The Effect of Varying Levels of Fiber on Inflammation, Hepatic Metabolism, and Regulation of the Growth Hormone-Insulin-like Growth Factor One Axis in Transition Dairy Cows

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Abstract

Fiber content of the post calving diet may have implications for both the energetic and inflammatory status of early lactation dairy cows. The objective of my thesis was to specifically examine the effect of high fiber dietary intervention on hepatic metabolism, namely hepatic gene expression of insulin-like growth factor one (IGF-I), growth hormone receptor (GHR), tumor necrosis factor alpha (TNFα), and TNFα receptor (TNFRSF1a) in lactating cows, with the goal of minimizing NEBAL and systemic inflammation during the early postpartum period. While examining the effect of diet on hepatic gene expression, I also wanted to analyze the effect of genotype and other information collected throughout the study related to energy balance, metabolism, and liver health on the hepatic expression of these genes. Multiparous Holstein cows (n=40) were fed a common prepartum ration and assigned randomly at calving to one of two postpartum diets differing in fiber content; high fiber (n=19; 35.3% NDF[1], 12.2% $uNDF_{240}$[2], 23.2% peNDF[3]) and low fiber (n=21; 32.8% NDF, 9.5% $uNDF_{240}$, 21.6% peNDF). Liver biopsies and blood samples were obtained on d 7 and d 14 postpartum. Liver tissue was analyzed for mRNA expression of IGF-I, GHR, TNFα, and TNFRSF1a by RT-qPCR. Blood samples were used for plasma metabolic profile analysis as well as to DNA genotype each cow for IGF-I, GHR, and TNFα single nucleotide polymorphisms (SNPs) by PCR-restriction fragment length methods. Data were analyzed by repeated measures ANOVA with fixed effects of dietary treatment,

[1] Neutral Detergent Fiber
[2] Undigested Neutral Detergent Fiber; remaining fiber after 240h in vitro digestion
[3] Physically effective Neutral Detergent Fiber; above 4mm screen when using Penn State Particle Separator

genotype for all genes, time, parity group, and all interactions. Correlations were computed between gene expression and 19 other variables related to production, metabolism and health. There were no effects of dietary treatment on hepatic gene expression of IGF-I, GHR, TNFα, or TNFRSF1a. Trends ($P<0.15$) towards differences in gene expression based on genotype were present, such that GHR genotype AB cows had higher IGF-I and GHR expression, and TNFα genotype AB cows had lower IGF-I expression. Hepatic expression of IGF-I and GHR was correlated positively ($P<0.05$) with variables related to increased energy balance and improved liver health. TNFα and TNFRSF1a correlated ($P<0.05$) positively with markers associated with decreased liver health. While there was no difference in hepatic gene expression of IGF-I, GHR, TNFα, or TNFRSF1a between dietary treatments, genotype appears to influence hepatic gene expression of IGF-I and GHR.

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Introduction

The transition period, defined as 3 weeks before to 3 weeks after calving (Grummer, 1995), is a time of major metabolic adaptation for the dairy cow. Transitioning from late pregnancy to early lactation is associated with a large increase in the demands for glucose, amino acids, and fatty acids in order to support the energetic costs of milk production (Bell 1995). Although a cow's dry matter intake (DMI) increases postpartum, it does not increase quickly enough to meet the demands for rising milk production, leaving her in a prolonged state of negative energy balance (NEBAL) in early lactation (Bell, 1995; Butler et al., 2003).

Negative energy balance is characterized by decreased blood glucose and insulin concentrations with elevated blood growth hormone (GH) concentrations, and this relationship between blood glucose, insulin, and GH forms the basis of the GH-IGF-I axis (Butler et al., 2003). Under positive energy balance in times of normal insulin, GH would bind to hepatic growth hormone receptor (GHR). This results in the production of insulin-like growth factor (IGF-I), a polypeptide that provides negative feedback for GH. The ability of IGF-I to decrease circulating GH concentrations, as well as bind to insulin receptors, enhances insulin sensitivity (Yakar et al., 2001). During NEBAL, the GH-IGF-I axis is uncoupled; plasma insulin levels are low, which lowers hepatic GHR concentrations and renders the liver resistant to circulating GH (Butler et al., 2003) resulting in lower IGF-I production. Decreased plasma insulin concentrations (Butler et al., 2003) and decreased insulin sensitivity due to lower IGF-I production (Yakar et al., 2001) promotes efficient glucose utilization by the non-insulin sensitive mammary gland as opposed to glucose being utilized by peripheral tissues (Bell and Bauman 1997). Lower circulating insulin and IGF-I with higher circulating GH concentrations also promotes adipose tissue mobilization,

increasing release of non-esterified fatty acids (NEFA), which help support the energy requirements of lactation (Butler et al., 2003).

In addition to NEBAL, systemic inflammation is another common characteristic of the transition period in dairy cattle. Inflammation frequently begins in the uterus during parturition, but can also affect other tissues such as the liver and adipose tissue (Loor et al., 2005; Bradford et al., 2015), instigating the acute phase response. This causes the pro-inflammatory cytokine tumor necrosis factor alpha (TNFα), produced by macrophages and mast cells in response to inflammatory stimuli, to induce the production and release of liver acute phase proteins (APP; Paltrinieri, 2008; Bannerman et al., 2009; Cray et al., 2009). Whereas some degree of inflammation is necessary to repair tissue damage from parturition and fight disease, prolonged or highly elevated levels of APP, such as haptoglobin, during this early lactation period have been shown to negatively affect animal health and productivity (Huzzey et al., 2009 and 2015).

Improving feeding and management strategies to support the metabolic adaptations in transition cows are critical steps to increase the productivity and profitability of a dairy through minimizing economic loss due to subclinical and clinical metabolic disorders (Overton and Waldron, 2004). Feeding higher starch diets is a common strategy to maximize energy intake, but may cause inflammation in the rumen due to a low rumen pH (Plaizer et al., 2007; Williams et al., 2015). One possible method of preventing this low rumen pH is to feed a high starch diet in combination with high fiber. Increased fiber intake could help play a role in keeping cows healthy by maintaining a healthy rumen, decreasing systemic inflammation, and improving metabolic status when fed in conjunction with a high starch diet (McCarthy et al., 2014). The objective of my thesis was to specifically examine the effect of feeding high fiber diets in combination with high starch on hepatic metabolism, namely hepatic gene expression of IGF-I, GHR, TNFα, and

TNFα receptor (TNFRSF1a) in lactating cows, with the goal of minimizing NEBAL and systemic inflammation during the early postpartum period. Since genotype data was available from a previous trial, I also wanted to analyze the effect of IGF-I, GHR, and TNFα genotype on hepatic gene expression. Finally, I wanted to determine if there was any significant correlation between hepatic gene expression and other variables related to energy balance, metabolism, and liver health.

Review of the Literature

Introduction

For decades, nutrients have traditionally been defined as fuel, building blocks, and enzymatic cofactors (Bradford, 2018). This understanding led to the construction of various determination systems to assess the nutrient requirements of livestock (Thornton, 2010), including the National Research Council's (NRC) first nutrient requirement tables for farm and laboratory animals published in 1944. These guidelines have been subsequently updated when deemed necessary, and used as the standard for animal diet formulation around the globe (Coffey et al., 2016). While this method of feeding livestock has certainly been beneficial, it fails to reflect the full impact of nutrients within an animal's body. Nutrients are not only basic fuels but also bioactive signals that exert direct effects on cellular function, chromatin structure, and overall animal physiology (Bradford, 2018). This occurs through interactions not merely within a cow's digestive system but throughout every system in her body.

At this point in time, all nutrient classes have been deemed capable to signal in an endocrine-like manner, resulting in a much more complex endocrine network than previously understood (Bradford, 2018). This network includes hormones released by adipose tissue, liver, muscle, and even bone to regulate appetite, nutrient partitioning, and nutrient mobilization. The liver particularly plays an essential role in the coordination of nutrient partitioning, allowing for the adjustment of metabolism to supply the needs of other tissues throughout the body (Drackley et al., 2001). The biggest impact of these discoveries lies in the potential to improve feeding strategies for physiologically stressed animals, including those that are heat-stressed, transitioning to lactation, or ill, to improve animal production and resilience (Bradford 2018). The following review of the literature will focus on the metabolic and physiological adaptations associated with the transition to lactation for dairy cattle, specifically in regards to hepatic metabolism of glucose, insulin, and GH during NEBAL. It will also review current and potential nutritional strategies to address and minimize the effects of NEBAL and systemic inflammation during the early postpartum period.

The Transition to Lactation

Milk production per cow in the United States over the last 100 years has steadily increased, mainly due to continuous improvements in nutritional management and genetic selection (VandeHaar and St-Pierre 2006). This trend is only expected to continue; in the last ten years alone, the average production per cow has increased 13%, leading the average cow to produce over 23,000 pounds of milk per year (USDA-NASS, 2019). While this drastic increase in production is

a huge accomplishment for dairy producers, it does not come without its challenges, especially during the transition to lactation.

The transition period, defined as 3 weeks before to 3 weeks after calving (Grummer, 1995), is a time of major metabolic adaptations throughout a cow's body. Predicted demands for glucose, amino acids, and fatty acids by the lactating mammary gland at four days in milk (DIM) are about 2.7, 2.0, and 4.5 times those of the gravid uterus during late pregnancy (Bell, 1995). A cow's dry matter intake (DMI) increases postpartum, but is insufficient to meet the immediate energetic costs of milk production, which leaves her in a prolonged state of NEBAL in early lactation (Bell, 1995; Butler et al, 2003).

Negative energy balance is characterized by extensive mobilization of stored adipose reserves into circulation as NEFA to meet the increased demand for nutrients (Morris et al., 2009; Ospina, 2010). These NEFA can be fully or partially oxidized in the liver (Newman and Verdin, 2017), stored as liver triglycerides (Gruenberg, 2019), used for milk fat synthesis, or used as an alternative fuel source for muscle tissues in times of low blood glucose (Drackley et al., 2001). Some hepatic lipid accumulation during the transition period is a normal adaptation to lactation (Grum et al., 1996), but excessive triglyceride accumulation in the liver has been demonstrated to impair hepatic metabolism (Strang et al., 1998).

