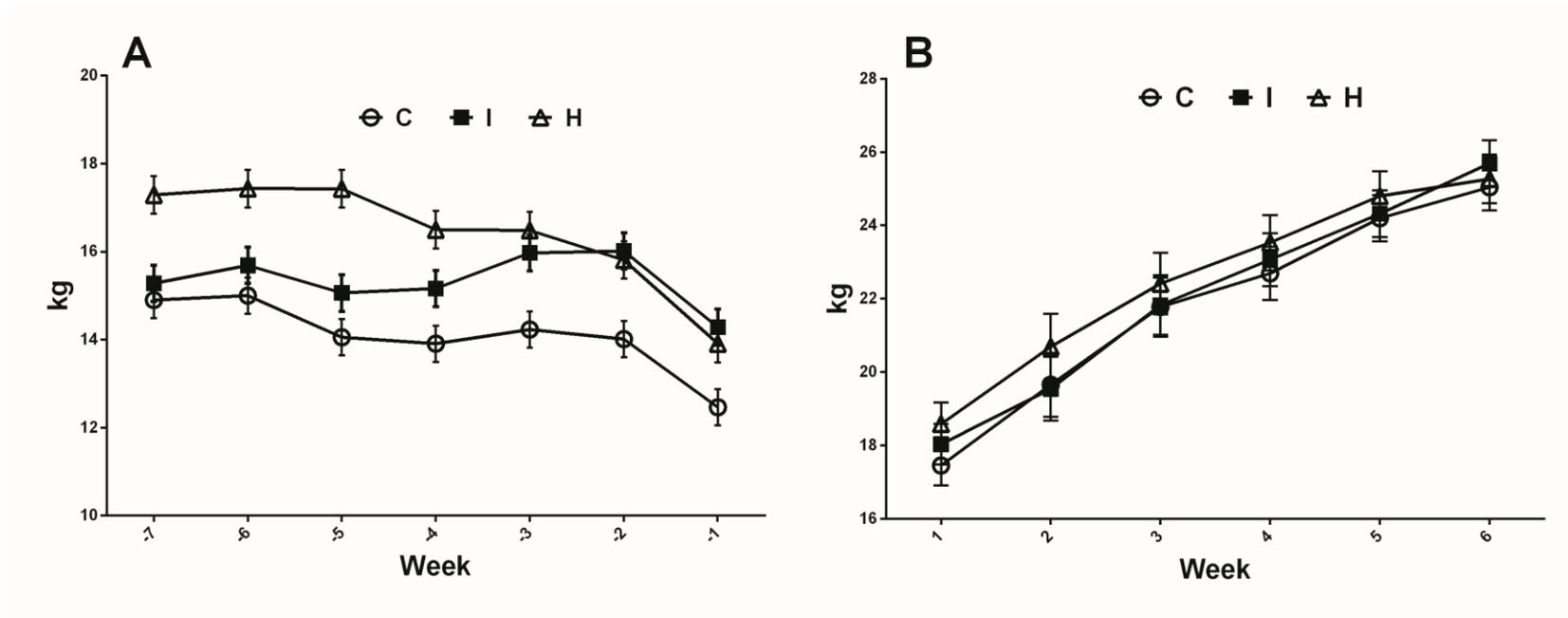


Figure 3.1. Continued

C= controlled energy prepartum diet, fed for ad libitum intake to control intake to 100% of energy requirements, I= intermediate energy prepartum diet, fed for ad libitum intake to control intake to 100% of energy requirements from 57 to 29 d before expected parturition and approximately 125% of energy requirements from 28 d before expected parturition until calving, H= high energy prepartum diet, fed for ad libitum intake to achieve energy intake at approximately 150% of requirements. At parturition, all cows were fed the same lactation diet. Data are presented as least squares means \pm SE. Prepartum: treatment, $P < 0.001$; time $P < 0.001$; parity, $P < 0.001$; treatment x time, $P = 0.03$; time x parity $P < 0.001$. Postpartum: treatment, $P = 0.99$; time, $P < 0.001$; parity, $P = 0.37$; treatment x time, $P = 0.75$; treatment x parity, $P = 0.04$. Parity was categorized into parity 2 or ≥ 3 . Interactions of main effects treatment, time and parity removed if $P \geq 0.05$ except treatment x time which was forced in the model.

Figure 3.2. Comparison of energy balance (percent of requirements) estimates.

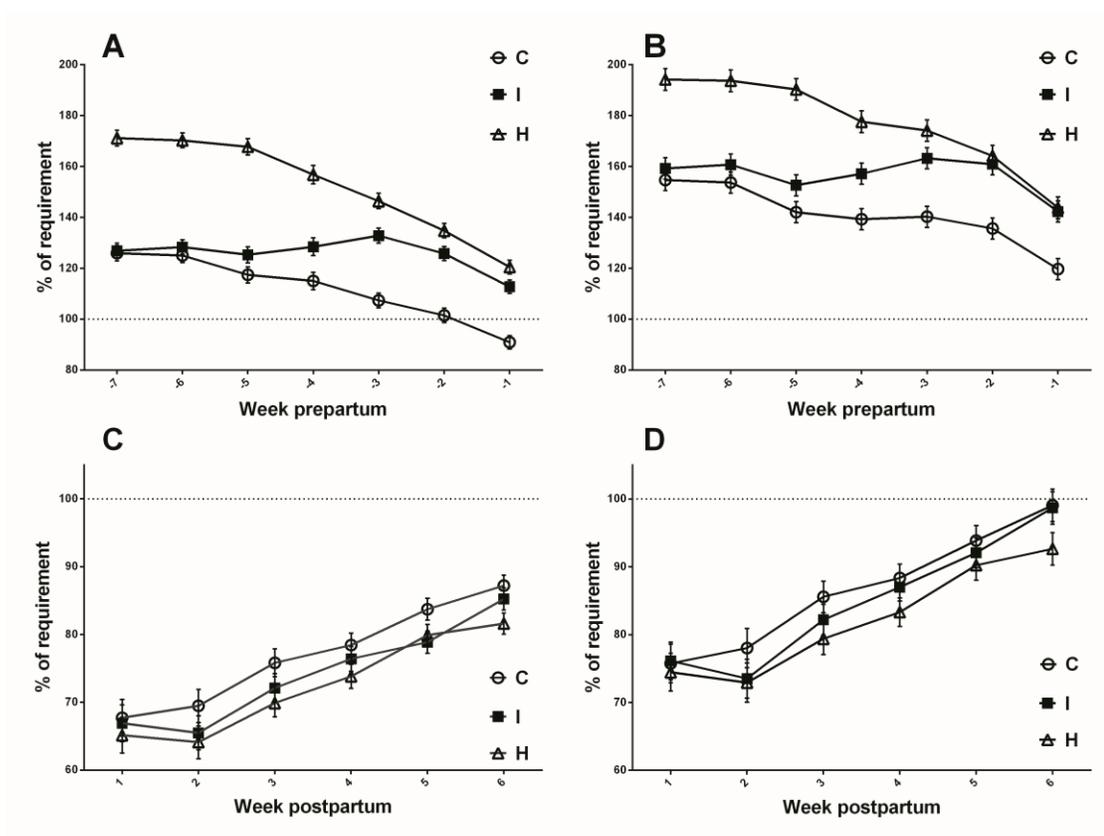


Figure 3.2. Continued

Estimations of prepartum (wk 7 to 1 prepartum) energy balance with the Cornell Net Carbohydrate and Protein System (CNCPS v.6.1) **(A)** or according to NRC (2001) **(B)** and postpartum (wk 1 to 6 postpartum) with CNCPS v. 6.1 **(C)** and NRC **(D)** models. C= controlled energy prepartum diet, fed for ad libitum intake to control intake to 100% of energy requirements, I= intermediate energy prepartum diet, fed for ad libitum intake to control intake to 100% of energy requirements from 57 to 29 d before expected parturition and approximately 125% of energy requirements from 28 d before expected parturition until calving, H= high energy prepartum diet, fed for ad libitum intake to achieve energy intake at approximately 150% of requirements. At parturition, all cows were fed the same lactation diet. Data are presented as least squares means \pm SE. (A) Prepartum CNCPS: treatment, $P < 0.001$; time, $P < 0.001$; parity, $P = 0.85$; treatment x time, $P < 0.001$; time x parity, $P = 0.005$ (B) Prepartum NRC: treatment, $P < 0.001$; time, $P < 0.001$; parity, $P = 0.53$; treatment x time, $P < 0.001$; time x parity, $P = 0.001$ (C) Postpartum CNCPS: treatment, $P < 0.09$; time, $P < 0.001$; parity, $P = 0.02$; treatment x time, $P < 0.75$ (D) Postpartum NRC: treatment, $P < 0.20$; time, $P < 0.001$; parity, $P = 0.06$; treatment x time, $P < 0.86$. Parity was categorized into entering parity 2 or ≥ 3 . Interactions of main effects treatment, time and parity removed if $P \geq 0.05$ except treatment x time which was forced in the model.

Figure 3.3. Concentration of metabolites in blood before morning feeding..

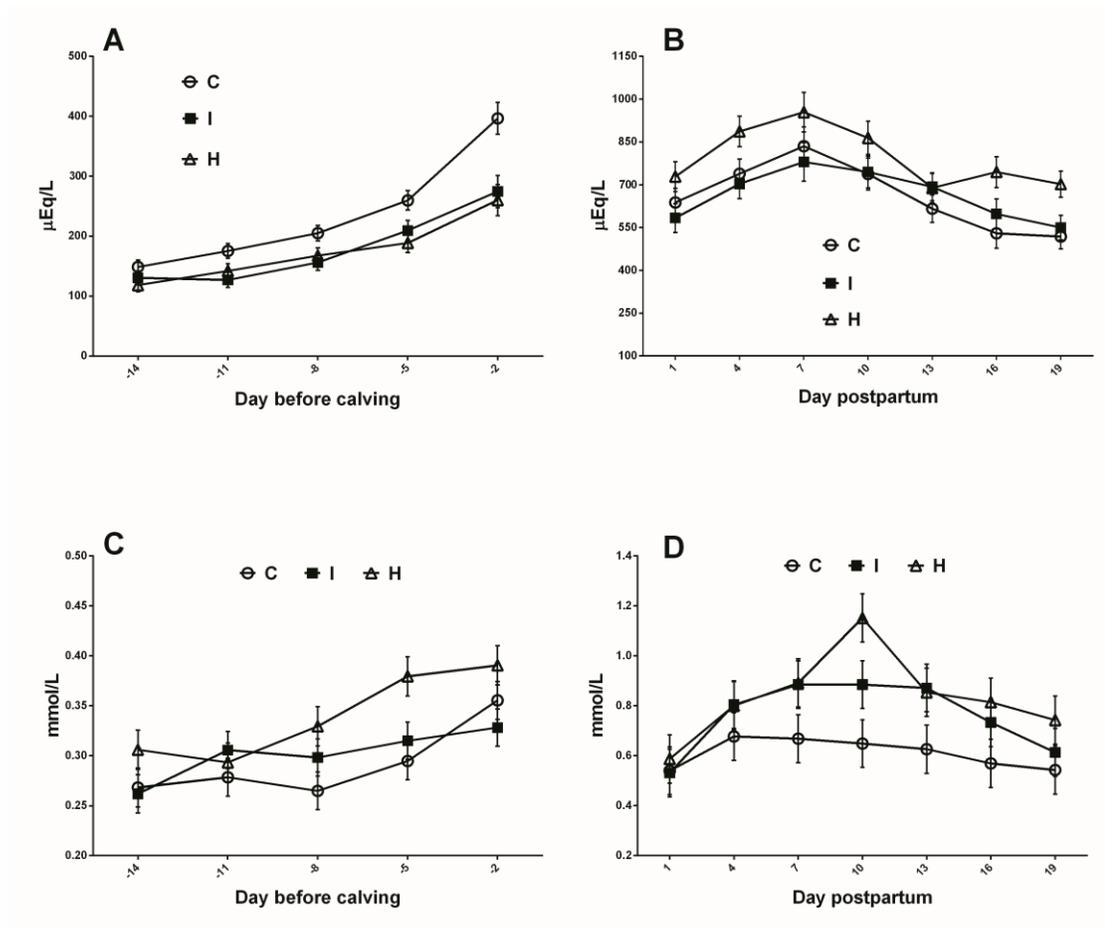


Figure 3.3. Continued

Concentration of serum NEFA ($\mu\text{Eq/L}$) (**A**) and BHBA (mmol/L) (**C**) for the last 2 wk before calving and for the first 3 wk postpartum (**B**: NEFA, **D**: BHBA). C= controlled energy prepartum diet, fed for ad libitum intake to control intake to 100% of energy requirements, I= intermediate energy prepartum diet, fed for ad libitum intake to control intake to 100% of energy requirements from 57 to 29 d before expected parturition and approximately 125% of energy requirements from 28 d before expected parturition until calving, H= high energy prepartum diet, fed for ad libitum intake to achieve energy intake at approximately 150% of requirements. At parturition, all cows were fed the same lactation diet. Data are presented as least squares means \pm SE. BHBA prepartum: treatment, $P = 0.04$; time, $P < 0.001$; parity, $P = 0.34$; treatment x time, $P = 0.03$; time x parity, $P = 0.04$. BHBA postpartum: treatment $P = 0.04$; time, $P < 0.001$; parity, $P = 0.03$; treatment x time, $P = 0.36$. NEFA prepartum: treatment, $P < 0.001$; time, $P < 0.001$; parity, $P = 0.07$; treatment x time, $P = 0.03$. NEFA postpartum: treatment, $P = 0.02$; time, $P < 0.001$; parity, $P = 0.01$; treatment x time, $P = 0.37$; treatment x parity, $P = 0.004$. Parity was categorized into entering parity 2 or ≥ 3 . Interactions of main effects treatment, time and parity removed if $P \geq 0.05$ except treatment x time which was forced in the model.

DISCUSSION

Our primary objective in this study was to investigate the effect of different prepartum feeding strategies differing in energy level on hyperketonemia. Our results show that animals overfed energy in the dry period had higher concentrations of both NEFA and BHBA postpartum, indicating a more severe negative energy balance in this group, as was also apparent in model estimates. These findings support the results reported by other investigators, concluding that it is advisable to prevent overfeeding energy in the dry period (Kunz et al., 1985; Dann et al., 2006; Janovick et al., 2011). Furthermore data indicate that supplying an intermediate energy diet during the close-up period alone can lead to at least some of the undesirable effects that were related to overfeeding a higher energy diet during the entire dry period. Our findings therefore support the application of a one-group controlled energy dry cow program.

Higher NEFA concentrations were measured prepartum in the controlled energy group. A higher concentration of NEFA in prepartum cows due to energy restriction has been reported previously (Douglas et al., 2006; Janovick et al., 2011; Schoenberg and Overton, 2011). Possible reasons for this are a higher degree of negative energy balance immediately before parturition when DMI drops (Radloff et al., 1966; Bell, 1995), or a smaller degree of insulin mediated inhibition of lipolysis due to a lower insulin concentration in blood. Differences in DMI prepartum, which were likely attributable to a difference in rumen fill (Allen and Piantoni, 2013), did not carry over to the postpartum period. As opposed to other studies (Douglas et al., 2006), we did not document an increase in DMI in the controlled energy group postpartum compared to the other treatment groups. The absence of an effect on early

postpartum DMI after feeding a controlled or restricted energy diet supports descriptions by other researchers (Agenas et al., 2003), although numerically higher intakes in prepartum energy restricted groups were described (Holtenius et al., 2003; Dann et al., 2006). Regardless of a lack of difference in DMI postpartum, the different planes of energy prepartum impacted fat mobilization differently as reflected by NEFA concentration, as well as elevation in BHBA concentration. DMI decreased in all groups in the immediate prepartum period as observed by others (Dann et al., 2006; Janovick et al., 2011; Vickers et al., 2013). Possible explanations are a rise in estrogen and other pregnancy-related factors as reviewed in Grummer et al. (2004), as well as interactions between those and nutritional factors (Roche et al., 2013). Despite this drop in DMI prepartum, animals in the controlled energy group showed a less pronounced drop in feed intake relative to prior intakes compared to both the intermediate and high energy group, maintaining DMI at a more constant level throughout the dry period. The change rather than the magnitude of DMI prepartum might have a greater effect on metabolic status of periparturient cows (Grummer et al., 2004).

In this study, monensin was included in all diets to more closely reflect feeding practices in modern dairy herds in the United States. Several authors have described the effect of monensin in the transition period (Pettersson-Wolfe et al., 2007; Mullins et al., 2012) as well as in early lactating dairy cows (Duffield et al., 2008). According to those results, monensin was able to lead to a reduction in the blood concentration of BHBA postpartum both when given in the dry period as well as in early lactation. Therefore, it is possible that metabolic effects of the different treatments would have

been more extreme in the absence of feeding monensin. Due to a lack of unsupplemented concurrent control groups, this remains speculation. Protein levels in the three treatment groups were formulated to assure a margin of safety so that none of the cows would be protein deficient. As intakes were higher than predicted, predicted MP levels exceeded the formulated 1,280 g/d in all groups prepartum but were similar across all groups.

Higher milk fat content in the high energy group was likely due to increased mobilization of fat depots in the first weeks of lactation (Palmquist et al., 1993). During this period, dietary supply of precursors for FA, such as acetate and glucose, are decreased and the mammary tissue synthesizes lower amounts of short chain fatty acids compared with a state of positive energy balance (Palmquist et al., 1993). Plasma NEFA can directly be taken up by the mammary gland as is reflected by a positive correlation between plasma NEFA concentration and milk fat percent (Pullen et al., 1989). The differences in milk fat as well as the absence of a difference in milk protein among treatment groups explain the numerical differences in ECM while treatment group did not affect milk production. Although fat mobilization can be due to various biological mechanisms and hormonal stimuli (Nielsen et al., 2014), we hypothesize that a higher degree of negative energy balance in the high energy group was associated with an increase in the rate of lipolysis as reflected by the higher concentration of NEFA (Rukkwamsuk et al., 1999). We found a higher concentration of the preformed FA C18:0 and *cis*-9 C18:1 in milk fat from cows being fed a higher energy diet during the dry period, while the amount of de novo FA was similar among treatment groups. Differences in C18:0 between cows being fed different planes of

energy during the dry period were also observed by Agenas et al. (2003) and the authors hypothesized them to be due to a higher supply of FA from body origin. *cis*-9 C18:1 is the desaturation product of C18:0 through the action of Δ (9)-desaturase and is the predominating FA in adipocytes (Nogalski et al., 2012). It is released during negative energy balance through lipolysis (Rukkwamsuk et al., 2000). The difference in the yield of *cis*-9 C18:1 inclusion in milk fat was present in both wk 2 and 4 postpartum, indicating that lipolysis and increased transfer of preformed FA persisted past the end of the transition period, typically considered as 21 DIM.

Animals in parity 2 had lower NEFA and BHBA concentrations postpartum compared to older animals. A higher odds of developing ketosis for parity 3 and higher has previously been reported using serum (Duffield et al., 1997), a handheld meter (McArt et al., 2013a; Suthar et al., 2013), or a milk-based test (Berge and Vertenten, 2014).

Although our intent was to best represent current feeding practices, there are limitations to our study that need to be considered. Cows on study were fed for ad libitum intake, but because of the housing system we could not account for possible effects of competition at the feed bunk as well as sorting behavior. These factors would need to be taken into account on most modern dairy farms with group housing and feeding. Moreover, because we set up a feeding model to also study glucose response and every cow received intravenous glucose at a dose of 0.25 g/kg body weight twice during the dry and fresh period after completion of the morning blood sampling (data not presented), levels of NEFA and BHBA could have been altered at least in a short term period and may have decreased the level of hyperketonemia in all

groups. Because all cows received the same treatments, this potential effect would have equally altered responses in all groups; therefore, results for the degree of hyperketonemia observed are likely conservative.

When applying different diet formulation model systems (CNCPS and NRC 2001), results differed both for energy density estimates of the diets as well as energy balance estimates. Regardless of the method chosen to estimate energy balance, the directional changes and magnitude of difference due to treatments were similar. However, the model systems yielded different estimates for the percentage of energy supplied. We believe that the difference can be attributed mostly to a difference in NDF digestibility used in the different model systems. To a smaller extent, differences could have been due to a disparity in the amount of energy requirements proposed for lactogenesis, supply of energy to the uterus and fetus in the dry period, as well as requirements for mammogenesis in the peripartum period. Drackley and Janovick Guretzky (2007) describe a similar discrepancy between different methods of energy density determination and recommend to focus on providing NDF and starch intake guidelines instead of energy density.

CONCLUSIONS

Results of this study showed an increased incidence of hyperketonemia in cows being fed a diet exceeding energy requirements during the whole dry period as well as in cows being fed a controlled energy diet in the far-off period and then a moderate energy diet in the close-up period. Milk production and postpartum DMI were not different among cows fed different planes of energy prepartum. Our findings support a one group controlled energy dry period feeding approach in order to minimize the degree of negative energy balance postpartum as well as hyperketonemia, possibly preventing negative health events shown to be correlated to an increase in BHBA concentration.

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CHAPTER 4

DRY PERIOD PLANE OF ENERGY: EFFECTS ON GLUCOSE TOLERANCE

IN TRANSITION DAIRY COWS

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ABSTRACT

Overfeeding energy in the dry period can affect glucose metabolism and energy balance of transition dairy cows with potentially detrimental effects on the ability to successfully adapt to early lactation. The objectives of this study were to investigate the effect of different dry cow feeding strategies on glucose tolerance and on resting concentrations of blood glucose, glucagon, insulin, non-esterified fatty acids (NEFA) and β -hydroxybutyrate (BHB) in the peripartum period. Cows entering second or greater lactation were enrolled at dry-off (57 d before expected parturition) into one of three treatment groups following a randomized block design: cows that received a TMR formulated to meet but not exceed energy requirements during the dry period (n=28, C), cows that received a TMR supplying approximately 150% of energy requirements during the dry period (n=28, H), and those that were fed the same diet as group C for the first 28 d after which the TMR was formulated to supply approximately 125% of energy requirements until calving (n=28, I).

Intravenous glucose tolerance tests (IVGTT) with rapid administration of 0.25 g/kg BW of glucose were performed 28 and 10 d before expected parturition, as well as 4 and 21 d after calving. Area under the curve (AUC) for insulin and glucose, maximal concentration and time to half-maximal concentration of insulin and glucose, and clearance rates were calculated. Insulin resistance (IR) indices were calculated from baseline samples obtained during IVGTT and Spearman rank correlations determined between IVGTT parameters and IR indices. Treatment did not affect IVGTT parameters at any of the four time-points. Correlation between IR indices and IVGTT parameters was generally poor. Overfeeding cows energy in excess of

predicted requirements by approximately 50% during the entire dry period resulted in decreased postpartum basal plasma glucose and insulin, as well as increased glucagon, BHB and NEFA concentrations after calving compared with cows fed a controlled energy diet during the dry period. In conclusion, overfeeding energy during the entire dry period or close-up period alone did not affect glucose tolerance as assessed by IVGTT but energy uptake during the dry period was associated with changes in peripartal resting concentrations of glucose, as well as postpartum insulin, glucagon, NEFA and BHB concentrations.

Key Words: dairy cow, transition period, energy, glucose, insulin

INTRODUCTION

The transition period represents a metabolic challenge to dairy cows because of the rapid increase of required nutrients, particularly for milk production. Excessive negative energy balance and hyperketonemia (defined as a blood concentration of BHB \geq 1.2 mmol/L) in the postpartum period are associated with detrimental effects on health and productivity of dairy cows (Duffield et al., 2009; McArt et al., 2013b). Feeding of high energy diets during the dry period increases the degree of lipid mobilization and ketogenesis postpartum (Dann et al., 2006; Janovick et al., 2011; Mann et al., 2015). A number of studies have attempted to elucidate the cause of this postpartum effect on ketogenesis in dairy cows overfed energy prepartum.

Schoenberg et al. (2011, 2012) investigated how plane of nutrition affected the response of dry cows to a glucose challenge in two experiments. In these experiments

cows were fed either approximately 90% or 160%, or 120 and 170% of predicted energy requirements during the dry period. Diet had no effect on the insulin response in both studies, but cows fed 90% of energy requirements tended to have higher glucose area under the curve, decreased glucose clearance and greater NEFA response compared with overfed cows. However, no measurements were taken postpartum, including glucose tolerance tests. Holtenius et al. (2003) found a numerically higher insulin peak and higher glucose clearance prepartum, whereas glucose clearance was reduced postpartum following a glucose challenge in cows overfed energy during the dry period compared with cows fed a lower energy diet. They hypothesized that the observed postpartum changes are evidence of a greater degree of insulin resistance (IR) in cows overfed energy during the dry period, leading to more lipolysis and higher blood NEFA concentration. However, sample size was relatively small and different genetic selection lines were used.

Several studies have aimed to describe how overfeeding in the dry period affects resting concentrations of insulin and glucose in the peripartum period. Overfeeding energy during the far-off and close-up dry period (Dann et al., 2006) and during the entire dry period (Holtenius et al., 2003; Douglas et al., 2006; Janovick et al., 2011) was associated with increased insulin concentrations prepartum compared with controlled or restricted fed cows. Overfeeding during the close-up period (Dann et al., 2006) or the whole dry period (Douglas et al., 2006) resulted in higher prepartum glucose concentrations compared with cows fed restricted energy. Cows fed a controlled energy diet during the whole dry period tended to have greater insulin concentrations postpartum compared with overfed cows (Janovick et al., 2011).

However, other studies showed no effect of overfeeding during the dry period on peripartal plasma glucose, glucagon and insulin (Selim et al., 2015) or postpartal glucose and insulin concentrations (Khan et al., 2014; Schulz et al., 2014). In light of the differences found in these studies, clear evidence is lacking if overfeeding during the entire dry period or during close-up alone leads to peripartal changes in glucose disposal, glucose availability, or both. Because excess energy intake affects insulin sensitivity in humans (Capurso and Capurso, 2012; Johnson and Olefsky, 2013), changes in resting concentrations of insulin as well as insulin response to a glucose challenge and glucose clearance are also of interest in this context in the bovine species.

Our objective was therefore to investigate the effect of different dry period planes of energy on glucose disposal by repeated intravenous glucose tolerance test in periparturient dairy cows and to evaluate the effect on resting concentrations of glucose, insulin, and glucagon as well as concentrations of blood NEFA and BHB.

MATERIALS AND METHODS

Animals, Feeding and Management

All procedures were approved by the Cornell University Institutional Animal Care and Use Committee. A detailed description of animals, feeding and management was reported previously (Mann et al., 2015). In brief, Holstein cows (n=84) entering second or greater lactation from the herd at the Cornell Teaching and Research Center were enrolled between September 2012 and April 2013. All animals were housed in

individual sawdust-bedded tie-stalls equipped with individual feed bins. Cows were allocated to one of three dry period dietary treatment groups using a randomized block design to control for time-dependent variation with 3 treatments in 28 blocks on the day of dry off (approximately 57 d before expected parturition). Blocking was based on expected calving date. Random sequence of allocation within the block was determined with a random number generator (Research Randomizer v.4.0, Urbaniak, 2012). Groups did not differ in BCS (on a scale of 1.0 to 5.0 according to Edmonson et al., 1989) of animals at enrollment or in the distribution of parity (Mann et al., 2015). Animals were enrolled in one of three feeding groups: a TMR formulated to meet 100 % of energy requirements at predicted ad libitum intake (C) a TMR formulated to supply 150% of energy requirements (H), and an intermediate group that received the same TMR as group C for the first 28 d after dry off and a TMR formulated to supply 125% of energy requirements from d 28 before expected parturition until calving (I, representing a 50:50 blend of both C and H diets). On a DM basis, conventional corn silage accounted for 28.5, 42.2, and 55.9% in diet C, I, and H, respectively, and wheat straw was included at 35.6, 24.0, and 12.4% of DM in groups C, I, and H. All cows received the same fresh cow TMR (F) from the onset of lactation until the end of the study period (42 DIM). Milk yield was measured at every milking (0900, 2100 h). Rations were formulated using the Cornell Net Carbohydrate and Protein System software (CNCPS, Cornell University, version 6.1). Samples of all TMRs were taken weekly and analyzed based on a monthly composite at a commercial laboratory with wet chemistry methods (Dairy One Cooperative Inc.,

Ithaca, NY). All diets were offered ad libitum, fed once daily at 0900 h and amounts fed were adjusted to allow for a minimum of 5% refusals.

Intravenous Glucose Tolerance Test

Intravenous glucose tolerance tests (IVGTT) were performed on all cows, targeting 28 and 10 d before expected parturition and 4 and 21 d postpartum. On sampling days, refusals were removed at 0700 h and cows were kept in box stalls after morning milking which was completed at 1000 h. A 14g x 140 mm catheter with a 305 mL/min capacity (Abboath-T, Hospira, Sligo, Ireland) was placed in the jugular vein. A 15 x 10-cm area was clipped, scrubbed with povidone iodine and 70% ethanol and 2 mL of 2% lidocaine (Vet One, Boise, ID) were injected subcutaneously. The area was scrubbed again and the catheter was placed approximately 10 min later and flushed with 10 IU heparin/mL of a 0.9% sterile saline solution (heparin: Sagent pharmaceuticals, Schaumburg, IL, saline solution: Abbott Animal Health, Abbott Park, IL). Cows were allowed to rest with access to water, but not feed, until the IVGTT was initiated.

Baseline samples were taken at 15 and 5 min before, as well as 0, 5, 10, 15, 20, 25, 30, 45, 60, 90 and 120 min after completion of an intravenous bolus infusion of 0.25 g/kg BW glucose (50% dextrose solution, Vet One, Boise, ID) into evacuated tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) with 158 USP units of sodium heparin. Glucose solution was pre-heated in a water bath to body temperature and infusion completed within approximately 2-3 min through a large bore infusion set (Jorgensen Laboratories, Loveland, CA). All samples were

immediately placed on ice, centrifuged within 30 min for 20 min at 2,800 x g, and plasma samples were stored as 1 mL aliquots in 1.7 mL microcentrifuge tubes (VWR, Radnor, PA) to prevent multiple freeze thaw cycles after snap freezing with liquid nitrogen. Samples were stored at -20°C until analysis.

Analysis of IVGTT blood samples

Plasma glucose (PGO enzyme preparation, Sigma Aldrich, St. Louis, MO) and NEFA (HR Series NEFA-HR (2); Wako Life Sciences, Mountain View, CA) concentrations were measured by colorimetric assay from all baseline samples. In addition, NEFA concentration was determined for all samples of each IVGTT at d 10 before and d 4 after parturition for animals in 19 out of 28 blocks only and plasma concentration of glucose was determined for all IVGTT samples at all time points.

Plasma concentration of insulin was determined for all time points of each glucose tolerance test for animals in 19 out of 28 blocks using a radioimmunoassay kit (PI-12K, Porcine Insulin RIA, EMD Millipore Corp, Billerica, MA). The concentration of BHB was determined for all baseline samples using a handheld device (Precision Xtra meter, Abbott Diabetes Care Inc., Alameda, CA) in whole blood immediately after obtaining the sample. Plasma glucagon concentration was determined for all baseline samples of IVGTTs from animals in 19 out of 28 enrollment blocks using a radioimmunoassay kit (Glucagon RIA kit, Millipore, St. Charles, MO).

On sampling days, subcutaneous and muscle biopsies were taken from the area of the paralumbar fossa (data not presented) and all animals received 2.2 mg/kg flunixin meglumine (Prevail, VetOne) i.v. for minor pain mitigation.

Weekly Blood sampling

Blood sampling in the peripartum period is described in detail in Mann et al. (2015). In brief, blood samples (10 mL) were taken three times per week before feeding (between 0600 and 0730 h) from the coccygeal vein or artery, placed on ice, separated within one h by centrifugation at 2,800 x g for 20 min at 4°C, snap-frozen in liquid N₂ and stored at -20°C. All samples were frozen in four aliquots to prevent multiple freeze thaw cycles. An animal was considered as hyperketonemic when BHB concentrations obtained during morning sampling reached 1.2 mmol/L (McArt et al., 2013a). Cows exhibiting a concentration of BHB \geq 2.5 mmol/L were treated with 250 mL of a 50% dextrose solution (Dextrose 50% inj, VetOne, i.v.) on two consecutive days as well as 300 mL (approximately 310 g) of propylene glycol orally for five consecutive days starting on the day of diagnosis. Subsequent episodes after this initial treatment were treated with another 5-d course of oral propylene glycol alone and this was repeated until BHB concentrations were determined to be < 2.5 mmol/L. Animals that had at least one episode of hyperketonemia within the first 21 DIM were considered as positive cows (HYK) for statistical analysis. In addition to the described analysis of IVGTT baseline samples, glucose, glucagon and insulin concentrations were determined in all weekly plasma samples from 7 d before until 7 d after parturition. Concentrations of BHB were determined 3 times per week in whole blood

from 3 wk before until 3 wk after parturition and NEFA concentrations were determined at the same time points in serum.

Analytical approach

Molar insulin to glucagon ratio and surrogate indices. The molar ratio of insulin and glucagon was computed after conversion of insulin from $\mu\text{U/mL}$ to pmol/L by multiplication with the factor 6.0 (Heinemann, 2010), and for glucagon from pg/mL to pmol/L by multiplying with the factor 0.287 (Banarer et al., 2002). A total of 7 baseline samples (1, 2, and 4 in group C, I and H, respectively) had an undetectable concentration of insulin as determined by RIA; for those samples, a value of 0 was used and samples were omitted from calculation of molar insulin: glucagon ratio (**IG**) ratio as well as surrogate index calculations.

