Effects of Peripartum Recombinant Bovine Somatotropin Treatment and Prepartum Stocking Density on Immune Responses, Metabolism, Health, and Performance of Dairy Cows

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Dedication

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Introduction

One of the greatest challenges in the modern dairy industry is dealing with health disorders of high milk producing cows and, at the same time, maintaining the industry profitable and sustainable. Diseases affect animal welfare and the profitability of dairy operations because of treatments costs, reduced milk production, compromised reproductive performance, increased mortality and culling, and increased replacement costs. Vulnerability to health disorders is increased during the transition period, from the last three weeks of pregnancy to the first three weeks of lactation, because of several physiological changes cows undergo in preparation for parturition and lactogenesis. Therefore, in the last decades, the transition period has been the focus of much research aiming to better understand physiological alterations during this critical period and seeking to identify best management practices to decrease susceptibility of dairy cows to health disorders and incidence of postpartum diseases.

Periparturient cows undergo physiological adaptations associated with changes in reproductive status, from pregnant to non-pregnant, lactational status, and feeding management. Such physiological adaptations include hormonal, metabolic, and body fat mass composition changes that negatively regulate dry matter intake (**DMI**), which decreases by approximately 30% during the prepartum period (Bertics et al., 1992; Hayirli et al., 2002; Ingvartsen and Andersen, 2000). Although feed intake starts to increase immediately after parturition, this increase is not sufficient to meet nutrient and energy requirements for lactogenesis. Thus, cows suffer from negative energy balance (**NEB**; Bell, 1995). During NEB fat depots are mobilized as alternative fuel source, increasing circulating non-esterified fatty acids (**NEFA**) and ketone bodies (Grummer, 2004). An orchestrated increase in growth hormone (**GH**) concentration and tissue insulin resistance and decrease in insulin-like growth factor-1 (**IGF-1**) concentration and pancreas insulin production enhance adipose tissue mobilization and nutrient partitioning to the mammary gland (Bell, 1995). Excessive lipolysis, however, results in excessive NEFA release from

the adipose tissue and may result in hyperketonemia and hepatic lipidosis, which are known to compromise liver and immune functions (Detilleux et al., 1994; Grummer, 2004; Kehrli et al., 1989a,b).

Immunosuppression in transition cows is a result of a sum of events causing immune cells to function inappropriately. Shortage in energy, nutrients and calcium impair immune cells' metabolism. Furthermore, immune cells' structures and components are altered in response to high levels of NEFA, cortisol, and products of oxidative stress (Burton et al., 1995; Contreras et al., 2010; Detilleux et al., 1994; Kehrli et al., 1989a,b). Thereafter, immunosuppressed periparturient cows are at higher risks for developing infectious and non-infectious health disorders (Cai et al., 1994; Hammon et al., 2006; Kimura et al., 2002; Romaniukowa, 1984).

Approximately 75% of diseases in dairy cows happen in the first month postpartum (LeBlanc et al., 2006). The majority of cases of milk fever, ketosis, retained fetal membranes (**RFM**), metritis, mastitis and displacement of abomasum occur in the first 30 d postpartum (LeBlanc et al., 2006). Metritis and subclinical ketosis, for example, are very common postpartum disorders with incidences ranging from 20 to 40% (LeBlanc et al., 2008) and 40 to 60% (Duffield et al., 1998; McArt et al., 2012) in US dairy farms, respectively. The estimated cost per case of metritis is $ 358 (Overton and Fetrow, 2008) and the estimated cost per case of ketosis is $ 289 (McArt et al., 2015). The high incidences and costs of such disorders draw attention to the importance of an appropriate transition period management that decreases incidence of health disorders and their negative effects on the milk production.

Consequently, management strategies that reduce the incidence and severity of diseases, increase profitability, improve animal well-being, and sustainability of dairy operations may prompt producers' interest. Strategies that improve metabolism and immune function of periparturient dairy cows are of particular interest because they may reduce incidence

of diseases and losses due to treatment. Six experiments were performed herein to test two main strategies: the use of recombinant bovine somatotropin (**rbST**) during the peripartum period and the reduction of prepartum pens' stocking density from 100 to 80% of headlocks.

Treatment of periparturient dairy cows with rbST has been associated with positive effects on glucose metabolism by increasing hepatic gluconeogenesis and by decreasing glucose oxidation by peripheral tissues and the insulin's inhibitory action on gluconeogenesis (Peel and Bauman, 1987; Bauman et al., 1989; Bauman and Vernon, 1993). Thus, it is plausible that treatment of periparturient cows with rbST could increase glucose and decrease NEFA and BHBA concentrations (Putnam et al., 1999). Concentrations of GH and IGF-1 are increased following treatment of periparturient cows with rbST (Vicini et al., 1991; Gulay et al., 2004a,b). Growth hormone plays an important role in immunoregulation stimulating especially cell-mediated and humoral responses (Kelley, 1990; Meazza et al., 2004). Similarly, IGF-1 plays an important role in the development of immune responses such as T-cell proliferation, chemotaxis, T-cell activation, apoptosis inhibition in several cell lines, and natural killer cell cytotoxicity (Weigent, 2013). Furthermore, polymorphonuclear leukocyte (**PMNL**) phagocytosis and oxidative burst activities were increased when PMNL were incubated with GH or IGF-1 and challenged with *E. coli* (Bjerknes and Aarskog, 1995; Inoue et al., 1998). Therefore, increasing GH and IGF-1 concentrations may improve innate and adaptive immune responses. Weaned and transported piglets treated with recombinant porcine somatotropin had greater concentrations of GH and IGF-1 and greater antibody concentrations (Sohmiya et al., 2005). We hypothesize that administration of rbST to periparturient dairy cows would increase GH and IGF-1 concentrations, improve metabolism and immune response and, consequently, decrease the incidence of postpartum diseases, increase milk yield and improve reproductive performance.

Overstocking prepartum cows is associated with changes in feeding behavior resulting in greater competition at the feed bunk (Hosseinkhani et al., 2008; Proudfoot et al., 2009). Dominant cows are at greater risk to develop ruminal acidosis as a result of increased feed sorting for energy/protein rich ingredients and fewer meals per day. Submissive cows are predisposed to metabolic disorders, ketosis and hepatic lipidosis because of reduced feed intake and possibly ingestion of low energy/protein dense ingredients (Hosseinkhani et al., 2008; Proudfoot et al., 2009). Oetzel et al. (2007) observed a decrease of 0.7 kg/d in milk yield of first lactation cows up to 80 d postpartum for every 10% increase in stocking density above 80% in the prepartum period., Parous and nulliparous animals were commingled. Reducing stocking density in prepartum pens might be a strategy to reduce disruptive behavior and consequently increase feed intake and improve metabolic status of peripartum cows. We hypothesized that increasing prepartum stocking density would negatively affect metabolic parameters, cause immunosuppression and, consequently, increase health disorders and impair productive and reproductive parameters of peripartum dairy cows.

CHAPTER I

Literature Review

Periparturient Feed Intake and Homeorhetic Adaptations

Feed intake has been reported to decrease from 2% of body weight (**BW**) in the beginning of the dry period to 1.4% BW in the last week before calving, reaching nadir at calving (Bertics et al., 1992; Hayirli et al., 1998; Robinson and Garrett, 1999). Although postpartum DMI starts to increase gradually as milk yield increases, the peak in milk yield typically occurs between 5 to 7 wk postpartum and the peak of intake happens at approximately 8 wk after calving; thus, cows suffer from NEB for up to 8 to 12 wk after parturition (Heinrichs et al., 1996). Regulation of DMI during the peripartum period is complex and involves changes in endocrine, nervous and immune systems (Ingvarsten and Andersen, 2000). Approximately 1 wk prior to parturition, cortisol and estradiol concentrations increase and progesterone concentration decreases in preparation for parturition, which exacerbates the decrease in DMI (Forbes, 1995). Body fat mass is also negatively correlated with DMI (Carpenter and Grossman, 1983). Overconditioned cows have greater circulating concentrations of NEFA, glycerol, and ketone bodies during periods of NEB; the oxidation of these lipogenic substrates can provide satiety signaling to the central nervous system (**CNS**) decreasing DMI (Langhans, 1996). Moreover, Bines and Morant (1983) suggested that thin cows have faster utilization of lipogenic substrates compared with fat cows. This prevents excessive accumulation of these substrates in the blood, enhancing the need for rumen absorption of nutrients and stimulating feed intake in thin cows. Leptin, a hormone produced by adipose tissue that acts on the CNS (Chlliard et al., 1998), suppresses feed intake by inhibiting neuropeptide Y, a stimulator of feed intake, and by stimulating pathways that inhibit feed intake such as the melanocortin-stimulating hormone pathway (Flier and Maratos, 1998). As cows gain BCS during the prepartum period, circulating leptin concentrations are expected to increase contributing to the decrease in DMI prepartum (Ingvarsten and Andersen, 2000). The immune system

regulates DMI through the production of cytokines by immune cells during acute phase inflammatory responses. Proinflammatory cytokines such as TNF-α, interleukin-1β (**IL-1β**), and interleukin-6 (**IL-6**) affect feed intake (Johnson, 1998; Langhans, 1996), in part, by inhibiting rumen motility and increasing leptin secretion (Finck et al., 1998; Van-Miert et al., 1992). Therefore, the occurrence of diseases and inflammatory processes may aggravate the decrease in DMI in transition cows.