Fatty acids that are partially oxidized in the liver can be released into circulation as beta-hydroxybutyrate (BHB) or other ketone bodies as energy sources for peripheral tissues when glucose supply is too low to meet energy needs (Newman and Verdin, 2017). Incomplete hepatic oxidation of NEFA can lead to an increase in reactive oxygen species (ROS) and oxidative stress, which can disrupt normal metabolism and can lead to immunosuppression in the already

compromised animal (Morris et al., 2009). High levels of circulating NEFA, BHB, as well as hepatic triglycerides characterizes ketosis (Xiliang et al., 2017), a metabolic disease common to the transition period. Given this increase of ROS and oxidative stress in combination with insufficient DMI, elevated circulating NEFA increases the risk for a number of other transition diseases as well, including displaced abomasum, retained placenta, and metritis (Ospina et al., 2010).

Energy Metabolism in the Transition Cow

Ruminants are obligate herbivores that use pregastric fermentative digestion to utilize cellulose and other fibrous feed components. During the process of microbial fermentation, rumen microbes consume much of the readily digestible nutrients in a cow's diet and produce volatile fatty acids (VFA), such as propionate, as a byproduct of pregastric fermentation (Bell and Bauman, 1997). These VFA are absorbed by the ruminal epithelium into portal venous blood and removed by the liver for metabolism (Elliot, 1980; Brockman, 1993). Hepatic supply of propionate is a principal determinant of hepatic glucose synthesis (Brockman, 1993). This means that there is a strong correlation between digestible energy intake and whole-body glucose production in the dairy cow. Moreover, the dairy cow depends almost entirely on hepatic gluconeogenesis from the byproducts of pregastric fermentation to meet tissue glucose requirements (Bell and Bauman 1997; Elliot, 1980; Brockman, 1993).

Requirements for glucose increase by 2.7 times after calving compared to late pregnancy (Bell, 1995), and the source of this demand shifts location from the gravid uterus to the mammary gland (Bell, 1995; Bell and Bauman, 1997). This shift in nutrient demand is largely due to the

particularly high glucose demand of the mammary gland to synthesize lactose, the main osmotic determinant of milk volume (Bell and Bauman, 1997). The liver must therefore adapt quickly to provide the increased glucose requirements for milk production, and to process high concentrations of NEFAs from adipose tissue mobilization during NEBAL (Drackley et al., 2001). To meet this high demand for glucose, hepatic gluconeogenesis must increase despite the lack of substantial increase in DMI (Paterson and Linzell, 1974; Bauman and Elliot, 1983). The difference between glucose from digestible energy intake and the cow's glucose demand must be made up from non-carbohydrate sources. These include intestinally absorbed amino acids and endogenous substrates such as amino acids, lactate, and glycerol (Drackley et al., 2001). All amino acids except for leucine and lysine are able to contribute to gluconeogenesis, but alanine and glutamine are the greatest contributors. Most fatty acids, on the other hand, are not able to be directly used for glucose synthesis. Instead, they oxidize long chain fatty acids such as NEFAs to ketone bodies, which provide ATP needed for gluconeogenesis (Drackley et al., 2001). The mammary gland's high demand for glucose, combined with a variety of limited sources of glucose and energy, makes proper nutrient partitioning critical to ensure that glucose produced by the liver is utilized primarily by the mammary gland and not peripheral tissues.

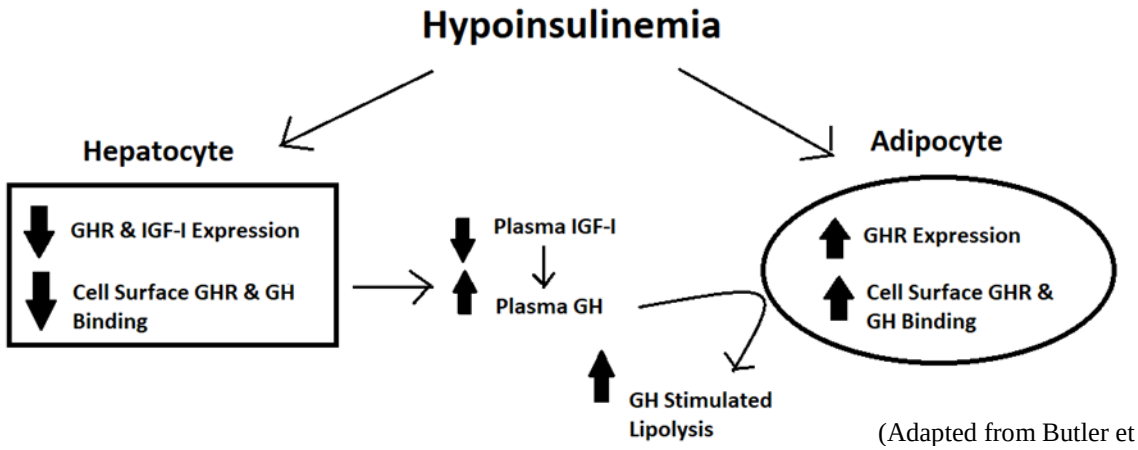
Endocrine Control of Nutrient Partitioning

Insulin is a key mediator of nutrient partitioning, helping to maintain metabolic equilibrium during variations in nutrient supply and demand. Plasma insulin levels specifically play a role in the mediation of chronic metabolic adaptations to pregnancy and lactation, including altering the hepatic production and peripheral utilization of glucose (Bell and Bauman, 1997). Lowering

plasma insulin levels around parturition is a metabolic tactic employed by the high producing dairy cow as a response to low blood glucose levels. This allows for the reduction of glucose uptake by insulin-responsive peripheral tissues, such as adipose tissue and muscle, in order to facilitate greater uptake of glucose by the non-insulin responsive mammary gland (Bauman and Elliot, 1983). Growth hormone is also key during this period, as it plays a role in altering tissue responses to insulin, providing further regulation of glucose utilization (Bell and Bauman, 1997). The coordination of low insulin, high GH, and low glucose is a characteristic of postpartum NEBAL (Butler et al., 2003). In general, these homeorhetic adaptations persist throughout early lactation, but decrease as voluntary feed intake increases several weeks after peak milk yield (Bell and Bauman, 1997), placing the cow once again in a positive energy balance.

A primary response to circulating GH is the production of IGF-I, a polypeptide produced primarily by the liver when GH binds to GHR on the surface of hepatocytes (Yakar et al., 2002; Butler et al., 2003; Jones and Clemmons, 1995). IGF-I has multiple biological effects, including regulating the metabolism of peripheral tissues (White, 2014) and enhancing insulin sensitivity (Yakar et al., 2001). Hepatic expression of IGF-I and GHR is therefore acutely responsive to the cow's nutritional status (Bornfeldt et al., 1989; Pell et al., 1993) and physiological state (Kobayashi et al., 1999). During NEBAL, plasma insulin levels are lowered, which lowers hepatic GHR concentrations and renders the liver resistant to circulating GH. As a result, hepatic IGF-I production is low despite high GH concentrations, uncoupling the GH-IGF-I axis (Figure 1; Butler et al., 2003). Decreased plasma insulin (Butler et al., 2003) and decreased insulin sensitivity due to lower IGF-I production (Yakar et al., 2001) allows for glucose uptake to be dominated by the noninsulin-sensitive mammary gland instead of insulin-sensitive peripheral tissues (Bell and Bauman, 1997).

Adipose tissue has the opposite response to hypoinsulinemia when compared to a hepatocyte; GHR expression is up-regulated, which results in GH-stimulated adipose tissue mobilization (Figure 1). High levels of circulating GH and low levels of insulin are common in early lactation cows in NEBAL, and these conditions favor adipose tissue mobilization in support of milk production (Butler et al., 2003). Mobilizing greater amounts of body reserves in early lactation increases the release of NEFA into circulation, placing cows at greater risk of transition diseases (Ospina et al. 2010).



(Adapted from Butler et al., 2003)

Figure 1. The effect of low plasma insulin in liver and adipose tissue. Low plasma insulin causes a reduction of cell surface growth hormone receptors (GHR), which reduces insulin-like growth factor (IGF-I) expression. Plasma IGF-I levels are reduced and plasma GH is raised as a result of this "GH-resistance" of the liver. Hypoinsulinemia has the opposite effect on adipose tissue, increasing GHR expression and the number of cell surface GHR, which encourages GH-stimulated lipolysis.

Systemic Inflammation during the Transition Period

In addition to NEBAL, systemic inflammation is another characteristic of the transition period in response to infection, tissue injury, and other stressors (Bradford et al., 2015). The

proinflammatory cytokine TNFα, which is produced by macrophages and mast cells during acute inflammation, is one of the most important cytokines that trigger the acute phase response as a reaction to inflammatory stimuli (Moshage, 1997; Bannerman et al., 2009). Immune system activation is an energetically demanding process, requiring a reallocation of nutrients from growth and productive functions (Bradford et al., 2015). Studies have associated the postpartum acute-phase response with negative lactation outcomes, and have also shown a delayed resolution of inflammation in cows with greater peak concentrations of inflammatory markers (Bionaz et al., 2007). It is therefore in the best interest of producers to minimize inflammation to not only be cognizant of animal welfare, but to increase the productive profitability of each animal transitioning to lactation.

Pathogen recognition from infections such as metritis and mastitis are among the most noticeable causes of inflammation during this transition period. Although there is a high incidence of morbidity during the first few weeks of lactation, a number of studies have shown that inflammatory and positive acute phase mediators are elevated in the days following parturition, even in the absence of disease (Bionaz et al., 2007; Huzzey et al., 2009; Graugnard et al., 2012; Mullins et al., 2012; Akbar et al., 2015 from Bradford 2015). This non-infectious inflammation can be a response to uterine damage during parturition, hepatic oxidative stress, or the need for adipose tissue mobilization during NEBAL.

Tissue damage in the endometrium and cervix during parturition is often the beginning of postpartum inflammation (Bradford et al., 2015; Bromfield et al., 2015). Even healthy animals experience some degree of inflammation to repair uterine tissue, which can lead to a systemic inflammatory condition postcalving (Bradford et al., 2015). This pattern of systemic inflammation from uterine injury has been documented in humans following parturition as well (Elliot et al.,

2001), and can affect the inflammatory status of other tissues throughout the body (Bradford et al., 2015). In addition to inflammation due to tissue repair, nearly half of all postpartum dairy cattle experience further inflammation from clinical metritis or endometritis (Bromfield et al., 2015).