The IR indices homeostasis model of insulin resistance (**HOMA-IR**) according to Muniyappa et al. (2008), quantitative insulin sensitivity check index (**QUICKI**) according to Katz et al. (2000) , revised QUICKI (**RQUICKI**) according to Perseghin et al. (2001), and revised QUICKI including BHB (**RQUICKI_{BHB}**) according to Balogh et al. (2008) were calculated as follows:

$$\text{HOMA-IR} = \{[\text{glucose (mmol/L)} \times \text{insulin } (\mu\text{U/mL})] / 22.5\}$$

$$\text{QUICKI} = \{1 / [\log \text{insulin } (\mu\text{U/mL}) + \log \text{glucose (mg/dL)}]\}$$

$$\text{RQUICKI} = \{1 / [\log \text{glucose (mg/dL)} + \log \text{insulin } (\mu\text{U/mL}) + \log \text{NEFA (mmol/L)}]\}$$

$$\text{RQUICKI}_{\text{BHB}} = \{1 / [\log \text{glucose (mg/dL)} + \log \text{insulin } (\mu\text{U/mL}) + \log \text{NEFA (mmol/L)} + \log \text{BHB (mmol/L)}]\}$$

Glucose clearance rate (**CR**) was calculated according to the method described by Kerestes et al. (2009): $\text{Glucose CR} = [(\ln \text{ glucose at timepoint 0} - \ln \text{ glucose at timepoint 60}) / 60] \times 100 = \%/ \text{ min}$

Insulin CR was calculated accordingly: $\text{Insulin CR} = [(\ln \text{ insulin at peak timepoint} - \ln \text{ insulin at timepoint 60}) / 60 - \text{peak timepoint}] \times 100 = \%/ \text{ min}$

Statistical Analysis. The primary outcome of this study was the description of measurements obtained during IVGTT and included resting blood concentration as well as glucose, insulin and NEFA response to the glucose challenge. Values from both baseline samples of each IVGTT were averaged to generate a single baseline value. Area under the curve (**AUC**) for insulin, glucose and NEFA concentrations was calculated in SAS (SAS Institute Inc, version 9.3, Cary, NC) as the positive incremental area adjusting for baseline according to the method described by Cardoso et al. (2011). Mixed effects ANOVA were carried out with PROC MIXED with the independent variables treatment group and HYK and dependent variables of IVGTT parameters. The interaction of treatment and HYK was tested for every ANOVA and included in the model if $P < 0.05$. Enrollment block was included as a random effect. Experiment-wise error rate for all ANOVA analyses was corrected with Tukey's test and reported P -values for comparisons among groups represent those corrected for multiple comparisons. Continuous data were tested for normality. Data for the outcome parameters IG ratio, insulin and BHB concentration on d 4 postpartum, BHB concentration on d 28 prepartum and d 21 postpartum, maximal glucose concentration, time to half maximal glucose concentration, AUC for glucose and insulin was log transformed to satisfy this assumption. Results of corresponding least squares means

of log transformed data were subsequently back transformed and are reported as geometric mean and 95% CI, results of untransformed data as mean and 95% CI. A chi-square test was performed with Proc FREQ for parity differences within treatment. The assumption of homoscedasticity was tested for each ANOVA with Levene's test. In cases that this assumption was violated, Welch's test was performed. The correlation between BHB and glucose concentration at day 4 postpartum was evaluated using Pearson correlation.

The analysis of secondary outcomes: repeated measures ANOVA was performed for the resting concentrations of glucose, glucagon, insulin, NEFA, BHB, and IG ratio from 7 d prepartum to 7 d postpartum in cows with and without hyperketonemia using PROC MIXED in SAS. Three covariance structures were tested (autoregressive 1, unstructured, compound symmetry) and the one yielding the smallest Akaike information criterion was chosen. Fixed effect was HYK group and the REPEATED statement was time. The interaction of HYK status and time was forced into every model. When results of the ANOVA analysis yielded a p-value of ≤ 0.05 , Tukey's posthoc test was used for comparison of means across all groups to control experiment wise error rate. Normality of residuals was tested after each model fit. Another secondary outcome was the correlation between the IR indices and AUC as well as curve parameters (time to maximal concentration, time to half maximal concentration and maximal concentration) which were tested for each sampling day using the Spearman correlation. Results were reported if $\rho > 0.50$. The correlation between IR indices on d 4 postpartum calculated from morning samples with those

calculated from IVGTT baseline parameters was also analyzed using Spearman correlation.

Descriptive statistical analysis using one-way ANOVA were carried out with Proc GLM for treatment differences in BW at enrollment, BCS, dry period length, milk production, sampling day relative to calving and IVGTT glucose dose. For the effect of time, corresponding ANOVA were carried out to analyze the differences in LSM for IVGTT parameters using time-period and group as fixed effects and including enrollment block as random effect.

Results are presented as means or geometric means and 95% CI unless otherwise stated.

RESULTS AND DISCUSSION

Cows were dry for an average of 55.5 d (C: 56.5 ± 3.1 , I: 55.0 ± 5.4 , H: 55.2 ± 4.3 ; $P = 0.4$). The average BW at enrollment was 672 (657-686) kg and was not different between groups ($P > 0.43$). A BCS score of 3.0 was predominant at dry-off (n=40, 48%) with 13 animals at BCS 2.75 (15%), and 27 animals with BCS of 3.25 or 3.50 (32%), the remaining 4 animals (5%) had a BCS of 3.75 (n=3) or 4.0 (n=1). On average, cows in group H gained an additional 0.27 (0.18-0.36) point in BCS score, compared with group C (0.15 [0.07-0.24], $P = 0.15$), and group I (0.11 [0.02-0.19], $P = 0.03$). However, it has to be taken into account that BCS may lack sensitivity for the detection of differences in visceral fat mass as recently described (Drackley et al., 2014) and that the small difference in BCS between the groups might underestimate

true differences in adipose tissue gain. Weight gain in the dry period was also highest in group H (90.4 [82.7-98.1] kg), compared with group C (72.1 [64.3-79.8] kg, $P = 0.004$) and group I (71.0 [63.3-78.8] kg, $P = 0.002$). The majority of cows ($n = 51$) were entering parity 2, and 33 animals were entering parity 3 and greater; the distribution of parity was not different between groups ($P = 0.34$). Selected items from the chemical analysis of rations and predicted MP supplies for pre-and postpartum diets are presented in Table 4.1. The complete list of ingredients of each diet, wet chemistry analysis results, as well as milk production and composition were previously described (Mann et al., 2015). Overall the averages of milk yield in the first two wk of lactation (C: 36.2 [33.8-38.6], I: 35.9 [33.6-38.2], H: 37.0 [34.7-39.3] kg; $P = 0.80$) as well as wk 3 and 4 of lactation (C: 47.7 [45.2-50.2], I: 46.2 [43.8-48.7], H: 45.8 [43.4-48.2] kg; $P = 0.53$) were not different among dietary treatment groups. Cows that became hyperketonemic in the first 21 DIM ($n = 29$) and those that did not ($n = 55$) had similar milk production in the first 2 wk (37.0 [34.6-39.5] vs. 35.6 [33.9-37.4] kg, $P = 0.35$) and in wk 3 and 4 after calving (46.9 [44.5-49.4] vs. 46.0 [44.2-47.8] kg, $P = 0.54$). Intra- and interassay coefficients of variation (CV) for glucose, NEFA, insulin, and glucagon measurements were 2.2 and 6.4%, 3.1 and 8.2%, 6.9 and 5.7% and 4.6 and 12.9%, respectively.

Glucose and Insulin Response during IVGTT

The primary objective of this study was to evaluate the effect of different dry period planes of energy on glucose tolerance in peripartum dairy cows. The dose of glucose administered during the IVGTT was smaller than the one used in a previous

study (Kerestes et al., 2009), but similar (Hove, 1978; Zachut et al., 2013), the same (Schoenberg and Overton, 2011; Schoenberg et al., 2012), or larger than in other studies (Holtenius et al., 2003), which should be taken into consideration when comparing results between them.

The mean and SD of actual sampling day for IVGTT was 26.6 ± 4.2 and 7.9 ± 4.0 d before calving, and 4.6 ± 1.2 and 21.0 ± 1.2 d postpartum. The average time point of glucose infusion was 1230 h (1120 – 1340 h) and approximately 5 h after morning blood sampling and feed removal.

Figure 4.1 and 4.2 depict the mean and SD of glucose and insulin concentrations during the IVGTT carried out at the four different time-points. Maximal concentration of insulin and glucose, the clearance rate of both, as well as time to half maximal values for both analytes, and total AUC were not different among groups for any of the time-points (Table 4.2). Time to maximal insulin concentration was also not different (Table 4.2). The findings of our study are similar to a previous study by Schoenberg and Overton (2011) where no differences were detected in IVGTT measurements of insulin and glucose between dry period energy treatment groups during late gestation. In a second study RQUICKI, IVGTT parameters and insulin sensitivity as assessed by the hyperinsulinemic euglycemic clamp tests did not differ between treatment groups of varying energy levels in the dry period (Schoenberg et al., 2012). Together these studies and the current experiment suggest that overfeeding during the dry period does not alter the ability of dairy cows to respond to a glucose challenge compared with cows being fed a controlled energy diet.

A number of measurements obtained from IVGTT were different between the four different sampling time-points of the pre- and postpartum period and when controlling for differences between treatment groups. Glucose clearance was highest at 21 d postpartum (2.05 [1.97-2.11] %/min), followed by rates on d 4 postpartum (1.85 [1.78-1.93] %/min), and both were different compared with both prepartum sampling time-points (28 d prepartum: 1.51 [1.44-1.59]; 10 d prepartum: 1.61 [1.54-1.69] %/min, $P < 0.0001$). The glucose CR between d 21 and 4 postpartum was also different ($P < 0.002$). Maximum concentrations for glucose 28 and 10 d prepartum [256 (247-266) and 266 (257-276) mg/dL, respectively], were higher than those on d 4 and 21 postpartum [207 (197-216) and 221 (212-230) mg/dL, $P < 0.0001$).

The increased glucose clearance is likely linked to the onset of lactation (Debras et al., 1989). For every kg of milk produced, 72 g of glucose are needed (Kronfeld, 1982). This higher basal glucose turnover in lactating cows has to be taken into account when comparing lactating cows to dry cows (De Koster and Opsomer, 2013), and this limits comparability of IVGTT findings between non-lactating and lactating animals. As opposed to previously reported findings (Kerestes et al., 2009), we saw both increased glucose and insulin clearance in cows postpartum. Numerically lower glucose CR in animals fed higher energy diet postpartum were observed previously and interpreted as increased IR (Holtenius et al., 2003). Discrepancies in study design, sample size, and genetics could be variables that account for the differences observed between this study and the current one.

Insulin clearance was highest at 21 and 4 d postpartum (21d postpartum: 4.35 [4.03-4.66]; 4 d postpartum: 4.13 [3.81-4.45] %/min) compared with both prepartum

time-points (28 d prepartum: 2.16 [1.85-2.47]; 10 d prepartum: 2.23 [1.92-2.53] %/min, $P < 0.0001$). Maximum concentration of insulin was 160 (144-178), 112 (101-124), 50 (45-55), and 67 (60-74) $\mu\text{U}/\text{mL}$ on d 28 and 10 prepartum, as well as 4 and 21 d postpartum, respectively, and concentrations at all four time-points were different from each other ($P < 0.0003$).

Zachut et al. (2013) hypothesized that the higher insulin AUC to clear the same dose of glucose in the prepartum period compared with the postpartum period indicates a degree of IR in late gestation as described by Bell (1995). Similar to our own findings, Bossaert et al. (2008) also reported lower insulin AUC in lactation and attributed this to reduced insulin secretion which could be part of the physiologic homeorhetic adaptation to early lactation. According to Malven et al. (1987), insulin uptake into the mammary gland after parturition is negligible and unlikely to play a role in the observed increase in clearance rate postpartum. Because insulin is predominantly metabolized in the liver (Ferrannini and Cobelli, 1987) and liver blood flow increases substantially with lactation, the increased insulin clearance could also be due to an increased rate of degradation (Lomax and Baird, 1983). However, peak insulin concentrations observed postpartum were greatly reduced compared with prepartum peak concentrations, and could help explain the observed changes in insulin clearance independently of changes in uptake by the mammary gland or increased metabolization rate by the liver.

Effect of Dry Period Plane of Energy on NEFA Response during IVGTT

Figure 4.3 shows the percentage of reduction from baseline of plasma NEFA concentrations at sampling time-points d 10 prepartum and d 4 postpartum which was not different among the treatment groups. This is in contrast to both studies by Schoenberg et al. (2011, 2012). Concentrations of NEFA reached a nadir around 45 min after glucose infusion which is similar to findings by Zachut et al. (2013). This represents the rapid inhibition of lipolysis by insulin (Ruan and Lodish, 2003) which we observed regardless of the differences in insulin concentration from pre- to postpartum time-points and regardless of the large increase in NEFA concentrations postpartum compared with prepartum. Circulating NEFA are readily removed from the blood stream through lipid storage or uptake by the liver, as well as by the mammary gland during lactation (Pethick, 2005). The absolute changes in NEFA concentrations expressed as AUC were not different on d 10 prepartum (C: -25.8 [(-19.4) – (-32.2)], I: -22.0 [(-15.6) – (-28.4)], H: -23.5 [(-17.1) – (-29.9)] mmol*120 min/L, $P = 0.70$) and d 4 postpartum (C: -62.7 [(-54.8) – (-70.5)], I: -56.7 [(-48.9) – (-64.6)], H: -67.6 [(-60.0) – (-75.5)] mmol*120 min/L, $P = 0.15$). The authors of a recent study investigating the effect of insulin on fatty acid metabolism in dry dairy cows differing in body condition found that inhibition of lipolysis remains intact in overconditioned cows and lower insulin concentrations are necessary for its action on FA metabolism compared with actions on glucose metabolism (De Koster et al., 2015). The results of our study would also suggest that even a lower insulin response to glucose challenge (smaller AUC, lower peak concentration) postpartum leads to a similar effect on reduction of NEFA concentration and therefore inhibition of lipolysis

as compared to prepartum time-points. In addition, the absence of a difference between the treatment groups in the present study indicates that inhibition of lipolysis and rate of removal of NEFA were not affected by the energy level and concurrent differences in body condition and weight gain during the dry period. These results together with an absence of differences in insulin response to a glucose challenge can be interpreted such that the increase in body condition score and weight in group H compared with groups C and I did not alter the insulin response to a glucose challenge or the ability of adipose tissue to respond to the insulin stimulus.

Resting Concentration of Pancreatic Hormones, Glucose and Markers of Negative Energy Balance

Measurement of baseline glucose, NEFA, insulin, glucagon, BHB and IG ratio was carried out as an alternate assessment of metabolic status pre-and postpartum and revealed notable differences on d 4 postpartum (Figure 4.4). Prepartum glucose concentrations were higher in cows in group H compared with both groups I and C at both prepartum time-points (d 28 prepartum: group H: 77.8 [74.6-81.1] mg/dL vs. C: 73.9 [70.8-77.1] mg/dL, $P = 0.07$; and I: 73.4 [70.3-76.5] mg/dL, $P = 0.04$; d 10 prepartum: group H: 75.9 [73.8-78.1] mg/dL vs. C: 69.6 [67.6-71.7] mg/dL, $P = 0.004$; and I: 70.6 [68.5-72.7] mg/dL, $P = 0.008$). The higher blood glucose concentration in prepartum cows fed a higher energy diet has been observed by others (Schoenberg and Overton, 2011), but could be due to increased propionate conversion in the liver (Janovick et al., 2011). We hypothesize that the higher prepartum plasma glucose concentration in group H could have been caused by availability of nutrients

in the form of starch in excess of requirements, resulting in greater availability of propionate for hepatic gluconeogenesis.

Glucose concentrations were lowest in group H (56.0 [53.4-58.5] mg/dL) and highest in group C (59.9 [57.4-62.4] mg/dL, $P = 0.08$) on d 4 postpartum. NEFA baseline values increased greatly on d 4 postpartum from prepartum values and were highest in group H (1.63 [1.52-1.74] mmol/L) compared with both groups C (1.37 [1.26-1.48] mmol/L, $P = 0.004$) and I (1.38 [1.27-1.50] mmol/L, $P = 0.008$). This is in accordance with a tendency for a more pronounced negative energy balance in group H compared with group C in the postpartum period which we previously described (Mann et al., 2015). These findings are of particular importance for future reproductive success as low blood glucose concentrations after calving are associated with subfertility due to its role as regulator of hormones (such as insulin and IGF-1) and metabolites (such as NEFA) controlling reproductive function (Garverick et al., 2013; Lucy et al., 2013).

Insulin baseline concentrations dropped from values measured on d -28 to reach the lowest concentration on d 4 postpartum. Insulin baselines on d 4 postpartum were lowest in group H (0.95 [0.55-1.62] μ U/mL) compared with group C (2.10 [1.22-3.58] μ U/mL, $P = 0.09$) and I (2.32 [1.38-3.92] μ U/mL, $P = 0.05$). Baseline glucagon concentration increased after calving and were different among groups on d 4 postpartum with the highest concentration in group H (137.2 [126.5-149.5] pg/mL) compared with groups C (115.4 [103.8-126.9] pg/mL, $P = 0.007$) and I (117.1 [105.3-128.8] pg/mL, $P = 0.009$). Cows experience a natural decrease in insulin concentration as part of the homeorhetic regulation to enable increased gluconeogenesis and

lipolysis (Bell, 1995; Nielsen et al., 2014) whereas glucagon is upregulated at the initiation of lactation (De Koster and Opsomer, 2013) and increases the oxidation of NEFA as well as plasma glucose concentrations (Bobe et al., 2003). The combination of decreased insulin and increased glucagon baseline concentrations allows for increased protein degradation (Rooyackers and Nair, 1997), and increased gluconeogenesis in the liver (Aschenbach et al., 2010). This physiological adaptation to the catabolic period (Holtenius and Holtenius, 1996) was observed in all treatment groups as a response to reduced glucose availability. However, the decrease in insulin and glucose concentrations and increase in glucagon concentration were most pronounced in cows in group H. This was reflected in a lower insulin: glucagon ratio in this group (Figure 4.4), indicating a more severe negative energy balance, leading to increased ketogenesis during this period when the capacity of gluconeogenesis becomes insufficient to provide energy substrates (Heitmann et al., 1987). This was further supported by the negative relationship of BHB with glucose concentrations on d 4 postpartum ($r = -0.53$, $P < 0.0001$) (Figure 4.6). Animals with resting BHB concentrations ≥ 1.2 mmol/L had lower concentrations of glucose on this day compared with those having BHB concentrations < 1.2 mmol/L (51.6 [48.6-54.6] vs. 60.2 [58.7-61.8] mg/dL, $P < 0.0001$).

Lower insulin concentrations in cows postpartum have previously been reported in cows overfed energy in the dry period (Janovick et al., 2011). Since glucose orchestrates whole animal metabolism through its effect on circulating concentrations of insulin and other hormones (Lucy, 2008), these changes were likely caused by the lower postpartum glucose concentrations in the overfed group.

Since no differences were found in DMI, milk production and lactose yield between these treatment groups (Mann et al., 2015) and based on the data presented here, we suggest that these differences in glucose concentrations were not caused by differences in glucose disposal (either into insulin-dependent tissues or the mammary gland), but rather represent a problem of limited glucose synthesis or availability of glucose precursors, or both, in cows overfed energy during the dry period. A number of studies have investigated the effect of overfeeding on markers of hepatic gluconeogenesis in cows fed different energy levels in the dry period. In the study by Rukkwamsuk et al. (1999), gluconeogenic enzyme activity was reduced in the liver of overfed cows from one week prepartum to two weeks postpartum. Decreased expression of key enzymes for gluconeogenesis in liver biopsied from 10 d before parturition until 10 d after calving were also found in cows overfed energy during the dry period (Selim et al., 2014). In the study by Murondoti et al. (2004) a decreased rate of gluconeogenesis in overfed cows was measured even before development of fatty liver, and was thought to be due to a larger amount of ileal-digestible but rumen-undegradable starch. Despite the fact that glucose transporters are present in the whole GI tract, only a few feedstuffs such as corn provide enough digestible starch to amount to significant quantities of absorbed glucose and between 5 to 20% of consumed starch is digested postruminally (Huntington, 1997; Aschenbach et al., 2010). Therefore, more work is needed to determine if direct absorption of glucose alters regulation of gluconeogenesis in cows overfed energy during the dry period.

Effect of Hyperketonemia on IVGTT measurements and Baseline Parameters

A secondary objective was the evaluation of IVGTT and baseline parameters in cows with and without postpartum hyperketonemia. In the first 21 d postpartum, 7, 10, and 12 animals in group C, I, and H became hyperketonemic. Complete information of all analytes was available for 20 hyperketonemic and 37 non-hyperketonemic animals. Characteristics of IVGTT for cows with and without postpartum hyperketonemia revealed an increase in glucose AUC, as well as reduced insulin CR postpartum (Table 4.2). NEFA AUC on d 4 was also not different for HYK versus non-HYK cows (-60.5 [-66.1 to -54.9] vs. -65.8 [-73.5 to -58.2] mmol*120 min/L, $P = 0.27$). Figure 4.5 shows the repeated measures least squares means of resting concentration of glucose, glucagon, insulin, NEFA and BHB, as well as molar IG ratio from 7 d prepartum to 7 d postpartum for cows that did and did not become hyperketonemic. Compared with animals that did not become hyperketonemic, glucose baseline of those animals that did become hyperketonemic within the first 21 DIM was decreased on d 4 (54.5 [51.9-57.0] vs. 60.4 [58.7-62.1] mg/dL, $P = 0.0002$) and on d 21 postpartum (56.5 [53.8-59.2] vs. 63.9 [62.1-65.8] mg/dL, $P < 0.0001$). Insulin baseline concentration was also decreased d 21 postpartum (3.81 [2.57-5.1 μ IU/mL]) compared with non-hyperketonemic animals (5.7 [4.7-6.6] μ IU/mL, $P = 0.02$) whereas glucagon (134.7 [123.0-146.4] vs. 120.0 [111.3-128.5] pg/mL, $P = 0.05$) and NEFA concentrations (1.6 [1.5-1.7] vs. 1.3 [1.3-1.4] mmol/L, $P = 0.01$) were increased on d 4 postpartum in this group.

In a study by Hove (1978), ketotic cows were characterized by lower insulin response to a glucose challenge and lower glucose concentrations preceding the

challenge. Insulin also increased to a smaller degree after feeding in hyperketonemic, hypoglycemic animals compared with non-hyperketonemic animals (Hove and Halse, 1978). The author hypothesized in both studies that the decreased secretion of insulin was possibly due to lower secretory capacity of the pancreas developed during the days of lower glucose concentrations in blood preceding the IVGTT and feeding. Sakai et al. (1996) also measured lower blood insulin concentration in ketotic cows after glucose infusion. The decreased insulin concentration allows for lipolysis during hypoglycemia and ketogenesis (Hove, 1974). Prolonged negative energy balance can lead to reduction in pancreatic islet cell and size, lower insulin secretion, hypoinsulinemia, lower glucose concentration as well as a lower glucose clearance rate (Hayirli, 2006).

IR Indices and Correlation with IVGTT parameters

Surrogate IR indices were evaluated to assess the association with IVGTT parameters. The results and statistical analysis of the calculations for surrogate indices for the treatment groups and hyperketonemia status are shown in Supplementary Table 4.1. We observed no differences in surrogate indices between treatment groups, but indices changed over time, reflecting the changes between time-points in glucose, insulin, NEFA and BHB concentrations as described.

Spearman correlation results between IR indices and IVGTT parameters with $\rho > 0.50$ included a positive correlation between NEFA AUC on d 10 prepartum and RQUICKI ($\rho = 0.75$, $P < 0.001$), as well as RQUICKI_{BHB} ($\rho = 0.76$, $P < 0.001$) on the

same d. None of the other tested correlations between IR indices and IVGTT parameters yielded a correlation coefficient above 0.50.

We observed that postpartum BHB concentrations increased from morning blood sampling to those concentrations measured in baseline samples of IVGTT (difference on d 4 postpartum: 0.20 [0.15-0.25], difference on d 21 postpartum: 0.44 [0.36-0.52] mmol/L). On d 4 postpartum, insulin concentrations in samples obtained during morning blood sampling were on average 2.82 (2.13-3.51) μ U/mL higher than concentrations measured in baseline samples. These differences were not affected by treatment group. Concentration of NEFA on this day increased substantially during this timeframe (0.65 [0.54-0.75] mmol/L) and increases were different for the three treatment groups (C: 0.45 [0.28-0.62], I: 0.68 [0.51-0.85], H: 0.83 [0.66-1.01] mmol/L, $P = 0.009$). The correlation between IR indices calculated on d 4 postpartum from samples taken in the morning and those taken on average 5 h later yielded the following Spearman correlation coefficients: $\rho = 0.58$ for HOMA-IR and QUICKI ($P < 0.0001$), 0.38 for RQUICKI_{BHB} ($P = 0.007$) and 0.37 for RQUICKI ($P = 0.008$). In comparison with the values obtained from the morning samples, HOMA-IR decreased by 0.34 (95% CI: -0.48 to -0.20), QUICKI increased by 0.12 (0.08 to 0.16), RQUICKI increased by 0.05 (0.01 to 0.08) and RQUICKI_{BHB} increased by 0.03 (-0.01 to 0.06). No treatment differences were observed for any of the IR indices on d 4 postpartum from morning samples ($P > 0.39$).

IR indices are widely used in human medicine and are interpreted such that increased HOMA-IR and decreased QUICKI indices represent an increase in IR. With the exception of RQUICKI_{BHB}, IR indices in the current study would be interpreted as

the highest degree of IR at 28 d prepartum with the smallest degree of IR on d 4 postpartum. Similar to findings reported by Schulz et al. (2014) we did not measure significant differences between RQUICKI in cows overfed energy in the dry period and those fed a normal control diet. IR indices are subject to potential variation caused by stage of lactation and pregnancy (De Koster and Opsomer, 2013) and usefulness in dairy cows is questionable because glucose and insulin kinetics are very different from those in human medicine where IR indices were established, especially after the onset of lactation (Schoenberg and Overton, 2011). The poor correlation observed in this study is in accordance to findings by Kerestes et al. (2009). Others have found better correlations between indices and parameters of clamp tests (Haarstrich, 2011), but cows were sampled in mid-lactation. An interesting finding of this study was the relatively poor correlation between samples taken at two different time-points on d 4 postpartum which can be explained by changes in insulin, NEFA, BHB over the course of several hours, and in this case potentially exacerbated by feed deprivation. Differences in measurements can be caused by diurnal changes in metabolites as well as changes induced by a fasted state (Nielsen et al., 2003; Schoenberg et al., 2012), such as increase in NEFA and decrease in insulin concentration (Schoenberg and Overton, 2011). It is also possible that increased handling of animals led to a higher degree of stress during IVGTT sampling compared with the morning sampling which could alter concentrations of certain metabolites such as NEFA (Gupta et al., 2005; Saco et al., 2008).

Table 4.1. Analyzed composition of diets. Mean and SD presented.

Component ²	Treatment ¹			
	Controlled	Intermediate	High	Fresh
MP, g/d ³	1490 (1272-1738)	1520 (1241-1808)	1520 (1223-1774)	2650 (1673-3009)
CP, % of DM	14.2 ± 1.6	13.9 ± 0.7	12.5 ± 0.9	17.8 ± 0.9
NDF, % of DM	48.4 ± 5.0	42.2 ± 4.5	41.0 ± 4.2	35.4 ± 2.3
ADF, % of DM	30.1 ± 4.2	28.5 ± 3.2	26.55 ± 3.3	21.3 ± 4.4
Starch, % of DM	15.0 ± 2.5	20.1 ± 3.6	23.7 ± 2.6	21.2 ± 2.3
Fat, % of DM	2.7 ± 0.2	2.9 ± 0.2	3.1 ± 0.1	4.1 ± 0.3

¹Treatments: Controlled energy prepartum diet, fed for ad libitum intake to control intake to 100% of energy requirements; intermediate energy prepartum diet, fed for ad libitum intake to control intake to 100% of energy requirements from 57 to 29 d before expected parturition and approximately 125% of energy requirements from 28 d before expected parturition until calving; high energy prepartum diet, fed for ad libitum intake to achieve energy intake at approximately 150% of requirements. All cows were fed the same fresh cow TMR.

²Chemical composition is presented as average of eleven monthly composites ± SD.

³Prediction of MP supply for average DMI (range given for ± 1 SD in DMI) in each treatment group for the last seven weeks before calving and for overall average intakes (± 1 SD in DMI) postpartum (CNCPS v.6.1).

Table 4.2. Results of mixed effects ANOVA for treatment differences in intravenous glucose tolerance tests parameters at 28 and 10 d prepartum (-28 and -10) as well as 4 and 21 d postpartum (+4 and +21) for treatment (T) groups and HYK² status. The interaction between treatment and HYK was tested and retained in the analysis if $P < 0.05$. Enrollment block included as a random effect. Results reported as mean or geometric mean and 95% CI.

Measure ment	T ¹			<i>P</i>	HYK ²		<i>P</i>	T x HYK
	Controlled	Intermediate	High		No	Yes		<i>P</i>
Maximal glucose concentration (mg/dL) ³								
-28 d	260 (243-278)	258 (243-275)	256 (242-272)	0.76	260 (250-271)	256 (242-272)	0.87	0.50
-10 d	259 (243-276)	261 (246-277)	269 (254-284)	0.54	268 (258-279)	258 (244-272)	0.66	0.61
+ 4 d	213 (203-225) ^a	209 (199-220) ^b	198 (188-209) ^c	0.08	211 (204-219)	203 (193-213)	0.20	0.10
+ 21 d	220 (208-233)	217 (206-228)	210 (200-221)	0.58	219 (211-227)	212 (202-223)	0.36	0.32
Time (min) to half maximal glucose concentration ³								
-28 d	34.7 (28.5-42.3)	42.5 (35.6-50.8)	37.4 (31.6-44.4)	0.10	36.7 (32.4-41.5)	39.5 (33.3-47.0)	0.56	0.51
-10 d	36.2 (30.9-42.4)	35.2 (30.5-40.5)	35.4 (30.9-40.6)	0.95	33.5 (30.3-36.9)	37.9 (33.1-43.5)	0.15	0.72
+ 4 d	27.4 (24.4-30.7)	29.7 (26.5-33.2)	31.8 (28.5-35.5)	0.20	28.3 (26.2-30.6)	30.8 (27.6-34.4)	0.22	0.02 ⁴
+21 d	24.3 (21.5-26.7)	24.1 (22.1-26.7)	27.4 (25.0-30.0)	0.10	25.1 (23.4-26.8)	25.3 (23.1-27.8)	0.77	0.63
Glucose clearance rate (%/min)								
-28 d	1.6 (1.5-1.8)	1.5 (1.3-1.6)	1.5 (1.4-1.7)	0.20	1.5 (1.4-1.6)	1.5 (1.4-1.7)	0.76	0.54
-10 d	1.6 (1.5-1.7)	1.6 (1.5-1.7)	1.6 (1.5-1.7)	0.99	1.6 (1.5-1.7)	1.6 (1.5-1.7)	0.77	0.87
+ 4 d	1.9 (1.7-1.9)	1.8 (1.7-1.9)	1.8 (1.7-1.9)	0.30	1.9 (1.8-2.0)	1.8 (1.7-1.9)	0.17	0.26
+21 d	2.1 (1.9-2.2)	2.1 (2.0-2.2)	2.0 (1.9-2.1)	0.47	2.1 (2.0-2.1)	2.0 (1.9-2.2)	0.91	0.75

Table 4.2. Continued

Glucose AUC (g*120 min/dL) ³								
-28 d	5.60 (5.05-6.21) ^{ab}	6.21 (5.64-6.83) ^a	5.33 (4.87-5.84) ^b	0.07	5.56 (5.21-5.94)	5.85 (5.34-6.40)	0.36	0.93
-10 d	6.01 (5.45-6.64)	5.94 (5.42-6.51)	5.83 (5.35- 6.36)	0.83	5.94 (5.58-6.33)	5.92 (5.43-6.45)	0.70	0.93
+4 d	3.85 (3.48-4.26)	3.92 (3.57-4.31)	3.86 (3.63-4.22)	0.75	3.69 (3.47-3.92)	4.02 (3.68-4.40)	0.11	0.50
+21 d	3.36 (3.05-3.70)	3.34 (3.06-3.64)	3.60 (3.31-3.91)	0.29	3.22 (3.02-3.43)	3.63 (3.33-3.94)	0.02	0.68
Maximal insulin concentration ³ (μIU/mL)								
-28 d	184 (148-227)	147 (120-182)	160 (130-197)	0.31	154 (133-180)	172 (141-211)	0.37	0.69
-10 d	124 (103-150)	103 (85-124)	110 (91-133)	0.34	110 (96-127)	115 (95-139)	0.74	0.93
+4 d	57 (45 -71)	51 (41-65)	42 (33-53)	0.13	54 (46-63)	43 (34-54)	0.08	0.56
+21d	73 (61-89)	65 (54-78)	60 (50-72)	0.24	69 (60-80)	63 (52-75)	0.33	0.54
Insulin AUC (mIU* 120 min/mL) ³								
-28 d	7.07 (5.58-8.97)	6.29 (5.07-7.79)	6.19 (5.00- 7.67)	0.58	5.99 (5.16-6.94)	7.06 (5.73-8.71)	0.19	0.65
-10 d	5.15 (4.24- 6.26)	4.36 (3.62- 5.26)	4.67 (3.87- 5.63)	0.49	4.47 (3.93-5.09)	4.98 (4.17-5.94)	0.33	0.94
+4 d	1.73 (1.42- 2.11)	1.44 (1.18- 1.76)	1.37 (1.13- 1.67)	0.18	1.56 (1.34- 1.81)	1.46 (1.20-1.77)	0.56	0.50
+21d	2.08 (1.71- 2.54)	1.93 (1.60- 2.33)	1.75 (1.45- 2.11)	0.31	1.88 (1.65-2.15)	1.94 (1.63-2.32)	0.74	0.35
Time (min) to half maximal insulin concentration ³								
-28 d	48.6 (42.1-55.2)	49.8 (43.2-56.3)	50.9 (44.4-57.4)	0.89	47.9 (43.3-52.5)	51.6 (45.6-57.9)	0.25	0.38
-10 d	49.4 (39.4-59.3)	56.2 (46.4-66.1)	54.1 (44.2-64.1)	0.51	51.4 (44.2-58.1)	55.1 (45.6-64.6)	0.30	0.07
+4 d	29.1 (25.0-33.8)	28.7 (24.7-33.3)	26.5 (22.7-30.9)	0.63	28.7 (23.2-30.5)	28.7 (27.9-33.2)	0.64	0.74
+21 d	29.4 (25.6-33.1)	29.6 (25.9-32.6)	29.3 (25.8-32.8)	0.23	27.8 (25.4-30.5)	28.9 (26.4-31.4)	0.48	0.48

Table 4.2. Continued

Time (min) to maximum insulin concentration								
- 28 d	12.4 (10.3-14.6)	11.5 (9.3-13.6)	12.0 (9.9-14.1)	0.59	10.4 (8.9-11.9)	13.5 (11.4-15.6)	0.05	0.71
- 10 d	14.6 (11.5-17.6)	12.0 (9.0-15.1)	13.9 (10.4-17.6)	0.41	12.3 (9.6-14.9)	14.5 (10.9-18.1)	0.08	0.74
+4 d	8.1 (6.6-9.6)	6.8 (5.3-8.2)	7.7 (6.2-9.1)	0.32	7.3 (6.3-8.5)	7.6 (6.1-9.1)	0.75	0.16
+21 d	7.3 (5.3-9.2)	7.4 (5.5-9.3)	6.9 (5.0-8.7)	0.92	7.1 (5.7-8.5)	7.3 (5.4-9.1)	0.88	0.95
Insulin clearance rate (%/min)								
- 28 d	2.4 (2.0-2.8)	1.9 (1.6-2.3)	2.2 (1.8-2.6)	0.21	2.2 (1.9-2.5)	2.0 (1.6-2.4)	0.34	0.73
- 10 d	2.5 (2.0-3.0)	1.9 (1.4-2.4)	2.3 (1.8-2.8)	0.23	2.3 (1.9-2.7)	2.0 (1.4-2.5)	0.15	0.31
+4 d	3.8 (3.2-4.5)	4.1 (3.4-4.7)	4.1 (3.4-4.7)	0.81	4.4 (3.9-4.9)	3.6 (2.9-4.2)	0.03	0.97
+21 d	4.2 (3.6-4.9)	4.5 (3.9-5.1)	4.1 (3.5-4.7)	0.52	4.6 (4.1-5.0)	3.9 (3.2-4.5)	0.04	0.52

Table 4.2. Footnotes

¹Treatments: Controlled energy prepartum diet, fed for ad libitum intake to control intake to 100% of energy requirements; intermediate energy prepartum diet, fed for ad libitum intake to control intake to 100% of energy requirements from 57 to 29 d before expected parturition and approximately 125% of energy requirements from 28 d before expected parturition until calving; high energy prepartum diet, fed for ad libitum intake to achieve energy intake at approximately 150% of requirements.