Fetal and placental energy demands for growth are estimated to be 3 to 5 Mcal of net energy per day in the last 100 days of gestation, whereas energy requirements for lactation are increased to approximately 26 Mcal of net energy (Bell, 1995). Major changes in metabolism occur to cope with this increase in energy requirements during the transition period, when DMI is low. These are homeorhetic changes that assure that cows continue to produce milk during periods of scarce nutrient availability (Bauman, 2000). Homeorhesis was defined by Bauman and Currie (1980) as 'the orchestrated or coordinated changes in metabolism of body tissues necessary to support a physiological state'. During the transition from pregnant-non-lactating to non-pregnant-lactating state, homeorhetic changes are designed to support lactation, the dominant physiological state (Bauman, 2000). The decoupling of the somatotropic axis coordinates the adaptations required for the preservation of metabolic homeostasis (Bell, 1995) and lactation (Bauman, 2000). The decoupling of the somatotropic axis is characterized by elevated concentrations of GH and reduced concentrations of IGF-1 because of the downregulation of expression of GH 1-α receptors in the liver (Rhoads et al., 2004; Lucy, 2008). The decrease in IGF-1 concentrations reduces the IGF-1 negative feedback effect on GH release by the pituitary gland maintaining elevated GH concentrations in the face of NEB (Lucy, 2008). The concentration of GH increases rapidly between the end of gestation and the initiation of lactation (Lucy, 2008). The decoupling of the somatotropic axis is important to promote the GH catabolic effect to mobilize nutrients from the diet and tissues for lactation. The main aspect of homeorhetic adaptations during early lactation is the prioritization of

nutrient partitioning for the mammary gland. As such, pancreas becomes insensitive to glucose around the time of parturition causing a reduction in insulin concentration and a state of insulin resistance by peripheral tissues is observed (Bauman, 2000). Consequently, liver gluconeogenesis, lipogenesis, skeletal muscle glucose uptake, and whole body glucose oxidation are decreased, resulting in more nutrients available to the mammary gland (Vernon and Sasaki, 1991; Williamson and Lund, 1994; Bell and Bauman, 1997; Vernon, 1998).

The combination of reduced feed intake, NEB, increased GH concentration, and insulin resistance result in increased lipolysis and elevated plasma concentrations of NEFA (Bertics et al., 1992; Grummer, 1995; Dyk and Emery, 1996). Exposure of cows to severe and prolonged NEB and extremely elevated concentrations of NEFA predisposes cows to hepatic lipidosis, compromised liver function, incomplete oxidation of NEFA, and elevated concentrations of BHBA (Grummer et al., 2004). Once lipolysis occurs, circulating NEFA are taken up by the liver and used to form triacylglycerol or are metabolized to acetyl-CoA. The acetyl-CoA has different pathways in the liver: it may be introduced into the Kreb's cycle, or it may be used as substrate for the synthesis of cholesterol, or for ketogenesis (Bruss, 1993). Whether the acetyl-CoA is introduced into the Kreb's cycle or not depends on the availability of oxaloacetate, which is predominantly derived from gluconeogenic precursors such as propionate, pyruvate, glycerol, or amino acids. During the NEB, the supply of these gluconeogenic precursors is inadequate, especially propionate derived from feed intake, and the availability of oxaloacetate for introducing acetyl-CoA into the Kreb's cycle is scarce. The lack of oxaloacetate leads to an excess of acetyl-CoA that is then used for ketogenesis and production of ketone bodies (Rukkwamsuk et al., 1999; Veenhuize et al., 1991). Under these conditions, the cow may develop subclinical (BHBA $\geq$ 1.2 and $<$ 3.0 mmol/L) or clinical (BHBA $\geq$ 3.0 mmol/L) ketosis (Oetzel, 2004; McArt et al., 2013). Increased NEFA uptake by the liver increases the activity of hepatic TG synthesizing enzymes, particularly phosphatidate phosphohydrolase and diacylglycerol acyltransferase

(Top et al., 1996). The TG are secreted by the liver in the form of very low density lipoproteins (**VLDL**) which are transported predominantly to the udder. The synthesis of VLDL in the liver requires apoliproteins, cholesterol, cholesterol esters, and phospholipids (Gibbons et al., 1995). Serum concentrations of total cholesterol and apolipoproteins are reduced in postpartum cows compared with non-lactating or lactating pregnant cows (Marcos et al., 1990), resulting in reduced plasma concentrations of low density lipoprotein (**LDL**) and VLDL (Rayssinguier, 1988). Consequently, fatty liver may develop when the rate of TG synthesis exceeds the rate of TG export as VLDL, which is specifically slow in ruminants compared with other species (Grummer, 1993).

Several experiments have demonstrated an important link between metabolic status and risk of diseases during the periparturient period. Ospina et al. (2010) demonstrated that prepartum and postpartum NEFA concentrations $\geq$ 0.29 and $\geq$ 0.57 mEq/L, respectively, could be used as an indicator for increased risk of displaced abomasum (**DA**), clinical ketosis, metritis and RFM. During the first 2 wk postpartum cows with BHBA > 1,000 or 1,200 μmol/L were at greater risk to develop metritis (Duffield et al., 2009), DA (LeBlanc et al., 2005) and had greater incidence and severity of mastitis (Kremer et al., 1993). In a large experiment conducted in 55 free-stall dairy herds across the United States and Canada, herds in which ≥ 25% of the cows had BHBA ≥ 1,400 μmol/L one week after calving had greater prevalence of DA (Chapinal et al, 2012).

In conclusion, peripartum decrease in feed intake and homeorhetic adaptations to prioritize nutrient partitioning to the mammary gland are natural and inevitable physiological events in mammalians. However, exacerbated NEB peripartum and NEFA mobilization lead to hyperketonemia and hepatic lipidosis that are predisposing factors to other health disorders (Dyk and Emery, 1996; Grummer, 1995). Peripartum management strategies that prevent excessive decrease in DMI and energy balance and improve liver function (i. e. increasing

gluconeogenesis and lipid transport from the liver) would potentially decrease risks for postpartum diseases.

Periparturient Immunosuppression and Health Disorders

Polymorphonuclear leukocyte phagocytosis and oxidative burst may decrease starting approximately 2 wk prepartum, reaching a nadir in the first week postpartum (Hoeben et al., 1997; Kehrli et al., 1989a; Moreira da Silva et al., 1998). Furthermore, lymphocyte blastogenesis and immunoglobulin production by B cells decreases from 1 wk pre- to 1 wk postpartum (Kehrli et al., 1989b; Nonnecke et al., 2003; Lacetera et al., 2005). The immunosuppression observed among dairy cows during the periparturient period is associated likely with hormonal changes and with shortages of major energetic fuels and minerals used by immune cells.

Five days before calving, concentrations of progesterone decrease whereas concentrations of cortisol, estradiol, prostaglandin $F_{2\alpha}$ (**$PGF_{2\alpha}$**), and prolactin increase to support lactogenesis and induce parturition (Stevenson, 2007). *In vitro* studies have demonstrated that high levels of progesterone, 6.56 μg/ml, can decrease PMNL oxidative burst capacity (Chaveiro and Moreira da Silva, 2010). Furthermore, fluctuations in serum progesterone and estradiol have been related to altered neutrophil gene expression (Madsen et al., 2002). Hoeben et al. (1998) observed *in vitro* that physiological concentrations of glucocorticoids depress PMNL oxidative burst capacity and cloning efficiency index of proliferating bovine bone marrow cells. Moreover, cortisol suppresses immune response by down regulating neutrophil expression of L-selectin and CD18, adhesion molecules involved in the trafficking of neutrophils through the endothelium to the site of inflammation (Burton et al., 1995; Weber et al., 2001). Cortisol secretion in response to stress is well characterized in many species and may in part serve to control the magnitude of the immune response (Blalock, 1994; Derijk and Sternberg, 1994). On the other hand, deficient

periparturient management of dairy cows that induce stress are expected to increase concentrations of cortisol and further compromise the immune function of transition cows.

The onset of milk production and the increased demands for energy, protein, and minerals are also associated with the diminished function of neutrophils and lymphocytes in the periparturient period (Kehrli et al., 1989b; Detilleux et al., 1994). Kimura et al. (1999) demonstrated that neutrophil activity, measured by myeloperoxidase activity, of mastectomized cows increased faster after calving compared with intact cows (Kimura et al., 1999). Thereby, situations that further reduce DMI and that exacerbate NEB and energy and nutrient deficits may compromise the immune function during the periparturient period. Cows experiencing NEB in early lactation have lower blood PMNL phagocytosis, chemotaxis and diapedesis than cows in mid-lactation (Stevens et al., 2011). The decreased glutamine and glucose concentrations observed during the periparturient period may further compromise immune function. Glutamine is a functional amino acid involved in modulation of cytokine production by immune cells, reactive oxygen intermediate production by neutrophils, cell division, phagocytosis, and can be used as energy source by PMNL (Newsholme et al., 1985; Ogle et al., 1994; Yassad et al., 1997; Wallace and Keast, 1992). Glucose is required by macrophages and PMNL for proliferation, survival, and differentiation and it is the preferred metabolic fuel during inflammation for activated PMNL, macrophages, and lymphocytes rather than fatty acids, amino acids or ketone bodies (Barghouthi et al., 1995; Gamelli et al., 1996; PithonCuri et al., 2004). Low circulating calcium concentrations during the postpartum period, as a consequence of the exacerbated calcium demands associated with colostrogenesis and lactogenesis, are also a predisposing factor for immunosuppression in the periparturient cow. Hypocalcemia results in failure of immune cells to become activated in the presence of bacteria (Kimura et al., 2006). Cytosolic calcium (Ca^{2+}) concentration in immune cells rises upon challenge with pathogens causing their activation and production of antibodies, bactericidal peptides, and other bactericidal substances (Lazzari et al., 1986; Cavicchioni et al., 2005). In

periparturient cows that developed hypocalcemia, the rise in cytosolic Ca^{2+} in peripheral blood mononuclear cells activated with anti-CD3 antibodies was blunted even before cows were diagnosed with hypocalcemia (Kimura et al., 2006). This mononuclear cells blunted cytosolic release of Ca^{2+} in hypocalcemic cows would likely predispose cows to postpartum diseases like metritis and mastitis (Goff, 2008).