The liver is another major tissue that experiences inflammation during the transition period. TNFα is an important pro-inflammatory cytokine in the liver, and has been shown to increase in hepatic expression for weeks leading up to and following parturition (Loor et al., 2005). Loor et al. (2005), showed a significant increase in hepatic TNFα expression from day -65 to day 1 relative to parturition, and another significant increase on day 49 compared to all previous time points. The acute phase response instigated by TNFα results in altered hepatic protein production; negative APP are those such as albumin that decrease in production during the acute phase response, while positive APP are those such as haptoglobin that are produced in greater quantities during the acute phase response. (Cray et al., 2009; Paltrinieri, 2008; Bertoni and Trevesi 2013). These APP perform many physiological functions within the immune system. Haptoglobin, for example, binds with high affinity to free hemoglobin, inhibiting toxic oxidative activity (Schaer et al., 2014). Whereas some degree of inflammation and APP production is necessary to repair tissues damage from parturition and to fight disease (Huzzey et al., 2009), elevated (>1 g/L) levels of haptoglobin were associated with 464 kg less milk during lactation and a twenty percent decrease in pregnancy risk during the first 150 days in milk (Huzzey et al., 2015).

Adipose tissue transcription of pro-inflammatory cytokines, such as TNFα, are upregulated during the transition period as well. Adipose TNFα transcription peaks when the surge in adipose tissue lipolysis, measured by free fatty acids in circulation, is observed (Sadri et al., 2009; Schmitt et al., 2011; Mann et al., 2016). Loor et al. (2005) showed that increased hepatic expression of TNFα and APP is positively correlated with lipid mobilization from adipose tissue (increased

plasma NEFA) and fatty acid oxidation (increased plasma BHB). While there is still significant work to be done in understanding the relationship between hepatic and adipose tissue TNFα expression, these correlations further support the combined challenge of NEBAL and systemic inflammation during the transition period.

Evidence from a variety of species indicates that TNFα not only affects systemic inflammation, but can alter the GH-IGF-I axis as well (Briard et al., 2000; Kushibiki et al., 2003; Blum et al., 2018). Pro-inflammatory cytokines, such as TNFα, are known to disrupt the GH-IGF-I axis in humans (Blum et al., 2018). This disruption comes from the interference of circulating IGF-I production, resulting in hepatic GH resistance (Zhao et al., 2014 Blum 2018 ref 22), and from cytokine interference with the action of locally produced IGF-I (Choukair et al., 2014). Briard et al. (2000) suggests that the acute phase response may combine with hypoinsulinemia in altering the GH-IGF-I axis in ruminants as well. Lipopolysaccharide (LPS) challenge induced a rapid increase in plasma concentration of inflammatory cytokines, including TNFα, resulting in increased circulating GH concentrations and decreased circulating IGF-I concentrations in sheep. Specifically in lactating dairy cattle, recombinant TNFα administered subcutaneously has been shown to decrease feed intake (Kushibiki et al., 2003). This places cows into greater NEBAL and can further uncouple the GH-IGF-I axis.

Dietary Interventions

Improving feeding strategies for physiologically stressed animals in the transition period is one of the most critical steps forward in increasing the productivity and profitability of a dairy. Complications during the transition period not only affect current health of cows but impedes

ability to perform to their potential well into lactation (LeBlanc, 2010). Like any animal, a dairy cow has limited resources; if output increases for lactation, other energetic processes that maintain health and fertility are downregulated (Oltenacu and Algers, 2005). Proper nutrition that successfully transitions a cow from the demands of late pregnancy to early lactation has the potential to mitigate many of the early lactation complications described above, through reducing the duration and severity of NEBAL as well as decreasing systemic inflammation.

Maximizing DMI immediately post calving logically mitigates NEBAL and therefore decreases the risk of periparturient disease. Feeding higher starch rations has been reported to not only increase energy availability due to the increased supply of glucose precursors supplied in the diet, but also increase overall DMI and decrease circulating NEFA in the early postpartum period (Anderson et al., 2003; Rabelo et al., 2003; McCarthy et al., 2015b and 2015c). This increase in DMI is a likely due to the reduced physical fill of high concentrate diets in combination with reduced NEFA mobilization for energy (Drackley et al., 2001). Although increasing starch can increase DMI, cows fed a higher starch diet during the first three weeks of lactation could also have a higher risk of sub-acute ruminal acidosis (SARA; Williams et al., 2015), a condition where ruminal pH is depressed due to accumulation of VFA and insufficient rumen buffering. Consequences of SARA can include decreased DMI, decreased fiber digestion, milk fat depression, diarrhea, laminitis, liver abscesses, and possible inflammation characterized by increased circulating APP (Plaizer et al., 2007).

One possible method of preventing SARA in cows fed high starch rations is to feed high starch in combination with different pools of fiber. Physically effective neutral detergent fiber

(peNDF[1]), defined as the fraction of fiber that stimulates chewing and contributes to the floating mat of particles in the rumen, increases chewing and rumination time (Mertens, 1997). Additional peNDF in a diet may help to minimize SARA through increased production of salivary bicarbonate (Stone, 2004), though its specific impact on the transition period has not been defined (LaCount et al., 2017). Undigested neutral detergent fiber ($uNDF_{240}$), defined as fiber remaining after 240 h of in vitro fermentation, has recently been examined as a possible metric related to DMI and rumen health in mid-lactation diets (Cotanch et al., 2014). A ration with too low $uNDF_{240}$ could compromise rumen health and increase risk for SARA, but one too high in $uNDF_{240}$ could limit intake due to physical fill (LaCount et al. 2017). Feeding a diet on either end of the $uNDF_{240}$ spectrum could place early lactation animals further into NEBAL. Case studies from our lab suggest that early postpartum cows fed a high starch diet in conjunction with higher $uNDF_{240}$ (10.7% of ration DM, intake 0.36% of BW) had higher DMI and improved health when compared to those fed low $uNDF_{240}$ (8.3% of ration DM, intake 0.27% of BW), as cows fed the lower $uNDF_{240}$ had higher incidences of metabolic disorders (McCarthy et al., 2014). The limited but promising evidence of the effect of uNDF, combined with peNDF impacts on intake and rumen dynamics, indicate that increased fiber could help play a role in keeping cows healthy through maintaining a healthy rumen environment and possibly decreasing inflammation, while improving metabolic status when fed in conjunction with a high starch diet. This improved metabolic status could help cows decrease NEBAL, bringing the uncoupled GH-IGF-I axis closer to what exists during times of positive energy balance.

[1] Estimated by calculating percent of particles that remain above the 4mm sieve when using the Penn State Particle Separator

Research Objectives

Understanding how to best use fiber pools in the early lactation ration has the potential to improve animal health and productivity during this stressful transition period. Feeding higher starch early lactation rations in combination with higher fiber could increase DMI and glucose availability while decreasing risk and impacts of SARA. This could decrease the extent and duration of NEBAL, improve metabolic status, and decrease systemic inflammation postpartum. The objective of my thesis was to specifically examine the effect of high fiber dietary intervention on hepatic metabolism of lactating dairy cows, namely hepatic gene expression of IGF-I, GHR, TNFα, and TNFα receptor (TNFRSF1a), with the goal of minimizing NEBAL and systemic inflammation during the early postpartum period. My hypothesis was that cows fed the high fiber diet would have reduced inflammation (marked by lower TNFα and TNFRSF1a expression), and an increase in overall energy balance (marked by an increase in IGF-I and GHR expression). While examining the effect of diet on hepatic gene expression, I also wanted to analyze the effect of genotype and other information collected throughout the study related to energy balance, metabolism, and liver health on the hepatic expression of these genes.

Methodology

Overview of the Animal Trial

This project involved analyzing liver samples collected from cows that were part of a previously conducted experiment studying the effects of varying undigested neutral detergent fiber

(uNDF240) and physically effective neutral detergent fiber (peNDF) content of fresh rations on cow performance and metabolism (LaCount et al., 2017). All procedures involving animals were approved by the Cornell University Institutional Animal Care and Use Committee. Multiparous Holstein cows (n=40) were fed a common prepartum ration beginning 28 d prior to expected parturition and assigned randomly at calving to one of two postpartum diets differing in fiber content. The high fiber (35.3% NDF, 12.2% uNDF240, 23.2% peNDF; n=19) and low fiber (32.8% NDF, 9.5% uNDF240, 21.6% peNDF; n=21) treatment diets were formulated for equivalent metabolizable protein and starch concentrations. Cows were fed their respective treatment diets from calving through d 28 postpartum. After calving, all cows were milked 3 times daily at 0600, 1400, and 2200 h.

Feed Management and Sampling

Cows were housed in tiestalls throughout the duration of the study. They were fed daily between the hours of 0700 and 0900 h, with individual feed intake measured on a daily basis through weighing feed delivered and feed refused. Refusal rates were targeted at 10% to allow for ad libitum intake throughout the study. Cornell Net Carbohydrate and Protein System (CNCPS, version 6.5, Cornell University, Ithaca, NY) was used to formulate rations. Ingredient composition and analyzed diet composition of all prepartum and postpartum treatment diets are presented in Tables 1 and 2, respectively.

Table 1. Formulated ingredient composition of the common prepartum diet and postpartum treatment diets formulated to differ in amount of undigested NDF at 240-h of *in vitro* fermentation ($uNDF_{240}$) and physically effective NDF (peNDF).

		Postpartum Diet[1]	
Ingredient, % of DM	Prepartum Diet	LF [1]	HF [1]
Conventional corn silage	45.19	42.31	38.46
Alfalfa hay	-	10.58	10.58
Straw	20.84	1.15	8.65
Corn meal	2.43	17.64	20.51
Soybean meal	-	6.03	4.72
Canola meal	3.48	4.52	3.88
Wheat middlings	-	4.82	1.62
Amino Plus[2]	5.91	4.35	5.32
Corn gluten feed	1.74	1.60	0.47
Blood meal	2.43	0.95	1.09
Soybean hulls	6.95	2.42	-
Citrus pulp	4.52	-	0.79
Energy Booster[3]	-	1.29	1.57
Rumensin[4]	0.10	0.05	0.05
Salt	-	0.48	0.47
Ca Carbonate	2.26	1.13	1.18
Mg Oxide	-	0.28	0.28
Animate[5]	3.13	-	-
Alimet[6]	0.07	-	-
Vitamin and mineral mix[7]	0.94	0.40	0.39

[1]Treatments: low fiber (**LF;** $uNDF_{240}$ = 9.5% of DM, peNDF = 21% of DM), high fiber (**HF;** $uNDF_{240}$ = 12.2% of DM, peNDF = 23% of DM). Treatment diets were fed from wk 1 to 4 after which all cows were switched to the LF diet.