² Hyperketonemia (HYK): Animals with a BHB concentration ≥ 1.2 mmol/L at any time-point within the first 21 DIM were considered as hyperketonemic for this analysis.

³ Geometric mean and 95% CI for log transformed data calculated as e^x .

⁴ For the interaction T x HYK, the least squares means (95% CI) were 27.4 (24.2-31.1), 31.1 (27.2-35.6), and 26.7 (23.1-30.8) min for the controlled, intermediate and high group without HYK, and 27.3 (22.0-33.9), 28.3 (23.6-33.9), and 37.9 (32.1-44.7) min for the same groups in case of HYK, respectively

^{a,b,c} Row means with different superscript letters differ (Tukey posthoc test $P < 0.10$)

Figure 4.1. Mean plasma concentration of glucose (mg/dL) during intravenous glucose tolerance test

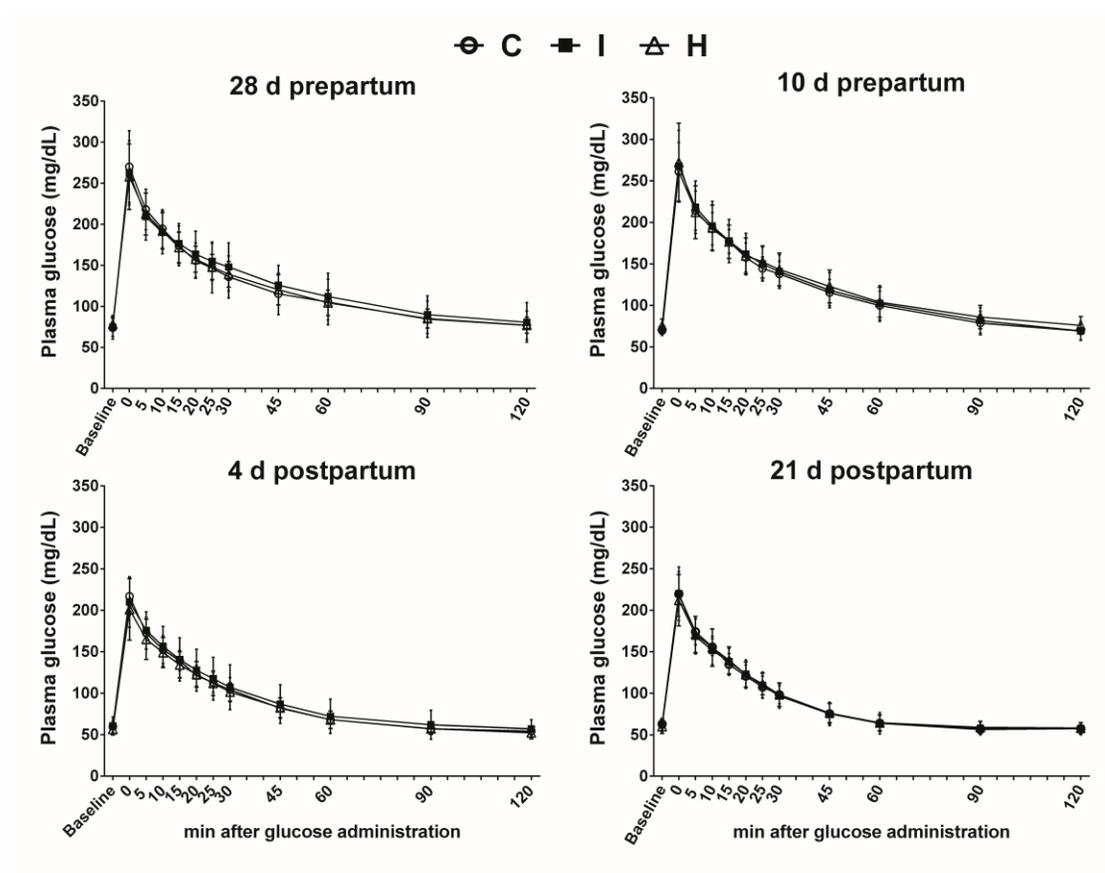


Figure 4.1. Continued

Mean plasma concentration of glucose (mg/dL) during intravenous glucose tolerance test (IVGTT) performed at 28 and 10 d prepartum, as well as 4 and 21 d postpartum. Error bars represent SD. Least squares means (95% CI) of area under the curve for time-points 28 and 10 d prepartum as well as 4 and 21 d postpartum were 5.7 (5.2-6.1), 5.9 (5.4-6.4), 3.7 (3.2-4.1), and 3.4 (2.9-3.8) g*120 min/dL, respectively. Baseline represents the average of samples taken 15 and 5 min before glucose infusion. Time-point 0 represents sample taken immediately after intravenous bolus administration of 0.25 g glucose per kg BW. C= Controlled energy prepartum diet, fed for ad libitum intake to control intake to 100% of energy requirements; I= intermediate energy prepartum diet, fed for ad libitum intake to control intake to 100% of energy requirements from 57 to 29 d before expected parturition and approximately 125% of energy requirements from 28 d before expected parturition until calving; H = high energy prepartum diet, fed for ad libitum intake to achieve energy intake at approximately 150% of requirements. Values based on 28 animals in each group.

Figure 4.2. Mean plasma concentration of insulin ($\mu\text{U}/\text{mL}$) during intravenous glucose tolerance test

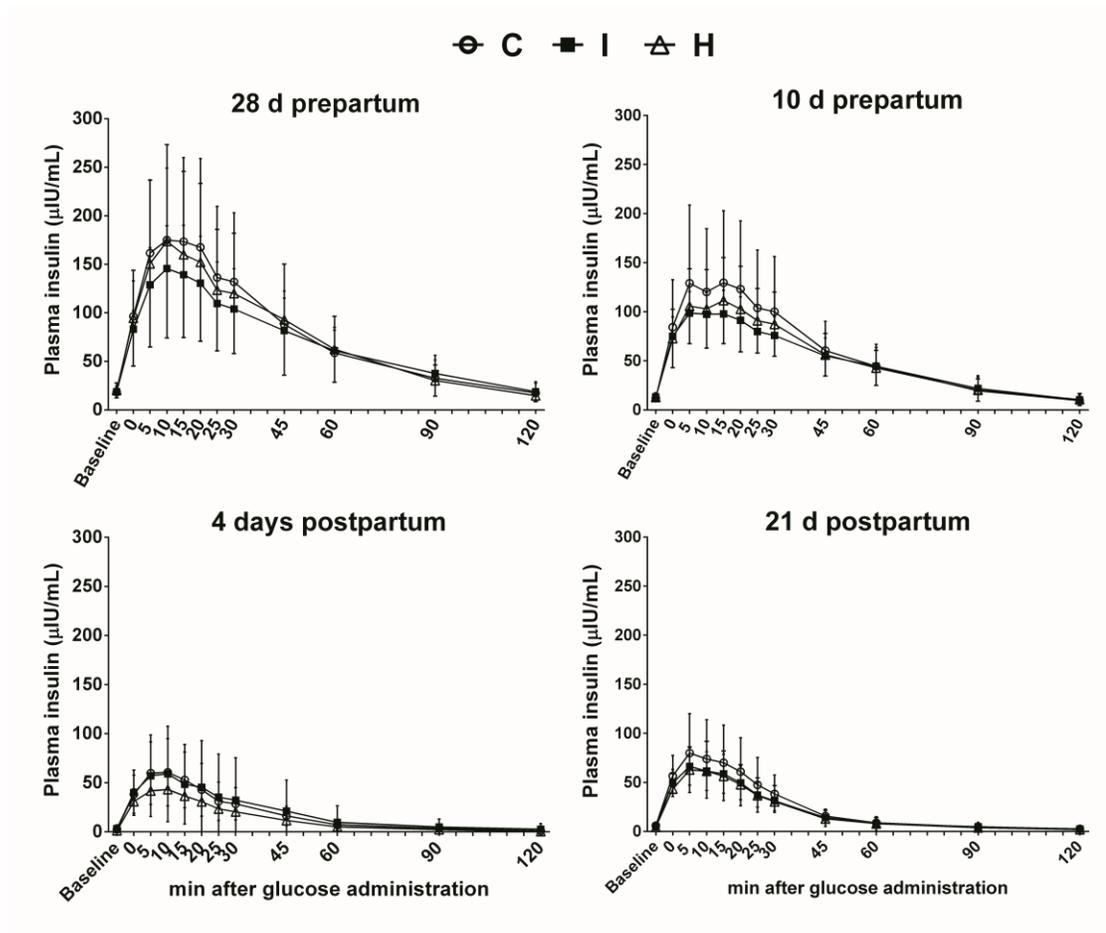


Figure 4.2. Continued

Mean plasma concentration of insulin ($\mu\text{U}/\text{mL}$) during intravenous glucose tolerance test (IVGTT) performed at 28 and 10 d prepartum as well as 4 and 21 d postpartum. Error bars represent SD. Least squares means (95% CI) of area under the curve for time-points 28 and 10 d prepartum as well as 4 and 21 d postpartum were 5.7 (4.7-6.8), 4.2 (3.5-5.0), 1.4 (1.2-1.7), and 1.7 (1.4-2.0) $\text{mU} \cdot 120 \text{ min}/\text{mL}$, respectively. Baseline represents the average of samples taken 15 and 5 min before glucose infusion. Time-point 0 represents the sample taken immediately after bolus administration of intravenous 0.25 g glucose per kg BW. C= Controlled energy prepartum diet, fed for ad libitum intake to control intake to 100% of energy requirements; I= intermediate energy prepartum diet, fed for ad libitum intake to control intake to 100% of energy requirements from 57 to 29 d before expected parturition and approximately 125% of energy requirements from 28 d before expected parturition until calving; H = high energy prepartum diet, fed for ad libitum intake to achieve energy intake at approximately 150% of requirements. Values based on 19 animals in each group.

Figure 4.3. Reduction of NEFA concentration (in % from baseline) during intravenous glucose tolerance

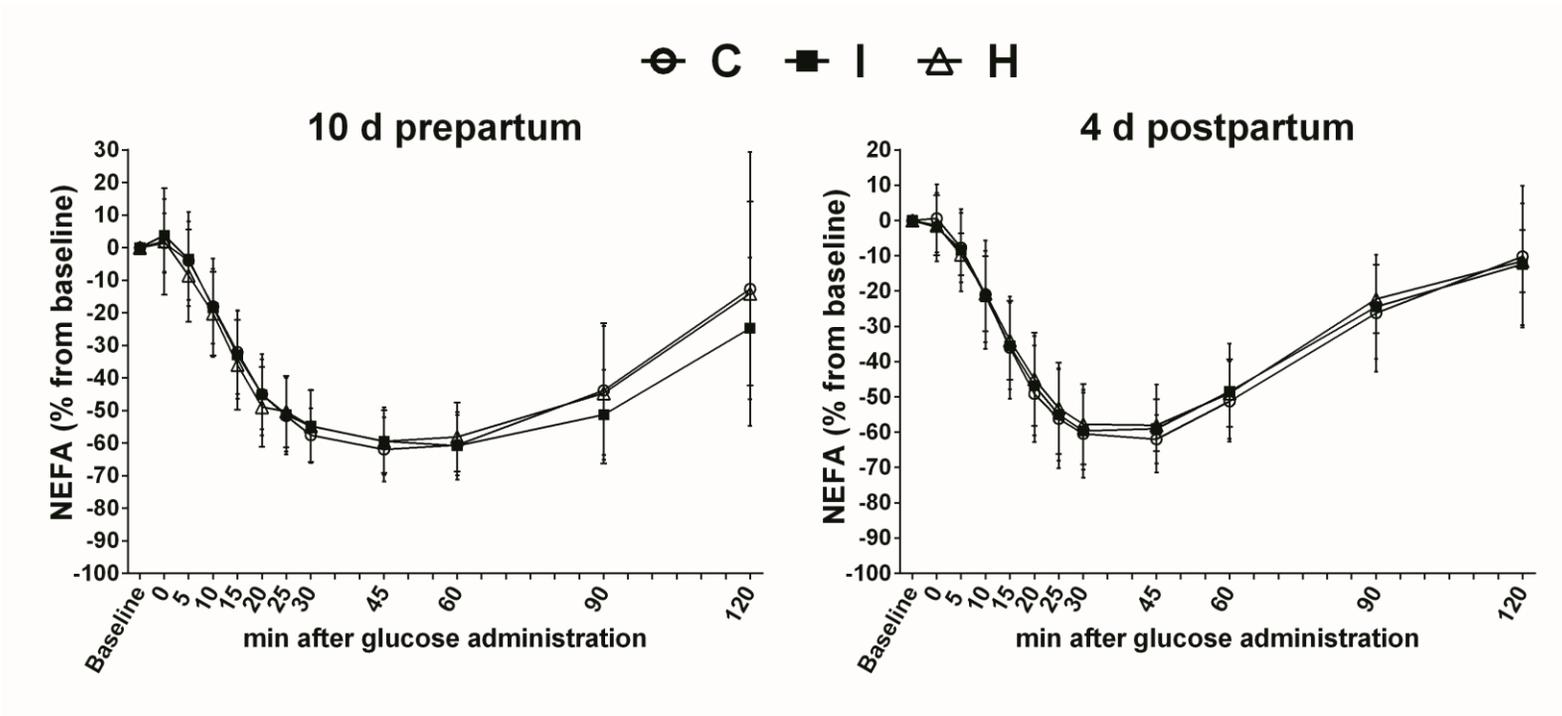


Figure 4.3. Continued

Reduction of NEFA concentration (in % from baseline) during intravenous glucose tolerance test (IVGTT) 10 d prepartum as well as 4 d postpartum. Error bars represent SD and time-point 0 the sample taken immediately after intravenous bolus administration of 0.25 g glucose per kg BW. Baseline represents the average of samples taken 15 and 5 min before glucose infusion. C= Controlled energy prepartum diet, fed for ad libitum intake to control intake to 100% of energy requirements; I= intermediate energy prepartum diet, fed for ad libitum intake to control intake to 100% of energy requirements from 57 to 29 d before expected parturition and approximately 125% of energy requirements from 28 d before expected parturition until calving; H = high energy prepartum diet, fed for ad libitum intake to achieve energy intake at approximately 150% of requirements. Values based on 19 animals in each group.

Figure 4.4. Least squares means of baseline concentrations of blood analytes

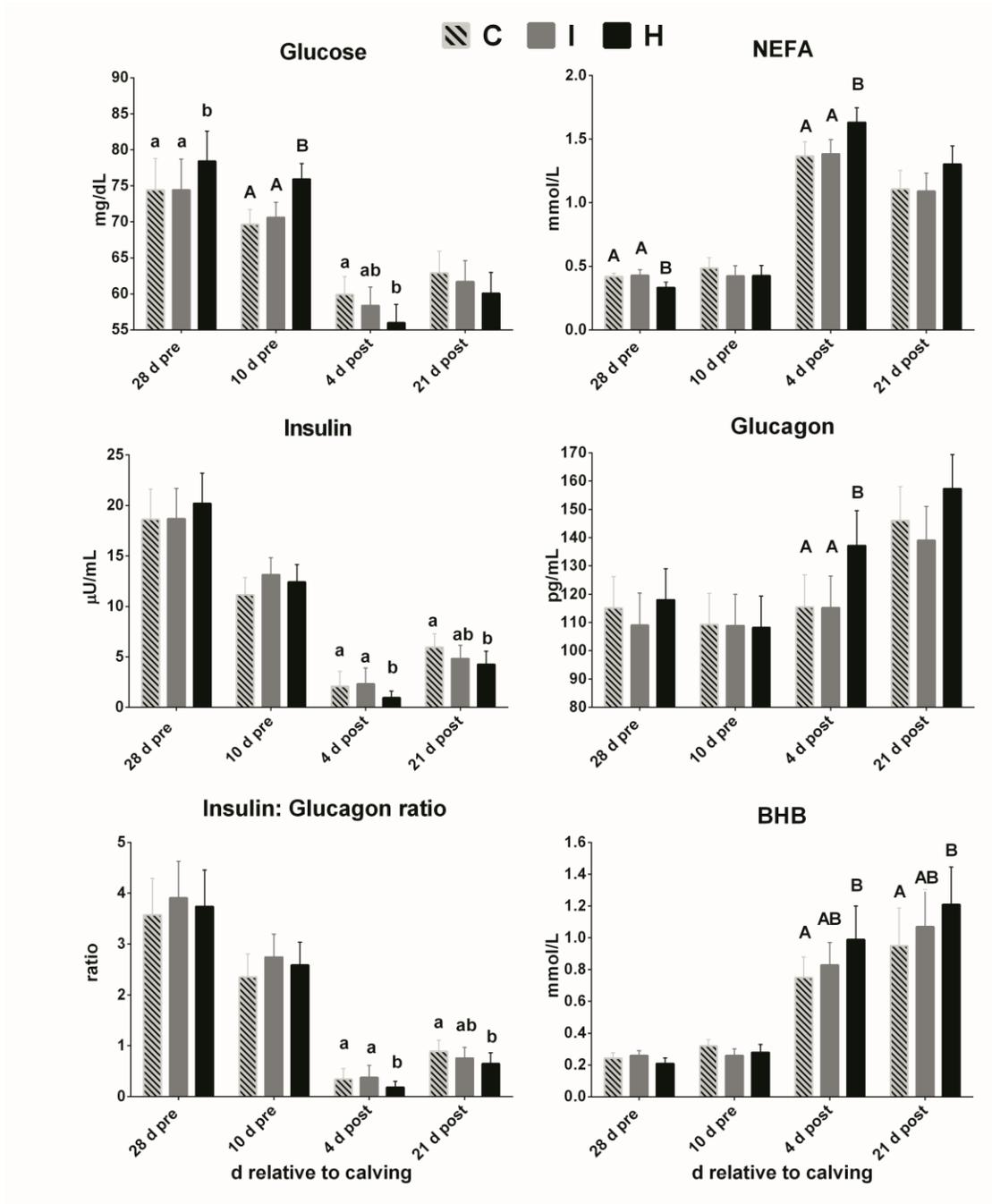


Figure 4.4. Continued

Least squares means of baseline concentration of glucose (top left), NEFA (top right), insulin (middle left), glucagon (middle right), molar insulin: glucagon ratio (bottom left) and BHB (bottom right) at 28 and 10 d before expected calving as well as 4 and 21 d after calving. Error bars represent 95% CI; enrollment block was included as a random effect. C= Controlled energy prepartum diet, fed for ad libitum intake to control intake to 100% of energy requirements; I= intermediate energy prepartum diet, fed for ad libitum intake to control intake to 100% of energy requirements from 57 to 29 d before expected parturition and approximately 125% of energy requirements from 28 d before expected parturition until calving; H = high energy prepartum diet, fed for ad libitum intake to achieve energy intake at approximately 150% of requirements. A,B Columns marked with different letters are different at a level of $P < 0.05$ in ANOVA and Tukey's posthoc test. a,b Columns marked with different letters are different at a level of $P < 0.10$ ANOVA and Tukey's posthoc test. Values based on 19 animals in each group except glucose which was based on 28 animals in each group.

Figure 4.5. Least squares means of repeated measures analysis of plasma analyse in cows with and without hyperketonemia

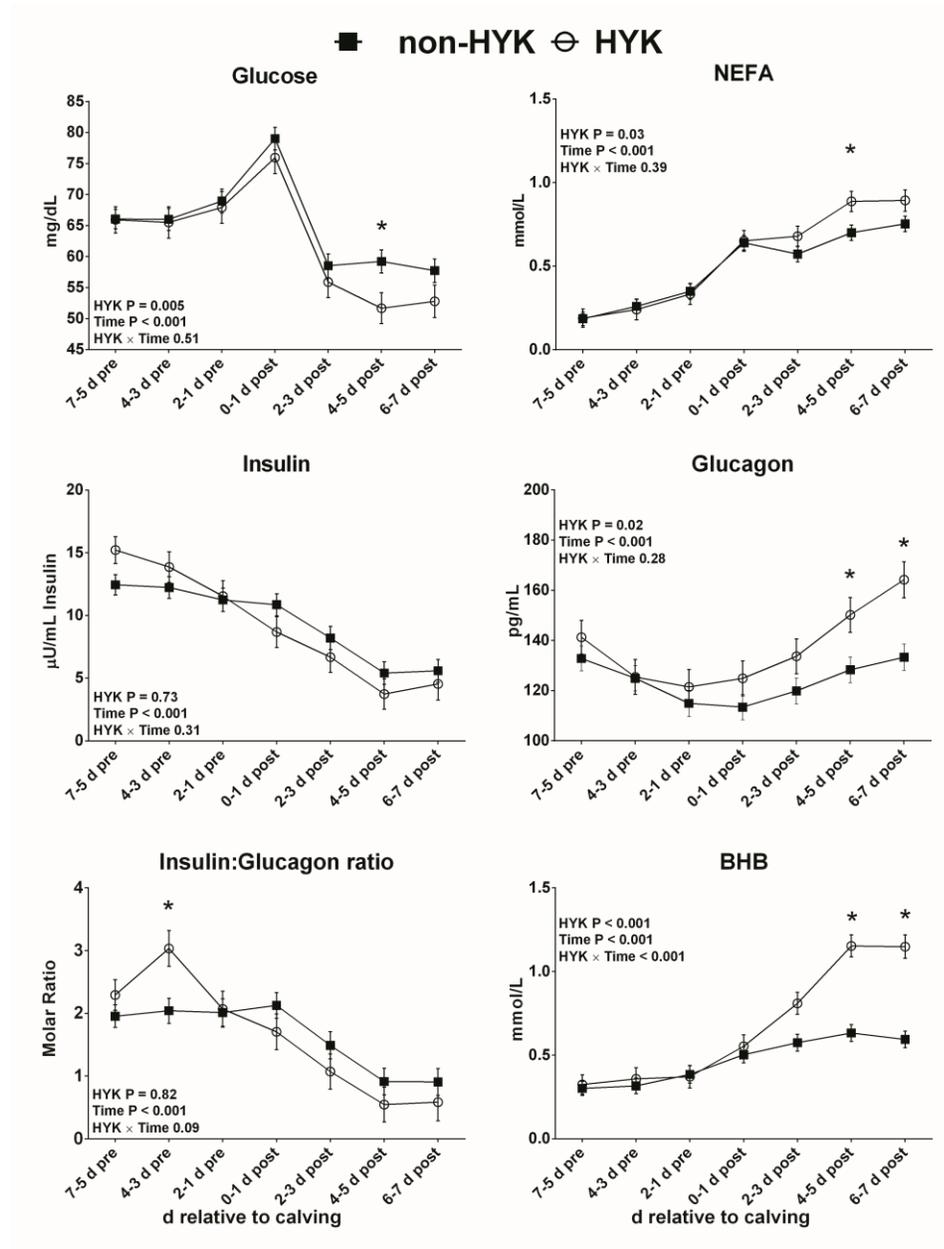
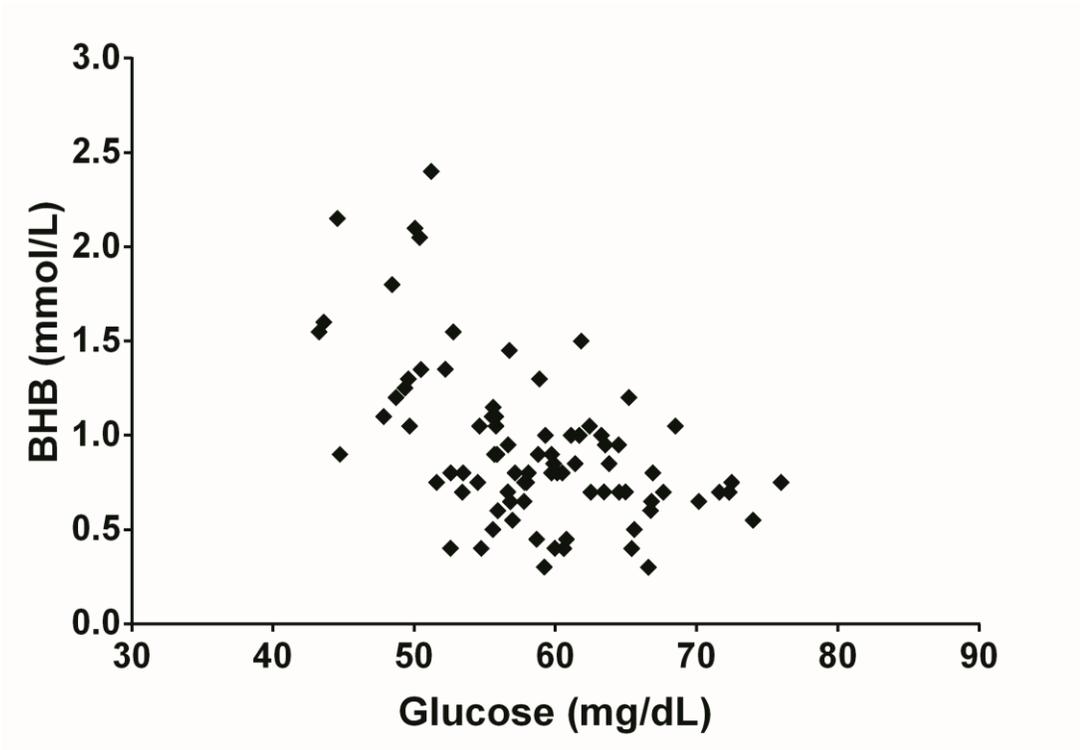


Figure 4.5. Continued

Least squares means of repeated measures analysis of plasma concentrations of glucose, NEFA, insulin, glucagon, molar insulin: glucagon ratio, as well as blood concentration of BHB from d 7 prepartum to d 7 postpartum for animals that became hyperketonemic (HYK; BHB \geq 1.2 mmol/L) or did not in the first 21 DIM. Error bars represent SE. Group differences at a level of $P < 0.05$ are marked by *. Values based on 20 animals in the hyperketonemic, and 37 animals in the non-hyperketonemic group. P -values for fixed effects of HYK, time and HYK x time interaction are given in the figure.

Figure 4.6. Scatterplot of BHB and glucose concentrations on d 4 postpartum



CONCLUSIONS

Overfeeding cows during the dry period was not associated with differences in glucose tolerance as assessed by IVGTT in this study, but feeding a high energy dry period diet did lead to differences in resting pre- and postpartum concentrations of glucose, as well as postpartum concentrations of insulin, NEFA, BHB and glucagon. The usefulness of IR indices in dairy cows is questionable and values obtained in different stages of lactation and pregnancy should not be compared with each other.

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CHAPTER 5

EFFECT OF DRY PERIOD DIETARY ENERGY LEVEL IN DAIRY CATTLE ON VOLUME, CONCENTRATION OF IMMUNOGLOBULIN G, INSULIN AND FATTY ACID COMPOSITION OF COLOSTRUM

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ABSTRACT

The objective was to investigate the effect of different dry cow feeding strategies on the volume, concentration of IgG and insulin, as well as fatty acid (FA) composition of colostrum. Our hypothesis was that different dry period diets formulated to resemble current feeding practices on commercial dairy farms and differing in plane of energy would have an effect on IgG and insulin concentration, as well as composition of FA of colostrum. Animals (n=84) entering parity 2 or greater were dried off 57 d before expected parturition and either fed a diet formulated to meet, but not greatly exceed energy requirements throughout the dry period (C), or a higher energy density diet, supplying approximately 150% of energy requirements (H). A third group received the same diet as group C from dry-off until 29 d before expected parturition. After this time-point, from 28 d before expected parturition until calving, they received a diet formulated to supply approximately 125% of energy requirements (I). Concentration of IgG and insulin in colostrum were measured by radial immunodiffusion and RIA, respectively. Composition of FA was determined by gas-liquid chromatography. IgG concentration was highest in colostrum of cows in group C (96.1 [95% CI: 83.3-108.9] g/L) and lowest in group H (72.4 [60.3-84.5] g/L), whereas insulin concentration was highest in group H (1105 [960-1244] μ U/mL) and lowest in group C (853 [700-1007] μ U/mL). Colostrum yield did not differ between treatments and was 5.9 (4.5-7.4), 7.0 (5.6-8.4), and 7.3 (5.9-8.7) kg in groups C, I and H, respectively. A multivariable linear regression model showed the effect of dietary treatment group on IgG concentration was independent of the effect of DM. Cows in groups C, I and H had an average colostrum fat % of 5.0 (4.1-5.9), 5.6 (4.8-

6.4), and 6.0 (5.2-6.8) % and an average fat yield of 289 (196-380), 406 (318-495), and 384 (295-473) g, respectively. Colostrum of cows fed to exceed predicted energy requirements during the last four weeks of the dry period (groups I and H) exhibited a higher concentration and yield of de novo FA and lower concentrations but similar yield of preformed FA compared with cows in group C.

Feeding a controlled energy diet during the dry period increased colostral IgG concentration in this study whereas overall colostrum yield was not affected. Exceeding energy requirements during the period of colostrogenesis led to higher concentrations of insulin and de novo FA in colostrum.

Key Words: dairy, colostrum, energy, insulin, fatty acids

INTRODUCTION

Quigley and Drewry (1998) stated that “the relationship between the prepartum diet and the acquisition of passive immunity merits further investigation.” Although research efforts have led to a substantial increase in knowledge on transition dairy cow management in the last decades, these studies have largely focused on health and productivity of the dam in relation to metabolic disorders. Studies on the effect of nutrition during late pregnancy in cattle on colostrum quality have concentrated primarily on feed restriction in beef cattle. These studies targeted either severe protein restriction (Blecha et al., 1981), or a combination of both protein and energy (Olson et al., 1981; Hough et al., 1990) fed below requirements. The results of such studies are poorly applicable to dairy cows because dairy rations are generally designed to provide adequate amounts of nutrients in the prepartum TMR and dairy cows face different metabolic challenges in the peripartum period.

Colostrum in dairy cattle has largely been regarded as a means of transferring passive immunity to the agammaglobulinemic newborn calf, as well as an excellent source of nutrients (Quigley and Drewry, 1998; Hammon et al., 2013). The fat component of colostrum plays an essential role in providing energy since energy reserves of the newborn are sparse and fat consumption increases the ability to thermoregulate (Quigley et al., 1994; Morrill et al., 2012). Besides these obvious functions, colostrum has a role in maturation of the neonatal gastrointestinal tract through provision of hormones and growth factors such as insulin (Shehadeh et al., 2006; Hammon et al., 2013). Previous studies have shown the systemic uptake of very high doses of insulin after oral administration exists in calves and rodents (Mosinger et

al., 1959; Pierce et al., 1964; Shehadeh et al., 2003). On the other hand, Gruetter and Blum (1991), using radio-immunologically-labeled insulin, were unable to confirm this finding at lower doses. Irrespective of a possible systemic uptake, insulin from colostrum likely plays a role as one of the components responsible for the improved oral glucose absorption in colostrum fed calves compared with those fed milk-based formula (Steinhoff-Wagner et al., 2011).

Because of the known detrimental effects of overfeeding during the dry period on peripartum metabolism (Dann et al., 2006; Janovick et al., 2011), it is of particular interest to study the effect of different dietary energy strategies on colostrum. Circulating glucose concentrations in cows overfed energy prepartum are higher than those of cows being fed a controlled or restricted energy diet (Janovick et al., 2011; Schoenberg and Overton, 2011). In humans, research has shown that colostrum of hyperglycemic mothers had lower levels of both IgA and IgG (Franca et al., 2012). Other studies in human medicine have also found a relation between maternal metabolic status and both hormone (Ley et al., 2012) as well as Ig concentration of colostrum (Morceli et al., 2011; Franca et al., 2012). Although placentation type and therefore transfer of IgG to the human fetus are different from the bovine species, these studies provide insight in possible influences of glucose metabolism on colostrum composition in mammalian species. Dietary energy level in the dry period has been shown to affect the metabolic status of dairy cows and alter blood glucose and insulin concentrations (Rukkwamsuk et al., 1999; Janovick et al., 2011). Our hypothesis was that metabolic changes in the prepartum period could lead to similar effects in the bovine species. The objective of this study was therefore to investigate

the effect of common dry period feeding strategies on colostral IgG concentration, as well as on colostrum volume, insulin concentration and fatty acid composition of colostrum.