The lipolysis observed during the periparturient period alters the fatty acid composition of different organs and cell populations, including blood, liver, adipose tissue, and mononuclear cells (Douglas et al., 2007; Contreras et al., 2010). Furthermore, increased NEFA concentrations modify vascular inflammatory responses by changing the expression of pro-inflammatory mediators, affecting traffic of leukocytes to the site of infectious tissues, which determines the efficiency of host inflammatory responses to infectious diseases (Contreras et al., 2012; Ley et al., 2007). Ster et al. (2012) demonstrated that proliferation of mononuclear cells was inversely correlated to NEFA concentrations and that NEFA negatively affected PMNL oxidative burst. Blood monocytes incubated with high NEFA concentration (1.0 mM) had suppressed DNA synthesis and IgM and interferon-γ secretion than monocytes incubated with lower (0.0625 and 0.125 mM) NEFA concentrations (Lacetera et al., 2004). In addition, PMNL incubated with high NEFA (2.0 mM) had a 48% increase in necrosis (Scalia et al., 2006). Similarly, bovine blood PMNL cultured in the presence of BHBA had reduced chemotactic capability (Suriyasathaporn et al., 1999) and respiratory burst activity (Hoeben et al., 1997). Moreover, incubation of lymphocytes in the presence of BHBA decreases proliferation, antibody secretion, and blastogenesis (Nonnecke et al., 1992; Sato et al., 1995; Targowski and Klucinski, 1983). Zerbe et al. (2000) evaluated the relationship between liver TG content and PMNL function in periparturient dairy cows. Polymorphonuclear cells from cows that had elevated liver TG content (> 40 mg/g) had reduced expression of the integrins adhesion molecules CD11a and CD11b compared with PMNL from cows with reduced TG content (Zerbe et al., 2000). Furthermore, PMNL from cows with elevated hepatic TG concentrations had reduced

antibody independent and antibody dependent cellular cytotoxicity postpartum compared with PMNL of cows with reduced hepatic TG concentration (Zerbe et al., 2000). Hill et al. (1985) observed that cows with fatty liver retained viable bacteria in their udders for longer periods than cows with normal livers. Finally, Wentink et al. (1997) demonstrated that cows with high hepatic TG concentrations had an impaired or delayed specific immunoreaction against tetanus toxoid compared with cows that had normal hepatic TG concentration.

Immune competence is a determinant factor in the development of postpartum diseases. The maternal immune system is responsible for the placenta detachment from the endometrium and preventing uterine infections. When the blood supply to the placenta ceases at parturition, the fetal membranes become a "foreign body" and the maternal immune system must recognize and attack it as foreign body to expel it (Gunnink, 1984). Cotyledons from cows that developed RFM had less leukocyte chemoattractant than cotyledons from cows that expelled the placenta normally after calving (Gunnink, 1984). Furthermore, *in vitro* assays demonstrated that leukocytes obtained from cows that developed RFM were less able to recognize cotyledon tissue in a chemotaxis assay before, at, and after calving than leukocytes obtained from cows that did not develop RFM (Gunnink, 1984). Polymorphonuclear cells from cows that develop RFM have decreased response to chemoattractants and reduced phagocytic capacity from the prepartum period until 1 to 2 wk after calving (Cai et al., 1994; Kimura et al., 2002; Romaniukowa, 1984). Delayed expulsion of fetal membranes increases the exposure of the uterus to environmental pathogens and contamination. Regardless of occurrence of RFM, the majority of cows have contamination of the uterus during the postpartum period (LeBlanc, 2010) and neutrophils plus acute phase proteins provide the first line of defense against this contamination, migrating from the bloodstream to the uterus. In immune compromised cows, the immune system is unable to promptly eliminate bacterial contamination and bacteria overgrowth in the uterus results in metritis (LeBlanc, 2010). Metritis is

characterized by a foul-smelling, red-brown watery discharge from the uterus within the first 14 days after calving and it may or may not be accompanied by systemic symptoms (i.e. pyrexia, anorexia, depression). Hammon et al. (2006) demonstrated that in cows diagnosed with puerperal metritis and subclinical endometritis PMNL function was impaired significantly during the periparturient period compared with healthy cows. Therefore, elevated incidence of RFM and metritis are considered manifestations of a poorly functioning immune system during the transition period (Goff, 2008).

Recombinant Bovine Somatotropin and Metabolism

Bovine somatotropin (**bST**), or growth hormone (**GH**), is a peptide hormone synthesized and secreted by the pituitary gland. Somatotropin participates in the control of nutrient partitioning directly through the regulation of physiological processes, including carbohydrate and lipid metabolism (Le Roith et al., 2001; Lupu et al., 2001), and indirectly through the regulation of IGF-1 secretion. Insulin like growth factor-1 is a somatomedin whose release by the liver is regulated by somatotropin (**ST**) activated JAK-STAT signaling pathways (Binder et al., 2007). Adipocytes and hepatocytes are major direct targets of ST action. In the adipose tissue ST acts chronically to facilitate lipolysis and decrease lipid synthesis (Boyd and Bauman, 1989). Somatotropin potencializes catecholamine-stimulated lipolysis, whereas it decreases insulin-dependent lipogenesis and suppresses adenosine's inhibitory effect on lipolysis (Houseknecht et al., 1995; Lanna et al., 1995; Lanna et al., 1992). In the liver ST increases gluconeogenesis and suppresses insulin's inhibitory effect on gluconeogenesis (Cohick et al., 1989; Gopinath and Etherton, 1989) through the regulation of enzymes such as phosphoenolpyruvate carboxykinase, a key enzyme in the conversion of oxaloacetate to phosphoenolpyruvate (Velez and Donkin, 2004). Furthermore, ST increased fatty acid oxidation to CO_2 in liver slices, but it had no effect on hepatic concentration of triacylglycerol (Pocius and Herbein, 1986). Recombinant forms of bST were developed to share similar biological functions as the pituitary-derived bST (Langley et al., 1987). The development and production of recombinant bovine

somatotropin (rbST) in large scale addresses the need of the dairy industry to increase production efficiency through the prioritization of nutrients to milk synthesis and increasing activity, number, and maintenance of secretory cells in the mammary gland (Bauman and Vernon 1993).

Strategies that increase plasma glucose concentrations in periparturient cows might result in reductions of circulating NEFA and BHBA concentrations and liver TG content (Studer et al., 1993). Because rbST was associated with positive effects on glucose metabolism (Peel and Bauman, 1987), its utilization in periparturient cows to prevent metabolic disorders and improve overall health and productivity has been explored (Gohary et al., 2014; Gulay et al., 2007; Gulay et al., 2004a,b; Gulay et al., 2003; Gulay et al., 2000; Putnam et al., 1999). Putnam et al. (1999) treated prepartum cows with 142.8 mg of rbST every 14 d and observed a reduction in NEFA and BHBA concentrations and an increase in glucose concentration around the time of calving. Cows treated with rbST also had greater DMI and produced 3.3 kg/d more milk in the first 42 d of lactation (Putnam et al., 1999). More recently, treatment of prepartum cows with 325 mg of rbST every 14 d increased peripartum glucose concentrations but NEFA and BHBA concentrations were slightly higher 1 wk postpartum for rbST treated cows compared with untreated controls (Gohary et al., 2014). Although rbST may increase adipose tissue mobilization, rbST-induced increase in uptake and oxidation of NEFA and reduced glucose uptake by peripheral tissues may prevent significant rises in circulating NEFA concentrations (Bauman, 1992). In other experiments, treatment of periparturient cows with low doses of rbST increased DMI, decreased the incidence of ketosis and clinical mastitis and increased milk yield (Gulay et al., 2007; Gulay et al., 2004a,b; Gulay et al., 2000; Putnam et al., 1999). The seemingly contradictory effects of rbST treatment of periparturient cows on metabolism, health, and performance described previously may be a consequence of different doses of rbST used among experiments and large intervals between doses (14 d). More experiments testing different doses of rbST with shorter intervals are needed.

Furthermore, to better understand the mechanisms of action of rbST in the liver of periparturient cows, experiments evaluating changes in hepatic mRNA gene expression of genes related to glucose and lipid metabolism during the periparturient period should be conducted.