[2] Heat-treated soybean meal, Ag Processing Inc., Omaha, NE.

[3] Commercial fat source, Milk Specialties Global, Eden Prairie, MN.

[4] Premix contained 26,400 g/t of monensin, Elanco Animal Health, Greenfield, IN.

[5] Anionic mineral supplement, Phibro Animal Health Corporation, Teaneck, NJ.

[6] 2-Hydroxy-4-(methylthio)-butanoic acid, Novus International, Saint Charles, MO.

[7] Prepartum mix contained 3,754 mg/kg Zn, 993 mg/kg Cu, 4,658 mg/kg Mn, 28.9 mg/kg Se, 82.8 mg/kg Co, 82.4 mg/kg I, 1,177 KIU/kg Vitamin A, 253 KIU/kg Vitamin D, and 55,784 IU/kg Vitamin E. Postpartum mix contained 25,560 mg/kg Zn, 7,154 mg/kg Cu, 21,958 mg/kg Mn, 214 mg/kg Se, 507 mg/kg Co, 331 mg/kg I, 3,704 KIU/kg Vitamin A, 922 KIU/kg Vitamin D, and 12,496 IU/kg Vitamin E (Central New York Feeds, Jordan, NY).

Table 2. Nutrient profile of all rations (mean ± SD), obtained through wet chemistry analysis and in vitro fermentation by Cumberland Valley Analytical Services (Hagerstown, MD), predicted by Cornell Net Carbohydrate and Protein System (version 6.5, Cornell University, Ithaca, NY), and obtained with a Penn State Particle Separator (Nasco Inc., Fort Watkinson, WI).

	Diet		
Item	Prepartum	Low Fiber (LF)[1]	High Fiber (HF)[1]
DM, % as fed[2]	45.9 ± 1.2	44.4 ± 2.2	47.4 ± 2.0
ADF, % DM	29.0 ± 0.5	21.3 ± 1.1	22.9 ± 2.1
aNDFom, % DM	43.1 ± 0.3	32.8 ± 1.4	35.3 ± 2.3
30-h NDFD[3], % DM	24.2 ± 0.5	20.2 ± 0.5	19.1 ± 1.7
30-h NDFD, % NDF	54.4 ± 1.5	59.5 ± 1.0	52.6 ± 2.2
$uNDF_{240}$[4], % DM	12.8 ± 0.5	9.5 ± 0.4	12.2 ± 1.6
CP, %	14.1 ± 0.8	15.5 ± 0.5	13.8 ± 1.4
Starch, %	15.7 ± 0.3	24.9 ± 1.7	24.6 ± 2.3
Sugar, %	3.5 ± 0.4	5.1 ± 0.7	3.9 ± 0.1
Fat, %	2.3 ± 0.2	3.3 ± 0.2	3.2 ± 0.2
peNDF[5], %	33.3[6]	21.6[7]	23.2[7]
MP[6], g/kg DM	89.0	112.1	108.0
NE_L, Mcal/kg	1.45 ± 0.02	1.65 ± 0.03	1.61 ± 0.02
Ca, %	1.46 ± 0.07	0.97 ± 0.12	0.98 ± 0.19
P, %	0.32 ± 0.02	0.41 ± 0.01	0.37 ± 0.01
Mg, %	0.51 ± 0.02	0.40 ± 0.04	0.39 ± 0.04
K, %	1.16 ± 0.02	1.24 ± 0.03	1.21 ± 0.04
S, %	0.39 ± 0.02	0.30 ± 0.02	0.26 ± 0.02
Na, %	0.13 ± 0.01	0.26 ± 0.02	0.23 ± 0.03
Cl, %	0.67 ± 0.02	0.49 ± 0.02	0.44 ± 0.04

[1] Treatments: low fiber (**LF;** $uNDF_{240}$ = 9.5% of DM, peNDF = 21% of DM), high fiber (**HF;** $uNDF_{240}$ = 12.2% of DM, peNDF = 23% of DM). Treatment diets were fed from wk 1 to 4 after which all cows were switched to the LF diet.

[2] Determined via wet chemistry from fresh weekly samples sent to Cumberland Valley Analytical Services for NIR analysis.

[3] NDF digestibility, determined via *in vitro* fermentation

[4] Undigested NDF at 240-h of *in vitro* fermentation

[5] Physically effective NDF

[6] Formulated value given by Cornell Net Carbohydrate and Protein System v. 6.5 using analyzed forage values and mean DMI for each period and treatment.

[7] Determined using a Penn State Particle Separator from two samples collected throughout the study. Value obtained by multiplying the percentage of sample retained on screens ≥ 4mm by the ration NDF content.

Samples of all feed ingredients and total mixed rations (TMR) were collected weekly for determination of dry matter (DM), and were dried at 40°C for 96 h in a forced-air oven. As-fed inclusion rates of all forages and grain ingredients were adjusted based on these weekly DM values. At the end of the experiment, dried samples were ground through a 2-mm screen in a Wiley mill and composited for later wet chemistry analysis.

Individual Animal Sampling

Liver tissue (3–5 g) was sampled via percutaneous trocar biopsy (Veenhuizen et al., 1991) on day 7 ± 1.1 and 14 ± 1.0 (mean ± SD) postpartum from cows under local anesthesia. After blotting the liver sample to remove excess blood and connective tissue the liver sample was snap frozen in liquid N_2 and stored at -80°C until analysis of glycogen, triglycerides, and RNA isolation for gene expression analysis.

Blood samples were collected between the hours of 0600 and 0730 daily from 0 to 7 DIM, and 3x/wk through 28 DIM. Samples were collected via coccygeal venipuncture using 10-mL sodium heparin evacuated tubes (158 USP, Becton Dickinson and Company, Franklin Lakes, NJ) and 20 G vacutainer needles (Becton Dickinson and Company) and placed on ice immediately after collection. Plasma was harvested after centrifugation at 2,000 × g for 20 min at 4°C, aliquoted into 1.7-mL microfuge tubes, snap frozen in liquid nitrogen, and stored at −20°C until later analysis.

Milk samples were collected at 3 consecutive milkings each week. After collection, they were mixed with a bronopol preservative and stored at 4°C until transportation within 72h of collection to a commercial laboratory (DairyOne, Ithaca, NY). Milk weights were recorded daily.

Daily milk yield was calculated as the sum of yields at all 3 milkings and used to determine average milk production per week, as well as total average milk production from d 0 through d 14.

Calculating Energy Balance

Energy balance was determined weekly according to NRC (2001) equations for the following: energy intake (Mcal/d) = weekly DMI average (kg/d) × diet net energy of lactation (NEL; Mcal/kg of DM), maintenance requirement (Mcal) = week metabolic body weight (BW) ($kg^{0.75}$) × 0.08 (Mcal/$kg^{0.75}$ per d), and lactation requirement (Mcal/d) = wk average milk yield (kg/d) × [(0.0929 × fat percentage) + (0.0563 × true protein percentage) + (0.0395 × lactose percentage)]. Weekly energy balance was calculated as follows:

> Postpartum NEL (Mcal/d) balance = energy intake (Mcal of NEL/d) – [maintenance requirement (Mcal of NEL/d) + lactation requirement (Mcal of NEL/d)].

Laboratory Methods

Composited feed samples were sent to a commercial laboratory (Cumberland Valley Analytical Services, Hagerstown, MD) for wet chemistry analysis of DM at 135°C (method 930.15, AOAC International, 2000), crude protein (CP; method 990.03, AOAC International, 2000), acid detergent fiber (ADF; method 973.18, AOAC International, 2000), neutral detergent fiber (NDF; Van Soest et al., 1991), starch (Hall, 2009), sugar (Dubois et al., 1956), ether extract (method 2003.05, AOAC International, 2006), and minerals (method 985.01, AOAC International, 2000). Values for NEL of TMR composite samples were calculated according to NRC (2001).

Milk samples were analyzed at a commercial laboratory (DairyOne, Ithaca, NY) for milk fat, protein, lactose, total solids (TS), and milk urea nitrogen (MUN) using mid-infrared techniques (method 972.16, AOAC International, 2006).

Plasma samples taken on the day corresponding with liver biopsy were sent to a commercial diagnostic lab for analysis (University of Guelph Animal Health Laboratory, Guelph, ON, Canada) of haptoglobin based on peroxidase activity (Makimura and Suzuki, 1982, Skinner et al., 1991), and aspartate aminotransferase (AST), cholesterol, glutamate dehydrogenase (GLDH), gamma-glutamyltransferase (GGT), bilirubin, total protein, albumin, globulin, and albumin:globulin ratio on a Roche Cobas 6000 c501 biochemistry analyzer (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions.

Plasma IGF-I concentrations were assayed using the radioimmunoassay procedure described by Butler et al. (2003) following the extraction procedure described in Butler et al. (2004). Analysis of plasma NEFA concentrations [HR Series NEFA HR (2), Wako Pure Chemical Industries, Osaka, Japan], and plasma beta-hydroxybutyrate (BHB) (Catachem Inc., Oxford, CT) were conducted in triplicate using commercial enzymatic kits, as described in Leno et al. (2017). Plasma glucose concentrations were determined by enzymatic analysis (glucose oxidase; protocol from kit 510-A; Sigma-Aldrich, St. Louis, MO) using commercial products (PGO Enzyme Preparation and o-dianisidine dihydrochloride, Sigma-Aldrich) as described in McCarthy et al. (2015c). Coefficients of variation for all assays (both inter- and intra-assay) were maintained below 10%.

Liver samples were analyzed for triglycerides using the Folch extraction method (Folch et al., 1957), followed by a colorimetric method for estimating serum triglyceride based upon the

Hantzsch condensation (Fletcher, 1968) with modifications described by Foster and Dunn (1973). Glycogen content of liver was determined by following procedures as described by Bernal-Santos et al. (2003).

DNA genotyping was completed for all IGF-I, GHR, and TNFα SNPs using PCR-restriction fragment length (PCR-RFLP) methods, as described in Hax et al., 2017 and Higuchi et al., 1999. Primers, annealing temperature, fragment lengths, and enzymes used can be found in Table 3. The genotype of each animal was determined by the fragment size reported as base pairs. The genotypes identified for IGF-I were: *Sna*BI (A/A) 249bp; *Sna*BI (A/B) 249bp, 226 bp; *Sna*BI (B/B) 226bp (Ge et al., 2011). The genotypes identified for GHR were: AluI (A/A) 747 bp, 75 bp; AluI (A/B) 747 bp, 602 bp, 145 bp, 75 bp; *Alu*I (B/B) 602 bp, 145 bp, 75 bp (Aggrey et al., 1999). The genotypes identified for TNFα were: Allele A: 1233bp; Allele B: 928 bp, 305 bp (Higuchi et al., 1999).