MATERIALS AND METHODS

Animals, Feeding and Management

All procedures were evaluated and approved by the Cornell University Institutional Animal Care and Use Committee (protocol no. 2011-0016). A detailed account of study animals, feeding management and diet composition was published previously (Mann et al., 2015). Briefly, Holstein cows (n=84) housed at the Cornell Teaching and Research Center entering their second or greater lactation were included in the study and enrolled between September 2012 and April 2013. All animals were housed in individual sawdust-bedded stalls equipped with feed bins. Cows were allocated to one of three dry period dietary treatment groups following a randomized block design on the day of dry off (approximately 57 d before expected parturition). Cows were blocked by expected day of calving. The treatment groups were: those that were fed a TMR formulated to meet but not greatly exceed energy requirements at predicted ad libitum intake (C), those that received a TMR formulated to supply 150% of energy requirements (H), and an intermediate group receiving the same TMR as group C for the first 28 d after dry off and a TMR formulated to supply 125% of energy requirements from d 28 before expected calving until parturition (I). For all dry cow rations, predicted MP was formulated to be 1280 g/d. Cows were fed once daily

at 0900 h and adjusted to allow for a minimum of 5% refusals. All animals received the same fresh cow ration after calving. Ration formulation and analysis was done as previously described (Bielmann et al., 2010). In brief, diets were formulated using the Cornell Net Carbohydrate and Protein System software (CNCPS, Cornell University, version 6.1). Monthly composites of all TMRs as well as one composite sample of all forages were sent to Dairy One (Ithaca, NY) for wet chemistry analysis. Energy density of the diets was determined by entering forage analysis results and grain composition into CNCPS. Energy balance was estimated in CNCPS for each week and individual by entering the weekly average of the animal's BW, DMI and days carried calf.

Individual animal sampling

Cows calved in sawdust-bedded individual box stalls under the supervision of farm personnel. Calving ease was recorded on a scale from 1 to 5 (1 = normal/no assistance, 2 = moderate assistance, provided by farm staff, 3= moderate assistance, but veterinarian assisted as a precaution, 4 = difficult calving, with assisted extraction, 5 = very difficult calving, with maximum veterinary assistance) by trained personnel. Calf birth weight (platform scale, Salter Scales, Fairfield, NJ) was determined prior to colostrum feeding. Calves were removed from their dams within 30 min after birth and not allowed to suckle their dams. Cows were milked within 60 min after calving with a bucket milker and colostrum weight was determined on a commercial scale (Pelouze Scale Co, Bridgeview, IL, model 4010). The composite sample was mixed

before, and aliquots either refrigerated at 4°C for IgG analysis, or frozen at -20°C for further analysis of insulin, FA, fat% and DM.

Blood samples were obtained three times weekly starting 21 days before expected parturition as described in detail in Mann et al. (2015). In brief, blood samples (10 mL) were taken before feeding (between 0600 and 0730 h) from the coccygeal vein or artery, placed on ice, separated within one h by centrifugation at 2,800 x g for 20 min at 4°C, snap-frozen in liquid N₂ and stored at -20°C. All samples were frozen in four aliquots to prevent multiple freeze thaw cycles.

Sample analysis

The refrigerated colostrum sample was submitted within 48 h to the New York State Animal Health Diagnostic Center (AHDC) at Cornell University. IgG concentration was measured via radial immunodiffusion (RID) according to standard procedures. In brief, colostrum samples were diluted with saline and set up on bovine IgG Veterinary Medical Research and Development (VMRD, Pullman, WA) plates. Serial dilutions of 1:2, 1:4, 1:8 and 1:16 were made for every colostrum sample. Bovine IgG standards were used according to the manufacturer's instructions and ranged from 400 to 3200 mg/dl. Values of the sample's ring diameter were read off the standard curve giving a mg/dL value of IgG. The second aliquot was frozen at -20°C for later analysis. For colostral insulin determination, frozen colostrum samples were thawed and centrifuged (2,400 x g for 20 min) and the fat layer was removed to prepare skim milk as described by Ley et al. (2012). Insulin was measured by RIA (PI-12K Porcine Insulin RIA, EMD Millipore Corp, Billerica, MA) used for diagnostic

testing and validated for bovine serological sample analysis by the AHDC Endocrinology Laboratory. The procedure was performed according to the manufacturer instructions with the standard curve range 3.13 to 200 $\mu\text{U}/\text{mL}$. Samples were diluted 1:40 to fall within an insulin concentration that corresponded to approximately 50% of total binding (range: 37.5-71.6). Linearity of serial dilution of five samples was tested and found to have an R^2 of 0.95 in the range of 20 to 170 $\mu\text{U}/\text{mL}$. For intra-assay precision, the CV calculations were based on 3 separate pools of colostrum tested 12 times within the assay. The % CV for the 3 samples with mean values of 19.4, 67.3, and 108.1 $\mu\text{U}/\text{mL}$ was 8.1, 3.4, and 7.2%, respectively.

Plasma concentration of insulin 1, 2 or 3 days before parturition was available for a subset of 56 animals using the same RIA kit as was used for colostrum samples. The intra- and interassay CV for determination of insulin concentration in plasma was 6.9 and 5.7%, respectively.

One aliquot of the stored colostrum was split for fat % and DM measurements. Total fat concentration was determined at Dairy One (Dairy One Cooperative Inc., Ithaca, NY) by ether extraction (method 989.05, AOAC 1995). One more aliquot was analyzed for FA composition as previously described (Lock et al., 2013). In brief, following extraction and methylation of lipids, GLC was used to determine the composition of FA methyl esters. Yield of individual FA in milk fat were calculated by correcting for glycerol content, and other milk lipid classes according to Piantoni et al. (2013). To describe the change of the concentration of major FA components from colostrum samples to samples obtained at wk 2 and 4 of lactation, data of FA

composition previously described for these two time-points in the same animals were used (Mann et al., 2015).

Dry matter of whole colostrum was determined according to the method described by Hooi et al. (2004) for the determination of moisture in milk. In brief, colostrum samples were thawed and warmed to 37°C in a water bath. Samples were mixed by inversion. Approximately 2.5 g of sample were weighed into dried aluminum dishes (Fisher Scientific, Waltham, PA). All samples were run in duplicate. Samples were dried at 100°C in a gravity convection oven (VWR, Radnor, PA, model 1305U). Duplicates were placed on different shelves within the oven to minimize influence of placement during drying. Samples were removed from the oven 20 h later, allowed to cool to room temperature and weighed again. Moisture was calculated as the difference between initial weight and weight after oven drying (Hooi et al., 2004).

Analytical approach

Because few animals entered parity 4 and 5, parity was dichotomized into parity 2 and ≥ 3 . A variable was created to dichotomize IgG concentration in either $< 100\text{g/L}$ or $\geq 100\text{g/L}$. Chi-square tests were generated with PROC FREQ in SAS (SAS 9.3, SAS Institute Inc., Cary, NC) for treatment differences in the dependent variables IgG $<$ or $\geq 100\text{g/L}$, calving scores, parity, and calf sex. Mixed effects ANOVA were carried out with PROC MIXED for treatment differences in the dependent variables energy balance prepartum, fat %, days dry, IgG, and insulin concentration as well as colostrum weight and FA composition including enrollment block as a random effect.

The assumption of equal variances was tested with Levene's test and Welch's ANOVA was performed in cases where the assumption was not met. Multiple comparisons were adjusted with Tukey's posthoc test. Because IgG concentration could be a function of the dry matter content of colostrum, a regression model with IgG concentration as the outcome and group as well as dry matter of colostrum as predictor variables was built (PROC MIXED). Two-way interactions between the predictor variables were investigated to determine if a possible group effect depended on dry matter of the colostrum. Enrollment block was included as a random effect. Residual analysis was performed and satisfied the assumptions of homoscedasticity and normality. Presence of highly influential observations was determined when Cook's $D > 0.5$. Repeated measures ANOVA was performed for FA composition over time using PROC MIXED in SAS. Three covariance structures were tested for each variable analyzed (compound symmetry, autoregressive order 1, and unstructured). The covariance structure with the smallest Akaike's information criterion was chosen. Fixed effects were treatment group and time and enrollment block was included as random effect. The relationship between plasma insulin concentration at 1, 2, or 3 d before parturition and the concentration of insulin in colostrum was assessed with Pearson correlation. Results are presented as mean and 95% CI unless otherwise stated.

RESULTS

Description of Study Population and Diets

Three cows were excluded from the analysis. One was dry for 99 d, and two other cows had perforating teat lesions prepartum leading to milk leakage. Cows were dry for an average of 55.5 (\pm 4.4 d) (C: 56.4 [54.6-58.2], I: 55.0 [53.3-56.7], H: 55.2 [53.5-56.9]; $P = 0.46$). The majority of cows were entering parity 2 (n=51, 62.9%), and 30 animals were entering parity 3 or greater (37.1%). There was no difference in parity between treatment groups ($P = 0.50$). The average BW at enrollment was 672 (657-686) kg and the average BCS was 3.1 (3.05-3.16). Weight and BCS were not different among groups at enrollment ($P > 0.33$). On average, cows in group H gained an additional 0.27 (0.18-0.36) point in BCS score, compared with group C (0.15 [0.07-0.24], $P = 0.15$), and group I (0.11 [0.02-0.19], $P = 0.03$). Weight gain in the dry period was also highest in group H (90.4 [82.7-98.1] kg), compared with group C (72.1 [64.3-79.8] kg, $P = 0.004$) and group I (71.0 [63.3-78.8] kg, $P = 0.002$). The majority of calving ease scores in all cows were score 1 and 2 (C: 23/25 (92.0%), I: 24/28 (85.7 %), H: 27/28 (96.4%)) and the distribution of all calving ease scores was not different among groups ($P = 0.62$). One animal in group I had twins. The mean weight of newborn calves was 44.2 ± 0.5 kg. Weight distribution by treatment group was 45.6 (44.0-47.3), 43.6 (42.0-45.1) and 43.2 (41.7-44.7) kg for C, I and H groups, respectively ($P = 0.07$). There were 17, 14, and 13 male and 11, 15, and 15 female calves in groups C, I, and H, respectively ($P = 0.49$). The analyzed composition of prepartum diets is summarized in Table 5.1. A detailed description of diet analysis and

results has previously been described (Mann et al., 2015). Weekly energy balance estimates for the last 4 wk prepartum based on weekly averages of daily DMI, weekly body weight and days carried calf is presented in Table 5.2. Predicted MP supply, expressed as a percentage of CNCPS predicted requirements, was 118, 123 and 120 % in the C, I and H group during the last four weeks of gestation, respectively.

Results of Colostrum Analysis

Differences in IgG concentration, insulin concentration, colostrum yield, dry matter and fat % and yield are summarized in Table 5.3. Cows in group C had the highest concentration of IgG and cows in group H the lowest concentration (95.5 [82.7-108.2] vs. 72.4 [60.6-84.1] g/L, $P = 0.03$). Insulin concentration was highest in group H (1105 [965-1244] $\mu\text{U}/\text{mL}$) and lowest in group C (882 [730-1033] $\mu\text{U}/\text{mL}$, $P = 0.09$). Values for both IgG concentration (88.2 [76.2-100.2] 6.1 g/L) as well as insulin (1046 [903-1189] $\mu\text{U}/\text{mL}$) for group I were in between those of the other groups. The correlation between insulin concentrations in plasma on d 1, 2, or 3 before parturition and concentrations in colostrum was $\rho = 0.54$ ($P < 0.0001$, Figure 5.1). When regarding IgG concentration as either below or above 100 g/L, 40.0, 21.4 and 10.7% of cows in group C, I and H had a colostral IgG concentration above 100 g/L, respectively ($P = 0.04$). The results of the regression analysis are shown in Table 5.4. There was no interaction between group and dry matter for the outcome of IgG concentration ($P = 0.44$), therefore, the effect of group was independent of the effect of dry matter. For every increase in one percent of DM, the model showed an increase in concentration of IgG by 2.9 g/L.

Fatty acid concentration and yield of colostrum samples are summarized in Table 5.5 and 5.6. Feeding a controlled energy diet during the dry period increased the concentration, but not yield, of preformed FA whereas the concentration of de novo FA was lower in comparison with cows that received a higher energy diet during the whole dry period (group H) or during the last four weeks of the dry period alone (group I), respectively. This difference was caused by an increase in C10:0, C12:0 and C14:0 FA in groups I and H in comparison with group C. The overall yield of de novo FA in colostrum was approximately 50% higher in both groups I and H compared with group C (Table 5.6).

Figure 5.2 shows the results of repeated measures analysis of preformed, mixed and de novo FA concentration in colostrum samples as well as samples in wk 2 and 4 of lactation. The concentration of preformed FA increased and the concentration of mixed FA decreased in all three groups in wk 2 and 4. The concentration of de novo FA in group C remained relatively stable, whereas the concentration of these FA dropped in groups I and H, especially in wk 2.

Table 5.1. Analyzed composition of diets

Component ¹	Treatment group			
	Controlled	Intermediate	High	Fresh
DM % ²	56.4 ± 3.8	49.8 ± 3.1	44.7 ± 3.9	46.8 ± 3.1
MP g/d ³	1490 (1272-1738)	1520 (1241-1808)	1520 (1223-1774)	2650 (1673-3009)
CP % of DM	14.2 ± 1.6	13.9 ± 0.7	12.5 ± 0.9	17.8 ± 0.9
NDF % of DM	48.4 ± 5.0	42.2 ± 4.5	41.0 ± 4.2	35.4 ± 2.3
ADF % of DM	30.1 ± 4.2	28.5 ± 3.2	26.55 ± 3.3	21.3 ± 4.4
Starch % of DM	15.0 ± 2.5	20.1 ± 3.6	23.7 ± 2.6	21.2 ± 2.3
Fat % of DM	2.7 ± 0.2	2.9 ± 0.2	3.1 ± 0.1	4.1 ± 0.3

¹Chemical composition is presented as average of eleven monthly composites ± SD (except DM).

²Average ± SD of 36 weekly DM measurements.

³Prediction of MP supply for average DMI (range given for ± SD in DMI) in each treatment group for the last seven weeks before calving and for overall average intakes postpartum. (CNCPS v.6.1).

Table 5.2. Energy balance in percent of requirement as estimated by the Cornell Net Carbohydrate and Protein System (CNCPS, v. 6.1) for each week in the last month prepartum. Enrollment block included as random effect, data presented as LSM (95% CI).

Week prepartum	Treatment group ¹			<i>P</i>
	Controlled	Intermediate	High	
4	115 (108-122) ^a	128 (121-135) ^b	156 (149-163) ^c	<0.0001
3	107 (102-113) ^a	133 (127-139) ^b	147 (141-153) ^c	<0.0001
2	102 (96-107) ^a	126 (120-131) ^b	135 (129-140) ^b	<0.0001
1	91 (86-97) ^a	113 (108-119) ^b	123 (117-128) ^b	<0.0001

^{a,b,c} Means in the same row with different superscripts differ ($P < 0.05$).

¹Cows were fed a TMR ad libitum to supply approximately 100, 125 and 150% of energy requirements in the controlled, intermediate and high energy diet, respectively during the last four weeks before expected calving.

Table 5.3. Effect of treatment group for the outcomes colostral IgG and insulin concentration, fat concentration and yield, as well as colostrum yield and DM. Data presented as LSM (95% CI), enrollment block included as random effect.

Measure-ment	Treatment group ¹			Overall mean	<i>P</i>
	Controlled	Intermediate	High		
IgG (g/L)	96.1 ^a (83.3-108.9)	88.2 ^{ab} (76.2-100.2)	72.4 ^b (60.3-84.5)	83.9 (76.6-91.1)	0.02
Insulin (μU/mL)	853 ^a (700-1007)	1054 ^{ab} (907-1202)	1105 ^b (960-1250)	1010 (924-1096)	0.04
Fat (%)	5.01 (4.12-5.90)	5.61 (4.77-6.41)	5.96 (5.15-6.78) ²	5.55 (5.07-6.01)	0.25
Fat yield (g)	289 (196-380)	406 (318-495)	384 (295-473) ²	393 (307-412)	0.08
Yield (kg)	5.94 (4.46-7.42)	7.00 (5.6-8.4)	7.27 (5.87-8.67)	6.77 (5.95-7.59)	0.40
DM (%)	26.52 (24.94-28.10)	25.84 (24.29-27.34)	25.73 (24.23-27.22)	26.01 (25.13-26.89)	0.74

^{a,b,c} Means in rows with different superscripts differ ($P < 0.05$) (Tukey's test).

¹Cows were fed a TMR ad libitum to supply approximately 100, 125 and 150% of energy requirements in the controlled, intermediate and high energy diet, respectively during the last four weeks before expected calving.

²One animal removed due high concentration and yield of fat leading to extreme observation (Cook's D > 0.5).

Table 5.4. Effect of treatment group and colostrum dry matter on the outcome IgG concentration (g/L) based on a multivariable linear regression model, enrollment block included as random effect.

Explanatory variable	Group ¹	LS Mean (95% CI)	β (95% CI)	<i>P</i>
Group	Overall	-	-	0.05
	Controlled	93.4 (80.9 to 105.6)	19.9 ² (2.8 to 36.8)	0.03
	Intermediate	90.0 (77.8 to 102.2)	16.5 ² (-0.29 to 33.2)	0.05
	High	73.5 (61.9 to 85.2)	Referent	-
DM (%)	-	-	2.9 ³ (1.1 to 4.7)	0.003

¹Cows were fed a TMR ad libitum to supply approximately 100, 125 and 150% of energy requirements in the controlled, intermediate and high energy diet, respectively during the last four weeks before expected calving.

²Change in IgG concentration compared with group H (=referent group).

³Change in IgG concentration for every increase in one percent of DM.

Table 5.5. Colostrum FA concentration (g/100g FA) of cows fed different dry period treatment diets (n=80).

FA concentration (g/100 g)	Treatment ¹			SEM	P
	C (n=25)	I (= 27)	H (n= 28)		
Summation by source ²					
<i>De novo</i>	20.6 ^a	22.6 ^b	22.4 ^b	0.38	0.001
Mixed	50.1	52.4	52.2	0.94	0.16
Preformed	29.4 ^a	25.0 ^b	25.4 ^b	1.07	0.001
Selected individual FA ³					
4:0	1.38	1.35	1.42	0.07	0.71
6:0	0.88	0.91	0.94	0.02	0.27
8:0	0.49	0.51	0.53	0.01	0.15
10:0	1.37 ^a	1.52 ^b	1.51 ^{ab}	0.04	0.03
12:0	2.43 ^a	2.73 ^b	2.67 ^{ab}	0.07	0.01
14:0	13.0 ^a	14.3 ^b	14.0 ^b	0.26	0.002
14:1 <i>cis</i> -9	1.11	1.29	1.26	0.08	0.14
16:0	46.8	49.2	49.0	0.83	0.07
16:1 <i>cis</i> -9	3.00	3.14	3.21	0.14	0.54
17:0	0.48 ^a	0.43 ^b	0.43 ^b	0.01	0.004
18:0	5.05	4.27	4.44	0.30	0.15
18:1 <i>trans</i> -4	0.007	0.006	0.006	0.0004	0.85
18:1 <i>trans</i> -5	0.006	0.006	0.006	0.0003	0.99
18:1 <i>trans</i> 6-8	0.13	0.12	0.13	0.006	0.45
18:1 <i>trans</i> -9	0.09	0.08	0.08	0.01	0.14
18:1 <i>trans</i> -10	0.14	0.12	0.11	0.007	0.12
18:1 <i>trans</i> -11	0.48 ^a	0.38 ^b	0.34 ^b	0.03	0.002
18:1 <i>cis</i> -9	16.3 ^a	13.1 ^b	13.5 ^b	0.67	0.002
18:1 <i>cis</i> -11	0.55 ^a	0.47 ^b	0.48 ^b	0.02	0.002
18:1 <i>cis</i> -12	0.12 ^a	0.12 ^a	0.14 ^b	0.01	0.02
18:2 <i>cis</i> -9, <i>cis</i> -12	2.01	2.02	1.95	0.07	0.25
18:2 <i>cis</i> -9, <i>trans</i> -11	0.24 ^a	0.20 ^b	0.17 ^b	0.01	< 0.0001
18:3 <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15	0.18	0.17	0.18	0.01	0.06

Table 5.5. Footnotes

^{a,b}Main effects of treatment in the same row with different superscripts differ ($P \leq 0.05$).

¹Cows were fed a TMR ad libitum to supply approximately 100, 125 and 150% of energy requirements in the controlled, intermediate and high energy diet, respectively during the last four weeks before expected calving.

²*De novo* FA originate from de novo synthesis in the mammary gland (<16 carbons), preformed FA originate from extraction from circulating plasma FA (>16 carbons), and mixed FA originate from both sources (C16:0 + *cis*-9 C16:1).

³A total of approximately 70 individual FA were quantified and used for calculations (summation by yield). Only select FA are reported.

Table 5.6. Colostrum FA yield of cows fed different dry period treatment diets (n=80).

Colostrum FA yield (g)	Treatment ¹			SEM	P
	C (n=25)	I (= 27)	H (n= 27) ²		
Summation by source ³					
<i>De novo</i>	55.5 ^a	86.4 ^b	80.2 ^{ab}	9.90	0.03
Mixed	132	193	187	21.8	0.04
Preformed	79.8	95.2	91.2	12.3	0.58
Selected individual FA ⁴					
4:0	3.79	5.14	5.14	0.68	0.19
6:0	2.37	3.44	3.35	0.41	0.08
8:0	1.31	1.95	1.87	0.23	0.06
10:0	3.68 ^a	5.78 ^b	5.37 ^{ab}	0.65	0.03
12:0	6.51 ^a	10.4 ^b	9.54 ^{ab}	1.18	0.03
14:0	35.1 ^a	55.0 ^b	50.6 ^{ab}	6.40	0.04
14:1 <i>cis</i> -9	2.75 ^a	4.74 ^b	4.37 ^b	0.43	0.004
16:0	125	181	176	20.7	0.05
16:1 <i>cis</i> -9	7.87 ^a	11.1 ^{ab}	11.2 ^b	1.04	0.04
17:0	1.30	1.64	1.53	0.19	0.34
18:0	14.1	16.5	16.0	2.32	0.71
18:1 <i>trans</i> -4	0.02	0.02	0.02	0.003	0.42
18:1 <i>trans</i> -5	0.02	0.02	0.02	0.002	0.40
18:1 <i>trans</i> 6-8	0.36	0.46	0.45	0.06	0.39
18:1 <i>trans</i> -9	0.24	0.29	0.29	0.03	0.48
18:1 <i>trans</i> -10	0.36	0.45	0.40	0.03	0.55
18:1 <i>trans</i> -11	1.30	1.44	1.21	0.20	0.70
18:1 <i>cis</i> -9	43.5	49.8	48.5	6.71	0.72
18:1 <i>cis</i> -11	1.50	1.75	1.72	0.22	0.61
18:1 <i>cis</i> -12	0.33	0.44	0.48	0.06	0.11
18:2 <i>cis</i> -9, <i>cis</i> -12	5.46	7.78	6.80	0.52	0.14
18:2 <i>cis</i> -9, <i>trans</i> -11	0.64	0.74	0.61	0.09	0.51
18:3 <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15	0.50	0.65	0.62	0.07	0.31

Table 5.6. Footnotes

^{a,b}Main effects of treatment in the same row with different superscripts differ ($P \leq 0.05$).

¹Cows were fed a TMR ad libitum to supply approximately 100, 125 and 150% of energy requirements in the controlled, intermediate and high energy diet, respectively during the last four weeks before expected calving.

²One animal removed due to extreme observation (Cook's $D > 0.5$).

³*De novo* FA originate from de novo synthesis in the mammary gland (<16 carbons), preformed FA originate from extraction from circulating plasma FA (>16 carbons), and mixed FA originate from both sources (C16:0 + *cis*-9 C16:1).

⁴A total of approximately 70 individual FA were quantified and used for calculations (summation by yield). Only select FA are reported.

Figure 5.1. Relationship between plasma insulin on d 1-3 parturum with colostrum insulin concentration (n =56). Pearson correlation coefficient $\rho = 0.54$, $P < 0.0001$.

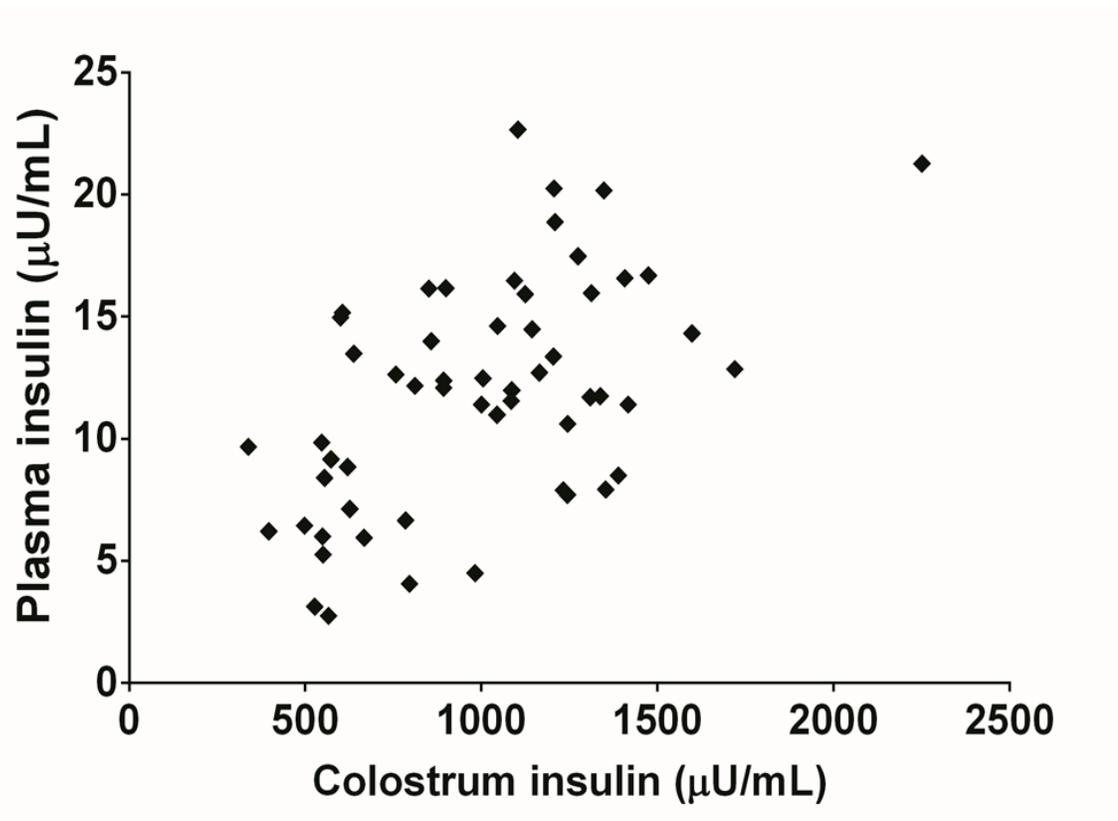


Figure 5.2. FA concentration in colostrum and milk of wk 2 and 4 postpartum.

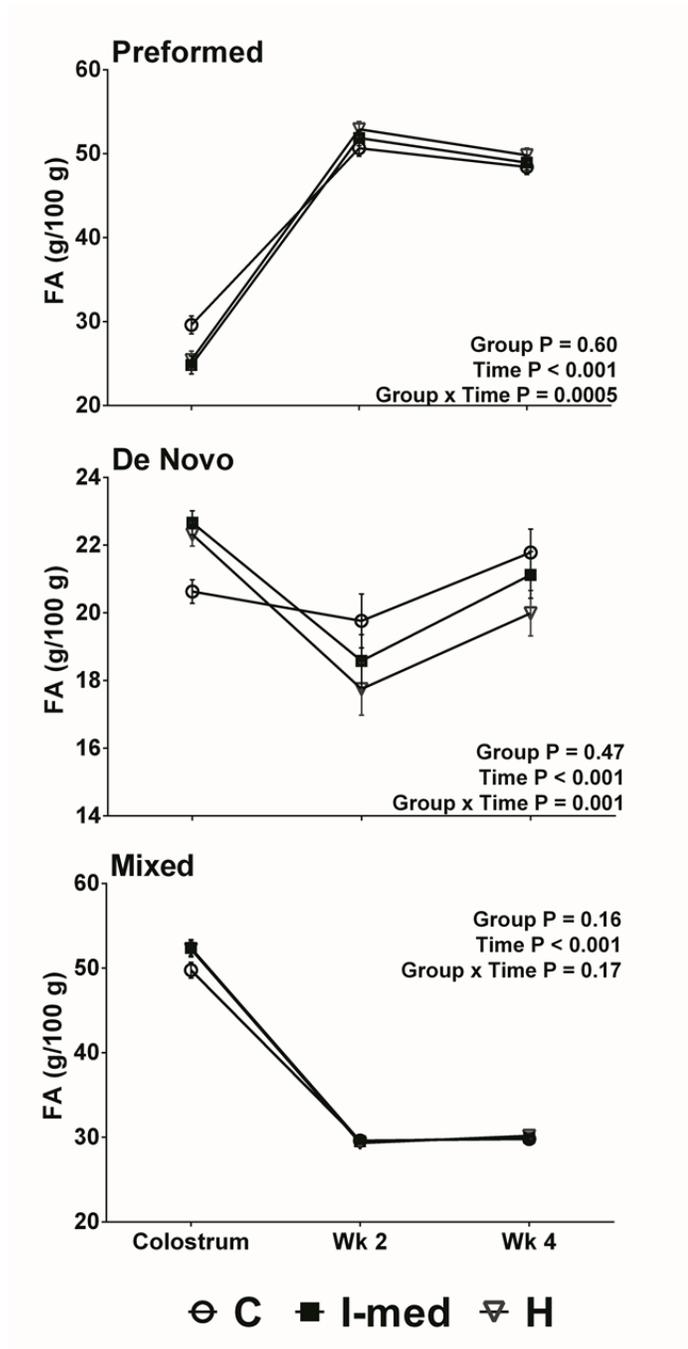


Figure 5.2. Continued

Least squares means of repeated measures ANOVA showing the sum by source of the concentration (g/100 g FA) of preformed (> 16 carbons), mixed (C16:0 + cis-9 C16:1) and de novo FA (< 16 carbons) of colostrum and milk in wk 2 and 4 postpartum. Error bars represent SE. Enrollment block included as a random effect. Cows were fed a TMR ad libitum to supply approximately 100, 125 and 150% of energy requirements in the controlled (C), intermediate (I-med) and high (H) energy diet, respectively during the last four weeks before expected calving.

DISCUSSION

Differences in IgG Concentration

In this study, cows fed a controlled energy diet during the dry period had a higher concentration of IgG in colostrum compared with cows fed higher energy density diets. Colostral Ig are derived from those circulating in plasma and are actively taken up by the mammary gland through binding to the neonatal FcRn receptors (Zhang et al., 2009). Lower concentrations in colostrum could therefore be caused by a decreased circulating level in the blood of the dam, a change in transfer capability, a difference in the rate of water inclusion or any combination of these as discussed in more detail below.

Jennbacken et al. (2013) describe that high concentrations of glucose caused a reduction in secretion of Ig from B-1 lymphocytes in an in vitro cell culture experiment. Lower IgG concentrations in colostrum or milk of prepartal hyperglycemic mothers have recently been reported (Morceli et al., 2011; Franca et al., 2012). Smilowitz et al. (2013) hypothesized that this difference is due to an impairment of B-cell function or a difference in circulating prolactin, which has a lower concentration in blood samples from women with mild hyperglycemia due to insulin-dependent diabetes (Botta et al., 1984). Several authors have reported higher blood glucose concentrations prepartum in cows overfed in the dry period (Janovick et al., 2011; Graugnard et al., 2012). To our knowledge, the biological significance of this difference and potential effects on B cell activity have not been investigated in the bovine species to date. Furthermore we did not assess plasma concentrations of IgG or

B cell function in the dams in the present study which limits our ability to evaluate differences in circulating blood concentrations of dams as a possible source of differences in colostral IgG concentration.

Human B lymphocytes are equipped with prolactin receptors and prolactin may play a role in regulating the immune response according to research carried out by Russell et al. (1985). Prolactin concentrations in study animals and the possible effects on B-cell function were not assessed in the present study and data on a possible dietary influence on prolactin concentration or onset of increased concentrations in dairy cows is lacking.

Colostral IgG concentration varies widely between individual animals (Kehoe et al., 2007; Morrill et al., 2012) suggesting differences in the total mass transferred. Moreover, individual concentration and mass differences of IgG₁ within quarters of individual cows exist (Baumrucker and Bruckmaier, 2014), further adding to the potential variability in IgG colostral mass. Concerning possible differences in transfer of Ig, Baumrucker et al. (2010) reported large differences in individual cows capacity to transfer IgG₁ that could be attributable to endocrine or genetic differences. Differences in rate of transcytosis via the FcRn receptor and individual variability in the onset of colostrogenesis may play a role (Baumrucker and Bruckmaier, 2014). We are currently unaware of a potential mechanism influencing IgG transfer capabilities in cows fed different planes of energy during the dry period.

In a study including over 900 colostrum samples, Pritchett et al. (1991) described that weight of first milking colostrum was most highly correlated with IgG concentration. In contrast, Quigley et al. (1994) found the correlation between IgG

concentration and colostrum volume to be small ($r = -0.19$). We addressed this possible influence of a simple dilution both by determining the dry matter content of colostrum samples and by using a multivariable linear regression model to account for both effects at once and to assess a possible interaction. Cows in the high energy group had on average a 1.3 kg higher colostrum yield and 0.80 % lower DM compared with the controlled energy group. Group had an effect on IgG concentration that was independent of the effect of colostral dry matter and there was no interaction between yield and group as independent variables. Also, the mean increase in colostral IgG concentration per one percent increase in dry matter as predicted by the model was 2.9 g/L. The numerical difference of an average of 0.80 % DM between the controlled and high energy group would therefore theoretically only explain a total average difference of less than 2.3 g. The authors of a recent study based on 700 cows in Ireland found that each increase in one kg of colostrum yield decreased the IgG concentration by 1.7 g/L (Conneely et al., 2013) which supports the magnitude of IgG concentration difference per increase in colostrum yield that we observed.

A recent study described the absence of an effect of dry period nutrition on colostral IgG concentration (Nowak et al., 2012) in dairy cattle. In this study, NDF content of the prepartum diets was 52% of DM in the higher energy group and 56% in the lower energy group. Although there might be a lack of comparability between forage sources and methods of analysis between the cited and our own study, both values are above our target value of 46% of NDF for the controlled energy diet. Starch concentrations were not reported in the study of Nowak et al. (2012). We assume that both experimental diets in the cited experiment would classify as controlled energy

diets when compared with values of our own study. Furthermore, the concentration of CP as percentage of DM was 11.4 and 11.5%, respectively, which is below the recommended minimal target for CP of 12.0% for dry cows (Lean et al., 2013). Diet formulation in our current study successfully assured that cows were not deficient in protein during the dry period (Mann et al., 2015). Predicted MP amounts were not different among groups and were on average well above the recommended average of 1300 g of predicted MP (Ji and Dann, 2013; Van Saun and Sniffen, 2014). Predicted MP amounts were not reported in the study of Nowak et al. (2012), but could have been deficient based on CP and NDF concentrations. Average IgG concentrations measured by ELISA were below 35 g/L in both groups. This difference in overall IgG concentrations could have been due to laboratory methods, but sample handling or differences in study animals, nutrition and management could also play a role. We measured IgG with RID which is considered the gold standard for this analysis (Bielmann et al., 2010; Bartier et al., 2015) in unfrozen, refrigerated samples and is used as the reference method for validation of other tests such as by colostrometer, turbidimetric immunoassay (TIA), Brix refractometry (Quigley et al., 2013), and newer methods such as quartz crystal microbalance (QCM, Crosson and Rossi, 2013). Gelsinger et al. (2015) recently described significant differences in measurements of IgG in colostrum depending on the laboratory method. Samples measured by ELISA yielded concentrations that were approximately 50% lower compared with those measured by RID. Therefore, when comparing colostrum IgG concentration between different studies, such as between the study by Nowak et al. (2012) and our own, the laboratory method used to generate the results should be taken into account.