Recombinant Bovine Somatotropin and Immune System

The neuroendocrine and the immune systems share common ligands and receptors facilitating the communication between the two systems (Blalock and Smith, 2007; Weigent and Blalock, 1995). The hypothalamic-growth hormone axis is one of the major pathways by which the CNS regulates the immune system (Blalock and Smith, 2007). Immune cells, particularly lymphocytes, have been shown to produce GH and IGF-1, which may act through autocrine and paracrine mechanisms, and to express receptors for GH and IGF-1 (Merimee et al., 1989; Rosenfeld et al., 1979). Several *in vivo* and *in vitro* experiments with humans and mice models have characterized the effects of GH and IGF-1 on immune cells. Treatment of immune cells with GH altered humoral and cellular immune functions, including immunoglobulin secretion of B-cells, natural killer (**NK**) cell activity, phagocytosis, and oxidative burst of neutrophils and macrophages (Kooijman et al., 1996; Weigent, 1996). Growth hormone treatment also increased expression of adhesion molecules (CD62L) in mice lymph nodes (Smaniotto et al., 2004) and enhanced adhesion of human neutrophils (Ryu et al., 2000). Furthermore, treatment of malnourished human patients with GH increased PMNL phagocytosis (Kotzmann et al., 2003) and GH replacement therapy of GH deficient humans increased release of superoxide anion by neutrophils (Reinisch et al., 1996). Treatment of GH deficient humans with ST increased IGF-1 concentrations and increased concentrations of granulocyte colony-stimulating factor and blood neutrophil counts (Sohmiya et al., 2005). Many of the effects of GH on the immune system may be mediated by IGF-1. Insulin-like growth factor 1 modulates hematopoiesis directly through induction of cell proliferation and anti-apoptotic signaling (Heemskerk et al., 1999). Moreover, incubation of PMNL with GH or IGF-1 increased

PMNL phagocytosis and oxidative burst after challenge with *E. coli* (Bjerknes and Aaskog, 1995; Inoue et al., 1998). Insulin-like growth factor-1 has been demonstrated to regulate immune cell proliferation (Merimee et al., 1989), chemotaxis (Tapson et al., 1988), T-cell activation (Johnson et al., 1992), inhibition of lymphocytes and neutrophils apoptosis (Baserga et al., 1997; Kooijman et al., 2002), and NK cell cytotoxicity (Kooijman et al., 1992). Moreover, IGF-1 has important function in increasing B-cell differentiation, maturation and proliferation (Funk et al., 1994; Gibson et al., 1993). Modulatory effects on inflammation can also be exerted by IGF-1 with anti-inflammatory functions by regulation of TNF-α, IL-1β, and IL-6 production (Guo et al., 2014).

Few studies evaluating the effects of ST on immune system of dairy cows have been reported. Treatment of healthy lactating cows with rbST induced an increase in PMNL and total leukocyte count in circulation (Burvenich et al., 1999). When PMNL retrieved from blood were treated *in vitro* with rbST PMNL activity responses were not altered; however, *in vivo* treatment of cows with rbST increased PMNL oxidative burst activity and NADPH-oxidase activity in the granules (Burvenich et al., 1999). These effects of rbST on immune response might be directly associated with rbST or indirectly through IGF-1 modulatory effects on the PMNL (Vandeputte-Van Messom and Burvenich, 1993).

Although the decoupling of the somatotropic axis results in increased concentrations of GH and decreased concentrations of IGF-1 during the periparturient period (Lucy, 2008; Rhoads et al., 2004), treatment of periparturient dairy cows with rbST increased IGF-1 concentrations (Gulay et al., 2004a; Gulay et al., 2004b; Vicini et al., 1991). Therefore, therapeutic strategies that increase circulating IGF-1 concentrations in periparturient dairy cows may sustain coupling of the somatotropic axis and improve innate and adaptive immune responses, via direct effects of GH and IGF-1 on immune cells or improved cow metabolism and consequent improvement in immune cells metabolism.

Social Behavior and Prepartum Stocking Density

Resting, feeding, and rumination are the most important natural behaviors of dairy cows and are related to health, welfare, and productivity (Grant, 2007). Situations of limited space, limited feed, elevated stocking density and frequent regrouping may increase the competition for resources and limit the cows' ability to behave naturally (Friend and Polan, 1974; Wechsler, 2007). Overstocking of freestall pens has been defined as housing a number of cows greater than the number of stalls available or decreasing the linear feeding space to < 60 cm per cow (Grant and Albright, 2001). Overstocking is a practice commonly used by dairy farms to increase herd size without increasing investments in facilities (Bewley et al., 2001).

Overstocking has been associated with decreased resting/lying time. Fregonesi et al. (2007) demonstrated that stocking densities of 109%, 120%, 133%, and 150% of stalls resulted in a linear reduction of lying time, relative to 100% stocking density. Similarly, when stocking density of pens of lactating dairy cows exceeded 113% (e.g. 131 and 142%) the lying time and the percentage of cows eating upon return from the milking parlor decreased, whereas the percentage of cows standing idly waiting for access to stalls increased (Hill et al., 2007). The increase in standing time can be detrimental to hoof health, may increase incidence of lameness because of the strain on the hoof from standing on concrete flooring (Cook, 2002) and because of softening of the hoof by more manure covering the alleyways that could predispose cows to hoof infections (Guard, 2002). Furthermore, cows deprived of lying had higher cortisol blood concentrations, which is an indicator of stress and is associated with immunosuppression (Munksgaard and Simonsen, 1996).

The effects of stocking density on feeding behavior have been examined. Linear increase in displacements from the feed bunk was observed when stall stocking density increased from 100% to 113%, 131%, and 142% (Krawczel et al., 2012). DeVries et al. (2004)

demonstrated that increasing the feed bunk space from 51 cm/cow to 102 cm/cow reduced the number of aggressive interactions per cow and increased the percentage of cows feeding during the 90 minutes following feed delivery (DeVries et al., 2004). Similarly, Huzzey et al. (2006) observed a reduction in feeding time and an increase in aggressive behavior as feed bunk space decreased from 81 cm/cow to 61, 41 or 21 cm. Reduced feeding space resulted in greater standing time idly waiting for access to feed (Huzzey et al., 2006; Olofsson, 1999; Singh et al., 1993). Proudfoot et al. (2009) and Hosseinkhani et al. (2008) demonstrated an interesting correlation between reduced feeding space and feeding behavior. Overstocking caused cows to have lower feeding time per visit and to eat at a faster rate (Proudfoot et al., 2009). Additionally, cows had fewer but longer meals per day and increased feed sorting when feed bunk space was reduced (Hosseinkhani et al., 2008). Such behaviors are proposed to predispose dominant cows to ruminal acidosis and submissive cows to reduced DMI, NEB, and metabolic disorders such as hyperketonemia and hepatic lipidosis (Proudfoot et al., 2009). Metabolic alterations can also be associated with stresses from social interactions by increasing epinephrine secretion and stimulating lipolysis, resulting in increased blood NEFA concentrations (Herdt, 2000). In agreement, Huzzey et al. (2012) observed greater plasma NEFA and fecal cortisol in cows housed at 200% stocking density compared with cows housed at 100% stocking density.

The effects of social behavior and stocking density on milk production, however, appear to be minimal or inexistent (Fregonesi and Leaver, 2002; Hill et al., 2007; Krawczel et al., 2008). The lack of effect of stocking density on milk yield may be explained by the limited number of animals used and the short duration of treatments. Oetzel et al. (2007) suggested that for every 10 percentage unit increase in stocking density above 80% of headlocks in the prepartum pen a 0.7 kg/d decrease in milk yield of first lactation cows would occur. The experiment by Oetzel et al. (2007), however, was not designed to evaluate the effects of stocking density on milk yield and parous and nulliparous animals were commingled. Interestingly, milk fat percentage was reduced by approximately 0.2% at 142% stocking

density compared with 100% stocking density and somatic cells count tended to increase for stocking densities above 113% (Krawczel et al., 2008).

Effects of stocking density on immune response of dairy cows are not well known. Dairy ewes housed in high stocking density had reduced anti-ovalbumin IgG concentration in response to an ovalbumin challenge compared with ewes housed in low stocking density conditions (Carporese et al., 2009). Additionally, ewes housed in high stocking density tended to have greater number of aggressive interactions, had reduced milk yield, and had increased milk somatic cell count compared with ewes housed in low stocking density (Carporese et al., 2009).

Overstocking cows in prepartum pens may predispose dominant and submissive cows to inadequate nutrient intake, higher cortisol levels, metabolic disorders, compromised immune function, and decreased milk yield. However, up to date, experiments conducted to evaluate the effects of stocking density on behavior, performance and metabolic and hormonal changes used a small number of experimental units or were not controlled experiments. Furthermore, no studies have evaluated the effects of stocking density during the prepartum period on innate and humoral immune responses, metabolic status, health, productive, and reproductive parameters in large dairy operations.