Table 3. Primers, annealing temperatures, and enzymes used to genotype insulin-like growth factor one (IGF-I), growth hormone receptor (GHR), and tumor necrosis factor alpha (TNFα) genes

Gene	Annealing T°	Fragment length	Enzyme	Reference
IGF-I primers				
FW: TTAAATAATTGGGTTGGAAGACTGC RV: ACCTTACCCGTATGAAAGGAATATACGT	58 °C	249 bp	*Sna*BI	Ge et al., 2011
GHR primers				
FW: TGCGTGCACAGCAGCTCAACC RV: AGCAACCCCACTGCTGGGCAT	66 °C	836 bp	*Alu*I	Aggrey et al., 1999
TNFα primers				
FW: GGGTGACTTGCTCTAACACTCATC RV: AGGCCTCACTTCCCTACATCCCTA	63 °C	1233 bp	*Rsa*I	Higuchi et al., 1999

Hepatic gene expression of IGF-I, GHR, TNFα, and TNFRSF1a was determined as a measure of relative quantification using quantitative PCR. Total RNA was isolated and purified for RT-qPCR using miRNeasy minicolumns and on-column ribonuclease-free deoxyribonuclease treatment (QIAGEN Inc., Hilden, Germany). Quantity and integrity of RNA was determined using the RNA Nano Lab chip kit and 2100 bioanalyzer system (Agilent, Santa Clara, CA).

Reverse transcriptase reactions were performed with 2 µg of RNA in a 20-µL volume using the high capacity cDNA reverse transcription kit with RNase Inhibitor (Applied Biosystems, Foster City, CA) according to the manufacturer's recommendation. Quantitative PCR reactions were prepared in duplicate according to recommendations for the use of Taqman Fast Advanced Gene Expression master mix on a StepOne Plus instrument (Applied Biosystems). Specifically, each well consisted of 10 µL of Taqman Fast Advanced master mix, 1 µL of Taqman assay, 7 µL of sterile H_2O, and 2 µL of 10-fold diluted cDNA for a total volume of 20 µL per well. A single control reaction using 2 µL of sterile H_2O was included for each gene on every plate. All Taqman probes for genes of interest were selected to span exon junctions and not detect genomic DNA and were purchased from Applied Biosystems (Foster City, CA): insulin-like growth factor one (IGF-I; assay Bt03252282), growth hormone receptor (GHR; assay Bt03216844), tumor necrosis factor alpha (TNFα; assay Bt03259156), and tumor necrosis factor alpha receptor super family one alpha (TNFSRF1a; assay Bt03215763). In addition, two references genes were selected: large ribosomal protein P0 (RPLP0; Bt03218086) and TATA box binding protein (TBP; Bt03241948).

Cycles were run on a StepOne Plus instrument (Applied Biosystems) with Taqman Fast Advanced master mix recommended settings. The instrument was set to 40 repeating cycles of 95°C for 1 second (denaturation) and 20 seconds at 60°C (annealing and extension), with a single uracil-N-glycosylase (UNG) incubation period of 2 min at 50°C and polymerase activation of 2

min at 95°C prior to cycling. A single pool of RNA from a liver sample harvested in this experiment was used to generate the calibrator cDNA used on all plates as a control following the $2^{-\Delta\Delta Ct}$ method, and results were expressed as a relative quantity.

Statistical Analysis

Daily means for DMI and milk yield were averaged by week prior to analysis. To standardize blood metabolite data, day of blood samples collected 2x / wk or 3x / wk were averaged to reference days prior to analysis. All statistical analyses were conducted using SAS (version 9.4, SAS Institute Inc., Cary, NC). All data collected over time were analyzed as repeated measures ANOVA using the REPEATED statement in the MIXED procedure of SAS (Littell et al., 1996). Fixed effects of treatment, time, parity group (2nd vs. 3rd and greater), genotype for all genes, and all interactions were included in the model. Cow nested within treatment was the random effect. Denominator degrees of freedom were estimated using the Kenward Rogers method. Five covariance structures were tested for each model: first-order autoregressive, heterogeneous first-order autoregressive, compound symmetry, heterogeneous compound symmetry, and unstructured; and the covariance structure with the lowest Akaike's information criterion was used (Littell et al., 1996). Residual analysis was performed to examine normality and homogeneity of residuals, when non-normality of residual variance was evident, data were log-transformed and analysis repeated. In the event of a significant effect of genotype comparison for differences among alleles was controlled for multiple comparisons with the Tukey procedure. All data presented are least squares means and standard error (SE), except in the case of transformed data where

geometric means with back-transformed 95% confidence intervals (CI) are reported. Significance was declared at $P \leq 0.05$, and trends discussed at $0.05 < P \leq 0.15$.

Nineteen variables (Table 4) related to liver health, energy balance, and production were chosen to test in Pearson correlation in SAS with gene expression of IGF-I, GHR, TNFα, and TNFRSF1a. Area under the curve (AUC) was calculated within the first 14 DIM for both NEFA and BHB concentrations using the trapezoidal rule as described by Cardoso et al (2011). Significance of correlations was declared at $P \leq 0.05$.

Table 4. Variables used in Pearson correlation analysis.

Variable
Measured on day 7 and day 14 to correspond with liver biopsies
Gene Expression- IGF-I
Gene Expression- Growth Hormone Receptor
Gene Expression- TNFα
Gene Expression- TNF Receptor Superfamily Member 1a
Liver Triglycerides
Liver Glycogen
Aspartate Aminotransferase
Cholesterol
Glutamate dehydrogenase
Gamma-glutamyltransferase
Haptoglobin
Bilirubin
Total Protein
Albumin
Globulin
Albumin:Globulin Ratio
Plasma IGF-I Concentrations
Averaged over wk 1 and wk 2
Average DMI
Average milk production
Calculated Energy Balance
Average Plasma Glucose Concentrations
Calculated total over 14 days
Area Under the Curve for blood NEFA
Area Under the Curve for blood BHBA

Results and Discussion

Effect of Dietary Treatment on Hepatic Gene Expression

Hepatic gene expression was measured in relative quantification (RQ) compared to a standard control. There were no differences (P>0.15) of dietary treatment on hepatic gene expression of IGF-I, GHR, TNFα, or TNFRSF1a (Figure 2). There was, however, a treatment by parity effect (P = 0.02), such that cows fed the high fiber diet that were in their third and greater lactation had lower hepatic IGF-I expression compared to second lactation cows fed the high fiber diet (Figure 3), while cows fed the low fiber diet exhibited no differences due to parity. GHR, TNFα, and TNFRSF1a expression did not have a treatment by parity effect. Although there was no significant difference in gene expression by treatment group, time did influence hepatic TNFα expression (P = 0.002), where d 14 biopsies had significantly higher levels of hepatic TNFα expression than d 7 biopsies.

The lack of an overall effect of treatment was not consistent with my hypothesis that high fiber - high starch, early lactation rations would reduce inflammation (marked by lower TNFα and TNFRSF1a expression), and increase overall energy balance (marked by an increase in IGF-I and GHR expression). The lack of treatment differences observed could have been due to unexpected poor forage quality during the trial, which resulted in increased fiber levels for both treatment groups; the high fiber treatment diet ended up containing very high fiber levels, while the low fiber diet ended up containing adequate fiber levels. Cows fed either diet were likely not at risk for SARA due to this increased effective fiber. Instead, the main impact of dietary treatment was that cows fed the high fiber diet were limited in intake starting in wk 2 postpartum. This reduction of

intake coincided with about 3.6 kg reduction daily milk yield on average during the first four weeks of lactation. More research will need to be done to determine if the lack of significant treatment groups was due to poor forage quality alone, or if we would have the same results regardless of forage quality.

This limit in intake of cows on the high fiber diet could have contributed to the observed treatment by parity effect of hepatic IGF-I expression; older cows generally have higher milk production and therefore are more likely to be in greater NEBAL than younger cows (Xu et al., 2018). This was consistent with data from our study, showing third and greater lactation animals to produce 4.3 kg more milk per day on average compared to second lactation animals during the first four weeks of lactation. The demands of higher milk production combined with the lack of available energy due to decreased intake would leave older cows in a greater energy deficit, marked by lower IGF-I expression, than younger cows limited by the same diet.

The increase in hepatic TNFα expression over time implies that as cows progress further into early lactation, they experience more of an inflammatory response. Milk production also increased significantly from wk 1 to wk 2 by about 11 kg and 13 kg on average per day in cows from the high fiber and low fiber treatment groups respectively. The liver's increase in TNFα expression may have been an inflammatory response to the nutrient demands of a drastic increase in production. Our results are somewhat consistent with the Loor et al. (2005) study, which reported a significant overall increase in hepatic TNFα expression from day 1 to day 49, but also had slight decrease in hepatic TNFα expression from day 1 to day 14. This slight decrease in expression could have been due to the small sample size (n = 5) of the study.