The absorption of Ig is linearly and positively related to concentration in colostrum (Stott and Fellah, 1983; Godden et al., 2009; Osaka et al., 2014). Although there might be a limitation to physiologic capacity of IgG absorption (Besser et al., 1985), higher concentrations of IgG in colostrum are likely always advantageous. Moreover, in a study by Hopkins and Quigley (1997) the efficiency of absorption did not change significantly as Ig intake increased. In our study, the proportion of animals with very high colostrum IgG concentration (≥ 100 g/L) was 4 times higher in animals fed the controlled energy diet compared with the high energy diet. In a recent study on US dairy farms by Morrill et al. (2007), 29.4% of all colostrum samples from 67 farms across 12 states had IgG concentrations < 50 g/L and were considered inadequate. Given the fact that colostral IgG concentration is highly variable on modern dairy farms (Conneely et al., 2013), achieving the highest possible concentration is important to optimize newborn health.

Differences in Fatty Acid Composition

The fat component in colostrum plays a major role in supplying energy to the newborn (Quigley and Drewry, 1998). Colostral fat can be derived through de novo synthesis in the mammary gland (4-16 carbons) from acetate and BHBA, from the feed and microbial activity in the rumen of the cow; and from dietary lipids and adipose tissue lipolysis (Mansson, 2008). The average colostral concentration of fat of 5.55% was lower compared with results by Kehoe et al (2007) reporting an average of 6.7% based on 55 samples from 55 farms in Pennsylvania. According to Quigley et al. (1994), the fat content of bovine colostrum is variable. Differences could have been

due to prepartum dietary composition, particularly fat content, but diet information was not available for the study by Kehoe et al. (2007). Fat content was numerically different for the three groups such that cows in both groups receiving higher energy density diets in the close-up period showed higher concentrations of fat in colostrum compared with the controlled energy group. This was also reflected in the numerically higher colostrum fat yield. Current literature suggests that overfeeding in the dry period increases expression of several genes involved in FA uptake and *de novo* lipogenesis. The expression of *LPL*, *ACACA*, *FASN* and *DGAT2* in adipose tissue was upregulated in response to overfeeding in the close-up period (Ji et al., 2012). The potential upregulation of genes involved in milk fat *de novo* synthesis could explain the observed difference in FA composition with an increased concentration and trend for higher yield of *de novo* FA in group I and H compared with group C. The greater concentration of *cis*-9 C18:1 in group C supports the greater relative contribution of FA from adipocytes (Rukkwamsuk et al., 2000) to colostrum FA in this group, whereas the total yield of preformed FA was similar in all groups. This suggests that the observed differences in the treatment groups are caused by differences in synthesis rates of *de novo* FA rather than differences in uptake of preformed FA. Garcia et al (2014) described the effect of supplementing fat during the dry period on colostrum FA profile. Cows receiving supplemental fat during the dry period had increased concentrations of C18:0, but *de novo* synthesis of short- to medium-chain FA was not affected by treatment. This is in contrast to our study where we observed differences in *de novo* FA concentration in groups overfed energy during the close-up period. This could be due to treatments differing in concentrations and composition of fat

supplements in the study by Garcia et al. (2014), but not in overall prepartum energy intake, and that differences in energy may be necessary to affect FA synthesis in the mammary gland during the dry period.

Based on a recent study where all animals were fed the same prepartum hay-based diet, milk FA composition of Holstein cattle changed over time in milk harvested starting at 24 h after parturition (Contarini et al., 2014). Lower amounts of short chain SFA and higher amounts of long chain SFA were found in milk from animals on the day of parturition compared with those samples that were obtained during the next four days, and five months later, respectively. In contrast, colostrum samples from our study exhibited a lower concentration of preformed FA, but increased concentration of de novo FA and FA from both sources (C16:0 and *cis*-9 C16:1) compared with samples obtained during wk 2 of lactation. Concentration of preformed FA increased substantially from concentrations found in colostrum. Differences could be due to energy level during the dry period as in the present study all animals were fed to either meet or exceed estimated energy requirements and would have limited mobilization of adipose tissue prepartum, whereas restriction of energy during the dry period could lead to a higher concentration of preformed FA from body fat mobilization as was previously shown (Dann et al., 2006) and which could have been the case in the study by Contarini et al (2014). However, estimation of energy balance for this study was not available.

Differences in Insulin Concentration

Increasing evidence in the current literature suggests that insulin is important in mammalian gut maturation (Buts et al., 1988; Shulman, 1990; Shehadeh et al., 2006). Malven et al. (1987) found that the ratio of colostrum: blood concentration of insulin in the immediate prepartum period was 41:1. Higher serum fasting glucose during pregnancy in women was associated with higher insulin concentrations in milk (Jovanovic-Peterson et al., 1989; Ley et al., 2012). Jovanovic-Peterson et al. (1989) also found that intravenously injected insulin appeared in milk with a lag time of 30-50 minutes. Several authors have documented a higher blood insulin concentration in cows being overfed energy prepartum (Dann et al., 2006; Janovick et al., 2011; Schoenberg and Overton, 2011) but to the best of our knowledge this is the first study describing the effect of prepartum energy on insulin concentration in colostrum. When investigating the relationship of circulating concentrations of insulin with those in colostrum, we chose to use the 1-3 d prepartum sampling time-point for plasma based on the greatest insulin uptake rate within the last few days before calving in dairy cows (Malven et al., 1987). The moderate positive correlation between plasma insulin concentration at this time and colostrum insulin concentration could be interpreted such that insulin uptake in colostrum is driven by a gradient, and that higher circulating concentrations of insulin would lead to higher concentrations in colostrum. However, the exact mechanism and control of insulin transport into the bovine mammary gland and rate of degradation during the colostrum phase require further research.

Since the aim of this study did not include follow-up of the calves and measurements of intestinal development and nutrient uptake capability, we are unsure

at this time of the biological significance of the observed difference in insulin concentration in cows being fed diets differing in energy levels during the dry period.

CONCLUSIONS

In this study feeding a dry period diet that was formulated to either meet or exceed energy requirements for Holstein affected the FA composition of colostrum, as well as colostral IgG and insulin concentrations. Cows overfed energy during the entire dry period exhibited lower colostral concentrations of IgG, and higher concentrations of insulin compared with cows fed a controlled energy diet. The composition of FA revealed that the concentration of de novo FA was increased in cows that exceeded energy intake above predicted requirements during the whole duration of the dry period or the close-up period alone compared with those fed a controlled energy diet. Prepartum dietary strategies influence the composition and quality of bovine colostrum. Further research is needed to study the possible effect of the differences in colostral components caused by different dry period feeding strategies on IgG uptake, gut maturation and metabolism of the newborn calf.

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CHAPTER 6

**INSULIN SIGNALING AND SKELETAL MUSCLE ATROPHY AND
AUTOPHAGY IN TRANSITION DAIRY COWS EITHER OVERFED
ENERGY OR FED A CONTROLLED ENERGY DIET PREPARTUM**

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ABSTRACT

During periods of negative energy balance, mobilization of muscle is a physiologic process providing energy and amino acids. This is important in transition dairy cows experiencing negative energy and protein balance postpartum. Overconsumption of energy during late pregnancy affects resting glucose and insulin concentrations peripartum and increases the risk for hyperketonemia postpartum, but the effects on muscle tissue are not fully understood. Skeletal muscle accounts for the majority of insulin-dependent glucose utilization in ruminants. Our objective was to study peripartal skeletal muscle insulin signaling as well as muscle accretion and atrophy in cows with high concentrations of BHB postpartum as a result of excess energy consumption prepartum. Skeletal muscle biopsies were obtained 28 and 10 d prepartum, as well as 4 and 21 d postpartum from 24 Holstein cows. Biopsies were taken immediately before and 60 min after intravenous glucose challenge causing endogenous release of insulin. Gene expression of IGF-1, myostatin, and atrogin-1, as well as immunoblot analysis of atrogin-1, muRF1, ubiquitinated proteins, LC3, and phosphorylation of AKT, ERK and mTORC1 substrate 4EBP1 was performed. Excess energy consumption in late pregnancy did not lead to changes in insulin-dependent molecular regulation of muscle accretion or atrophy compared with the controlled energy group. In both groups, phosphorylation of AKT and mTORC1 substrate were significantly decreased postpartum whereas proteasome activity and macroautophagy were upregulated. This study showed that in addition to the proteasome pathway of

muscle atrophy, macroautophagy is upregulated in postpartum negative energy and protein balance regardless of dietary energy strategy prepartum.

Keywords: dairy cow, negative energy balance, ketosis, muscle, insulin pathway, muscle atrophy

INTRODUCTION

Skeletal muscle atrophy during negative energy balance or starvation is associated with upregulation of multiple proteolytic pathways which culminate in ubiquitin proteasome mediated degradation of myofibrillar units (Medina et al. 1995). This process requires calpain activation and poly-ubiquitin tagging of proteins via muscle specific E3 ubiquitin ligases before targeting of the substrate to the proteasome. More recently, alternative pathways to muscle atrophy have been associated with activation of the autophagy system, a process by which cell components get degraded in lysosomes (Stipanuk 2009).

As dairy cows transition from late gestation to early lactation, nutritional demands for milk production increase substantially and lead to negative energy balance in the immediate postpartum period. At the same time, most animals exhibit a reduction in DMI which further exacerbates the negative energy and protein balance. This situation provides a unique and interesting model to study the effect of negative energy balance on skeletal muscle atrophy, particularly since the exact physiological mechanisms that contribute to loss of muscle mass in periods of energy deprivation are not fully characterized (Carbone et al. 2012). In order to provide both energy and amino acids to support milk production during this period, cows mobilize not only adipose, but also substantial amounts of muscle tissue in the weeks following calving (Bell et al. 2000). Amino acids released from muscle tissue through proteolysis can be directed towards milk protein synthesis, and towards direct oxidation, gluconeogenesis, and ketogenesis in the liver. Increased need for gluconeogenesis

postpartum may be an underlying reason for amino acid concentrations to fall immediately after parturition (Kuhla et al. 2011).

Circulating insulin concentrations are low immediately postpartum as part of the homeorhetic adaptation to lactation (Bauman and Currie 1980) and reflect the reduced glucose availability during this period. Insulin has a central role in regulating skeletal muscle hypertrophy and atrophy in all mammals via the AKT and mammalian target of rapamycin (mTORC1) signaling pathway, and through E3 ubiquitin ligases, such as atrogen-1 and muscle RING-finger protein-1 (muRF1) (Glass 2010; Sandri 2008). The central component of the insulin signaling cascade is mTORC1 which is an important nutritionally regulated signaling component, and regulates protein synthesis and mRNA translation through the translation repressor protein 4E-binding protein (4EBP1) and ribosomal protein S6 kinase (Carbone et al. 2012).

Excessive negative energy and protein balance can lead to hyperketonemia in dairy cattle postpartum expressed as high blood concentrations of β -hydroxybutyrate (BHB). Overconsumption of energy during the prepartum period is associated with an increased risk for hyperketonemia (Mann et al. 2015). However, the exact mechanisms for hyperketonemia are poorly understood and may involve changes in insulin signaling in insulin-dependent tissues such as muscle and adipose. Hyperketonemic animals have a more pronounced drop in insulin concentrations in the first weeks after calving (Kerestes et al. 2009) and could therefore experience a change in the rate and regulation of muscle atrophy and muscle specific insulin signaling compared with cows that maintain nutrient balance more adequately. Moreover, availability of amino acids in the immediate postpartum period can be decreased in dairy cows, including

leucine (Kuhla et al. 2011), an amino acid that together and independently of insulin acts as a regulator of the mTORC1 pathway in bovine tissues (Appuhamy et al. 2011). The regulation of the mTORC1 pathway during the precarious energy and protein balance in postpartum dairy cows is of particular interest. Moreover, the mTORC1 pathway in turn regulates the rate of macroautophagy, an alternative pathway to muscle mobilization that is increased in nutrient-deficient conditions in other species (Stipanuk 2009). Alternatively to the AKT pathway which accounts for most of the metabolic effects downstream of insulin receptor binding, insulin signals through the MAPK/ERK pathway (Taniguchi et al. 2006).

As described above, protein synthesis and degradation are coordinately regulated by pathways in response to mechanical stress, physical activity, availability of nutrients and growth factors that also include insulin-like growth factor I (IGF-I) and myostatin (Sandri 2008). Besides insulin, IGF-1 is an inducer of muscle hypertrophy through the AKT pathway (Glass 2003) and can act in an autocrine fashion after release from muscle tissue whereas myostatin is a negative regulator of muscle growth (Solomon and Bouloux 2006).

The objective of our study was to describe changes in insulin signaling and regulation of muscle hypertrophy and atrophy *in vivo* during the peripartal period in dairy cattle with different degrees of ketonemia postpartum as a result of different dietary planes of energy fed prepartum with a particular focus on the AKT/mTORC1 signaling pathway. Our hypothesis was that a higher degree of postpartum ketonemia is associated with the degree of muscle accretion and proteolysis as well as insulin signaling in muscle tissue.

MATERIALS AND METHODS

Animals, feeding and management

A detailed description of study animals, diets and feed analysis as well as energy balance and metabolizable protein (MP) estimation was described previously (Mann et al. 2015). In brief, animals entering second or greater parity were either fed a total mixed ration (TMR) formulated to meet, but not greatly exceed energy requirements at predicted ad libitum intake (C, n=28), or received a TMR formulated to supply 150% of energy requirements (H, n=28) beginning at dry-off approximately 57 d before expected parturition. After analysis of feed composite samples with wet chemistry analysis (Dairy One Cooperative Inc., Ithaca, NY), estimation of energy balance and MP supply as a percentage of predicted requirements was carried out with the Cornell Net Carbohydrate and Protein System software (CNCPS; Cornell University, version 6.1). Analyzed diet composition, weekly weight and DMI averages were entered, as well as days carried calf for prepartum estimates, and weekly averages of milk yield as well as milk fat, protein and lactose concentration for postpartum estimates. Animals for this study were selected from the larger study population based on the concentration of BHB over time during the first 3 weeks postpartum based on the resulting BHB area under the curve (AUC). The calculation of AUC was based on three weekly samples of whole blood for determination of the BHB concentration during the first 3 weeks postpartum (9 samples total) and following the method described by Cardoso and coauthors (2011) using SAS v. 9.3 (SAS Inc., Cary, NC).

Samples of animals with the highest total area under the curve (AUC) of BHB concentration in group H (n=12) and those with the lowest area under the curve in group C (n=12) were recruited for this study in order to achieve the largest contrast in BHB concentrations. In each of the selected groups C and H, 7 and 5 animals were entering second and third lactation, respectively.

Ultrasound measurements

At enrollment (57 d before expected parturition), on days 28 and 10 before expected parturition, and 4, 21 and 42 d after parturition, ultrasonographic measurements of longissimus dorsi muscle diameter were carried out with a modification of the method previously described by van der Drift et al. (2012). In brief, muscle diameter was measured on the right side perpendicular to the spine at the height of the 4th transverse process as the largest diameter between the muscular fascial layers at that site. The hair in the area corresponding to the exact placement of the ultrasound probe as determined during the first examination was clipped to assure repeatable probe placement. The skin surface was brushed and 70% alcohol (VetOne, Boise, ID) was applied as a coupling agent before measurements were performed using the IBEX Pro portable ultrasound (E.I. Medical Imaging, Loveland, CO) with a 8.5-MHz linear array transducer. Each measurement was performed three times, avoiding pressure as to not compress the underlying tissue, and the average of the three measurements used for analysis at each time point.

Muscle tissue samples

Biopsies of the external oblique muscle were taken 28 and 10 d before expected parturition and 4 and 21 d after calving, immediately before and 60 min after rapid intravenous infusion of glucose at a dose of 0.25 g/kg body weight (Mann et al. 2016), alternating sides. The biopsy sites were prepared by clipping the hair in the paralumbar fossa and scrubbing the skin with iodine soap (Pivodine Scrub 7.5% Iodine, VetOne, Boise, ID) and water. The area was rinsed with water and dried. The biopsy site was then aseptically prepared with iodine scrub and 70% alcohol (VetOne, Boise, ID) before local anesthesia with 10 ml of a 2% lidocaine solution (lidocaine 2% HCL, VetOne, Boise, ID) was performed following the pattern of an inverted L-block at a distance of at least 10 cm from the biopsy site. The surgical area was again scrubbed by alternating iodine scrub and alcohol as described above. A vertical incision of approximately 5 cm length was made through the skin of the paralumbar fossa approximately 20 min after the application of local anaesthesia, and the subcutaneous adipose layer was removed to expose the underlying muscle layer. After resection of the fascial layer covering the external oblique muscle, approximately 500 mg of muscle tissue were removed with forceps and scalpel and immediately snap frozen by immersion in liquid nitrogen in 2 mL cryovials (Nalgene, VWR, Radnor, PA) containing 500 µl of RNA buffer solution (RNA later, Qiagen, Hilden, Germany) for later extraction of mRNA, or in the same cryovials without addition of buffer for immunoblotting. The skin incision was closed with a single cruciate suture (metric 5, Supramid Extra II, S.Jackson Inc., Alexandria, VA). Samples were transported in liquid nitrogen to the laboratory and stored at -80°C until processing.

Preparation of mRNA and real-time quantitative reverse-transcription PCR

Approximately 50 mg of frozen muscle tissue from each sample were transferred to 500 µL of Trizol reagent (Life Technologies, Thermo Fisher Scientific, Waltham, MA), placed in the pre-chilled adapter of a tissue disruptor (TissueLyser LT, Qiagen, Hilden, Germany) and homogenized at maximum oscillation for 3 min with sterile 5 mm stainless steel beads. Total RNA was extracted and treated in-column with DNase I (Direct-Zol RNA MiniPrep, Zymo Research, Irvine, CA). The optical density 260:280 ratio was measured with a spectrophotometer (NanoVue Plus, GE Healthcare, Life Sciences, Little Chalfont, UK) for quality control of integrity and quality of RNA. For cDNA synthesis, up to 1 µg of RNA was reverse-transcribed (SuperScript III First-Strand Synthesis SuperMix, Life Technologies, Thermo Fisher Scientific, Waltham, MA) and stored at -20°C until analysis. Bovine-specific primer probe sets with exon spanning probes were purchased for the control gene and all genes of interest (*EIF3K*: Bt03226565, *FBXO32/atrogen-1*: Bt03234620, myostatin (*MSTN*): Bt03217980, *IGF-1*: Bt03252282; TaqMan Gene Expression Assays, Applied Biosystems, Thermo Fisher Scientific, Waltham, PA). Real-time quantitative reverse –transcription PCR (qRT-PCR) was performed using a 5-fold dilution of cDNA at 10% of the final reaction volume and each sample was analyzed in triplicate using a StepOne Plus system and 2x master mix (TaqMan Gene Expression Master Mix, Applied Biosystems, Thermo Fisher Scientific, Waltham, PA). The PCR protocol consisted of denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec (denaturation) and 1 min at 60°C (annealing and extension). For the calibration reference sample, aliquots of cDNA obtained from muscle tissue of a cow

at 28 d before expected parturition was used on every plate. Eukaryotic translation initiation factor 3K (*EIF3K*) was chosen as the housekeeping gene (Bonnet et al. 2013). Results were analyzed using the comparative quantification algorithms-standard curve method ($\Delta\Delta C_t$ method, StepOne Software, v 2.3). Results were expressed as relative quantity ($RQ = 2^{-\Delta\Delta C_t}$).

Protein extraction and immunoblotting

Frozen muscle tissue was homogenized under liquid nitrogen by pestle and mortar and approximately 50 mg transferred to 1 mL of ice-cold lysis buffer containing 25 mM Tris pH 7.4, 100 mM NaCl, 1 mM EDTA and 1% Triton X-100 and 1 mM PMSF with the addition of a phosphatase and protease inhibitor cocktail (Halt, Thermo Fisher Scientific, Waltham, PA), kept on ice for 20 min and centrifuged at 10,000 g and 4°C for 10 min. The supernatant was transferred and the protein concentration determined by use of the Bradford technique (Bradford 1976) using a commercially available reagent (Coomassie Protein Assay, Thermo Scientific, Rockford, IL). Lysates were adjusted to a protein concentration of 4 $\mu\text{g}/\mu\text{L}$ and western blot analysis was performed with either 10 or 14% Tris-Glycine SDS-polyacrylamide gels with loading amounts of 40 μg of protein per well or 4-20% Tris-Glycine gradient gels (Novex, Life Technologies, Thermo Fisher Scientific, Waltham, MA) for ubiquitin immunoblotting only. After wet transfer on ice to PVDF membrane (Immobilon-P, EMD Millipore, Billerica, MA) for 2 hours at constant amperage (500 mA), membranes were blocked in 5% nonfat milk and incubated with the primary antibody at 4°C overnight. Antibodies were purchased from Cell Signaling

Technology (Danvers, MA; p-ERK 1/2 [Thr202/Tyr204], AKT, p-AKT [Ser473], β -Tubulin, non-phospho 4EBP1 [Thr476], total 4EBP1, p-4EBP1 [Thr 37/46], LC3B, ubiquitin), Abcam (Cambridge, MA. muRF1), BD Biosciences (pan ERK) and ECM biosciences (Versailles, KY: atrogin-1). For antibodies without documented cross-reactivity with the bovine species, epitope homology was 100% except for total 4EBP1 which was 93% homologous. All primary antibodies were diluted 1:1,000 in TBST, except for LC3B and muRF1 which were diluted 1:2,000. After incubation with primary antibodies, membranes were washed three times with TBST and incubated for 1 hour at room temperature with the appropriate 1:2,000 dilution of anti-rabbit or anti-mouse HRP-linked secondary antibodies (both Cell Signaling Technology). Blots were again washed three times in TBST, exposed to enhanced chemiluminescent substrate (Clarity Western ECL Substrate, Biorad, Hercules, CA), imaged sequentially at 10, 20, 30, 50 and 60 sec intervals (BioSpectrum Imaging System, UVP, Upland, CA) and densitometry was performed using VisionWorks software (VisionWorks LS software, v. 8.1.2, UVP, Upland, CA).

Proteasome assay

The activity of the 20S proteasome was measured in muscle cell lysate using a commercially available assay (EMD Millipore, Billerica, MA) based on the detection of 7-amino-4-methylcoumarin (AMC) fluorescence in muscle lysate after cleavage of the substrate LLVY-AMC. A standard curve was established with AMC and a serial dilution of a 20 S positive control as well as a pooled control muscle lysate sample as internal control, were included on every plate. Muscle lysates were prepared as

described above and used at 100 µg of protein per well. All reactions were performed in duplicate. To improve detection of proteasome activity, the addition of ATP (Sigma Aldrich, St. Louis, MO) was tested at concentrations of 10-100 µM (Powell et al. 2007). The final concentration was set at 10 µM. Fluorescence data was obtained at 380 nm excitation and 460 nm emission wavelengths with a Spectra Max M3 plate reader (Molecular Devices, Sunnyvale, CA). Intra-assay coefficient of variation determined from 12 samples was 4.5%.

Statistical methods

Repeated ultrasonographic measurements of the diameter of *M. longissimus dorsi*, proteasome activity and baseline gene and protein expression before glucose infusion, as well as the change after infusion were analyzed using repeated measures ANOVA (Proc MIXED, SAS, v. 9.3, Cary, NC) with the fixed effects group, time and including a group x time interaction, and including the dry-off measurement as a covariate. Five covariance structures were tested (unstructured, autoregressive 1, variance components, compound symmetry and Toeplitz) and the one resulting in the lowest Akaike Information Criterion was chosen. The degrees of freedom were approximated with the Kenward-Roger method. Model assumptions were assessed by evaluation of homoscedasticity and normality of residuals. To satisfy this assumption, data of the proteasome activity assay, gene expression data, and immunoblot densitometry were log transformed, resulting least squares estimates were subsequently back transformed and presented as geometric mean and 95% CI. Wilcoxon rank test was performed to assess the difference of BHB AUC between the

two groups (JMP, v. 11.0, SAS Institute, Cary, NC). Differences between groups regarding milk production, weight, glucose and insulin concentrations were assessed by Student's t-test (JMP, v.11.0, SAS Institute, Cary, NC). Muscle diameter change per day between from day 10 prepartum to day 21 postpartum was estimated from the slope of a linear regression line fit through the time points -10, +4, and +21 and expressed as decrease in muscle diameter per day; differences in the obtained values between groups were subsequently analyzed by Student's t-test. Graphs were created with GraphPad Prism (v. 6.04, La Jolla, CA).

RESULTS

The actual average sampling day relative to calving was -26.6 (-25.2 to -27.9), -7.5 (-6.0 to -9.0), +5.1 (+4.6 to +5.6), and +21.2 (+20.7 to +21.6) d. By design, the area under the curve for BHB in the first 21 DIM was different for the selected cows and approximately twice as high in group H compared with group C (group H: 22.6 [17.2- 28.0] mmol/L*21d⁻¹; group C 10.4 [5.0-15.7] mmol/L*21d⁻¹, $p = 0.0003$). The average estimated energy balance for the last four weeks prepartum was higher in group H (145.4 [138.6-152.3] %) compared with group C (100.5 [93.6-107.3] %, $p < 0.001$). During the first three weeks postpartum, predicted energy balance was below requirements for both groups and did not differ between them (group H: 64.8 (59.9-69.8), group C:70.5 (65.4-75.7), $p = 0.11$). As formulated, the predicted MP balance exceeded requirements for the prepartum period in both groups and also did not differ between them (group H: 137 [132-141]; group C: 132 [128-137] % of predicted requirements, $p = 0.10$). Predicted MP requirements were not met for both groups

postpartum and estimates did not differ between groups (group H: 72.4 [67.7-77.1]; group C: 74.9 [70.2-79.7] %, $p = 0.34$). At the end of the study period (6 wk after calving), estimated MP requirements were met or exceeded for only 7 out of 24 animals (29.1%; 3 and 4 animals in group H and C, respectively).

Figure 6.1 shows the least squares means of the repeated ultrasonographic measurements of the M. longissimus dorsi diameter. Muscle diameter was different for both groups with a more rapid increase in diameter prepartum as well as a more rapid decrease postpartum in group H compared with group C. In fact, the estimated daily loss of muscle diameter was greater on average in group H (0.35 [0.27-0.44] mm) compared with the average in group C (0.19 [0.11-0.27] mm, $p = 0.008$).

Figure 6.2 depicts the gene expression for *IGF-1*, *atrogen-1* and myostatin (*MSTN*) at the four different time points. We observed no differences for gene expression measurements between the groups, but baseline gene expression of *IGF-1* and *atrogen-1* increased, whereas gene expression of myostatin decreased from prepartum to postpartum samples. The differences in gene expression after glucose infusion were not different between the groups ($p > 0.30$) and we found no difference in gene expression from baseline for IGF-1 and myostatin 60 min after glucose challenge ($p > 0.05$). A significant decrease from baseline was measured for the expression of atrogen-1 in group H at 4 d postpartum, as well as for both groups on d 21 postpartum ($p < 0.05$, data not shown). The ratio of absorbance (260 nm / 280 nm) of the RNA samples was on average 2.044 (2.026-2.061).

The proteasome activity was not different between groups, but increased from values measured at d 28 prepartum to those measured on d 10 prepartum as well as d 4

and 21 postpartum (Figure 6.3). Figure 6.4 shows densitometry results for ubiquitinated proteins from all cows at the four time-points. The total amount of ubiquitin-tagged proteins increased significantly from both prepartum values to the postpartum time-points ($p < 0.05$) with the highest amount on day 21 postpartum.

The densitometry analysis of E3-ligase protein expression (atrogen-1 and muRF-1) before and after glucose infusion (Figure 6.5) did not differ between groups ($p > 0.15$). Figure 6.6 summarizes the densitometry analysis for the ratios of pAKT: AKT, pERK: ERK, as well as non-phosphorylated 4EBP1: 4EBP1. Infusion of glucose led to a general increase in the measured ratio of pAKT: AKT, but the magnitude of this increase decreased postpartum compared with prepartum values (Time $p = 0.004$). Differences in baseline concentrations over time were noted for the phosphorylation of AKT which was significantly reduced on d 4 postpartum compared to prepartum time-points in both groups (Time $p = 0.0002$). This difference translated to an overall increase in non-phosphorylated mTORC1 substrate 4EBP1. The ratio of non-phosphorylated 4EBP1 to total 4EBP1 significantly increased for both postpartum time-points compared with values on d 28 prepartum (Time $p = 0.0002$). There was no effect on the phosphorylation status of ERK1/2 over time or 60 min after glucose infusion.

Figure 6.7 depicts the ratio of LC3 showing group differences for the ratio of LC3-II: LC3-I which was higher in group H compared with group C on d 28 and 10 prepartum, as well as d 4 postpartum. The ratio of LC3-II: LC3-I increased from prepartum to postpartum time-points in both groups (Time $p < 0.0001$) but was continuously higher in group H compared with group C, except for day 21 postpartum,

when the groups were not different. Whereas infusion of glucose led to a reduction in the ratio of LC3-II: LC3-I in both groups, this change was more pronounced in group H at all time-points (Group $p = 0.04$).

Table 6.1. Baseline characteristics of the study population. Data presented as mean/geometric mean and 95% CI

	C (n=12)	H (n=12)	<i>P</i>
Total milk production 2-49 DIM ^a (kg)	2177 (1970-2405)	2216 (2014-2437)	0.79
Weight at enrollment (kg)	666 (620-711)	671 (626-716)	0.87
Weight increase from dry-off to calving (kg)	73.5 (59.5-87.5)	93.9 (79.9-107.9)	0.04
Weight loss from 0 to 21 DIM (kg)	23.6 (6.2-41.0)	41.2 (22.3-60.2)	0.17
Glucose concentration on d of biopsy (mg/dL)			
28 d prepartum	71.9 (68.4-75.3)	76.5 (73.0-79.9)	0.06
10 d prepartum	69.8 (66.2-73.4)	74.9 (71.4-78.4)	0.05
4 d postpartum	61.1 (58.1-64.1)	53.2 (50.2-56.2)	0.001
21 d postpartum	65.0 (60.4-69.8)	56.9 (52.3-61.6)	0.02
Insulin concentration on d of biopsy (μIU/mL)			
28 d prepartum	18.3 (14.7-21.9)	17.6 (13.9-21.4)	0.80
10 d prepartum	10.2 (7.9-12.5)	11.7 (9.6-13.8)	0.31
4 d postpartum	2.0 (1.2-2.8)	0.9 (0.2-1.6)	0.05
21 d postpartum	5.9 (4.2-7.5)	3.2 (1.6-4.8)	0.02
Maximum insulin concentration after glucose challenge (μIU/mL) ^b			
28 d prepartum	186 (133-295)	152 (110-209)	0.37
10 d prepartum	137 (101-187)	113 (84-151)	0.92
4 d postpartum	55.8 (42-75)	38 (29-50)	0.06
21 d postpartum	76 (58-100)	57.8 (45-75)	0.14

^a Days in milk ^b Geometric mean

Figure 6.1. Muscle diameter of M. longissimus dorsi Muscle diameter of M. longissimus dorsi.

Least squares means (mm) of repeated measures ANOVA showing the ultrasonographic measurements of the diameter of M. longissimus dorsi at the height of the 4th transverse process. Error bars show 95% CI. Time-points with distinct letters show differences at a level of $p < 0.05$ in repeated measures ANOVA and Tukey's posthoc test . * Difference between groups at each time-point at a level of $p < 0.05$. Group P = 0.004; Time $p < 0.001$, Group x Time $p = 0.02$.