CHAPTER II

Effects of recombinant bovine somatotropin during the periparturient period on innate and adaptive immune responses, systemic inflammation, and metabolism of dairy cows

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OUTLINE

The aim of this experiment was to determine effects of treating peripartum dairy cows with body condition score ≥ 3.75 with recombinant bovine somatotropin (rbST) on immune, inflammatory, and metabolic responses. Holstein cows (253 ± 1 d of gestation) were assigned randomly to 1 of 3 treatments: untreated control (n = 53), rbST87.5 (n = 56; 87.5 mg of rbST), and rbST125 (n = 57; 125 mg of rbST). Cows in the rbST87.5 and rbST125 treatments received rbST weekly from −21 to 28 d relative to calving. Growth hormone, insulin-like growth factor 1, haptoglobin, tumor necrosis factor α, nonesterified fatty acids, β-hydroxybutyrate, glucose, and cortisol concentrations were determined weekly from −21 to 21 d relative to calving. Blood sampled weekly from −14 to 21 d relative to calving was used for hemogram and polymorphonuclear leukocyte (PMNL) expression of adhesion molecules, phagocytosis, and oxidative burst. Cows were vaccinated with ovalbumin at −21, −7, and 7 d relative to calving, and blood was collected weekly from −21 to 21 d relative to calving to determine IgG anti ovalbumin concentrations. A subsample of cows had liver biopsied −21, −7, and 7 d relative to calving to determine total lipids, triglycerides, and glycogen content. Growth hormone concentrations prepartum (control = 11.0 ± 1.2, rbST87.5 = 14.1 ± 1.2, rbST125 = 15.1 ± 1.3 ng/mL) and postpartum (control = 14.4 ± 1.1, rbST87.5 = 17.8 ± 1.2, rbST125 = 21.8 ± 1.1 ng/mL) were highest for rbST125 cows. Cows treated with rbST had higher insulin-like growth factor 1 concentrations than control cows (control = 110.5 ± 4.5, rbST87.5 = 126.2 ± 4.5, rbST125 = 127.2 ± 4.5 ng/mL)

only prepartum. Intensity of L-selectin expression was higher for rbST125 than for control and rbST87.5 cows [control = 3,590 ± 270, rbST87.5 = 3,279 ± 271, rbST125 = 4,371 ± 279 geometric mean fluorescence intensity (GMFI)] in the prepartum period. The PMNL intensities of phagocytosis (control = 3,131 ± 130, rbST87.5 = 3,391 ± 133, rbST125 = 3,673 ± 137 GMFI) and oxidative burst (control = 9,588 ± 746, rbST87.5 = 11,238 ± 761, rbST125 = 12,724 ± 781 GMFI) were higher for rbST125 cows than for control cows during the prepartum period. Concentrations of serum IgG anti-ovalbumin tended to be higher for rbST125 cows than for control cows (control = 0.75 ± 0.11, rbST87.5 = 0.94 ± 0.10, rbST125 = 1.11 ± 0.11 optical density) in the prepartum period. Haptoglobin concentration was significantly reduced 7 d postpartum for rbST125 treatment compared with control and rbST87.5 treatments (control = 2.74 ± 0.28, rbST87.5 = 2.81 ± 0.28, rbST125 = 1.87 ± 0.28 optical density). Although treatment tended to affect postpartum β-hydroxybutyrate (control = 747.5 ± 40.2, rbST87.5 = 753.2 ± 40.1, rbST125 = 648.8 ± 39.7 μmol/L), it did not affect liver contents of total lipids, triglycerides, or glycogen. Incidence of metritis among rbST125 cows was reduced compared with that in control cows (control = 23.1, rbST87.5 = 18.0, rbST125 = 7.8%). Treatment of dairy cows with 125 mg of rbST improved innate immune responses and IgG concentration, with limited effects on metabolism.

Key words: dairy cow, somatotropin, immune response

INTRODUCTION

A large proportion of dairy cows are immunosuppressed during the periparturient period. Phagocytosis and oxidative burst of PMNL may decrease starting approximately 2 wk prepartum, reaching a nadir in the first week postpartum (Hoeben et al., 1997; Kehrli et al., 1989a; Moreira da Silva et al., 1998). Furthermore, decreases in lymphocyte blastogenesis and immunoglobulin production by B cells have been observed from 1 wk pre- to 1 wk postpartum (Kehrli et al., 1989b; Nonnecke et al., 2003; Lacetera et al., 2005). It is not surprising, therefore, that a large proportion of dairy cows present infectious diseases such

as metritis and mastitis during the early stages of lactation (Burton et al., 2001; Hammon et al., 2006).

Dairy cows have increased concentrations of growth hormone (**GH**) and decreased IGF-1 concentrations immediately before parturition and during the postpartum period (Rhoads et al., 2004; Lucy, 2008). Insulin-like growth factor 1 is an important cell growth and differentiation factor associated with regulation of innate and adaptive immune functions (Heemskerk et al., 1999). Culture of granulocytes in the presence of IGF-1 reduces granulocyte apoptosis and increases phagocytosis, oxidative burst, and expression of adhesion molecules (Inoue et al., 1998; Kooijman et al., 2002). Furthermore, IGF-1 inhibits gene expression of mesenchymal stem cells and production of tumor necrosis factor α (**TNF-α**), IL-1β, and IL-6 (Guo et al., 2014). Growth hormone-deficient humans treated with recombinant somatotropin had increased concentrations of IGF-1 and granulocyte colony-stimulator factor and blood neutrophil counts (Sohmiya et al., 2005). Piglets treated with recombinant somatotropin before weaning and transportation had greater antibody concentrations (Kojima et al., 2008). Therefore, therapeutic strategies that increase circulating IGF-1 concentrations in periparturient dairy cows may improve their innate and adaptive immune responses.

Treatment of periparturient dairy cows with recombinant bovine somatotropin (**rbST**) increases IGF-1 serum concentrations during the periparturient period (Vicini et al., 1991; Gulay et al., 2004a,b) and has been associated with positive effects on glucose metabolism (Peel and Bauman, 1987). Recombinant bST may improve glucose metabolism by increasing hepatic gluconeogenesis, by decreasing glucose oxidation, and by blocking the inhibitory action of insulin on hepatic gluconeogenesis (Peel and Bauman, 1987; Bauman et al., 1989; Bauman and Vernon, 1993). Furthermore, treatment of periparturient cows with low doses of rbST was shown to increase DMI, decrease the incidence of ketosis and clinical mastitis, and increase milk yield (Putnam et al., 1999; Gulay et al., 2000, 2004b,

2007). Cows with elevated BCS could be ideal candidates for treatment with rbST during the periparturient period because obese cows have reduced postpartum IGF-1 concentrations (Kasimanickam et al., 2013). Thus, obese cows are more likely to be immunosuppressed (Samartin and Chandra, 2001) and are more susceptible to infectious diseases (Fronk et al., 1980; Treacher et al., 1986) during the peripartum period compared with thin cows. Furthermore, obese cows have suppressed DMI peripartum (Hayirli et al., 2002) and increased mobilization of NEFA (Treacher et al., 1986). Consequently, obese cows have impaired hepatic metabolism, are predisposed to fatty liver during early lactation, and are more likely to develop ketosis than thin cows (Mills et al., 1986; Smith et al., 1997).

The hypotheses of the current experiment were that administration of rbST to peripartum dairy cows with BCS $\geq$ 3.75 would increase GH and IGF-1 concentrations, improve immune responses (e.g., leukocyte count, phagocytosis, oxidative burst, and expression of adhesion molecules by PMNL, increased IgG concentration), reduce systemic inflammation (e.g., haptoglobin and TNF-α concentrations), and improve metabolism (e.g., reduced NEFA, BHBA, and liver total lipids and triglycerides, and increased glucose and liver glycogen). Therefore, the objectives of the current experiment were to evaluate the effects of peripartum rbST treatment of obese dairy cows on concentrations of GH and IGF-1, immune parameters, inflammatory response, and metabolism.

MATERIALS AND METHODS

The procedures conducted during this experiment were approved by the Institutional Animal Care and Use Committee from the University of Minnesota (protocol # 1306-30734A).

Animals, Housing, and Nutrition

One hundred sixty-six Holstein cows (≥ first lactation) were enrolled in the experiment at 253 ± 1 d of gestation. Cows with BCS ≥ 3.75 and locomotion score ≤ 2 from a commercial freestall dairy herd located in northwest Wisconsin were enrolled in the experiment from September 2012 to April 2013 and were followed until October 2013. From approximately 244 d of gestation to 21 d postpartum, cows were housed in a naturally ventilated freestall barn. As cows demonstrated signs of calving (discomfort, restlessness, tail twitching, and allantoic sac visible through the vulva), they were moved to a box stall. Within 12 h after calving, cows were moved to a postpartum pen (1 to 14 d postpartum) for daily observation and examination for diagnosis of postpartum diseases. Cows free of clinical diseases were then moved to a pen in which they stayed from approximately 14 to 28 d postpartum. At 28 d postpartum, cows were moved to 1 of 2 naturally ventilated freestall dairies (0.5 and 10 miles away) in which they remained until the end of their lactation.

From enrollment to calving, a TMR was fed ad libitum once a day at 0900 h; from calving to 21 d postpartum, a TMR was fed ad libitum once a day at 0400 h. Composition of TMR fed in the prepartum (far-off and close-up) and immediate postpartum (1 to 21 d postpartum) periods are described in Table 1.

Treatments

At enrollment (−28 d relative to calving), each cow's BW was estimated using a Holstein dairy cow weigh tape (The Coburn Company Inc., Whitewater, WI) and body condition was scored (1 = emaciated and 5 = obese; 0.25-unit increments; Ferguson et al., 1994). Cows with BCS ≥ 3.75 were balanced for weight, BCS, lactation number, previous lactation 305-d mature equivalent milk yield, and previous days open. Cows were then assigned randomly to 1 of 3 treatments: untreated control (n = 53), 87.5 mg of rbST (**rbST87.5**; n = 56), or 125 mg of rbST (**rbST125**; n = 57). Recombinant bovine somatotropin (Posilac/Elanco, Greenfield, IN) injections were administered every 7 d from

−21 to 28 d relative to calving subcutaneously in the neck area using 1-mL tuberculin syringes and hypodermic 16-gauge and 5/8-inch-long needles. Commercially available ready-to-use syringes of rbST were used. To administer the proper dose of rbST to each cow, on the day before treatments, the contents of commercially available syringes of rbST were dispensed into sterile containers and the appropriate volume of rbST was aspirated into sterile syringes that were kept refrigerated until treatment of cows on farm.