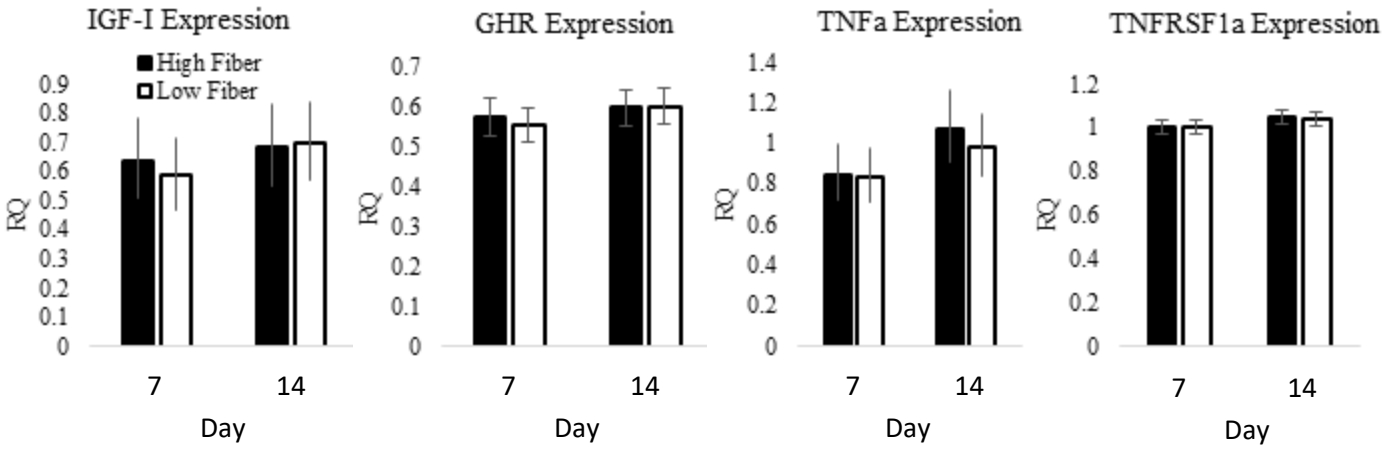


Figure 2. The hepatic expression of insulin-like growth factor one (IGF-I; P = 0.2772), growth hormone receptor (GHR; P = 0.4794), tumor necrosis factor alpha (TNFα; P = 0.521), and TNFα receptor superfamily 1a (TNFSRF1a; P = 0.9233) of cows fed diets differing in amount of undigested NDF at 240-h of in vitro fermentation (uNDF240) and physically effective NDF (peNDF) in the postpartum period. Treatments were low fiber (uNDF240 = 9.5% of DM, peNDF = 21% of DM) and high fiber (uNDF240 = 12.2% of DM, peNDF = 23% of DM). Gene expression is measured in relative quantification (RQ) compared to a standard control. Day 7 corresponds to the liver biopsy taken on d 7 ± 1.1 (mean ± SD), while day 14 corresponds to the liver biopsy taken on d 14 ± 1.0 (mean ± SD). IGF-I and TNFα expression are reported as geometric means and back transformed 95% confidence intervals. GHR and TNFRSF1a expression are reported as least square means and standard error.

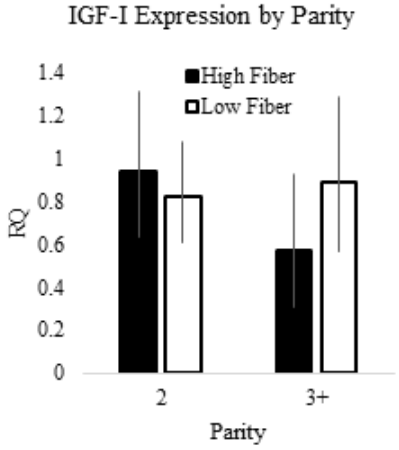


Figure 3. Hepatic gene expression of insulin-like growth factor one (IGF-I; P = 0.0257) by parity (2nd vs 3rd and greater) for cows fed diets differing in amount of undigested NDF at 240-h of in vitro fermentation (uNDF240) and physically effective NDF (peNDF) in the postpartum period. Treatments were low fiber (uNDF240 = 9.5% of DM, peNDF = 21% of DM) and high fiber (uNDF240 = 12.2% of DM, peNDF = 23% of DM). Gene expression was measured in relative quantification (RQ) compared to a standard control. Data are reported as geometric means and back transformed 95% confidence intervals.

Effect of Genotype on Hepatic Gene Expression

The effect of Single Nucleotide Polymorphisms (SNP) in IGF-I, GHR, and TNFα on hepatic gene expression of IGF-I, GHR, TNFα, and TNFSRF1a was examined. The number of cows with each genotype is recorded in Table 5. Because the main purpose of the study was to analyze the effect of dietary treatment on hepatic gene expression, we did not genotype the cows ahead of time to ensure an equal representation of each genotype. While we still feel like the results of the effect of genotype on gene expression are worth sharing, they should be interpreted with caution given the small representation of some genotypes.

There was no effect ($P>0.15$) of IGF-I genotype on hepatic gene expression of IGF-I, GHR, TNFα, or TNFSRF1a (Figure 4A). Cows with GHR SNP AB tended ($P<0.15$) to have higher hepatic IGF-I gene expression compared to cows with GHR SNP AA or BB. GHR genotype AB cows also tended ($P<0.15$) to have a higher GHR expression than cows with genotype AA or BB. GHR genotype did not affect ($P>0.15$) TNFα or TNFSRF1a hepatic gene expression (Figure 4B). Cows with TNFα SNP AB tended ($P<0.15$) to have lower hepatic IGF-I gene expression compared to those with TNFα SNP BB. TNFα genotype did not affect ($P>0.15$) hepatic GHR, TNFα, or TNFSRF1a gene expression (Figure 4C).

The mirrored relationship between IGF-I and GHR expression in regards to GHR genotype is not surprising considering the direct effect of GHR concentration on IGF-I expression (Butler et al., 2003). The higher hepatic IGF-I expression for GHR genotype AB, however, is inconsistent with results from a previous study (Schnider et al., 2013) analyzing associations of GHR polymorphisms and the fertility of Holstein cows, where cows with GHR genotype AA had significantly higher levels of plasma IGF-I expression. Plasma IGF-I levels are directly influenced

by hepatic IGF-I expression (Butler et al., 2003); it would follow that genotype AA cows in this study from Schieder et al. (2013) likely had higher hepatic IGF-I expression as well. Considering the larger (n = 94) and more evenly distributed sample size in this genotype study compared to our own, there is opportunity for additional research analyzing the effect of both GHR genotype and IGF-I genotype on hepatic GHR and IGF-I gene expression to gain a more accurate understanding of the role genotype plays.

It is unclear why TNFα genotype in our study did not influence hepatic TNFα expression. Limited evidence of the relationship between TNFα and IGF-I expression, however, could explain the observed effect of TNFα genotype on hepatic IGF-I expression. In Briard et al. (2000), an LPS challenge induced a rapid increase in plasma concentration of inflammatory cytokines such as TNFα, resulting in increased circulating GH concentrations and decreased circulating IGF-I concentrations in sheep. It is possible that TNFα genotype AB could result in an increased circulation of TNFα, thus further decreasing hepatic IGF-I expression and ultimately decreasing circulating IGF-I when compared to TNFα genotype AA or BB. Again, our results should be interpreted with caution given the small and uneven distribution of genotypes.

Table 5. Total number of cows determined with each single nuclear polymorphism genotype for insulin-like growth factor one (IGF-I), growth hormone receptor (GHR), and tumor necrosis factor alpha (TNFα)

Allele Type	IGF-I Genotype	GHR Genotype	TNFα Genotype
AA	22	16	5
AB	11	1	13
BB	2	17	14

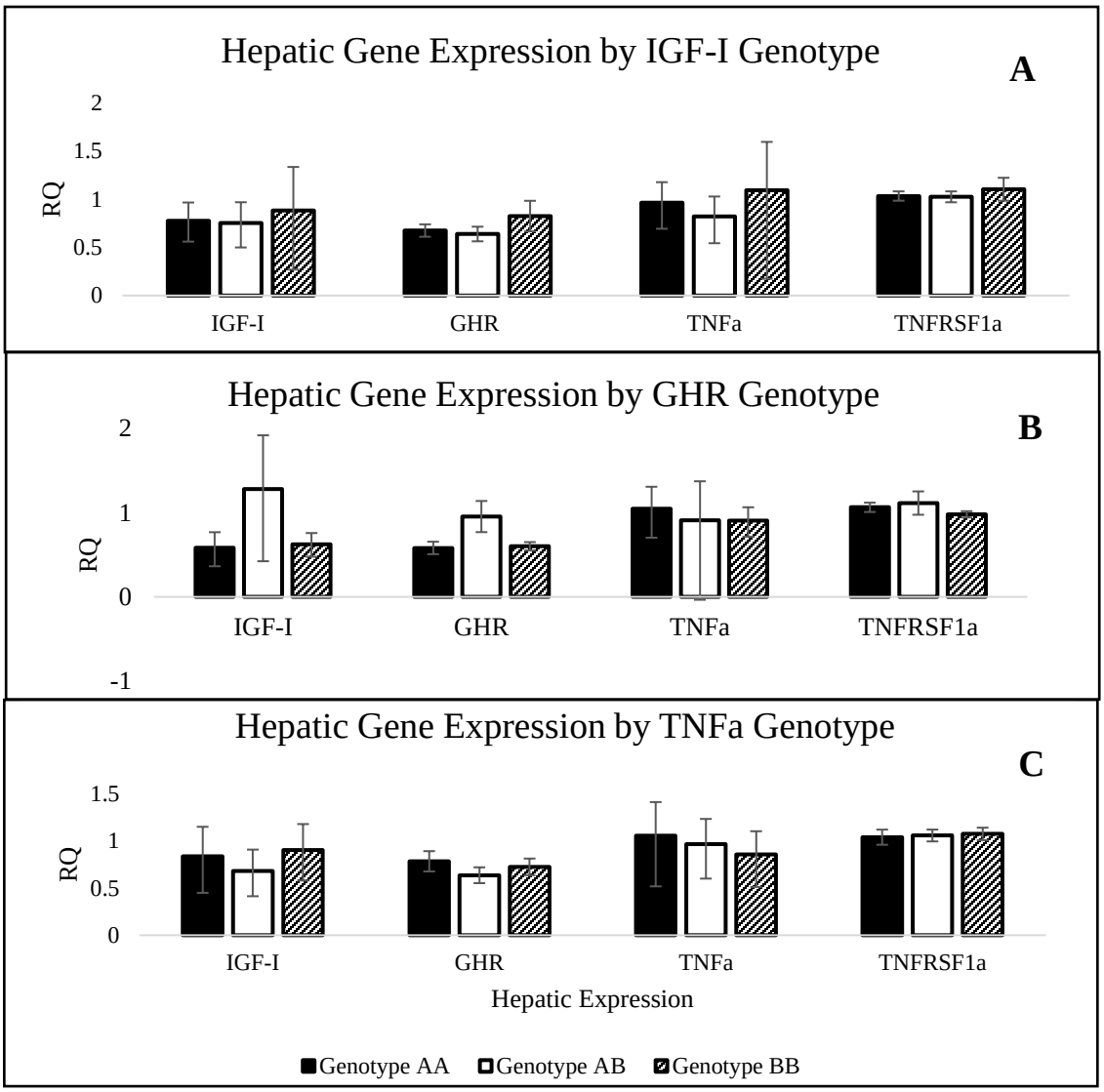


Figure 4. The effect of IGF-I (A), GHR (B), and TNFα (C) genotype on hepatic expression of insulin like growth factor one (IGF-I), growth hormone receptor (GHR), tumor necrosis factor alpha (TNFα), and TNFα Receptor Superfamily 1a (TNFRSF1a). IGF-I and TNFα gene expression are reported as geometric means and back transformed 95% confidence intervals. GHR and TNFRSF1a gene expression are reported as least square means and standard error. P-values are as follows: IGF-I expression: IGF-I genotype (P = 0.8496), GHR genotype (P = 0.0623), TNFα genotype (P = 0.1068). GHR expression: IGF genotype (P = 0.4595), GHR genotype (P = 0.1167), TNFα genotype (P = 0.2011). TNFα expression: IGF-I genotype (P = 0.3367), GHR genotype (P = 0.5039), TNFα genotype (P = 0.3629). TNFRSF1a expression: IGF-I genotype (P = 0.776), GHR genotype (P = 0.269), TNFα genotype (P = 0.8498).