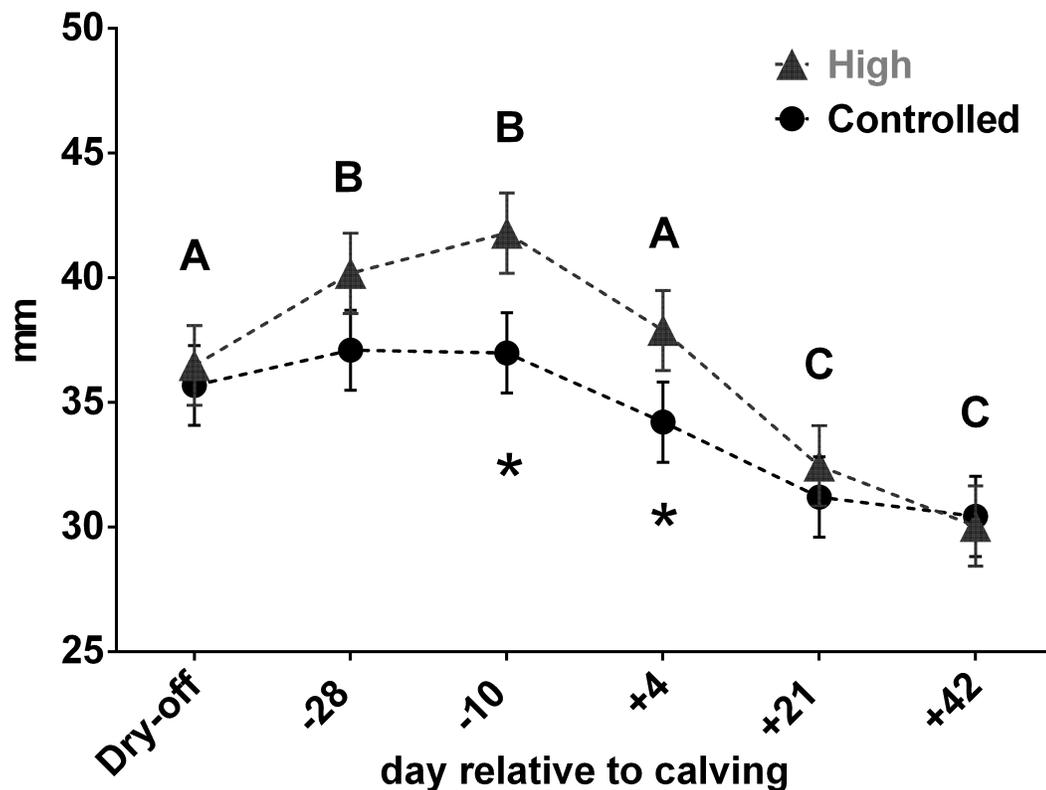


Figure 6.2. Gene expression of insulin-like growth factor (IGF-1), atrogen-1 and myostatin in external oblique muscle biopsies

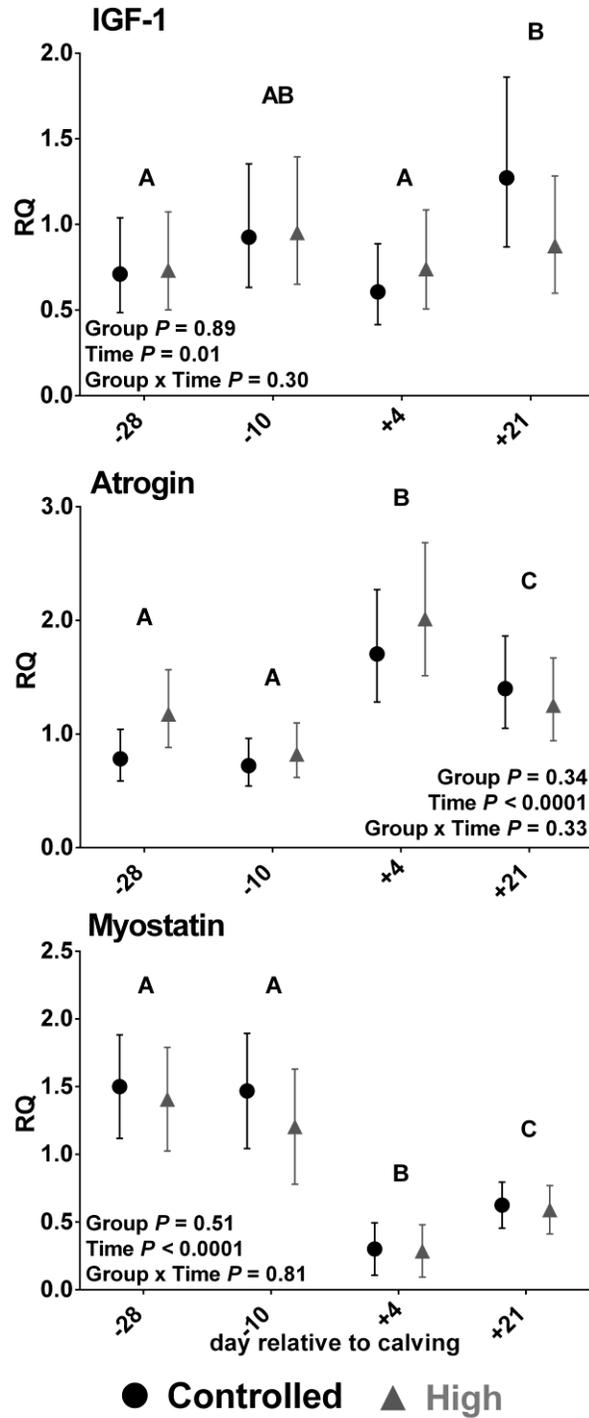


Figure 6.2. Continued

Gene expression of insulin-like growth factor (IGF-1), atrogen-1 and myostatin in external oblique muscle biopsies of peripartum dairy cows at four different time-points relative to calving. Sample days: -28 and -10 = 28 and 10 days prepartum, +4 and +21 = 4 and 21 days postpartum, respectively. Least squares means and 95% CI error bars presented. Time-points with distinct letters show differences at a level of $p < 0.05$ in repeated measures ANOVA and Tukey's posthoc test (p -values for fixed effects group, time and group x time depicted in figure).

Figure 6.3. Proteasome activity of external oblique muscle biopsy lysates

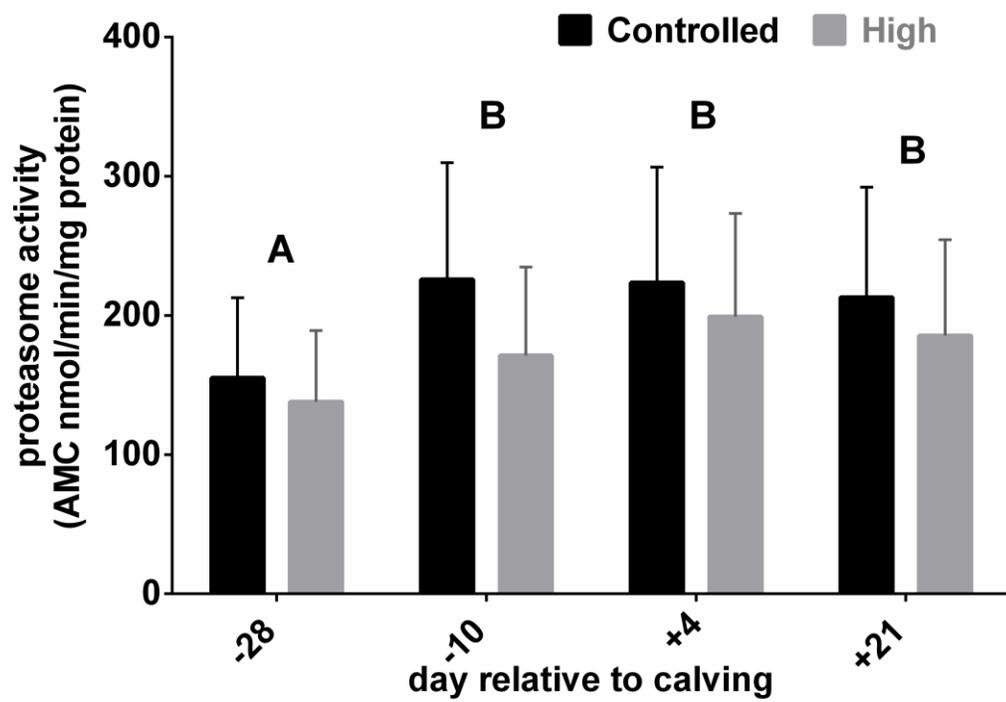


Figure 6.3. Continued

Least squares means of repeated measures ANOVA showing the proteasome activity of external oblique muscle biopsy lysates. Sample days: -28 and -10 = 28 and 10 days prepartum, +4 and +21 = 4 and 21 days postpartum, respectively. Error bars show 95% CI. Time-points with distinct letters show differences at a level of $p < 0.05$ in repeated measures ANOVA and Tukey's posthoc test (p-values for fixed effects in the model: group $p = 0.39$, time $p = 0.003$, group x time $p = 0.84$)

Figure 6.4. Densitometry analysis of immunoblots for ubiquitin from external oblique muscle biopsies

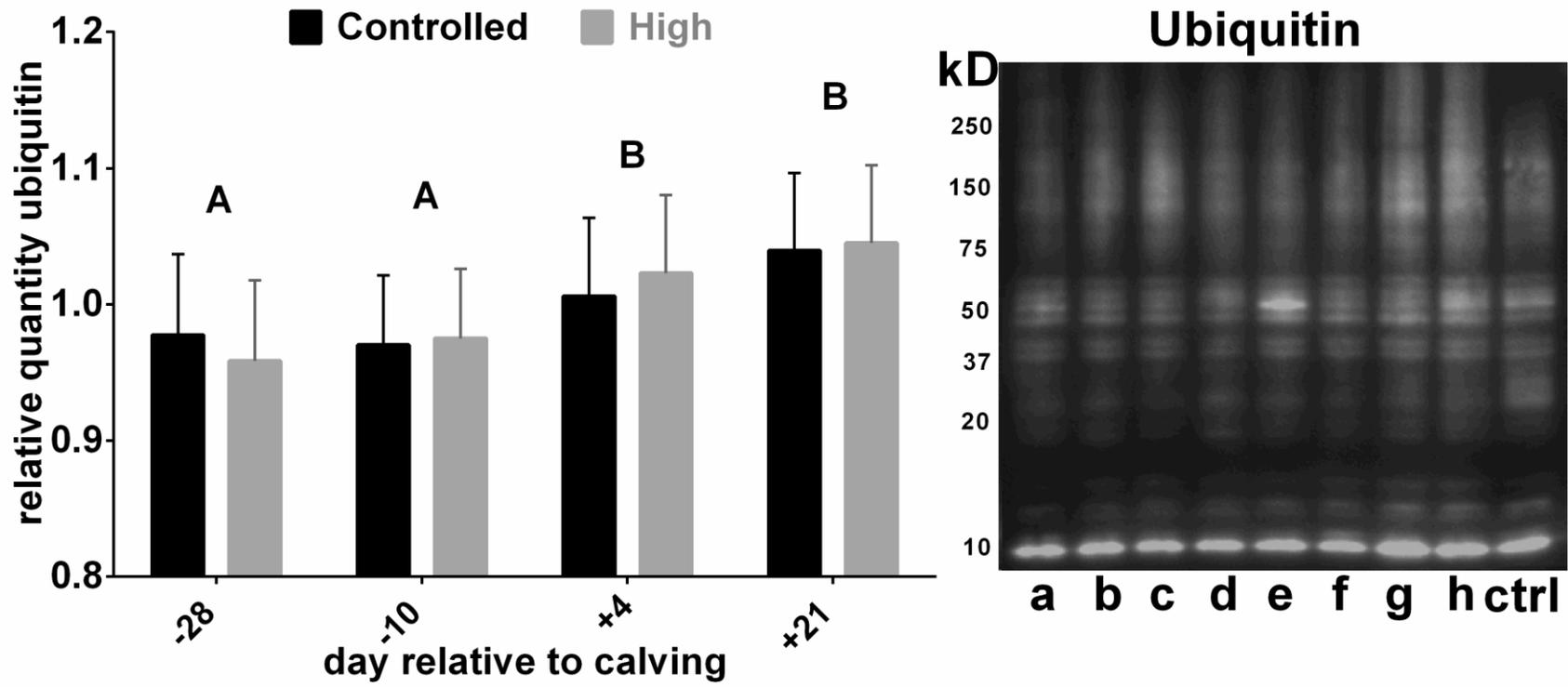


Figure 6.4. Continued

Least squares means of repeated measures ANOVA of densitometry analysis of immunoblots for ubiquitin from external oblique muscle biopsies of peripartum dairy cows (left) as well as a immunoblot of baseline samples from two representative animals (right). Sample days: -28 and -10 = 28 and 10 days prepartum, +4 and +21= 4 and 21 days postpartum, respectively. Error bars show 95% CI. Time-points with distinct letters show differences at a level of $p < 0.05$ in repeated measures ANOVA and Tukey's posthoc test. *P*-values of fixed effects in the model: group $p = 0.95$; time $p = 0.0002$, group x time $p = 0.62$ Lanes: a, e: 28 d prepartum, b, f: 10 d prepartum, c, g: 4 d postpartum, d, h: 21 d postpartum, ctrl= control sample

Figure 6.5. Protein expression of the E-3 ligases atrogin-1 and muRF1 in external oblique muscle biopsies

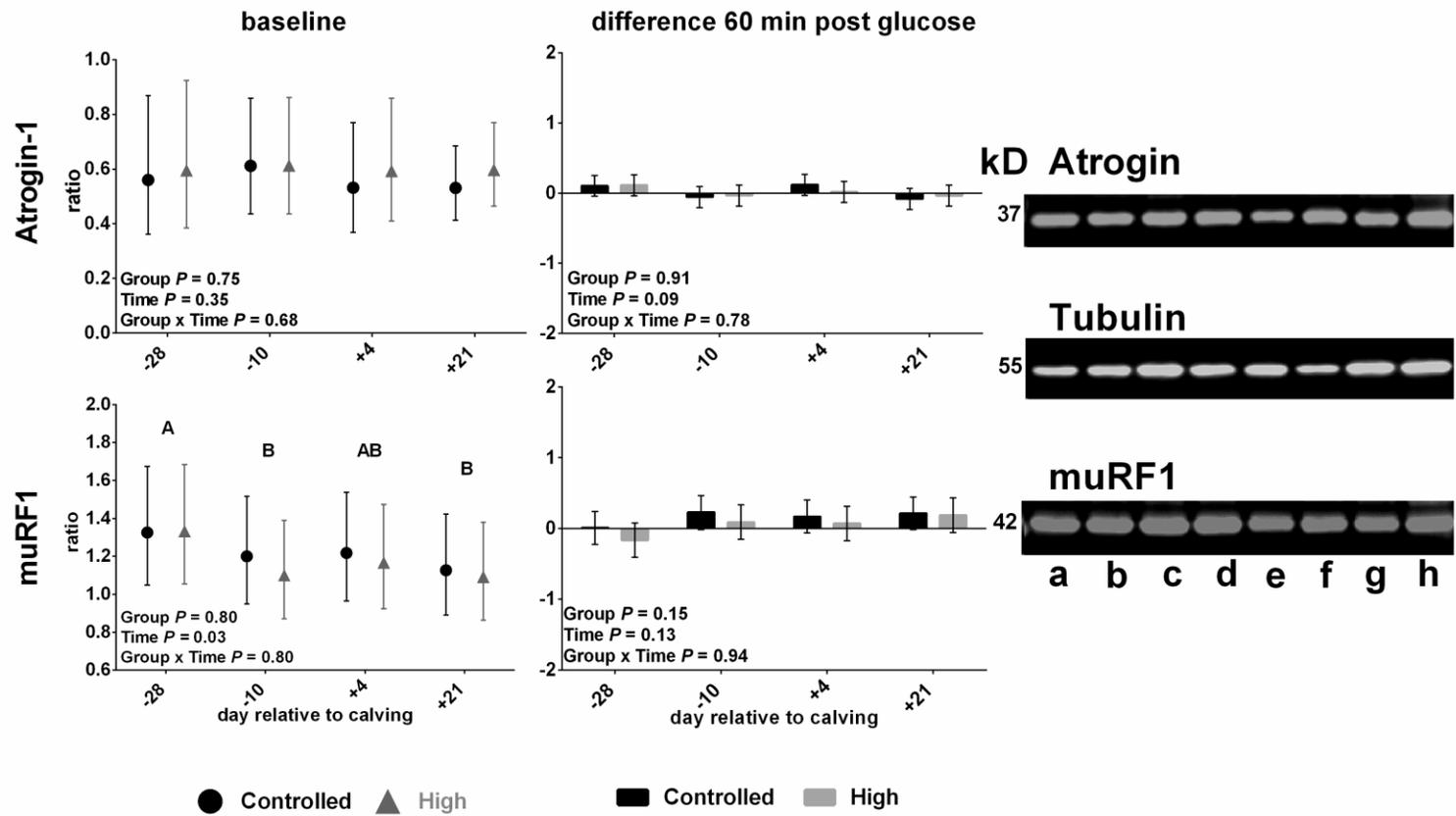


Figure 6.5. Continued

Protein expression of the E-3 ligases atrogin-1 and muRF1 in external oblique muscle biopsies of peripartum dairy cows with β -tubulin as loading control. Least squares means and 95% CI error bars presented in left and middle panel. Sample days: -28 and -10 = 28 and 10 days prepartum, +4 and +21 = 4 and 21 days postpartum, respectively. **Left panel:** Baseline protein expression in biopsies obtained before glucose challenge. Time-points with distinct letters show differences at a level of $p < 0.05$ in repeated measures ANOVA and Tukey's posthoc test. **Middle panel:** Differences in protein expression from baseline in external oblique muscle one hour after rapid intravenous infusion of 0.25 g/kg of glucose. **Right panel** shows representative immunoblots of one animal. Lanes a: 28 d prepartum before challenge, b: 28 d prepartum after challenge, c: 10 d prepartum before challenge, d: 10 d prepartum after challenge, e: 4 d postpartum before challenge, f: 4 d postpartum after challenge, g: 21 d postpartum before challenge, h: 21 d postpartum after challenge

Figure 6.6. Ratios of pAKT to total AKT, unphosphorylated 4EBP1 to total 4EBP1, pERK to total ERK in external oblique muscle

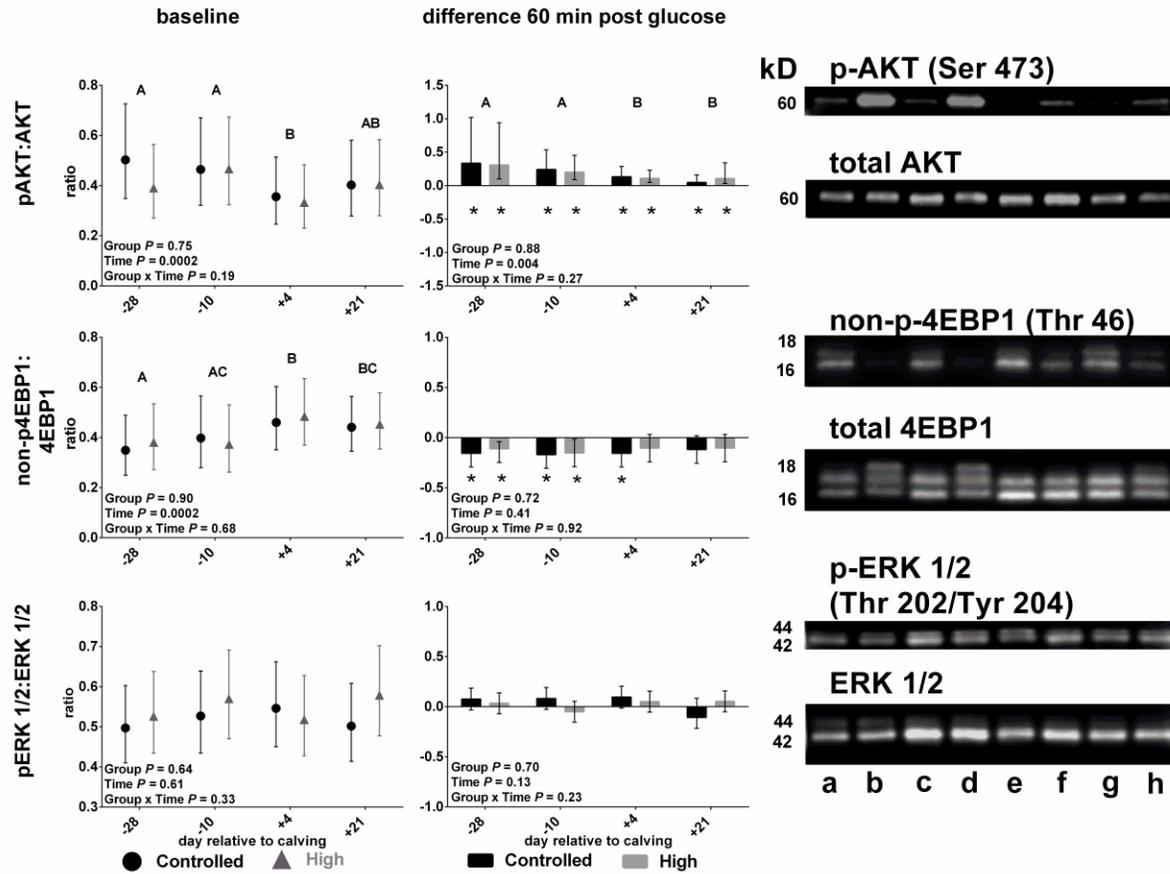


Figure 6.6. Continued

Ratios of pAKT (Ser 473): total AKT; unphosphorylated 4EBP1 (Thr 46):total 4EBP1; pERK 1/ 2 (Thr 202/Tyr 204): total ERK1/2 in external oblique muscle biopsies of peripartum dairy cows. Sample days: -28 and -10 = 28 and 10 days prepartum, +4 and +21= 4 and 21 days postpartum, respectively. Least squares means and 95% CI error bars presented in left and middle panel. **Left panel:** baseline protein expression. Time-points with distinct letters at each time-point show differences at a level of $p < 0.05$ in repeated measures ANOVA and Tukey's posthoc test. **Middle panel:** differences in protein expression from baseline in external oblique muscle one hour after rapid intravenous infusion of 0.25 g/kg of glucose. * change from baseline is different from 0 ($p < 0.05$). **Right panel:** Representative immunoblots from one animal. Lanes a: 28 d prepartum before challenge, b: 28 d prepartum after challenge, c: 10 d prepartum before challenge, d: 10 d prepartum after challenge, e: 4 d postpartum before challenge, f: 4 d postpartum after challenge, g: 21 d postpartum before challenge, h: 21 d postpartum after challenge

Figure 6.7. Protein expression of LC3B in external oblique muscle biopsies of peripartum dairy cows

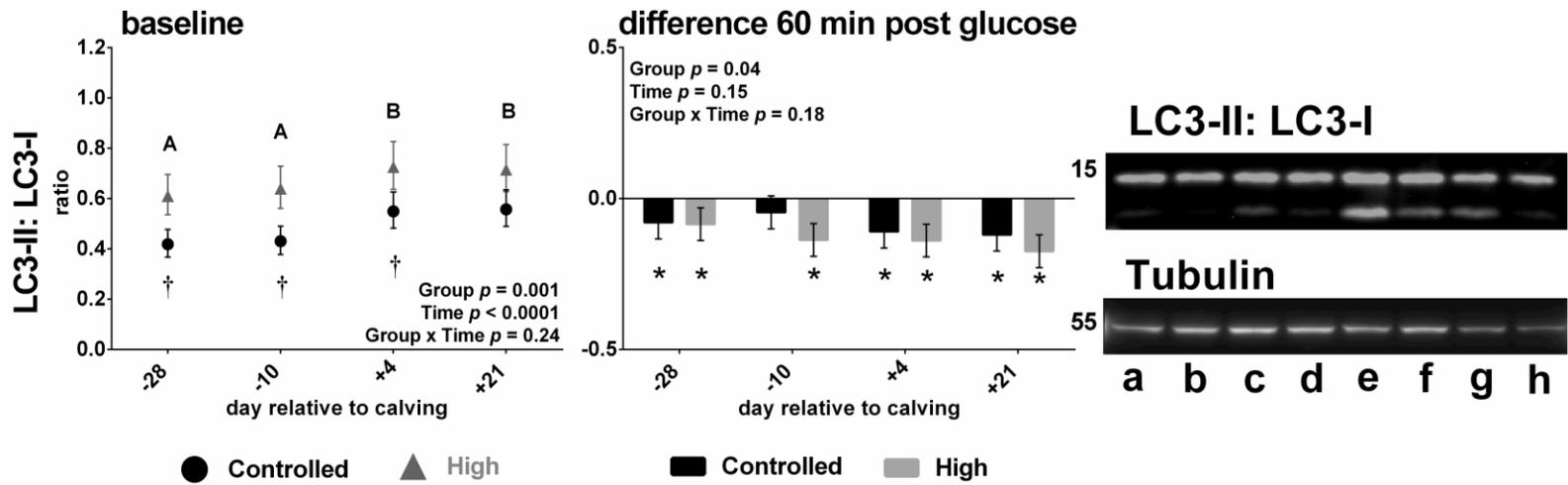


Figure 6.7. Continued

Protein expression of LC3B in external oblique muscle biopsies of peripartum dairy cows. Least squares means and 95% CI error bars presented in left and middle panel. **Left panel:** baseline protein expression in biopsies obtained before glucose challenge. Time-points with distinct letters show differences at a level of $p < 0.05$ in repeated measures. † = treatment group differences in repeated measures ANOVA and Tukey's posthoc test $p < 0.05$. **The middle panel** shows differences in protein expression from baseline in external oblique muscle one hour after rapid intravenous infusion of 0.25 g/kg of glucose. *change from baseline is different from 0 ($p < 0.05$). **Right panel** shows representative immunoblots of one animal. Upper band represents LC3B-I (approx. 16 kD), lower band LC3B-II (approximately 14 kD). Loading control β -tubulin. Lanes a: 28 d prepartum before challenge, b: 28 d prepartum after challenge, c: 10 d prepartum before challenge, d: 10 d prepartum after challenge, e: 4 d postpartum before challenge, f: 4 d postpartum after challenge, g. 21 d postpartum before challenge, h.21 d postpartum after challenge

DISCUSSION

Regulators of muscle atrophy and hypertrophy: Myostatin and IGF-I

During the prepartum period we observed an increase in muscle mass which corresponds to the greater rate of protein synthesis during positive energy balance. In addition, the increase in the diameter of the longissimus dorsi muscle prepartum was larger in group H, and the daily average muscle diameter loss was higher between day 10 prepartum and 21 days postpartum in group H. A more rapid muscle diameter loss was previously observed in cows overfed energy during the far-off period (Kokkonen et al. 2005). However, ultrasonographic data on muscle diameter as a measurement of change in muscle mass has some limitations such as the inability to account for intramuscular adipose deposits that would increase the ultrasonographically assessed diameter. Despite the greater rate of muscle accretion in group H and the greater availability of energy in this group, ratios of phosphorylated AKT and expression of *IGF-I* did not differ between groups. The absence of differences in *IGF-I* expression can thus partially explain the absence of differences in AKT phosphorylation since the activation of the AKT pathway is downstream of the IGF-1-receptor (Glass 2010; Velloso 2008). Furthermore, the glucose induced insulin release following glucose challenge did not lead to a measurable increase in IGF-1 gene expression in muscle tissue in both groups. In a previous study, continuous infusion of glucose at a dose of 1,000 g/d increased circulating IGF-1, whereas at a dose of 500 g/d no increase was measured (Lucy et al. 2013). Despite the fact that contribution of the liver to the circulating IGF-1 concentration would have been significant (Velloso 2008), our study

shows that the bolus dose of less than 200 g of glucose was insufficient to increase expression of IGF-1 in muscle tissue itself.

Gene expression of myostatin was reduced in postpartum samples with the most significant drop on day 4 postpartum in this study. This prominent decrease in myostatin expression has not been documented in peripartum muscle samples to the best of our knowledge. Therefore we can only speculate about the possible reasons for this change. Myostatin expression is negatively regulated by growth hormone (Liu et al. 2003) which has a potential role in regulation in transcription, translation, and post-translational processing of myostatin (Oldham et al. 2009). Concentrations of growth hormone in circulation show a distinct peak at parturition in dairy cattle and decline to moderately elevated concentrations through early lactation (Bell 1995). The downregulation of myostatin in the immediate postpartum period has also been demonstrated in mouse mammary tissue. In a study by Manickam et al. (2008) myostatin mRNA levels were reduced to minimal levels by the onset of lactation. It can therefore be hypothesized that the expression profile of myostatin correlates not only with the growth and differentiation phases of mammary ductal growth and epithelial differentiation as proposed by Manickam et al., (2008), but more generally with the homeorhetic changes of parturition and early lactation. The role of myostatin in the adaptation to lactation and the reasons for the observed downregulation of this gene during periods of negative energy and protein balance remain unclear and a direct association with growth hormone profiles remains to be tested.

Insulin signaling pathway and muscle atrophy

We found that the glucose-induced increase in circulating insulin concentration *in vivo* led to the activation of the AKT pathway in muscle tissue, and that the difference in resting insulin concentrations between groups did not translate into a difference in the baseline phosphorylation status of AKT. However, because densitometry of immunoblot data remains a semi-quantitative way of assessing the phosphorylation status of proteins, small differences with biological significance could have remained undetected. Appuhamy et al. (2011) showed in an *in vitro* experiment on bovine mammary epithelial cells that insulin alone was able to increase the phosphorylation of AKT and mTORC1, including its direct substrate 4EBP1.

The kinase mTORC1 is an important nutritionally regulated cellular signaling component since it controls protein synthesis and mRNA translation through 4EBP1 and p70 ribosomal protein S6 kinase (Carbone et al. 2012; Velloso 2008). Reduced phosphorylation of AKT and 4EBP1 were previously demonstrated in muscle samples during acute, moderate energy deficit in healthy adults (Pasiakos et al. 2010). The function of mTORC1 requires sufficient concentration of amino acids, particularly leucine (Huang and Fingar 2014), hence the reduced ability to phosphorylate its substrate 4EBP1 as shown in this study in postpartum samples could be due to a combination of reduced availability of insulin and amino acids at the same time, since both increase mTORC1 activity.

Four pathways contribute to skeletal muscle proteolysis, the autophagy/lysosomal, as well as the calpain-dependent and caspase-mediated pathways that add proteins to the pool targeted for the ubiquitin proteasome system

(Carbone et al. 2012). Among those, the ubiquitin proteasome system and autophagosome-lysosome system are the most important cellular proteolytic systems in muscular protein turnover (Bonaldo and Sandri 2013). Chibisa et al. (2008) showed that gene expression of m- and μ - calpain were upregulated in samples obtained from longissimus dorsi muscle samples of dairy cows 15 d after calving compared with samples taken approximately two weeks before calving, whereas the amount of ubiquitin mRNA was not different.

During reduced AKT-phosphorylation, forkhead box O transcription factors (FOXO) migrate to the nucleus to induce a number of atrophy-related genes which include atrogin-1 and muRF1 (Carbone et al. 2012; Glass 2010). Decreased AKT-phosphorylation postpartum as a result of the decreased stimulation of the insulin pathway increased the gene expression of atrogin-1 in bovine muscle tissue samples from cows with and without high concentrations of BHB postpartum. However, the amount of protein measured by immunoblot was not different over time. This lack of coordination of mRNA and protein concentrations is disconcerting, however it may be due to either mRNA not being translated at the same rate into protein as the rate of transcription suggests, or atrogin-1 protein being degraded at a higher rate postpartum, or a combination of both. The relationship between AKT activation and atrogin-1 gene expression was also seen by the reduction in atrogin-1 mRNA quantity in biopsies obtained after glucose challenge when AKT was activated as shown by an increase in the ratio of phosphorylated to total protein.

Proteasome activity increased in samples from the immediate peripartum period as compared with the sample obtained approximately one month before

calving. This indicates that upregulation of proteasome activity occurred before calving and extended into the third week postpartum and that the process initiated prepartum even in cows fed in excess of energy requirements. The onset of mobilization is in accordance with data that shows an increase in plasma and urinary 3-methylhistidine concentration in the last two weeks before (Doepel et al. 2002) and corresponds to the decreasing concentration of circulating insulin at this time since insulin potently inhibits body protein degradation (Tesseraud et al. 1993). Together these data indicate that muscle mobilization, like adipose tissue mobilization, initiates well before the day of calving. The upregulation of the proteasome system postpartum was further confirmed by an increase in the amount of ubiquitin-labelled protein at both time-points after calving and likely coincides with the greater amino acid need for milk protein synthesis at the onset of lactation (Meijer et al. 1995).

The activation of mTORC1 also suppresses macroautophagy during positive energy balance and abundance of amino acids (Huang and Fingar 2014). The conversion of LC3-I to the lower migrating form LC3-II has been used as an indicator of autophagy (Stipanuk 2009). Macroautophagy in muscle tissue lysates was increased postpartum as compared with prepartum samples regardless of the energy status prepartum. This was reflected by the increase in the ratio of the lower migrating form of LC3-II to LC3-I as a marker of mature autophagosome formation (Stipanuk 2009). To the best of our knowledge this is the first study showing a role of the macroautophagy mechanism of proteolysis in the homeorhetic adaptation to negative energy and protein balance during the transition period in dairy cattle.

The ratio of LC3-II to LC3-I was consistently higher in group H compared with group C, indicating an overall higher rate of macroautophagy. This might be due to the fact that autophagy supports cell remodeling and proliferation (Stipanuk 2009; Cecconi and Levine 2008) and the higher rate of both accretion and mobilization of muscle tissue in this group as reflected by ultrasound measurements with the observed changes resulting in potentially larger losses of lean mass over time in group H.

We did not detect any difference in the ratio of phosphorylated ERK kinase either over time, between groups or after glucose challenge. Stimulation of ERK1/2 can be achieved by growth factors including insulin and IGF-1 (Roskoski 2012). We demonstrated the increase in circulating insulin following glucose infusion; however, an increase in IGF-1 could not be shown based on the gene expression data we presented. The results of our study can be interpreted such that insulin is either not a potent stimulant of ERK1/2 activation, or that the change in phosphorylation in ERK1/2, which may be biologically relevant, is minimal and undetectable by a semi-quantitative method such as immunoblotting.

It has to be acknowledged that all measurements were done on the basis of the same amount of protein lysate, which does not account for the difference in whole body muscle mass that we observed between groups. Therefore, although we did not detect differences in the cellular regulation of muscle degradation per unit of muscle tissue, the sum of changes on the animal level could differ and this not assessed in the current study.

CONCLUSIONS

This study showed skeletal muscle changes due to parturition and lactation, particularly in the phosphorylation status of AKT at baseline and in response to an endogenous insulin surge. These changes were not driven by prepartum plane of nutrition or concentration of BHB postpartum. We identified the potential role of macroautophagy in skeletal muscle proteolysis during negative energy balance in postpartum dairy cows in addition to the well characterized proteasome pathway of degradation. The changes in myostatin expression relative to the onset of lactation were pronounced, yet counterintuitive to myostatin's traditional regulatory role.

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Conflict of interest: The authors declare that they do not have any conflict of interest.

Ethical approval: All procedures were approved by the Cornell University Institutional Animal Care and Use Committee (protocol no. 2011-0016) and were in accordance with the ethical standards of the institution at which the studies were conducted.

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CHAPTER 7

INSULIN SIGNALING, INFLAMMATION AND LIPOLYSIS IN TRANSITION DAIRY COWS EITHER OVERFED ENERGY DURING THE PREPARTUM PERIOD OR FED A CONTROLLED ENERGY DIET

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ABSTRACT

Adipose tissue mobilization is a hallmark of the transition period in dairy cows. Cows overfed energy during the dry period have higher concentrations of nonesterified fatty acids (NEFA) and BHB compared with cows fed a controlled energy diet prepartum. The reason for an increase in blood NEFA concentrations at the level of adipose tissue in cows overfed energy has not been fully elucidated. One hypothesis is that cows with high BHB concentrations suffer from adipose tissue specific insulin resistance, leading to higher rates of adipose tissue mobilization in the postpartum period.