Concentrations of GH and IGF-1

Concentrations of GH and IGF-1 were determined weekly from −21 ± 3 to 21 ± 3 d relative to calving. Serum GH and IGF-1 concentrations were determined in triplicate using a modified RIA from an ovine GH assay (Lalman et al., 2000). The intra- and interassay coefficients of variation (**CV**) for the assays were <5%.

Hemogram

Blood samples collected from the coccygeal vein or artery into 3-mL evacuated tubes containing EDTA (Kendall Monoject, Mansfield, MA) were used for the hemogram. Samples were collected −14 ± 1, −7 ± 1, 0 ± 1, 7 ± 1, 14 ± 1, and 21 ± 1 d relative to calving. Blood samples were analyzed within 10 h of sample collection using a Vet Scan HM2 (Abaxis, Union City, CA) hemogram machine.

Innate Immune Response Assays

Blood samples collected into 10-mL heparinized evacuated tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) −14 ± 1, −7 ± 1, 0 ± 1, 7 ± 1, 14 ± 1, and 21 ± 1 d relative to calving were used for determination of ex vivo innate immune responses (Hulbert et al., 2011). Although treatment with rbST started on d −21 relative to calving, a decision was made to start evaluating innate immune responses at −14 d relative to calving because of budgetary issues.

Briefly, indirect immunofluorescence staining was used to determine expression of adhesion molecules L-selectin (also known as CD62L) and β2 integrins (also known as CD18) by peripheral PMNL. The assay consisted of incubating 200 μL of whole blood at 4°C for 30 min with 200 μL (5 μg/mL) of monoclonal anti-bovine CD62L antibody (BAQ92A; Monoclonal Antibody Center, Washington State University, Pullman, WA) or 200 μL (2.5 μg/mL) of monoclonal anti-bovine CD18 antibody (BAQ30A; Monoclonal Antibody Center, Washington State University). Erythrocytes were lysed with hyperconcentrated PBS to isolate PMNL before a second 30-min incubation of cells with an anti-mouse IgG-fluorescein isothiocyanate (FITC) secondary polyclonal antibody (AbD Serotec, Raleigh, NC) diluted (1:400) in a PBS solution (Sigma-Aldrich, St. Louis, MO). Cells were washed, resuspended in PBS solution, and immediately analyzed by flow cytometry. The hemogram of all cows was performed before innate immune assays, and blood from cows with normal hemogram parameters was used as positive and negative controls in all assays. Negative controls were incubated with 200 μL of PBS solution instead of monoclonal antibodies.

Phagocytic and oxidative burst activities of peripheral PMNL were determined upon challenge with enteropathogenic bacteria (*Escherichia coli* 0118:H8). Briefly, the assay consisted of incubating 200 μL of whole blood with 100 μ*M* dihydrorhodamine 123 (Molecular Probes/Invitrogen, Eugene, OR), an oxidative-sensitive indicator, and 40 μL of fluorescently labeled bacteria (10^9 cfu/mL) at 38.5°C for 15 min, with surface bacteria fluorescence removed using Trypan Blue solution (0.4%; Sigma-Aldrich). After washing with MilliQ water (Millipore, Billerica, MA) to remove excess dye, erythrocytes were lysed by the addition of hyperconcentrated PBS solution and resuspended in PBS solution for immediate flow cytometry analyses. Blood from cows with a normal hemogram was used as positive and negative controls. Unlabeled bacteria were used as negative controls for the phagocytosis assay and samples that received no dihydrorhodamine 123 served as negative controls for the oxidative burst assay. Flow cytometry was carried out on a BD

FACSCanto II (BD Biosciences, Franklin Lakes, NJ) and analyzed using FlowJo 7.6.4 software (Tree Star Inc., San Carlos, CA). The PMNL population was identified on basis of forward- and side-scattered properties. After strictly gating the PMNL population, forward scatter, side scatter, and log fluorescence data were recorded.

Data reported herein is referent to percentages of PMNL positive for phagocytosis and oxidative burst, and expressing CD18 and CD62L molecules. Furthermore, data referent to intensity of phagocytosis, oxidative burst, and expression of CD18 and CD62L molecules are reported herein as geometric mean fluorescence intensity (**GMFI**). Intensity of phagocytosis and intensity of expression of adhesion molecules are indirect indicators of the number of bacteria phagocytized by PMNL and the number of adhesion molecules expressed by PMNL, respectively. On the other hand, oxidative burst intensity is an indirect indicator of the amount of reactive oxygen species produced via oxidation of dihydrorhodamine 123.

Ovalbumin Challenge, Antibody Concentration Assay, and Colostrum IgG Concentration

Cows were immunized with 0.5 mg of chicken egg ovalbumin (Type VII; Sigma-Aldrich) diluted in 0.5 mL of PBS and emulsified in Quil A adjuvant (0.5 mg of Quil A/0.5 mL of PBS; Accurate Chemical & Scientific Corp., Westbury, NY) by subcutaneous injections in the neck area. Cows were vaccinated with ovalbumin −21 ± 3, −7 ± 3, and 7 ± 3 d relative to calving. Blood sampled weekly from d −21 to 21 into 10-mL evacuated tubes without anticoagulant (Becton Dickinson Vacutainer Systems) was used to determine IgG anti-ovalbumin concentration. Blood tubes were placed on ice until centrifugation (1,200 × g for 15 min at 4°C) and serum was stored at −32°C until analysis.

Immunoglobulin G anti-ovalbumin concentration in serum was determined by ELISA and quantified based on optical density (Mallard et al., 1997). Briefly, 96 well plates were

coated for 48 h at 4°C with ovalbumin solution (1.4 mg of ovalbumin/mL; Type VII, Sigma-Aldrich) diluted in carbonate-bicarbonate buffer (pH 9.4) and then washed with PBS and 0.05% Tween 20 (Sigma-Aldrich) solution (pH 7.4). Plates were blocked with 4% BSA (Santa Cruz Biotechnology Inc., Santa Cruz, CA) solution for 2 h and washed; then, control and diluted test sera (1/200) were added for an additional 2 h. The negative control included a pool of samples of nonimmunized cows' sera, and the positive control included a pool of samples collected from cows 14 d after the second immunization. After washing the plates, conjugated antibody anti-bovine IgG (KPL Inc., Gaithersburg, MD), diluted (1/2,000) in 10 m*M* Tris-HCl buffer (Sigma-Aldrich) was added to the plates and incubated for 1 h. Substrate (5 mg/mL) *p*-nitrophenyl phosphate (Sigma-Aldrich) was added and, after a 30-min incubation, plates were read at 410 and 540 nm using a Eon plate reader (BioTek Instruments Inc., Winooski, VT). The intra- and interassay CV were 4 and 8%, respectively.

Farm personnel were instructed to collect colostrum samples immediately after parturition. Samples were stored at −32°C until analyzed by RIA (University of Saskatchewan, Saskatoon, Canada) for total IgG concentrations. Although farm personnel were instructed to collect samples from all cows enrolled in the experiment, samples from only 15, 6, and 16 cows from the untreated control, rbST87.5, and rbST125 treatments, respectively, were collected.

Haptoglobin and TNF-α Assays

Blood sampled weekly from d −21 ± 3 to 21 ± 3 into 10-mL evacuated tubes with EDTA (Becton Dickinson Vacutainer Systems) was used to determine concentrations of haptoglobin and TNF-α. Blood tubes were placed on ice until centrifugation (1,200 × *g* for 15 min at 4°C) and plasma was stored at −32°C until analysis. Haptoglobin concentrations were determined by a colorimetric procedure (Hulbert et al., 2011) and absorbance was measured using a plate reader (BioTek Instruments Inc.). A pool of plasma samples from

cows at 7 d postpartum was used as control. The intra- and interassay CV were 4 and 3%, respectively.

Concentrations of TNF-α were determined by ELISA using a protocol described and validated for bovine plasma (Farney et al., 2011). Eight cows, not enrolled in the experiment, received intravenous infusion of LPS and had blood collected 4 h after infusion. A pool of plasma from the LPS-challenged cows was used as positive control (elevated TNF-α concentration). A pool of plasma samples from non-LPS-infused cows was used as negative control (reduced TNF-α concentration). The intra- and interassay CV were 5 and 3%, respectively.

Metabolite and Cortisol Concentrations and Liver Biopsies

Blood samples were collected weekly from −21 ± 3 to 21 ± 3 d relative to calving to determine concentrations of NEFA, BHBA, and glucose. Samples were collected from the coccygeal vein or artery into empty evacuated tubes and evacuated tubes containing K2 EDTA (Becton Dickinson Vacutainer Systems) immediately after feeding while cows were restrained in self-locking headlocks. Tubes were placed in ice until centrifugation for plasma separation (1,200 × *g* for 15 min at 4°C). Plasma was aliquoted into microcentrifuge tubes and stored at −32°C until analysis. Concentrations of NEFA were determined using a colorimetric assay (Wako Chemicals USA, Richmond, VA; Ballou et al., 2009), and concentrations of BHBA were determined enzymatically (Ranbut, Randox Laboratories, Antrim, UK; Ballou et al., 2009). Control serum (Randox Laboratories) was used for the NEFA and BHBA assays. The intraassay CV were 7 and 11% for the NEFA and BHBA assays, respectively, and interassay CV were 4 and 8% for the NEFA and BHBA assays, respectively. Glucose concentration was determined by enzymatic reaction (Stanbio Laboratory, Boerne, TX). The intra- and interassay CV were 4 and 3% for the glucose assay, respectively. Serum cortisol was measured by a solid phase RIA using a

commercially kit (Coat-A-Count Cortisol; Siemens Medical Solutions Diagnostics, Los Angeles, CA). The intra- and interassay CV were 7 and 6%, respectively.