Correlations between Gene Expression and Other Data Collected

Pearson correlation analysis was completed between IGF-I, GHR, TNFα, and TNFRSF1a hepatic gene expression and 19 other variables related to health and production; these data are shown in tables 6-8).

Correlations between Gene Expressions

Hepatic IGF-I expression had a strong, positive correlation with hepatic GHR expression on both d 7 and d 14 (Table 6), implying that cows with higher GHR expression also had a higher IGF-I expression during the first two weeks of lactation. This is consistent with the understanding that the number of hepatic surface GHR has a direct effect on hepatic IGF-I production (Butler et al., 2003). Hepatic TNFα expression also had a strong positive correlation with the hepatic expression of its receptor, TNFRSF1a, on d 7 and d 14 (Table 6). This relationship was expected, given that TNFα signals through TNFRSF1a to activate and induce inflammatory responses (Mohankrishnan et al., 2018; Kondo and Sauder 1997).

Table 6. Pearson correlations[1] between hepatic gene expression of insulin-like growth factor one (IGF-I), growth hormone receptor (GHR), tumor necrosis factor alpha (TNFα), and TNFα receptor superfamily 1a (TNFRSF1a); d 7 and d 14 correspond to samples taken on liver biopsy day 7 and day 14.

	IGF-I		GHR		TNFα		TNFRSF1a	
	d 7	d 14	d 7	d 14	d 7	d 14	d 7	d 14
IGF-I d 7	1.000	0.601 <.0001	0.534 0.0004	0.458 0.003	0.044 0.786	-0.270 0.092	0.037 0.821	0.085 0.602
IGF-I d 14		1.000	0.297 0.062	0.609 <.0001	-0.220 0.173	-0.259 0.106	0.073 0.654	0.110 0.500
GHR d 7			1.000	0.647 <.0001	0.029 0.857	-0.233 0.148	-0.038 0.815	-0.156 0.337
GHR d 14				1.000	-0.069 0.672	-0.069 0.672	-0.012 0.942	-0.137 0.399
TNFα d 7					1.000	0.417 0.008	0.552 0.0002	0.205 0.205
TNFα d 14						1.000	0.096 0.556	0.529 0.0004
TNFRSF1a d 7							1.000	0.294 0.065
TNFRSF1a d 14								1.000

[1]Correlation coefficient (r) is the top number, with corresponding P-value beneath.

Correlations with Energy Status, Milk Production, and Metabolism

Plasma IGF-I: Plasma IGF-I had a strong positive correlation with hepatic IGF-I on d 7 and d 14 (Table 7) as expected, confirming that hepatic IGF-I gene expression does relate to plasma measurements of IGF-I. Plasma IGF-I was also correlated positively with GHR expression on d 7 and d 14 (Table 7), providing additional support for our understanding of the GH-IGF-I axis. As GHR expression in the liver increases, more IGF-I is produced in the liver (Butler et al., 2003) and released into circulation.

Energy Balance, DMI, and Production: Hepatic expression of IGF-I and GHR on d 7 had a positive correlation with wk 1 energy balance, but had no significant correlation with weekly average DMI. There was a negative correlation between hepatic GHR expression on d 7 and milk production in wk 1. Hepatic TNFRSF1a expression on d 7 had a strongly negative correlation with average milk production in wk 2, and hepatic TNFα expression correlated negatively with average blood glucose during wk 2 (Table 7).

The positive correlation between hepatic IGF-I and GHR expression and energy balance without a corresponding positive correlation with DMI suggests that the improvement in energy balance from increased IGF-I and GHR expression was not due to increased dietary energy, but likely due to reduced body reserve mobilization (Butler et al., 2003), lower milk production (Xu et al., 2018), or a likely combination of these factors. This could explain why we observed a negative correlation between hepatic GHR expression and milk production during the first week of lactation. Hepatic TNFRSF1a expression's negative correlation with average milk production in wk 2 suggests that those individuals with increased inflammation due to an increased TNFRSF1a expression (Mohankrishnan et al., 2018; Kondo and Sauder 1997) may have had lower milk production. This is consistent with findings that increased inflammation decreases milk production (Bionaz et al., 2007; Bradford et al., 2015). It is unclear why hepatic TNFα expression did not also have a negative correlation with milk production, but its negative correlation with average blood glucose during wk 2 suggests that cows in more severe NEBAL with lower blood glucose levels also had a higher inflammatory response.

Liver Metabolism: Hepatic IGF-I expression on d 7 correlated positively with liver glycogen on d 7 and d 14, and hepatic GHR expression on d 7 was correlated positively with glycogen on d 14. There was no significant correlations between IGF-I or GHR expression and

liver triglycerides, blood NEFA, or blood BHB. There was, however, a positive correlation between TNFα on d 14 and liver triglycerides on d 7 (Table 7).

Cows release hepatic glycogen stores to increase blood glucose during times of NEBAL (McCarthy et al., 2015a). The presence of higher glycogen stores in the liver would indicate cows that have a reduced state of NEBAL, as they would not need to release these stores into circulation to maintain blood glucose. This is consistent with our findings that cows with increased hepatic IGF-I and GHR expression also had increased liver glycogen stores. Given the known inverse relationship between energy balance and liver triglycerides, plasma NEFA, and plasma BHB (Strang et al., 1998; Butler et al., 2003, Xiliang et al., 2017), we also expected to see a negative correlation between IGF-I expression and these indicators of hepatic metabolism, as well as between GHR expression and these indicators. Our results, however, did not show any significant correlations between hepatic IGF-I or GHR expression and these variables. Increased plasma NEFA concentration has also been associated with impaired immune function and a proinflammatory effect (Gruenberg, 2019), which could explain the observed correlation between TNFα and liver triglycerides.

Table 7. Pearson correlations[1] between insulin-like growth factor one (IGF-I), growth hormone receptor (GHR), tumor necrosis factor alpha (TNFα), and TNFα receptor superfamily 1a (TNFRSF1a) expression and variables related to energy status, intake, and milk production; d 7 and d 14 correspond to samples taken on liver biopsy day 7 and day 14.

	IGF-I		GHR		TNFα		TNFRSF1a	
	d 7	d 14	d 7	d 14	d 7	d 14	d 7	d 14
Plasma IGF-I d 7	0.806	0.499	0.411	0.372	-0.115	-0.211	-0.179	-0.037
	<0.0001	0.002	0.012	0.024	0.498	0.210	0.288	0.828
Plasma IGF-I d 14	0.585	0.608	0.457	0.492	-0.022	-0.297	0.131	-0.085
	0.0001	<0.0001	0.004	0.002	0.895	0.074	0.440	0.618
EBAL[2] wk 1	0.409	0.280	0.324	0.152	0.058	-0.191	0.222	0.166
	0.013	0.098	0.054	0.375	0.737	0.264	0.192	0.335
EBAL wk 2	0.325	0.141	0.213	0.132	0.218	-0.131	0.262	0.225
	0.065	0.434	0.234	0.464	0.223	0.466	0.142	0.209
Avg[3] DMI[4] wk 1	0.108	0.029	0.096	0.163	-0.031	0.016	-0.042	0.197
	0.512	0.862	0.562	0.321	0.850	0.924	0.801	0.231
Avg DMI wk 2	0.064	0.008	0.140	0.162	-0.176	-0.272	-0.233	-0.007
	0.700	0.964	0.394	0.324	0.284	0.093	0.153	0.966
Avg Milk wk 1	-0.259	-0.195	-0.347	-0.071	0.058	0.280	-0.258	0.065
	0.111	0.233	0.030	0.667	0.724	0.085	0.112	0.695
Avg Milk wk 2	-0.261	-0.104	-0.128	0.101	-0.204	-0.052	-0.528	-0.202
	0.109	0.528	0.437	0.542	0.213	0.751	0.001	0.218
Avg Glucose[5] wk 1	0.286	0.143	0.235	0.057	0.107	-0.239	0.105	-0.116
	0.077	0.386	0.150	0.731	0.518	0.142	0.526	0.482
Avg Glucose wk 2	0.300	0.262	0.234	0.185	0.056	-0.334	0.210	-0.030
	0.063	0.108	0.152	0.259	0.736	0.038	0.200	0.855
Liver glycogen d 7	0.514	0.069	0.190	-0.018	-0.002	-0.297	-0.095	0.091
	0.001	0.674	0.240	0.914	0.992	0.063	0.561	0.575
Liver glycogen d 14	0.410	0.297	0.366	0.353	0.056	-0.245	0.032	-0.088
	0.009	0.063	0.020	0.026	0.732	0.127	0.846	0.591
Liver triglycerides	-0.175	0.118	-0.054	0.093	-0.028	0.332	-0.181	0.021
d 7	0.280	0.469	0.741	0.568	0.865	0.036	0.264	0.896
Liver triglycerides	-0.142	0.085	0.073	0.155	-0.084	0.257	-0.198	-0.108
d 14	0.381	0.602	0.653	0.340	0.608	0.109	0.221	0.507
NEFA AUC[6]	-0.267	-0.038	-0.091	0.008	-0.188	0.159	-0.309	-0.221
	0.100	0.819	0.583	0.962	0.253	0.334	0.056	0.176
BHB AUC[7]	-0.101	0.026	-0.134	0.016	-0.100	0.063	-0.154	0.029
	0.541	0.876	0.418	0.922	0.547	0.701	0.349	0.862

[1]Correlation coefficient (r) is the top number, with corresponding P-value beneath.