To test this hypothesis, subcutaneous adipose tissue biopsies of cows overfed energy in excess of predicted requirements by 50% in the dry period, and that had high concentrations of blood BHB postpartum (group H; n=12) were used. Findings were compared with results of biopsies from cows fed a controlled energy diet and with low BHB concentrations postpartum (group C; n=12). Subcutaneous adipose tissue biopsies were obtained before and 60 min after an intravenous glucose challenge (0.25g/kg glucose) at 28 and 10 d before expected calving as well as on d 4 and 21 postpartum. Phosphorylation of protein kinase B (AKT), extracellular signal-regulated kinase (ERK) and hormone sensitive lipase (HSL) was determined before and after glucose infusion by western blot. Western blot was also used to assess the baseline protein abundance of PPAR γ and insulin receptor β -subunit. In addition, gene expression of fatty acid synthase (FASN), adiponectin (ADIPOQ), monocyte chemoattractant protein 1 (MCP1/CCL2) and tumor necrosis factor alpha (TNF α) was

determined by qRT-PCR. Backfat thickness was determined in the thurl area by ultrasonography.

Cows in group H showed a greater degree of lipogenesis prepartum, but no differences were found in lipolytic enzyme activity postpartum compared with cows in group C. Baseline plasma insulin concentrations were decreased and serum NEFA concentrations increased postpartum in group H. Insulin signaling through AKT, quantity of insulin receptor, markers of inflammation and PPAR γ in adipose tissue were not different between the groups, but expression of adiponectin was increased in adipose tissue of cows in group H during the immediate peripartum period.

In conclusion, differences in serum concentrations of NEFA between cows overfed energy prepartum and high blood concentrations of BHB are likely due to greater negative energy balance postpartum reflected in lower circulating concentrations of glucose and insulin and an increase in the total amount of mobilized adipose tissue mass rather than due to changes in adipose tissue insulin signaling.

Keywords: adipose, insulin, transition, energy

INTRODUCTION

The transition period in dairy cattle is characterized by changes in DMI and energy balance that affect adipose tissue metabolism as well as the rate of adipose tissue synthesis and mobilization. Mobilization of adipose tissue, which is reflected by the rise of serum concentration of nonesterified fatty acids (NEFA) in the peripartum period and especially during the first weeks after calving, is part of the physiological process of homeorhetic adaptation to lactation (Bauman and Currie, 1980; Bell, 1995). Epidemiological data provides evidence that an excessive increase in circulating NEFA concentration predisposes cows to negative health events, loss of reproductive success and milk production (Ospina et al., 2010; Garverick et al., 2013; McArt et al., 2013). High concentrations of blood NEFA also contribute to increased oxidative stress and dysfunctional inflammatory response during the postpartum period (Sordillo and Raphael, 2013) as well as development of fatty liver (Bobe et al., 2004).

In a number of studies, overfeeding cows energy during the dry period was associated with higher concentration of circulating NEFA and BHB in the postpartum period compared with cows fed a controlled or restricted energy diet prepartum (Janovick et al., 2011; Khan et al., 2014; Mann et al., 2015). The underlying mechanism of this increase in NEFA concentration and ketogenesis is not fully understood since a difference in DMI is often absent postpartum (Rabelo et al., 2003; Mann et al., 2015). An increase in serum NEFA concentration can be due to an increase in lipolysis, a decrease in the re-esterification rate after release of NEFA from adipose stores, or a decrease in uptake of circulating NEFA (Bell, 1995). Both lipolysis and lipogenesis are influenced by circulating insulin concentrations as well as

by the responsiveness and sensitivity of the adipose tissue to insulin (De Koster and Opsomer, 2013). Recent evidence suggests that cows overfed during the dry period have lower circulating concentrations of insulin in the immediate postpartum period (Janovick et al., 2011; Mann et al., 2016) which may directly affect adipose tissue lipolysis because of a decrease in insulin's direct and indirect anti-lipolytic effect (Corl et al., 2006; Fruhbeck et al., 2014). However a difference in resting concentrations of insulin postpartum between groups fed different dietary planes of energy prepartum was not observed in other similarly conducted studies (Dann et al., 2006; Douglas et al., 2006). Prepartum overfeeding was associated with increased plasma glucose (Mann et al., 2016) or insulin (Douglas et al., 2006) concentration prepartum, or an increase of both (Holtenius et al., 2003; Douglas et al., 2006; Janovick et al., 2011). These results have led to the hypothesis that excess intake of energy during the dry period may affect prepartal insulin response and interfere with adipose tissue signaling and lead to a potential carry-over effect into lactation (Ji et al., 2012; Selim et al., 2015), predisposing cows to excessive mobilization of adipose tissue and an increase in ketogenesis postpartum.

Adipokines, such as adiponectin and tumor-necrosis factor alpha (**TNF α**), and increased adipose tissue inflammation have been implicated as mediators in the development of insulin resistance during pregnancy in human medicine (Barbour et al., 2007). Clear evidence for a potential effect of dry period energy level and excessive body condition at calving on adipose tissue inflammation and immune cell infiltration in dairy cows is still lacking (Bradford et al., 2015).

Our objectives were to investigate peripartal adipose tissue insulin signaling by measuring the activity of protein kinase B (**AKT**) and extracellular signal-regulated kinase (**ERK**), as well as the activity of hormone-sensitive lipase (**HSL**) before and after a glucose challenge-induced endogenous insulin response in cows fed a high energy dry period diet with high blood concentrations of BHB postpartum compared with cows fed a controlled energy dry period diet and with low concentrations of BHB. Furthermore, gene expression of fatty acid synthase (*FASN*), adiponectin (*ADIPOQ*), *TNF α* and monocyte-chemoattractant protein 1 (*MCPI/CCL2*), as well as protein abundance of insulin receptor β -subunit (**INSR**) and peroxisome proliferator-activated receptor gamma (**PPAR γ**) was determined.

MATERIALS AND METHODS

Animals, Feeding and Management

A detailed description of study animals, diets and feed analysis as well as energy balance was described previously (Mann et al., 2015). In brief, animals entering second or greater parity were either fed a TMR formulated to meet, but not greatly exceed energy requirements at predicted ad libitum intake (C, n=28), or received a TMR formulated to supply 150% of energy requirements (H, n=28) beginning at dry-off approximately 57 d before expected parturition. After analysis of feed composite samples with wet chemistry analysis (Dairy One Cooperative Inc., Ithaca, NY), estimation of energy balance and MP supply as a percentage of predicted requirements was carried out with the Cornell Net Carbohydrate and Protein System

software (CNCPS; Cornell University, version 6.1). Analyzed diet composition, weekly weight and DMI averages were entered, as well as days carried calf for prepartum estimates, and weekly averages of milk yield as well as milk fat, protein and lactose concentration for postpartum estimates. BCS was determined at enrollment and weekly throughout the study based on a 0 to 5 point scale as described by Edmonson et al. (1989). Animals for this study were specifically selected from the larger study population described in Mann et al. (2015) based on the concentration of BHB over time during the first 3 weeks postpartum. To achieve this, area under the curve (AUC) was based on three weekly BHB concentrations during the first 3 weeks postpartum (9 samples total) which were determined in whole blood using a handheld meter (Precision Xtra meter, Abbott Diabetes Care Inc., Alameda, CA). The calculation was carried out following the method described by Cardoso et al. (2011) using SAS v. 9.3 (SAS Inc., Cary, NC). Samples of animals with the highest total area under the curve (AUC) of BHB concentration in group H (n=12) and those with the lowest area under the curve in group C (n=12) were recruited for this study in order to achieve the largest contrast in BHB concentrations. In each of the selected groups C and H, 7 and 5 animals were entering second and third lactation, respectively.

Ultrasound Measurements

At enrollment (57 d before expected parturition), on d 28 and 10 before expected parturition, and d 4, 21 and 42 d after parturition, ultrasonographic measurements of the subcutaneous fat thickness in the thurl area were carried out using a modification of the method previously described by Schröder and Staufienbiel

(2006) and Joshi and Herdt (Joshi and Herdt, 2009). In brief, fat thickness was measured on the right side of the animal as the distance between the skin and the profound fascia above the gluteus medius muscle on a line between the tuber coxae and tuber ischia, approximately 10 cm cranial to the latter. Backfat thickness (BFT) was expressed excluding the measurement of the skin and values given are therefore actual fat thickness. The hair in the area corresponding to the exact placement of the ultrasound probe as determined during the first examination was clipped to the size of the probe to assure repeatable probe placement. The skin surface was brushed and 70% alcohol (VetOne, Boise, ID) was applied as a coupling agent before measurements were performed using the IBEX Pro portable ultrasound (E.I. Medical Imaging, Loveland, CO) with a 8.5-MHz linear array transducer. Each measurement was performed in triplicate, avoiding pressure as to not compress the underlying tissue, and the average of the three measurements was used for analysis.

Blood samples

Venous blood samples were taken at the time of biopsy according to the methods previously described (Mann et al., 2016). In brief, two samples were taken from a jugular catheter 10 min apart, centrifuged within 30 min and aliquots of plasma and serum stored at -20°C until analysis for serum NEFA (HR Series NEFA-HR (2), Wako Life Sciences, Mountain View, CA) and plasma insulin concentrations (Porcine Insulin RIA, Millipore, St. Charles, MO). Concentration of BHB in whole blood was determined cow-side using a Precision Xtra meter (Abott Diabetes Care Inc., Alameda, CA).

Adipose tissue samples

Biopsies of the subcutaneous adipose tissue were taken 28 and 10 d before expected parturition and 4 and 21 d after calving, immediately before and 60 min after rapid intravenous infusion of glucose at a dose of 0.25 g/kg body weight (Mann et al., 2016). Samples were taken from the deepest point of the paralumbar fossa approximately 15-25 cm behind the last rib and 10-30 cm below the lumbar transverse processes. The biopsy sites were prepared by clipping the hair in the paralumbar fossa and scrubbing the skin with iodine soap (Pivodine Scrub 7.5% Iodine, VetOne, Boise, ID) and water. The area was rinsed with water and dried. The biopsy site was then aseptically prepared with iodine scrub and 70% alcohol (VetOne, Boise, ID) before local anesthesia with 10 ml of a 2% lidocaine solution (lidocaine 2% HCL, VetOne, Boise, ID) was performed following the pattern of an inverted L-block at a distance of at least 10 cm from the biopsy site. The surgical area was again scrubbed by alternating iodine scrub and alcohol as described above. A vertical skin incision of approximately 3-5 cm length was made and 2-4 g of the subcutaneous adipose layer was removed with sterile forceps and scalpel. Tissue samples were immediately snap frozen by immersion in liquid nitrogen in 2 mL cryovials (Nalgene, VWR, Radnor, PA) containing 500 µl of RNA buffer solution (RNA later, Qiagen, Hilden, Germany) for later extraction of mRNA, or in the same cryovials without addition of buffer for immunoblotting. Before the skin incision was closed with a single cruciate suture (metric 5, Supramid Extra II, S.Jackson Inc., Alexandria, VA), a biopsy of the external oblique muscle was taken (data not presented). Samples were transported in liquid nitrogen to the laboratory and stored at -80°C until processing.

Preparation of mRNA and real-time quantitative reverse-transcription PCR

Approximately 100 mg of frozen adipose tissue from each sample were transferred to 500 μ L of ice-cold Trizol reagent (Life Technologies, Thermo Fisher Scientific, Waltham, MA), placed in the pre-chilled adapter of a tissue disruptor (TissueLyser LT, Qiagen, Hilden, Germany) and homogenized at maximum oscillation for 3 min with sterile 5 mm stainless steel beads. Total RNA was extracted and treated in-column with DNase I (Direct-Zol RNA MiniPrep, Zymo Research, Irvine, CA). The optical density 260:280 ratio was measured with a spectrophotometer (NanoVue Plus, GE Healthcare, Life Sciences, Little Chalfont, UK) for quality control of integrity of RNA. For cDNA synthesis on the same day, up to 500 ng of RNA was reverse-transcribed (SuperScript III First-Strand Synthesis SuperMix, Life Technologies, Thermo Fisher Scientific, Waltham, MA) and stored at -20°C until analysis. Eukaryotic translation initiation factor 3K (EIF3K) was chosen as the housekeeping control gene as recommended recently (Bonnet et al., 2013). Bovine-specific primer probe sets with exon spanning probes were purchased for the control gene and all genes of interest (EIF3K: Bt03226565, FASN: Bt03210491, CCL2/MCP-1: Bt03212322, TNF α : Bt03259156; TaqMan Gene Expression Assays, Applied Biosystems, Thermo Fisher Scientific, Waltham, PA) except for adiponectin (ADIPOQ) where an exon spanning primer probe set was not available (Bt03292341; TaqMan Gene Expression Assays, Applied Biosystems, Thermo Fisher Scientific, Waltham, PA). Real-time quantitative reverse-transcription PCR (qRT-PCR) was performed using a 2-fold dilution of cDNA at 10% of the final reaction volume and each sample was analyzed in triplicate using a StepOne Plus system and 2x master

mix (TaqMan Gene Expression Master Mix, Applied Biosystems, Thermo Fisher Scientific, Waltham, PA). The PCR protocol consisted of denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec (denaturation) and 1 min at 60°C (annealing and extension). For the calibration reference sample, aliquots of cDNA obtained from adipose tissue of a healthy cow at 28 d before expected parturition was used on every plate. Results were analyzed using the comparative quantification algorithms-standard curve method ($\Delta\Delta C_t$ method, StepOne Software, v 2.3). Results were expressed as relative quantity ($RQ = 2^{-\Delta\Delta C_t}$). Gene names are written in capital and italic letters to differentiate them from the protein that they encode for.

Protein Extraction and Immunoblotting

Approximately 100 mg of frozen adipose tissue were homogenized with a tissue homogenizer (VDI 12, VWR, Radnor, PA) for approximately 60 sec in 500 μ L of ice-cold RIPA lysis buffer containing 150 mM sodium chloride, 1% Triton X, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris, and 1 mM PMSF with the addition of a phosphatase and protease inhibitor cocktail (Halt, Thermo Fisher Scientific, Waltham, PA), sonicated (sonicator) for 6 sec, and kept on ice for 20 min before centrifugation at 14,000 g and 4°C for 15 min. The fat layer was removed and the supernatant transferred. Then the protein concentration was determined by use of the Bradford technique (Bradford, 1976) using a commercially available reagent (Coomassie Protein Assay, Thermo Scientific, Rockford, IL). Lysates were adjusted to a protein concentration of 4 μ g/ μ L and western blot analysis was performed with 8%

Tris-Glycine SDS-polyacrylamide gels with loading amounts of 40 µg of protein per well.

On each gel a control sample obtained from a healthy cow at d 28 before expected parturition was loaded and results standardized to the densitometry results for the control sample in order to minimize variability in the results between gels. After wet transfer on ice to PVDF membrane (Immobilon-P, EMD Millipore, Billerica, MA) for 2 h at constant amperage (500 mA), membranes were blocked in 5% nonfat milk for 1 h. Membranes were incubated with primary antibodies to-ERK (Thr202/Tyr204), AKT, p-AKT (Ser 473), HSL, p-HSL (Ser563), p-HSL (Ser660), PPAR γ , and the β -subunit of the insulin receptor (Cell Signaling Technology, Danvers, MA), to pan-ERK (BD Biosciences, Franklin Lakes, NJ) overnight at 4°C. For antibodies without documented cross-reactivity with the bovine species, epitope homology was 100% except for HSL, pHSL (Ser563), and pHSL (Ser660), for which homology was 89, 92, and 92%, respectively. Use of antibodies for HSL, pHSL (Ser563) and pHSL (Ser660) in bovine tissues was previously described (Elkins and Spurlock, 2009; Locher et al., 2011). All primary antibodies were diluted 1:1,000 in TBST, except for pHSL (Ser563) and pHSL (Ser660) which were diluted 1:2,000. After incubation with primary antibodies, membranes were washed three times with TBST and incubated for 1 hour at room temperature with the appropriate 1:2,000 dilution of anti-rabbit or anti-mouse HRP-linked secondary antibodies (Cell Signaling Technology, Danvers, MA). Blots were again washed three times in TBST, exposed to enhanced chemiluminescent substrate (Clarity Western ECL Substrate, Biorad, Hercules, CA), imaged sequentially at 10, 20, 30, 50 and 60 sec intervals

(BioSpectrum Imaging System, UVP, Upland, CA). Densitometry was performed using VisionWorks software (VisionWorks LS software, v. 8.1.2, UVP, Upland, CA).

Statistical methods

Repeated ultrasonographic measurements of the backfat thickness, baseline gene expression and protein measurements before glucose infusion, as well as the change of protein phosphorylation of AKT, ERK and HSL after glucose infusion were analyzed using repeated measures ANOVA (Proc MIXED, SAS, v. 9.3, Cary, NC) with the fixed effects group, time and including a group x time interaction, actual sampling d at time-point -28 was included in the model as a covariate. Five covariance structures were tested (unstructured, autoregressive 1, variance components, compound symmetry and Toeplitz) and the one resulting in the lowest Akaike Information Criterion was chosen. The degrees of freedom were approximated with the Kenward-Roger method. Model assumptions were assessed by evaluation of homoscedasticity and normality of residuals. To satisfy this assumption, gene expression data, and immunoblot densitometry data were log transformed, and resulting least squares means estimates were subsequently back transformed and presented as geometric mean and 95% CI. Differences between BCS and BW, as well as the concentration of NEFA, BHB, glucose and insulin in blood samples and measurements obtained during IVGTT were analyzed by one-way ANOVA using the Proc MIXED procedure in SAS. Differences in the proportion of animals with increase in phosphorylation of AKT postpartum was analyzed using Fisher's exact test. The association of AKT phosphorylation and resting insulin concentrations was

assessed by Spearman correlation. Graphs were created with GraphPad Prism (v. 6.04, La Jolla, CA). All results are presented as mean or geometric mean and 95% CI unless otherwise stated. All P - values given are those controlled for multiple comparisons with Tukey's HSD test.

RESULTS

Energy balance, Blood Concentration of Analytes and Backfat Thickness

Energy balance in % of predicted requirements for wk 1 to 7 prepartum and wk 1 to 6 postpartum as well as milk production in the first 6 wk after calving is depicted in Figure 7.1. The energy density of the diets as estimated with CNCPS (v.6.1) was 1.98, 2.12, 2.23, and 2.24 Mcal ME/kg DM for the C, I, H, and F diets, respectively (Mann et al., 2015). As intended, prepartum energy balance was higher at every time-point in group H (wk 1 to 7 prepartum: 157 [150-164] %) compared with group C (109 [102-116], $P < 0.0001$). Energy balance was lower in group H postpartum (71 [67-75] %) compared with group C (76 [73-80] %, $P = 0.05$). No differences were observed in average milk production during the first 6 wk of lactation (group C: 45.2 [41.4-48.1], group H 46.6 [43.5-50.0] kg/d, $P = 0.60$). By design, the area under the curve for BHB in the first 21 DIM was different for the selected cows and approximately twice as high in group H compared with group C (H: 22.6 [17.2- 28.0] mmol/L; C 10.4 [5.0-15.7] mmol/L , $P = 0.0003$).

The actual average biopsy sampling day relative to calving was -26.6 (25.2 to -27.9), -7.5 (-6.0 to -9.0), +5.1 (+4.6 to +5.6), and +21.2 (+20.7 to +21.6) d. Resting blood concentrations of glucose, insulin, NEFA and BHB at the time of biopsy are

listed in Table 7.1. No prepartum differences were observed in concentrations of NEFA, insulin and BHB ($P > 0.22$) but glucose concentrations were higher in group H at both prepartum time-points ($P = 0.05$ and 0.07 , respectively). Group differences were observed for all four analytes on d 4 and 21 postpartum with higher concentrations of NEFA and BHB, as well as lower concentrations of insulin and glucose in group H compared with group C.

Average BCS at enrollment was 3.04 (2.90-3.19) and 3.10 (2.90-3.25) at enrollment ($P = 0.53$) and was not different between groups at any time ($P > 0.10$). Average BW at enrollment was 666 (620-711) and 670 (626-716) kg in group C and H, respectively ($P = 0.87$). Cows in group C gained on average 73.5 (59.5-87.5) kg whereas cows in group H gained 93.9 (79.9-107.9) kg BW during the dry period ($P = 0.04$). Average weight loss was 24.3 (5.9-42.5) and 40.1 (21.9-58.3) kg in group C and H, respectively ($P = 0.21$). Results for measurements of backfat thickness (BFT) and BW are depicted in Figure 7.2. An interaction between group and time ($P = 0.04$) existed in repeated measures analysis of BFT with a more rapid increase in group H until the time of calving and a more rapid decrease after calving whereas measurements at dry-off and 42 d postpartum were not different (Figure 7.2).

Adipose Tissue Gene Expression

The average ratio of absorbance (260/280 nm) of the RNA samples was 2.00 (1.98-2.02). Figure 7.3 shows the gene expression of FASN, ADIPOQ, CCL2 and TNF α at 28 and 10 d prepartum, as well as 4 and 21 d postpartum. We found a treatment group difference for relative expression of FASN ($P = 0.001$) with the

largest difference on d 10 prepartum (group C: RQ = 0.05 [0.02-0.10], group H: RQ = 0.79 [0.35-1.78], P = 0.003). Expression over time was different in both postpartum samples compared with prepartum samples (P < 0.05) such that in both groups expression dropped to low values postpartum (RQ < 0.01). An interaction was noted for gene expression of ADIPOQ (P = 0.04) with a lower expression in group C compared with group H on d 10 prepartum (RQ: 1.7 [0.8-2.6] vs. 3.7 [2.8-4.6]), as well as d 4 (RQ: 2.3 [1.4-3.3] vs. 4.2 [3.3-5.1]) and d 21 (RQ: 1.3 [0.3-2.2] vs. 3.1 [2.2-4.0]) postpartum. The expression of CCL2/MCP1 and TNF α changed over time. Expression of CCL2 was highest on d 4 postpartum (RQ: 3.9 [2.2-6.7]) compared with the lowest expression on d 28 prepartum (RQ: 1.8 [1.1-3.2], P = 0.03) and the highest expression of TNF α was measured on d 21 postpartum (RQ: 1.9 [1.4-2.7]) compared with the lowest expression on d 28 prepartum (RQ: 1.0 [0.7-1.4], P = 0.03). We did not detect group differences or interactions between group and time for expression of these two inflammatory genes (P > 0.22).

Adipose Tissue Immunoblotting

The total amount of AKT and HSL in relation to β -actin quantified by densitometry changed over time. The ratio of AKT: β -actin declined from 1.3 (1.2-1.5) on 28 d prepartum to 1.2 (1.0-1.4) and was 1.0 (0.9-1.2) on d 4 and 1.1 (1.0-1.2) on d 21 postpartum (P = 0.02). The ratio of HSL: β -actin also declined from 1.0 (0.9-1.2) on d 28 prepartum to 0.7 (0.6-0.9) on d 10 prepartum. 0.7 (0.6-0.8) on d 4 and 0.8 (0.7-0.9) on d 21 postpartum (P = 0.003). In contrast, total amounts of ERK showed

no change over time ($P = 0.30$). Group differences and interactions between time and group were not observed ($P > 0.36$).

The ratio of pAKT: AKT, pERK: ERK, pHSL (Ser660): HSL is presented in Figure 7.4. The ratio of pAKT: AKT changed over time with the lowest values on d 4 and 21 postpartum compared with both d 28 and 10 prepartum. We noted an interaction between group and time ($P = 0.02$) with a higher ratio of phosphorylated protein in group H compared with group C on d 10 prepartum (group C: 1.0 [0.7-1.3], group H: 1.2 [0.9-1.4]), whereas there was a lower ratio on d 4 postpartum (group C: 0.60 [0.37-0.83], group H: 0.26 [0.05-0.50]). The correlation coefficient between resting concentrations of insulin and phosphorylation of AKT was 0.64 ($P < 0.0001$). The increase in the ratio of phosphorylated AKT 60 min after glucose infusion also changed over time with the greatest increase on d 28 prepartum (1.43 [0.83-2.03]), and the smallest increase on d 21 postpartum (0.16 [-0.01-0.32]) but no differences were observed between the groups ($P = 0.82$). On all time-points the average difference from baseline was greater than 0 at a level of $P < 0.05$ except on d 21 postpartum in group H. However, 4 cows on d 4 postpartum (2 in group C, 2 in group H) and 10 cows on d 21 postpartum (4 in group C, 6 in group H) had no detectable increase in phosphorylation ratio of AKT 60 min post-glucose ($P > 0.68$). No differences in BW or BCS loss within the first 3 and 6 wk, respectively, were observed for these cows in comparison with cows that did have an increase in AKT phosphorylation ($P > 0.60$).

The ratio of pERK: ERK was not different over time or between treatment groups ($P > 0.13$) (Figure 7.4). The ratio of pHSL (Ser660): HSL changed over time ($P = 0.001$) with the largest ratio on d 4 postpartum (1.46 [1.14-1.87]) in both groups

compared with d 10 prepartum (0.66 [0.47-0.96], $P = 0.002$) but no difference was observed between groups ($P = 0.81$) (Figure 7.4). A difference from baseline in the ratio of pHSL (Ser660) after glucose infusion was only detected on d 4 postpartum in group H. Similarly, the ratio of pHSL (Ser563) was also not different between groups ($P = 0.56$), but the ratio increased from 1.03 (0.72-1.45) on d 28 prepartum and 0.63 (0.31-1.21) on d 10 prepartum to 4.07 (2.50-6.68) on d 4 postpartum and 2.59 (1.03-6.51) on d 21 postpartum. The ratios on d 28 and 10 prepartum were lower than on d 4 postpartum ($P < 0.002$), and the ratio on d 10 prepartum was lower than on d 21 postpartum ($P = 0.02$).

We found no group differences or interactions between group and time in densitometry results for the ratio of INSR: β -actin and PPAR γ : β -actin ($P > 0.51$) but a difference over time was noted with a decrease in INSR on d 10 prepartum (0.80 [0.62-1.04]), and d 21 postpartum (0.80 [0.62-1.04]) compared with d 28 prepartum (1.37 [1.06-1.77] $P < 0.004$). The ratio of PPAR γ was lower on d 4 (1.49 [1.06-2.08]) compared with d 21 (2.59 [1.85-3.63], $P = 0.01$) postpartum.

Table 7.1. Blood concentrations of BHB, NEFA, glucose and insulin in study animals on the day of biopsy and during intravenous glucose tolerance test. Results presented as mean and 95% CI.

Item	Group ¹		<i>P</i>
	Controlled (n=12)	High (n=12)	
NEFA (mmol/L)			
-28	0.37 (0.30-0.44)	0.35 (0.29-0.42)	0.74
-10	0.40 (0.29-0.51)	0.44 (0.33-0.55)	0.63
+4	1.35 (1.18-1.52)	1.82 (1.64-1.99)	0.001
+21	1.02 (0.82-1.22)	1.44 (1.24-1.63)	0.005
BHB (mmol/L)			
-28	0.23 (0.17-0.28)	0.24 (0.18-0.30)	0.73
-10	0.32 (0.25-0.40)	0.25 (0.18-0.33)	0.22
+4	0.69 (0.45-0.93)	1.20 (0.96-1.44)	0.005
+21	0.63 (0.18-1.08)	1.49 (1.04-1.94)	0.01
Glucose (mg/dL)			
-28	71.9 (68.4-75.4)	76.4 (73.0-79.9)	0.07
-10	69.8 (66.2-73.4)	74.9 (71.4-78.4)	0.05
+4	61.1 (58.1-64.1)	53.2 (50.2-56.2)	0.001
+21	65.1 (60.4-69.8)	56.9 (52.3-61.6)	0.01
Insulin (μU/mL)			
-28	17.7 (13.9-21.4)	18.3 (14.8-21.9)	0.80
-10	11.7 (8.7-14.8)	11.7 (8.6-14.7)	0.99
+4	2.5 (1.4-3.5)	0.9 (0.2-1.9)	0.03
+21	5.9 (4.2-7.6)	3.2 (1.6-4.8)	0.02

¹ Controlled (n=12): cows fed a controlled energy diet during the prepartum period, supplying approximately 100% of predicted energy requirements and with the low BHB concentrations postpartum. High (n=12): cows fed a high energy diet during the prepartum period, supplying approximately 150% of predicted energy requirements and with high BHB concentrations postpartum.

Figure 7.1. Energy balance (EBAL in % of predicted requirements) and milk production of study animals.

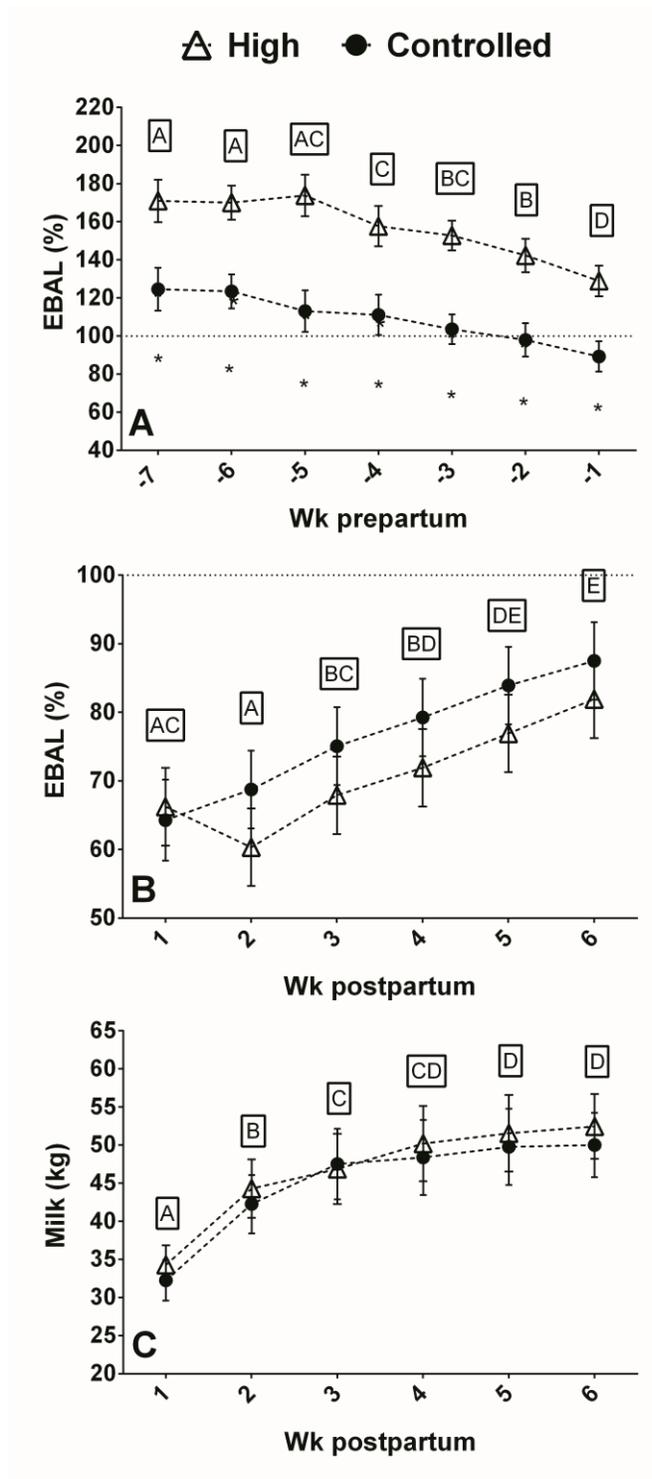


Figure 7.1. Continued

Energy balance (EBAL in % of predicted requirements) and milk production of study animals. Least squares means of prepartum (A) and postpartum (B) energy balance in % of predicted requirements (CNCPS v. 6.1). Least squares of daily milk production in kg (C). Data presented as means and 95% CI. Time-points marked with different superscript letters differ at a level of $P < 0.05$ in repeated measures ANOVA and Tukey's posthoc test. EBAL prepartum: group $P < 0.0001$, time $P < 0.0001$, group x time $P = 0.12$; EBAL postpartum: group $P = 0.05$, Time $P < 0.0001$, group x time $P = 0.25$; Milk kg: group $P = 0.46$, time $P < 0.0001$, group x time $P = 0.38$. Controlled (n=12): cows fed a controlled energy diet during the prepartum period, supplying approximately 100% of predicted energy requirements and with the low BHB concentrations postpartum. High (n=12): cows fed a high energy diet during the prepartum period, supplying approximately 150% of predicted energy requirements and with high BHB concentrations postpartum.

Figure 7.2. Least squares means of backfat thickness in the thurl area

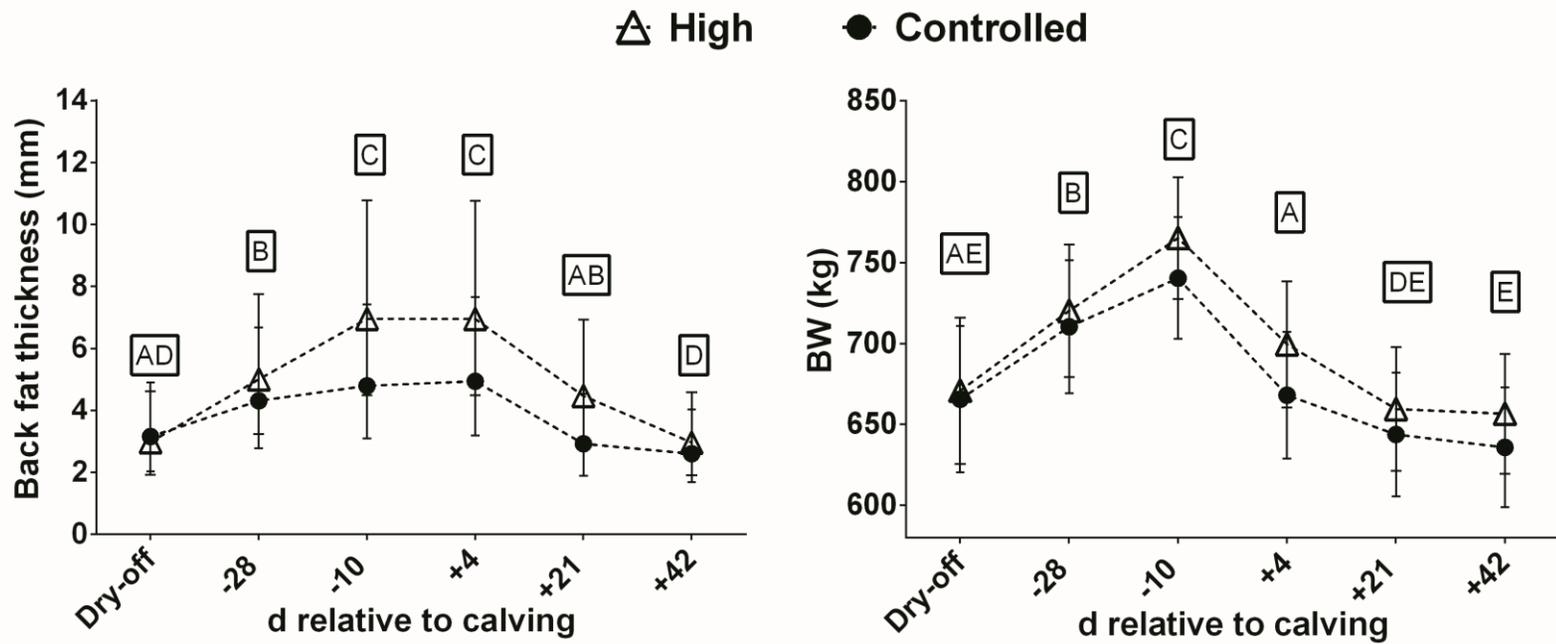


Figure 7.2. Continued

Least squares means of backfat thickness (BFT) in the thurl area in mm (left) and body weight in kg (right) of study animals at six different time-points throughout the study. Time-points marked with different superscript letters differ at a level of $P < 0.05$ after Tukey's HSD test. Error bars represent 95% CI. P-values derived from repeated measures ANOVA: BFT group $P = 0.42$, time $P < 0.0001$, group x time $P = 0.04$; Weight group $P = 0.50$, time $P < 0.0001$, group x time $P = 0.11$. Controlled (n=12): cows fed a controlled energy diet during the prepartum period, supplying approximately 100% of predicted energy requirements and with the low BHB concentrations postpartum. High (n=12): cows fed a high energy diet during the prepartum period, supplying approximately 150% of predicted energy requirements and with high BHB concentrations postpartum.