A subsample of cows (n = 10/treatment) had liver samples collected at −21, −7, and 7 d relative to calving to determine liver content of total lipids, triglycerides, and glycogen. Liver biopsies were placed in nitrogen immediately after harvesting and later stored at −80°C until analysis. Total lipids and triglycerides extracted from liver tissues were measured by a colorimetric procedure (Fletcher, 1968; Foster and Dunn, 1973; Hara and Radin, 1978). The intra- and interassay CV for the total lipids and triglycerides assay were 2 and 3%, respectively. Glycogen content in the liver was determined by colorimetric assay (Lo et al., 1970). The intra- and interassay CV were 5 and 1%, respectively. Liver total lipids, triglycerides, and glycogen data are reported herein in percentage of wet weight.

Clinical Examination, Disease Definitions, BCS, and Productive Parameters

Farm personnel were instructed to evaluate cows within 24 h after calving to determine and record the occurrence of retained fetal membranes. Unfortunately, occurrence of retained fetal membranes was not recorded consistently and this information was not used in the current experiment. Study personnel palpated cows per rectum daily from d 2 to 21 postpartum for the diagnosis of metritis. Metritis was defined as cows having watery, pink/brown, and fetid uterine discharge (LeBlanc, 2010). Ketosis was defined as cows having circulating BHBA concentrations ≥ 1,400 μmol/L (Duffield et al., 2009). Percentage of cows removed from the herd was recorded for the first 60 d postpartum. Cows' body condition was scored every 14 d from −28 to 70 d relative to calving and at 90 d postpartum.

Cows were milked thrice daily and started to receive full doses of rbST (500 mg) every 11 d between 55 to 60 d postpartum and remained in the rbST treatment until 220 to 225 d of gestation. Daily milk yield was recorded for individual cows (Afimilk Ltd., Kibbutz

Afikim, Israel) starting at approximately 21 d postpartum and recorded on DairyComp305 software (Valley Ag. Software, Tulare, CA). Milk yield was not measured in the first 21 DIM because the transition cow facility did not have the capability to do so. Milk data reported herein are weekly milk yield averages from 21 to 150 DIM.

Statistical Analysis

Cows that calved before receiving at least 2 prepartum doses of rbST were removed from the experiment and statistical analyses. All statistical analyses were conducted using SAS version 9.3 (SAS/STAT, SAS Inst. Inc., Cary, NC). In all models, cows were used as experimental unit. Binomial data were analyzed by logistic regression using the LOGISTIC procedure and the FREQ procedure of SAS with the Chi-Square and Fisher's exact tests. Continuous data were analyzed by ANOVA using the MIXED procedure of SAS. For analyses of repeated measurements, the repeated statement was used and treatment (control vs. rbST87.5 vs. rbST125), time, and the interaction between treatment and time were included in the model as fixed effects. The structure of covariance (auto-regressive, unstructured, or compound symmetry) was chosen according to the Bayesian information criterion (BIC). The assumption of normality was assessed using the UNIVARIATE procedure of SAS. A log-transformation of the dependent variables granulocyte:lymphocyte ratio and TNF-α concentration was required to achieve a normal distribution. The prepartum and postpartum periods were analyzed separately because of inherent physiological changes that occur during calving.

Analysis of covariance was used to analyze the effect of treatment on liver contents of total lipids, triglycerides, and glycogen at different time points relative to calving using the GLM procedure of SAS. The model included treatment as a fixed effect. In the model to determine the effect of treatment on liver contents on d −7 relative to calving, liver content on d −21 relative to calving was used as a covariate.

Two statistical analyses were conducted to evaluate the effects of treatment on milk yield. The first analysis considered the period from 3 to 21 wk postpartum. The second statistical analysis considered the period from 3 to 7 wk postpartum because in wk 8 of lactation, all cows started to receive 500 mg of rbST every 11 d.

When the effect of treatment on dependent variables was $P \leq 0.15$, the contrast statement of SAS was used to compare control versus rbST87.5, control versus rbST125, and rbST87.5 versus rbST125 treatments. In addition, the contrast statement of SAS was used to test whether rbST dose had a linear or a quadratic effect on dependent variables using the IML procedure of SAS and the ORPOL function to adjust and obtain the appropriate contrast coefficients when testing unequally spaced dose treatments. Statistical significance was defined as $P \leq 0.05$ and tendency was considered if $0.05 < P \leq 0.15$. When treatment affected or tended to affect a dependent variable, the Bonferroni multiple comparison correction was used and statistical significance was defined as $P \leq 0.015$ and tendency was considered if $0.015 < P \leq 0.05$.

RESULTS

Seven cows were removed from the analyses (rbST87.5 = 3, and rbST125 = 4) for the following reasons: not pregnant at enrollment (1 cow) and calved before receiving at least 2 doses of rbST (6 cows).

At enrollment, lactation number (control = 2.36 ± 0.14, rbST87.5 = 2.34 ± 0.14, rbST125 = 2.37 ± 0.14 lactations; $P = 0.98$), BW (control = 777.6 ± 10.7, rbST87.5 = 777.8 ± 10.7, rbST125 = 777.8 ± 10.7 kg; $P = 0.99$), and BCS (control = 4.03 ± 0.03, rbST87.5 = 3.98 ± 0.03, rbST125 = 3.99 ± 0.03; $P = 0.49$) were not different among treatments. Treatments did not differ regarding previous lactation 305-d mature-equivalent milk yield (control = 12,224 ± 331, rbST87.5 = 12,986 ± 331, rbST125 = 12,409 ± 331 kg; $P = 0.24$) and

previous lactation interval from calving to pregnancy (control = 152.2 ± 11.2, rbST87.5 = 143.7 ± 11.2, rbST125 = 147.6 ± 11.2 d; $P = 0.87$).

Average number of days that cows stayed in the close-up pen did not differ among treatments (control = 25.4 ± 0.7, rbST87.5 = 26.8 ± 0.7, rbST125 = 26.2 ± 0.7 d; $P = 0.41$). The percentages of cows calving male calves tended ($P = 0.14$) to be different among treatments, as rbST125 cows tended? to have higher ($P = 0.05$) incidence of male calves than control cows. No differences were observed in incidence of male calves between control and rbST87.5 ($P = 0.17$) or between rbST87.5 and rbST125 ($P = 0.56$) treatments (Table 2). The percentage of cows calving twins was not ($P = 0.39$) different among treatments (Table 2).

GH and IGF-1 Concentrations

Treatment tended ($P = 0.06$) to affect prepartum GH concentrations (quadratic effect: $P = 0.05$; Figure 1A). The contrasts among treatments demonstrated that rbST125 cows tended ($P = 0.02$) to have higher prepartum GH concentrations than control cows. No differences were observed in prepartum GH concentrations between control and rbST87.5 ($P = 0.08$) or between rbST87.5 and rbST125 ($P = 0.60$; Figure 1A). During the postpartum period, treatment affected ($P < 0.01$) GH concentrations (quadratic effect: $P < 0.01$; Figure 1A). The rbST125 cows had ($P < 0.01$) and tended to have ($P = 0.02$) greater postpartum GH concentrations than control and rbST87.5 cows, respectively, and rbST87.5 tended ($P = 0.04$) to have higher GH concentrations than control (Figure 1A). Prepartum IGF-1 concentrations were affected by treatment ($P = 0.01$; quadratic effect: $P = 0.04$; Figure 1B). Insulin-like growth factor 1 concentration was greater for rbST87.5 ($P = 0.01$) and rbST125 ($P = 0.01$) cows compared with control cows but no difference ($P = 0.88$) was observed between rbST87.5 and rbST125 cows. Treatment did not affect postpartum IGF-1 concentrations ($P = 0.38$; Figure 1B).

Hemogram Parameters

Prepartum (P = 0.57) and postpartum (P = 0.25) percentages of leukocytes classified as granulocytes were not affected by treatment (Table 3). Treatment did not affect percentages of leukocytes classified as lymphocytes during the prepartum (P = 0.49) or postpartum periods (P = 0.34; Table 3). Prepartum (P = 0.52) and postpartum (P = 0.25) granulocyte:lymphocyte ratios were not affected by treatment (Table 3).

Innate Immune Parameters

Although treatment (P = 0.16) did not affect percentage of PMNL expressing CD62L (L-selectin) during the prepartum period, treatment tended (P = 0.15) to affect CD62L expression during the postpartum period (quadratic effect: P = 0.05; Table 3). Percentage of PMNL expressing CD18 during the prepartum period tended (P = 0.08) to be affected by treatment (linear effect: P = 0.03; Table 3). Cows in the rbST87.5 treatment tended (P = 0.04) to have a lower percentage of PMNL expressing CD18 during the prepartum period compared with control cows. We detected no differences in percentage of PMNL expressing CD18 during the prepartum period between control and rbST125 (P = 0.87) or between rbST87.5 and rbST125 (P = 0.07) cows. No differences among treatments (P = 0.59) were observed for the percentage of PMNL expressing CD18 during the postpartum period (Table 3). Treatment affected (P = 0.02) intensity of PMNL expression of CD62L during the prepartum period (quadratic effect: P = 0.01; Figure 2A). Contrasts revealed that rbST125 cows had (P < 0.01) and tended to have (P = 0.05) greater intensity of CD62L expression by PMNL during the prepartum period than rbST87.5 and control cows, respectively. Intensity of CD62L expression by PMNL during the prepartum period did not (P = 0.42) differ between control and rbST87.5 cows. No differences in intensity of CD62L expression by PMNL among treatments (P = 0.77) were observed in the postpartum period (Figure 2A). During the prepartum (P = 0.43) and postpartum (P = 0.96) periods, treatment did not affect intensity of expression of CD18 by PMNL (Figure 2B).