[2]Energy balance, [3]Average [4]Dry matter intake [5]Plasma glucose, [6]Area under the curve for plasma non-esterified fatty acids (NEFA), [7]Area under the curve for plasma beta-hydroxybutyrate (BHB)

Correlations with Indicators of Liver Health

Hepatic Damage Markers: Hepatic IGF-I, GHR, or TNFα expression did not correlate with serum levels of the hepatic damage markers aspartate aminotransferase (AST), glutamate dehydrogenase (GLDH), and gamma-glutamyl transferase (GGT). There was, however, a negative correlation between TNFRSF1a expression and GGT on d 7, as well as TNFRSF1a expression on d 14 with AST and GLDH on d 7 (Table 8). Excessive lipid accumulation in the liver results in the overproduction of reactive oxygen species, which alters hepatic cellular integrity and induces apoptosis (Schieber and Chandel 2014). Cows with severe oxidative stress have been reported to have increased serum levels of hepatic damage markers, including AST, GLDH, GGT (Xiliang et al., 2017). With this understanding of the effects of NEBAL on hepatic damage markers, it is unusual that we did not find negative correlations between IGF-I and GHR expression and these markers. The negative correlations between TNFRSF1a expression and these hepatic damage markers further complicate our previous understanding of the relationship between inflammation and oxidative stress; one of the primary responsibilities of TNFα is to induce apoptosis (Idriss and Naismith, 2000), so it would be expected that cows with greater oxidative stress and higher levels of circulating hepatic damage markers would also have higher hepatic TNFα and TNFRSF1a expression.

The Acute Phase Response: Hepatic expression of IGF-I was correlated positively with plasma concentrations of both albumin and cholesterol. Hepatic expression of IGF-I on d 7 and GHR on d 14 were correlated negatively with plasma globulin and positively with albumin to globulin ratio in plasma. Hepatic TNFRSF1a expression showed a negative correlation with plasma albumin and a positive correlation with plasma haptoglobin. Plasma concentrations of

bilirubin showed a few significant correlations with our genes of interest; hepatic TNFRSF1a expression had a negative correlation with bilirubin on d 14, and hepatic IGF-I expression had a negative correlation with bilirubin on d 7. There was no significant correlation between total protein and hepatic expression of our genes of interest (Table 8).

During the acute phase response, the activity of the liver is diverted from the synthesis of usual proteins that are considered negative APP, to the synthesis of undesirable positive APP (Bertoni and Trevisi, 2013). This makes liver protein synthesis a valuable indicator to assess liver health during the transition period. Albumin is a negative APP that is frequently used to determine inflammatory response; in nearly all species, albumin decreases in blood concentration during the acute phase reaction (Kaneko, 1997, from Cray 2009). Precursors to cholesterol are also considered negative APP (Bertoni and Trevesi, 2013), which means that cholesterol should also decrease during the acute phase reaction. The positive correlation of hepatic IGF-I expression and plasma concentrations of both negative APP albumin and cholesterol suggests that cows in greater energy balance have improved liver health. This would be expected, considering that the cow must depend almost entirely on hepatic gluconeogenesis to meet tissue glucose requirements (Elliot, 1980; Brockman, 1993; Bell and Bauman, 1997), and a liver in better metabolic condition would be more suited to complete this task. The negative correlation between plasma albumin, a negative APP, and TNFRSF1a expression suggests that cows with a greater acute phase inflammatory response have a higher expression of TNFRSF1a. The positive correlation between plasma haptoglobin and hepatic TNFRSF1a expression further supports our understanding of the positive relationship between inflammation and positive APP.

Bilirubin behaves similarly to haptoglobin during the acute phase reaction; while bilirubin is not an APP, enzymes that clear bilirubin behave in a pattern of negative APP. During the acute

phase response, the production of these enzymes are lowered. This leaves a higher concentration of bilirubin in circulation, making increased plasma bilirubin levels a marker of decreased liver health (Bertoni and Trevesi, 2013). The negative correlation between hepatic IGF-I expression and plasma bilirubin suggests that cows in greater NEBAL also have decreased liver health and function. The negative correlation between hepatic TNFRSF1a expression and bilirubin was unexpected given our understanding of increased bilirubin during the acute phase response (Bertoni and Trevesi, 2013).

Globulins are considered to be all serum proteins that are not albumin. Therefore, globulin = total protein – albumin (Cornell Vet Med., 2013). The albumin to globulin to ratio is consequently a helpful indicator of overall liver health and inflammatory status. A higher albumin to globulin ratio implies increased liver health, while a lower ratio implies decreased serum albumin and therefore decreased liver health during the acute phase reaction. The negative correlation between plasma globulin and hepatic expression of IGF-I and GHR, as well as the positive correlations between albumin to globulin ratio and hepatic expression of IGF-I and GHR, suggests that cows in more positive energy balance also have improved liver health. We would expect hepatic TNFα expression to negatively correlate with the albumin to globulin ratio considering a higher ratio means a reduced inflammatory status (Bertoni and Trevesi, 2013; eClinpath Cornell Vet Med, 2013), but we did not find such correlations.

Table 8. Pearson correlations[1] between insulin-like growth factor one (IGF-I), growth hormone receptor (GHR), tumor necrosis factor alpha (TNFα), and TNFα receptor superfamily 1a (TNFRSF1a) expression and plasma variables related to liver health; d 7 and d 14 correspond to samples taken on liver biopsy day 7 and day 14.

	IGF-I		GHR		TNFα		TNFRSF1a	
	d 7	d 14	d 7	d 14	d 7	d 14	d 7	d 14
AST[2] d 7	-0.090	-0.059	-0.002	0.031	-0.173	-0.131	-0.273	-0.354
	0.579	0.718	0.988	0.850	0.285	0.419	0.089	0.025
AST d 14	-0.068	-0.040	-0.020	-0.013	-0.234	-0.171	-0.210	-0.296
	0.677	0.808	0.904	0.937	0.146	0.290	0.192	0.064
Cholesterol d 7	0.431	0.356	0.199	0.171	-0.110	-0.033	0.006	0.070
	0.006	0.024	0.217	0.293	0.497	0.842	0.968	0.668
Cholesterol d 14	0.312	0.243	0.061	0.117	-0.184	-0.081	0.063	-0.0003
	0.050	0.131	0.710	0.470	0.256	0.620	0.700	0.999
GLDH[3] d 7	-0.036	0.133	0.173	0.254	-0.218	-0.238	-0.254	-0.347
	0.826	0.413	0.286	0.113	0.176	0.139	0.114	0.028
GLDH d 14	-0.110	-0.135	-0.071	-0.180	-0.175	-0.187	-0.270	-0.230
	0.501	0.408	0.665	0.268	0.281	0.247	0.092	0.154
GGT[4] d 7	0.285	0.084	0.122	0.055	-0.199	-0.289	-0.360	-0.227
	0.075	0.606	0.452	0.735	0.218	0.070	0.023	0.159
GGT d 14	0.101	0.031	-0.057	-0.179	-0.255	-0.256	-0.274	-0.134
	0.534	0.852	0.726	0.268	0.112	0.110	0.088	0.410
Haptoglobin d 7	-0.273	0.047	-0.035	-0.136	-0.011	0.0001	0.326	0.007
	0.088	0.774	0.832	0.404	0.946	0.999	0.040	0.965
Haptoglobin d 14	-0.184	-0.047	-0.039	-0.234	0.069	0.140	0.220	0.373
	0.255	0.773	0.812	0.146	0.673	0.391	0.172	0.018
Bilirubin d 7	-0.357	-0.166	-0.113	-0.015	0.258	0.400	-0.031	-0.186
	0.024	0.306	0.487	0.929	0.108	0.011	0.851	0.251
Bilirubin d 14	-0.160	-0.243	-0.098	-0.195	-0.046	0.070	-0.383	-0.257
	0.323	0.132	0.548	0.228	0.777	0.670	0.015	0.110
Total Protein d 7	-0.182	-0.273	-0.083	-0.282	-0.069	0.138	-0.254	-0.047
	0.261	0.089	0.612	0.077	0.674	0.395	0.114	0.771
Total Protein d 14	-0.249	-0.151	-0.065	-0.224	-0.067	0.043	0.209	0.088
	0.121	0.352	0.691	0.165	0.680	0.794	0.196	0.591
Albumin d 7	0.451	-0.017	0.226	0.180	0.081	-0.155	-0.210	-0.321
	0.004	0.918	0.161	0.267	0.621	0.340	0.193	0.044
Albumin d 14	0.223	0.026	0.150	0.231	-0.109	-0.159	0.032	-0.207
	0.167	0.875	0.356	0.152	0.502	0.326	0.846	0.199
Globulin d 7	-0.316	-0.241	-0.151	-0.315	-0.089	0.177	-0.158	0.065
	0.047	0.135	0.353	0.047	0.585	0.275	0.330	0.690
Globulin d 14	-0.317	-0.163	-0.107	-0.293	-0.040	0.087	0.207	0.146
	0.046	0.315	0.510	0.066	0.805	0.594	0.200	0.368

Albumin to Globulin ratio d 7	0.379 0.016	0.176 0.279	0.199 0.220	0.309 0.053	0.141 0.385	-0.162 0.317	0.123 0.450	-0.120 0.462
Albumin to Globulin ratio d 14	0.436 0.005	0.224 0.166	0.223 0.167	0.414 0.008	-0.002 0.989	-0.126 0.437	-0.096 0.555	-0.156 0.336

[1]Correlation coefficient (r) is the top number, with corresponding P-value beneath.

[2]Aspartate aminotransferase, [3]Glutamate dehydrogenase, [4]Gamma-glutamyltransferase,

Conclusions

While we did not observe a difference in hepatic gene expression of IGF-I, GHR, TNFα, or TNFRSF1a based on varying dietary fiber treatments alone, there was a treatment by parity effect, where cows in their third lactation or greater on the high fiber diet had lower IGF-I expression than cows in their second lactation on the high fiber diet. This might indicate that older, higher producing cows are more prone to the impacts of dietary restrictions and NEBAL.

There was an observed effect of genotype on gene expression, where the GHR genotype AB tended to have higher IGF-I and GHR expression, and the TNFα genotype AB tended to have lower IGF-I expression, though these results should be interpreted with caution given low sample numbers for certain alleles. Given these findings with a small and unevenly distributed sample size, the effect of IGF-I, GHR, and TNFα genotype on hepatic gene expression with a larger population of cattle is an area of future research.

Finally, hepatic expression of IGF-I and GHR generally correlated positively with variables related to improved energy balance and liver health, while hepatic expression of TNFα and TNFRSF1a correlated positively with markers associated with decreased energy balance and liver health. This further supports the concept that NEBAL, metabolism, and inflammation are all interrelated.

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