Figure 7.3. Gene expression measured by qRT-PCR in subcutaneous adipose tissue biopsies at four time-points in the peripartum period

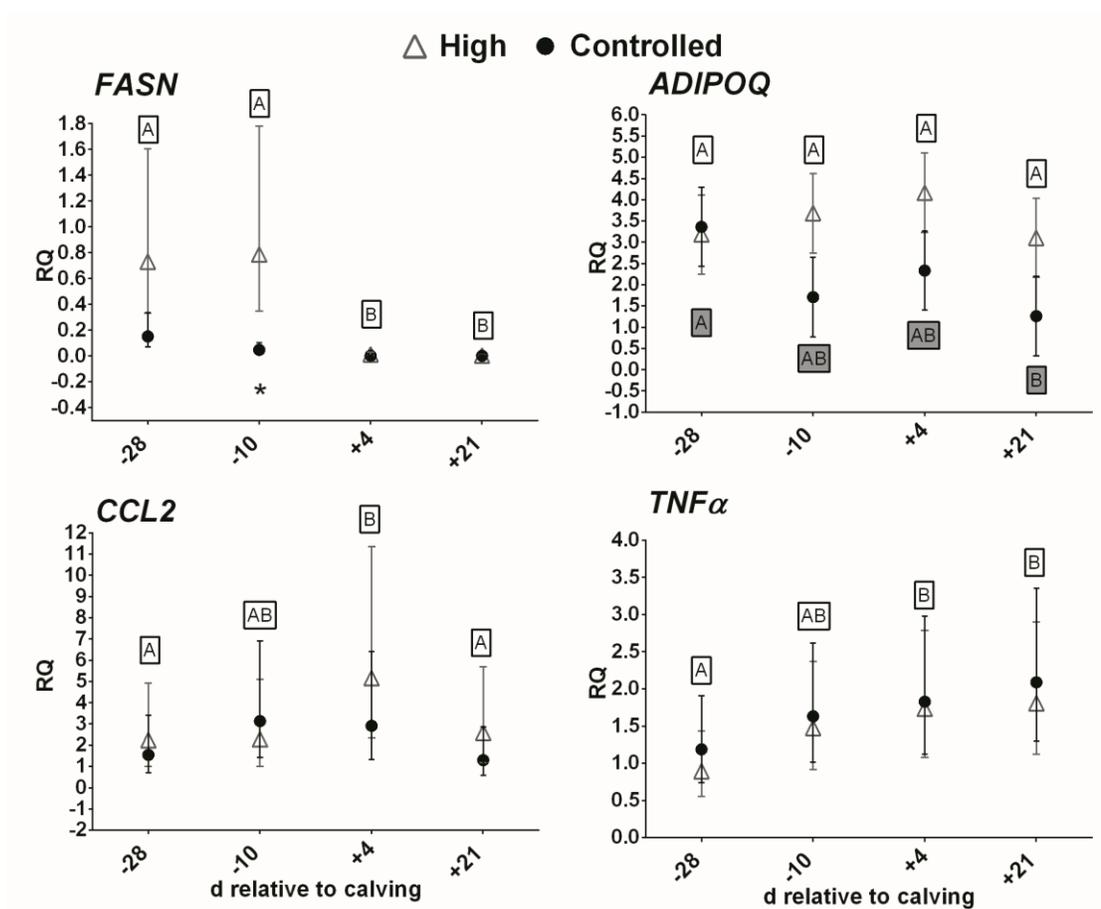


Figure 7.3. Continued

Gene expression measured by qRT-PCR in subcutaneous adipose tissue biopsies at four time-points in the peripartum period. Results presented as geometric least squares means and 95% CI. Differences over time are marked with different superscript letters differ at a level of $P < 0.05$ in repeated measures ANOVA and Tukey's test; differences between groups are depicted as * $P < 0.05$. Interaction between group and time is depicted in superscript letters for group H and shaded superscript letters for group C. *FASN* = fatty acid synthase, group $P < 0.001$, Time $P < 0.0001$, group x time $P = 0.08$; *ADIPQ* = adiponectin, group $P = 0.004$, Time $P = 0.04$, group x time $P = 0.04$; *CCL2* = chemokine ligand 2/monocyte chemotactic protein-1, group $P = 0.50$, time $P = 0.01$, group x time $P = 0.22$, *TNF α* = tumor necrosis factor alpha, group $P = 0.43$, time $P = 0.01$, group x time $P = 0.89$. Controlled (n=12): cows fed a controlled energy diet during the prepartum period, supplying approximately 100% of predicted energy requirements and with the low BHB concentrations postpartum. High (n=12): cows fed a high energy diet during the prepartum period, supplying approximately 150% of predicted energy requirements and with high BHB concentrations postpartum.

Figure 7.4. Results of immunoblot densitometry and resulting ratio for phosphorylated proteins of interest

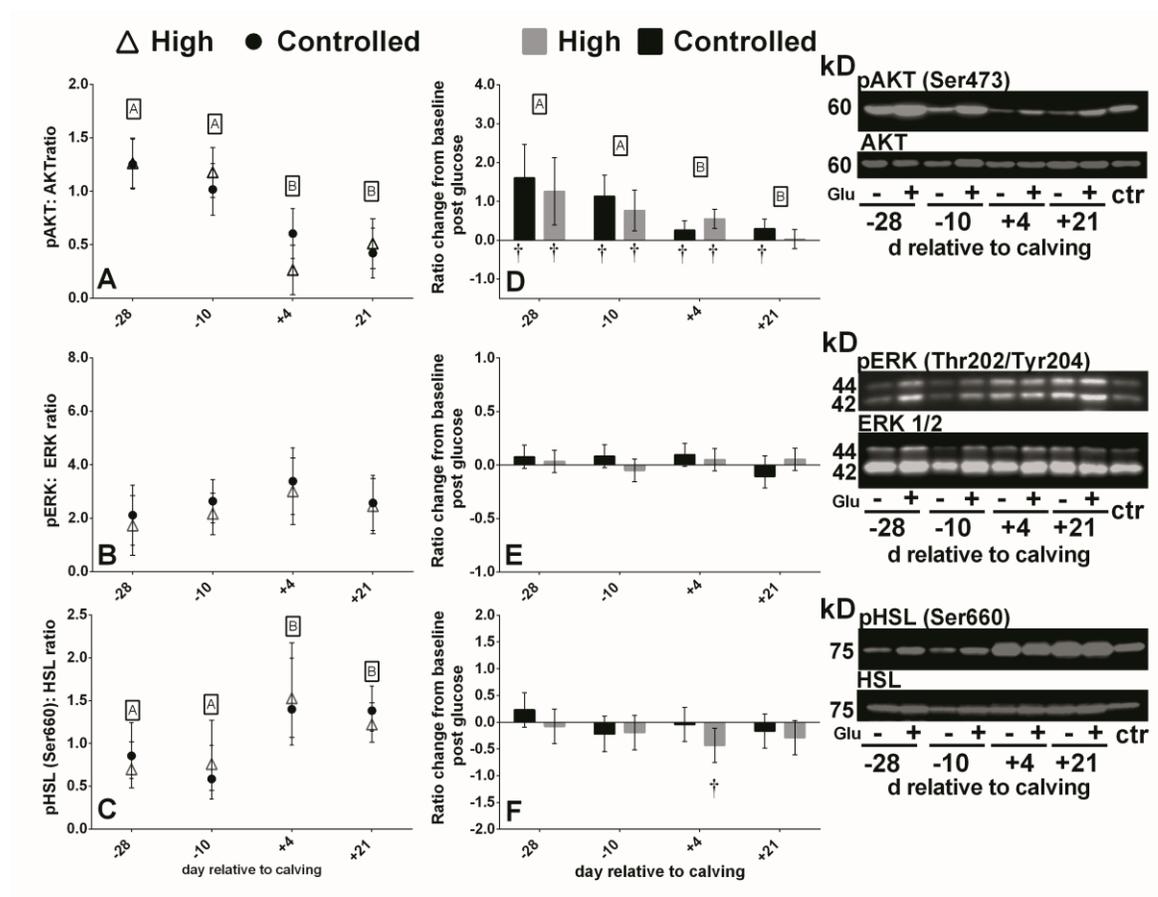


Figure 7.4. Continued

Results of immunoblot densitometry and resulting ratio for phosphorylated proteins of interest before (left panel) and after glucose infusion (middle panel) and representative blots of adipose tissue in the peripartum period (right panel). Least squares geometric means of the ratio of pAKT(Ser473):AKT (**A**), pERK(Thr202/Tyr204):ERK (**B**), and pHSL(Ser660):HSL (**C**) proteins before glucose infusion and difference in the ratio of pAKT(Ser473):AKT (**D**), pERK(Thr202/Tyr204):ERK (**E**), and pHSL(Ser660):HSL (**F**) 60 min after glucose infusion (glu). Results presented as geometric mean and 95% CI. Time-points marked with different superscript letters differ at a level of $P < 0.05$ in repeated measures ANOVA and Tukey's test; † marks difference from baseline is different from 0 ($P < 0.05$). Controlled (n=12): cows fed a controlled energy diet during the prepartum period, supplying approximately 100% of predicted energy requirements and with the low BHB concentrations postpartum. High (n=12): cows fed a high energy diet during the prepartum period, supplying approximately 150% of predicted energy requirements and with high BHB concentrations postpartum. P -values of repeated measures ANOVA: pAKT:AKT: group $P = 0.85$, time $P < 0.001$, group x time $P = 0.02$; pERK:ERK group $P = 0.48$, time $P = 0.15$, group x time $P = 0.97$; pHSL:HSL group $P = 0.97$, time $P = 0.001$, group x time $P = 0.72$; change in pAKT:AKT group $P = 0.48$, time $P = 0.001$, group x time $P = 0.14$; change in pERK:ERK group $P = 0.33$, time $P = 0.55$, group x time $P = 0.62$; change in pHSL660:HSL group $P = 0.20$, time $P = 0.09$, group x time $P = 0.46$.

Figure 7.5. Least squares means of immunoblotting densitometry results for insulin receptor and peroxisome proliferator-activated receptor- γ

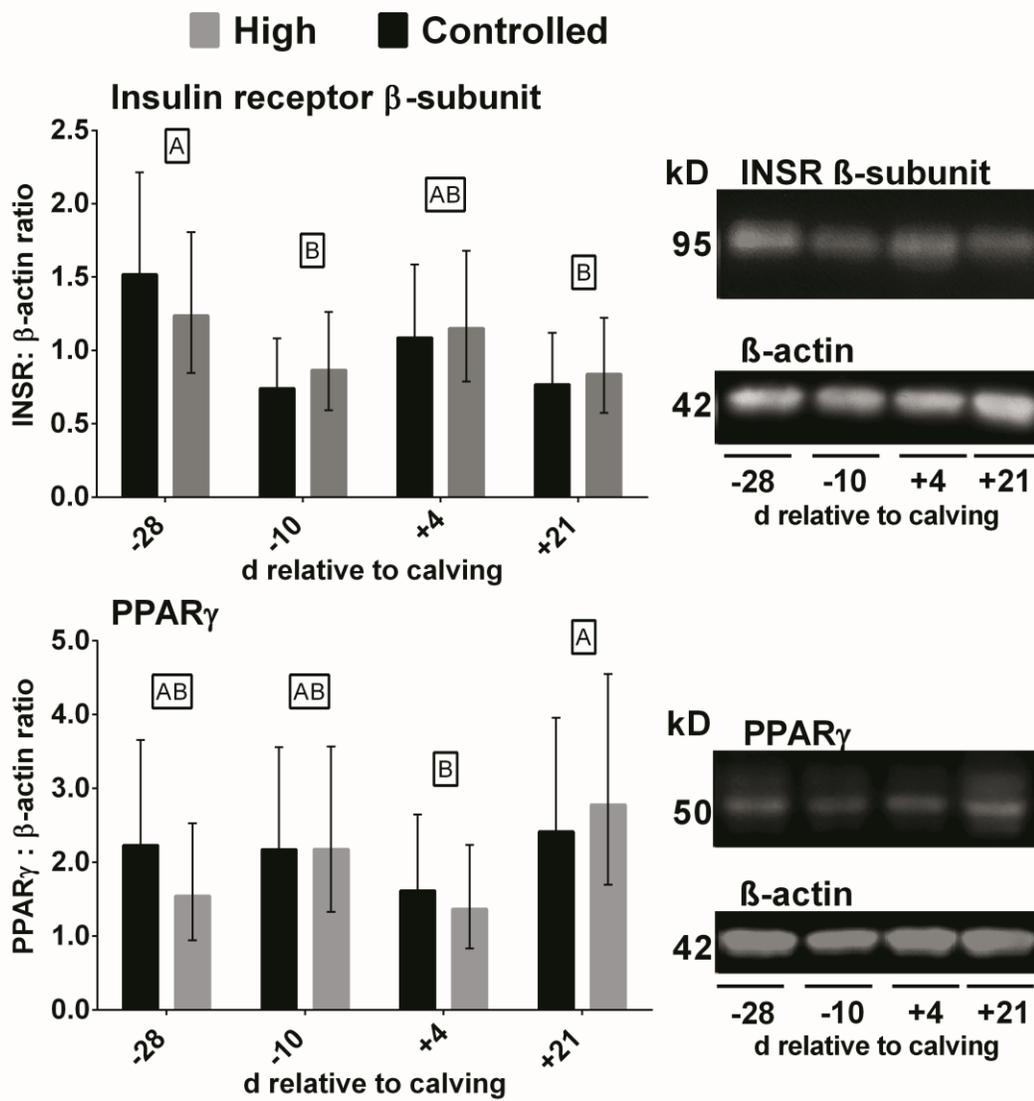


Figure 7.5. Continued

Least squares means of immunoblotting densitometry results for insulin receptor-(INSR)- β (top) and peroxisome proliferator-activated receptor (PPAR)- γ (bottom) as well as representative blots for each protein, β -actin served as loading control. Results presented as geometric mean and 95% CI. Time-points marked with different superscript letters differ at a level of $P < 0.05$ in repeated measures ANOVA and Tukey's test. P -values of repeated measures ANOVA: INSR: group $P = 0.91$, time $P = 0.001$, group x time $P = 0.65$; PPAR γ : group $P = 0.75$, time $P = 0.02$, group x time $P = 0.47$

DISCUSSION

Adipogenesis and lipolysis

Expression of the classic lipogenic gene FASN in cows overfed energy during the dry period was increased compared with cows fed a controlled energy diet. Ji et al. (2012) found a similarly greater expression of FASN in subcutaneous adipose tissue of moderately overfed cows at d 10 prepartum. Upregulation of expression of this gene was also found in subcutaneous adipose tissue of overfed non-pregnant, non-lactating cows compared with those fed a controlled energy diet (Hosseini et al., 2015). This upregulation is likely due to the greater energy balance and blood glucose concentrations in the overfed group (Carra et al., 2013) and led to the numerically larger increase in BFT in the prepartum period in the overfed group. Based on those differences and the greater increase in weight prepartum, it is likely that adipose tissue mass was greater in cows in group H compared with group C despite an absence in differences in BCS. In a recent study, Drackley et al (2014) showed that visceral adipose tissue mass increased due to overfeeding in non-lactating dairy cows compared with those fed a low energy diet with approximately double the mass in omental, mesenteric and perirenal fat, but that BCS was not different at the end of the feeding period.

Phosphorylation of HSL at serine residues 563 and 660 is induced by catecholamines and plays a role in the activation of this enzyme which facilitates triglyceride hydrolysis together with adipocyte TAG lipase (Yeaman et al., 1994; Fruhbeck et al., 2014). Phosphorylation of HSL is increased in the postpartum period

in dairy cattle (Locher et al., 2011). We did not observe a difference in phosphorylation levels of HSL (both Ser563 and Ser660) between the two groups at any time-point despite higher concentrations of serum NEFA during the postpartum period in cows in group H. However, both serine residues had significantly higher phosphorylation ratios postpartum, indicating increased HSL activity on d 4 and 21 postpartum irrespective of prepartum dietary treatment. This corresponds with the increase in β -adrenergic receptor activity in adipose tissue of postpartum dairy cows (Jaster and Wegner, 1981; Bell, 1995). Feeding diets differing in concentrate ratio postpartum did not lead to a detectable effect on HSL phosphorylation ratios of subcutaneous adipose tissue in the study by Locher et al. (2011) but phosphorylation ratios were increased on d 1 and 21 in subcutaneous and retroperitoneal adipose tissue in both groups. Taken together, the study by Locher et al. (2011) and our study found no association between HSL phosphorylation in the peripartum period and dietary energy level either during the pre- or postpartum period.

We also observed a decrease in the total amount of HSL protein after d 28 prepartum in both groups. This finding is in accordance with the results of Locher et al. (2011) who also observed a reduced quantity of this protein in subcutaneous adipose tissue and which they attributed to a possible feedback mechanism due to increased blood NEFA concentrations. We observed the decrease in HSL starting on d 10 prepartum, before a large increase in NEFA concentration took place. This makes a direct feedback regulation in response to increased NEFA concentrations less likely. Locher et al. (2011) hypothesized that BHB might have an inhibitory effect on phosphorylation of HSL at Ser563. However, in the present study, we observed no

difference in the ratio of pHSL (Ser 563) between cows with high and low BHB concentrations postpartum.

Insulin signaling

We found an interaction between group and time at the level of baseline insulin signaling through AKT due to the numerically lower phosphorylation ratio in group H on d 4 postpartum. Based on the correlation between baseline concentrations of insulin and AKT phosphorylation we attributed this to the lower resting blood concentrations of insulin on this d in group H. Zachut et al. (2013) did not find an increase in AKT phosphorylation following a glucose challenge in subcutaneous adipose tissue in 4 out of 8 Holstein cows pre- as well as postpartum and identified these cows as being prone to high weight loss postpartum. All animals were fed the same diet prepartum (1.46 Mcal NEL/ kg DM). The authors concluded that animals with increased body condition loss and increased milk production were suffering from adipose-tissue specific insulin resistance. In the present study the average AKT phosphorylation 60 min after glucose challenge was different from baseline for all groups and sampling time-points except d 21 postpartum in group H. No difference in weight loss was found between animals that did and did not have an increase in the ratio of AKT phosphorylation after glucose challenge in our study. The difference between the study by Zachut et al. (2013) and our own is that all 24 cows in our present study showed an increase in AKT phosphorylation prepartum whereas the 4 cows in the study by Zachut et al. (2013) already exhibited an absence of increase in phosphorylation prepartum. We therefore assume that differences existed between

cows used in these two studies, either due to genetics, body condition, or due to differences in nutrition and management. In addition, we took samples 60 min after glucose infusion whereas in the study by Zachut et al. (2013) samples were taken between 25 and 30 min after glucose infusion. Although maximal insulin concentrations are reached between approximately 7 to 13 minutes in lactating and dry cows, respectively (Mann et al., 2016), the increased timespan that adipose tissue was exposed to high circulating concentrations of insulin in vivo in our study could have resulted in the observed differences.

As reported previously (Mann et al., 2016), neither glucose clearance, insulin response to the glucose challenge, nor reduction in NEFA concentration during the IVGTT were different at any time between the groups. In accordance with this data, Ji et al. (2012) studied the effect of moderate overfeeding during the dry period on peripartal gene expression of adipose tissue insulin signaling, adipogenesis, and lipolysis, and concluded that rather than compromising the insulin signaling pathway in subcutaneous adipose tissue, signaling was increased in overfed cows, particularly in late pregnancy.

No difference was found in ERK phosphorylation ratios over the transition period, following a glucose challenge, or between groups. This could be interpreted either that phosphorylation ratios of ERK do not change detectably after binding of insulin to its receptor, such as after an insulin stimulus in bovine adipose tissue, or that activation of ERK plays a secondary role in insulin-induced signaling compared with AKT.

Changes in insulin receptor abundance have previously been assessed on the level of gene expression in cows fed different energy levels in the dry period (Ji et al., 2012) and in accordance with our study no difference was found between groups overfed energy or fed a controlled energy diet in the dry period. We observed a decrease in the protein amount of the β -subunit of the insulin receptor from d 28 prepartum compared with d 10 prepartum and d 21 postpartum which we interpret as part of the adaptation to lactation and the reduced concentrations of circulating insulin. In contrast to our findings, Ji et al. (Ji et al., 2012) and Sadri et al. (Sadri et al., 2010) did not observe changes of insulin receptor mRNA abundance in the peripartum period. Differences between these and our own study could be due to the choice of sampling time-points and a discrepancy between transcription and translation as well as post-translational modifications.

PPAR γ , Adiponectin and Markers of Inflammation

A central regulator of adipocyte biology and energy homeostasis is the transcription factor PPAR γ which induces adipogenic gene expression, remodeling of adipose tissue, and adipokine secretion (Astapova and Leff, 2012). In accordance with the results of Selim et al. (2014) who measured expression of PPARG in subcutaneous adipose tissue, no difference between groups fed a high or controlled energy diet in the dry period was observed for PPAR γ . In contrast, Ji et al. (2012) found a greater expression of PPARG in subcutaneous adipose tissue of moderately overfed cows at d 10 prepartum and d 7 postpartum. The amount of PPAR γ was lower early postpartum compared with 21 d postpartum in both groups in the current study. Saremi et al.

(2014) measured the expression of PPARG over time in subcutaneous adipose tissue and liver of two groups of cows (fed conjugated linoleic acid or a control diet after calving) and found a decrease in expression of PPARG in both tissues shortly after parturition, followed by an increase after the first sampling time-point. Since activation of PPAR γ increases glucose uptake into adipose tissue (Ahmadian et al., 2013), the decrease in expression might reflect the reduced availability of glucose during this time. Fasting dramatically decreased the amount of PPARG in adipose tissue in mice (Vidal-Puig et al., 1996).

We observed an interaction between group and time for expression of ADIPOQ with a decrease of expression in the controlled energy group after d 28 prepartum compared with the high energy group. A tendency for greater gene expression of ADIPOQ in cows overfed energy during the dry period has previously been observed by Ji et al. (2012) and the difference was greatest on d -10 and 7 relative to parturition which corresponds to the time-points when we observed differences in the present study. The authors of the study by Ji et al. (2012) hypothesized that this difference might have been driven by the greater expression of PPARG in their study and allowed the adipose tissue to retain insulin sensitivity despite overfeeding of energy. An upregulation of ADIPOQ in the absence of concurrent increase in PPARG in subcutaneous adipose tissue of non-pregnant, non-lactating cows was reported recently by Hosseini et al. (2015), but the authors observed a numerical increase in protein expression of PPAR γ in the overfed group. In contrast, our study did identify a difference between ADIPOQ, but not PPAR γ , between the treatment groups as described above. This difference could have been due

to the fact that we semi-quantitatively determined PPAR γ protein expression whereas Ji et al. (2012) measured difference on the level of gene expression. In a study investigating the change of mRNA expression in subcutaneous adipose tissue in high-yielding dairy cows, Lemor et al. (2009) did not observe changes of ADIPOQ between the week prepartum to 3 weeks postpartum. In contrast, ADIPOQ expression in subcutaneous adipose tissue of cows fed a controlled energy diet during the dry period was reduced from 28 d prepartum to 21 d postpartum and was significantly lower than that of the high energy group in our study. Differences between our study and the findings of Lemor et al. (2009) could be due to the fact that we had one additional time-point (28 d prepartum) before down-regulation of mRNA expression occurred. Furthermore differences could have been due to prepartum dietary energy level which was not reported in the study by Lemor et al. (2009) and could have resembled the high energy group in our study rather than the controlled energy group where we observed a reduction in expression. In accordance with our results, Giesy et al. (2012) found no reduction of mRNA expression in subcutaneous adipose tissue from late pregnancy to early lactation in cows fed a high energy TMR (1.63 Mcal NEL/kg DM) ad libitum during the dry period. However, circulating blood concentrations were decreased in the immediate peripartum period which is in accordance with the results reported by others (Mielenz et al., 2013; Kabara et al., 2014; Singh, 2014). Hosseini et al, (2015) observed that gene expression of ADIPOQ in subcutaneous adipose tissue did not correlate with blood concentrations. Taken together these studies suggest that cows fed a high energy diet prepartum might not experience the same degree of down-regulation of ADIPOQ expression as cows fed a controlled energy diet, but that

circulating concentrations in blood may still decrease in the immediate postpartum period.

In human and rodent models, excess energy intake and obesity are associated with a proinflammatory state of the adipose tissue which is thought to play a role in the development of metabolic dysfunction and insulin resistance (Bai and Sun, 2015). In humans, hypertrophic adipocytes secrete low levels of TNF α and the stromal vascular fraction of adipose tissue increases expression of CCL2/MCP1, leading to infiltration of macrophages and a low-grade inflammatory status of the tissue (Capurso and Capurso, 2012). The results of our study showed that expression of adipose tissue CCL2 /MCP1 was not different between groups suggesting that a high prepartal plane of energy, increased rate of mobilization of NEFA and high concentrations of BHB did not affect the production of this chemokine compared with the controlled energy group. However, the presence of inflammatory cells in adipose tissue biopsies in the present study was not measured directly. Akter et al. (2012) assessed the proportion of infiltrating macrophages of various adipose tissue locations (subcutaneous and visceral) in early lactation Holstein cows as well as nonpregnant, nonlactating Holstein heifers by immunohistochemistry and concluded that the extent of obesity in early lactation did not contribute to increased infiltration of phagocytic cells and that the overall proportion of samples with phagocytic immune cell infiltration was low in cows in early lactation.

In accordance with our study, Ji et al. (2014a) did not observe a difference in the expression of TNF α between nonpregnant nonlactating Holstein cows overfed energy or fed a controlled energy diet and concluded that overfeeding did not induce

an overt inflammatory response in adipose tissue. Selim et al. (Selim et al., 2014) measured expression of TNF α in subcutaneous adipose tissue of cows either overfed energy or fed a controlled energy diet for the last 6 wk prepartum and found no effect of energy level throughout the transition period. Saremi et al (2014) found only a weak or no correlation between expression of TNF α in adipose tissue and blood concentration of NEFA, BHB, as well as BFT and BCS. Together these studies suggest that the degree of metabolic changes that is caused by overfeeding during the dry period did not produce a proinflammatory response as is observed in obese rodents and humans. A limitation of our study is that we measured the expression of TNF α in subcutaneous adipose tissue only and that expression of this gene might differ from visceral adipose tissue, potentially underestimating an effect of overfeeding and increased lipolysis on adipose tissue inflammation. Two recent studies however found expression of TNF α to be greater or equally as large in subcutaneous compared with mesenteric, omental, or retriaperitoneal (Saremi et al., 2014), or greater than in both mesenteric and omental adipose tissue (Ji et al., 2014b).

CONCLUSION

Cows that were overfed energy during the dry period and exhibited high concentrations of BHB postpartum showed a greater degree of lipogenesis prepartum whereas there were no differences in lipolytic enzyme activity compared with cows fed a controlled energy diet prepartum that had low concentrations of BHB postpartum. Baseline insulin concentrations were decreased and NEFA concentrations

increased postpartum in the overfed group. Baseline insulin signaling through AKT was lower in the overfed group on d 4 postpartum but no difference existed in the ability to activate AKT phosphorylation following glucose challenge, or in the quantity of insulin receptor, markers of inflammation and PPAR γ in adipose tissue between the groups. Expression of adiponectin was increased in the immediate peripartum period in the overfed group. Based on these findings we conclude that overfeeding energy during the dry period and having high BHB concentrations postpartum does not alter the response to a glucose-induced endogenous insulin stimulus in subcutaneous adipose tissue compared with cows fed a controlled energy diet and having low BHB concentrations postpartum.

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CHAPTER 8

OVERALL CONCLUSIONS AND FUTURE DIRECTIONS

Dairy cows undergo tremendous metabolic changes as they transition from the pregnant, non-lactating period into early lactation when nutrient demands increase substantially. Nutritional management during the dry period plays a fundamental role in the prevention of excessive negative energy balance (NEB) during this time and thereby can help prevent the risk for early lactation negative health. This research explored the effect of different feeding strategies on energy balance in the far-off, close-up and fresh period, on colostrum quality, and on milk production. Furthermore, glucose tolerance, as well as adipose and muscle tissue insulin signaling were investigated.

OVERALL CONCLUSIONS

The first objective was to evaluate the effect of three different dry period feeding strategies on energy balance and colostrum and milk production. Cows that were fed a controlled energy diet during the dry period had equal milk yields in early lactation compared with cows overfed energy either during the close-up period alone or during the entire dry period but tended to have an improved energy balance during the first six weeks postpartum. Concentrations of markers of negative energy balance (NEFA and BHB) in the postpartum period were lower in cows fed a controlled

energy diet prepartum and colostral IgG concentration was increased, whereas insulin concentrations were highest in colostrum of overfed cows.

When evaluating the effect of different feeding strategies on systemic glucose tolerance and insulin response to an intravenous glucose bolus, no differences were found in the ability to respond to this challenge between cows fed a controlled energy diet or those overfed energy in the dry period. Dry period plane of energy was associated with changes in resting concentrations of glucose, insulin, and glucagon in the postpartum period. Cows fed a controlled energy diet prepartum had higher concentrations of glucose and insulin, as well as lower glucagon concentrations immediately postpartum.

The third objective was to evaluate the effect of different feeding strategies on adipose and muscle tissue accretion and loss as well as tissue-specific insulin signaling. Cows overfed energy had a higher rate of muscle and adipose tissue accretion during positive energy balance, and a higher rate of loss of both tissues postpartum. However, this was not associated with a change in insulin signaling, mechanisms of muscle atrophy or regulation of lipolysis.

When regarding all of these findings together, feeding a controlled energy diet had beneficial effects on peripartum metabolic adaptations without affecting early lactation milk yield. Changes in insulin signaling were unable to explain the observed differences in postpartum concentrations of NEFA and BHB and the collective data of this work does not imply that a change in insulin sensitivity on a whole body or tissue level is the underlying cause. However, higher concentrations of resting glucose during the time of the most precarious glucose supply can only be regarded as a more

stable glucose balance and may be directly associated with the higher insulin concentrations postpartum. Both are beneficial to limit the degree of adipose and lean muscle tissue loss because mechanisms of lipolysis and muscle atrophy are intricately linked with glucose and insulin availability. With resting glucose concentrations being higher in the controlled energy prepartum diet group, this either means an increased influx/production (in the postpartum dairy cow almost exclusively by hepatic gluconeogenesis) into the circulatory system on a whole body basis, or a decreased efflux/uptake into the periphery and therefore out of the bloodstream. No differences in the rate of clearance of glucose from the blood were detected between cows overfed energy and those fed a controlled energy diet. Furthermore, no difference in milk production was observed and it is thus unlikely that a larger amount of glucose was taken up by the mammary gland for the synthesis of lactose in those cows that had lower glucose concentrations in blood. This means that based on the data presented here, there is no indication for a greater mass of glucose exiting the available pool. It is therefore possible that the observed differences may be interpreted as a differentially regulated gluconeogenic capacity in cows fed different planes of energy.

In order to fill the requirements in light of less available *de novo* glucose, alternate fuels from lipolysis and muscle breakdown may fill some of this nutrient gap and an increase in the concentrations of NEFA and BHB would happen concurrently. However, in the absence of data on gluconeogenic capacity in the cows in this study the contribution of such a change in hepatic gluconeogenic capacity remains to be determined.

FUTURE DIRECTIONS

Following the conclusions I could draw from my dissertation, one future direction of research is to elucidate the effect of prepartum overfeeding on hepatic gluconeogenic capacity in the postpartum period. This work may have to involve measurement of net liver nutrient flow as a direct assessment of hepatic release using sophisticated splanchnic catheterization techniques in cows either overfed energy or fed a controlled energy diet. Such work, albeit a daunting task for most research groups, would provide information beyond what is currently known in the literature.

Another area that future research can address is how the changes in glucose availability affect other tissues besides adipose and muscle tissue. I am thinking particularly about the extension of some of the findings of this work to the immune system. Energy status plays a central role in immune cell proliferation and differentiation and the regulation of inflammatory status in other species. Much interest exists in learning more about the inflammatory state of the transition cow and which aspects are responsible for some animals to be able to transition through this phase with a physiologically adequate inflammatory response and others being unable to do so. Amino acid status, glucose and insulin concentrations among others partake in this regulation and this certainly will be an exciting area of research in the next future.

As we learn more about the role of colostrum beyond conferring transfer of passive immunity the effect of dry cow feeding strategies on colostrum composition will be very interesting to pursue. This work showed that prepartum energy level affects not only IgG concentration, but also fatty acid composition and insulin

concentration. It can be speculated that other components of colostrum, such as growth factors, cytokines and lipid mediators might be differentially regulated depending on the dam's energy status as well.

Research in transition cow metabolism and prevention strategies will remain an exciting and challenging area that will require the synthesis of a number of research fields to move forward. These will include the traditional animal and veterinary sciences, but also molecular biology and genetic methods, particularly to address the variability in responses to different nutritional strategies and prevention efforts. I hope that this dissertation and my future work can add substantial findings to the body of knowledge in this area to help prevent disease of transition dairy cows to improve their wellbeing and the lives of those who care for them.