Treatment did not (P = 0.21) affect the percentage of PMNL positive for phagocytosis and oxidative burst during the prepartum period (control = 66.8 ± 1.8, rbST87.5 = 69.1 ± 1.8, rbST125 = 71.5 ± 1.9%). Similarly, treatment had no (P = 0.88) effect on the percentage of PMNL positive for phagocytosis and oxidative burst in the postpartum period (control = 64.9 ± 2.0, rbST87.5 = 64.7 ± 2.0, rbST125 = 66.0 ± 2.0%). Treatment affected (P = 0.02) PMNL phagocytosis intensity during the prepartum period (quadratic effect: P < 0.01; Figure 3A). According to the contrast analyses, during the prepartum period, intensity of phagocytosis by PMNL from rbST125 cows was greater (P < 0.01) than that from control cows but no differences were observed between control and rbST87.5 (P = 0.17) or between rbST87.5 and rbST125 (P = 0.14) treatments. During the postpartum period, treatment did not (P = 0.35) affect PMNL phagocytosis intensity (Figure 3A). Treatment affected (P = 0.02) PMNL oxidative burst intensity during the prepartum period (quadratic effect: P < 0.01; Figure 3B). Intensity of oxidative burst by PMNL from rbST125 cows during the prepartum period was higher (P < 0.01) compared with the control treatment. We found no differences, however, in PMNL oxidative burst intensity during the prepartum period between control and rbST87.5 (P = 0.13) or between rbST87.5 and rbST125 (P = 0.18) treatments. No differences were observed among treatments in PMNL intensity of oxidative burst during the postpartum period (P = 0.43; Figure 3B).

Adaptive Immune Parameters

Prepartum concentrations of serum IgG anti-ovalbumin tended (P = 0.09) to be affected by treatment (quadratic effect: P = 0.04; Figure 4). According to the contrast analyses, during the prepartum period, concentrations of IgG tended (P = 0.03) to be greater for rbST125 cows compared with control cows. No differences in IgG concentration during the prepartum period were observed between control and rbST87.5 (P = 0.20) or between rbST87.5 and rbST125 (P = 0.28) cows. Treatment did not (P = 0.83) affect the IgG concentration during the postpartum period (Figure 4). Treatment did not (P = 0.28) affect

colostrum IgG concentration (control = 98.5 ± 9.0, rbST87.5 = 86.1 ± 13.7, rbST125 = 111.1 ± 8.4 g/L).

Haptoglobin and TNF-α Concentrations

Haptoglobin concentrations during the prepartum (P = 0.74) and postpartum (P = 0.45) periods were not different among treatments (Figure 5A). We observed a tendency (P = 0.08), however, for the interaction between treatment and day to affect haptoglobin concentrations during the postpartum period because rbST125 cows had reduced haptoglobin concentration on d 7 postpartum compared with control (P = 0.03) and rbST87.5 (P = 0.02) cows (Figure 5A). Treatments did not affect prepartum (P = 0.56) and postpartum (P = 0.27) TNF-α concentrations (Figure 5B).

NEFA, BHBA, Glucose, and Cortisol Concentrations

Treatment did not affect prepartum (P = 0.85) or postpartum (P = 0.43) NEFA concentrations (Table 4). Concentration of BHBA was not affected by treatment during the prepartum period (P = 0.84) but tended (P = 0.12; quadratic effect: P = 0.05) to be affected during the postpartum period (Table 4). Treatment did not affect prepartum (P = 0.99) or postpartum (P = 0.58) glucose concentrations (Table 4). Similarly, treatment did not affect prepartum (P = 0.66) or postpartum (P = 0.71) cortisol concentrations (Table 4).

Liver Total Lipids, Triglycerides, and Glycogen Contents

Percentage of total lipids in the liver was not different among treatments on d −21 (P = 0.45), d −7 (P = 0.33), or d 7 (P = 0.85) relative to calving (Figure 6A). Percentage of liver triglycerides tended to be different among treatments on d −21 (P = 0.14), but no differences were observed on d −7 (P = 0.65) or d 7 relative to calving (P = 0.21; Figure 6B). Finally, treatment did not affect percentage of glycogen in the liver on d −21 (P = 0.97), d −7 (P = 0.31), or d 7 (P = 0.48; Figure 6C).

BCS, Incidence of Metritis, Ketosis, Removal from the Herd Within 60 DIM, and Milk Yield

Treatment did not affect prepartum BCS ($P = 0.83$) but tended ($P = 0.10$; linear effect: $P = 0.08$) to affect postpartum BCS, as control cows had slightly higher BCS on d 42 postpartum than rbST87.5 and rbST125 cows (Figure 7). Treatment tended ($P = 0.12$; quadratic effect: $P = 0.04$) to affect the incidence of metritis (Table 2). According to the contrast analyses, cows in the rbST125 treatment tended ($P = 0.04$) to be less likely to be diagnosed with metritis than control cows, but no differences were observed between control and rbST87.5 cows ($P = 0.53$) or between rbST87.5 and rbST125 cows ($P = 0.14$). Treatment did not affect incidence of ketosis ($P = 0.52$; Table 2). Percentage of cows removed from the herd within 60 d postpartum was not ($P = 0.26$) different among treatments (Table 2).

Milk yield from 3 to 7 wk postpartum was not ($P = 0.33$) affected by treatment (control = 43.4 ± 1.4, rbST87.5 = 44.9 ± 1.5, rbST125 = 46.4 ± 1.4 kg/d). Milk yield from 3 to 21 wk postpartum was not affected ($P = 0.77$) by treatment (control = 45.9 ± 1.25, rbST87.5 = 45.8 ± 1.35, rbST125 = 47.0 ± 1.33 kg/d; Figure 8). The interaction between treatment and week, however, affected ($P = 0.02$) milk yield because there was a tendency for rbST125 cows to produce more milk than control cows in wk 3 ($P = 0.09$) and wk 5 ($P = 0.06$).

DISCUSSION

Dairy cows undergo uncoupling of the somatotropic axis, characterized by reduced IGF-1 concentrations despite increasing GH concentrations, during the periparturient period (Lucy, 2008). Obese cows have reduced feed intake (Hayirli et al., 2002) and are more likely to lose BCS and BW (Fronk et al., 1980; Treacher et al., 1986) during the periparturient period than thinner cows. Increased BCS 1 wk prepartum was associated with more dramatic reductions in IGF-1 concentrations compared with 1 wk postpartum (Kasimanickam et al., 2013). In the current experiment, concentrations of GH and IGF-1

were increased during the prepartum period in rbST-treated cows. In contrast, during the postpartum period, only GH concentration was increased by rbST treatment. Gulay et al. (2004a) observed a slight increase in IGF-1 concentration during the postpartum period when peripartum cows received 142.8 mg of rbST every 14 d from −21 to 42 d relative to calving. Visual inspection of Figure 1B indicates that the pattern of IGF-1 concentration appears to differ between control and rbST125 cows starting on d 14 postpartum. Therefore, we speculate that no differences in IGF-1 concentrations were observed in the current experiment during the postpartum period because treatment ended 28 d postpartum.

In an observational experiment, Kasimanickam et al. (2013) demonstrated that concentrations of IGF-1 were reduced from 1 wk prepartum to 1 wk postpartum among cows diagnosed with metritis. Insulin-like growth factor-1 stimulates growth and differentiation of cells of the immune system (Heemskerk et al., 1999). Therefore, it is likely that one of the mechanisms associated with more dramatic immunosuppression among obese cows during the periparturient period (Samartin and Chandra, 2001; Sauerwein et al., 2014) is their reduced IGF-1 concentration.

Percentages of granulocyte in blood were not different among treatments. Treatment of GH-deficient adult humans with rbST increased IGF-1 concentration and neutrophil count (Sohmiya et al., 2005). Insulin-like growth factor 1 modulates hematopoiesis directly through induction of cell proliferation and antiapoptotic signaling (Heemskerk et al., 1999). Frostad et al. (1998) demonstrated that IGF-1 increased production of neutrophils from cultures of CD34+ cells from umbilical cord in a dose-dependent manner. This dose-dependent effect of IGF-1 on neutrophil count leads us to speculate that the doses of rbST administered in the current experiment did not increase IGF-1 concentrations sufficiently to modulate hematopoiesis. Although in vitro and in vivo experiments have demonstrated that GH stimulates lymphocyte proliferation and stimulates function of human and mice thymic cells (Bozzola et al., 1988–1989; Savino, 2003), percentages of lymphocytes in

blood did not differ among treatments in the current experiment. It is possible that the concentrations of IGF-1 and GH reached through the treatments applied herein were insufficient to affect lymphocyte count.

Neutrophil adhesion and migration to the inflammatory site are important initial steps during the inflammatory process. Although no differences in percentages of PMNL expressing L-selectin (CD62L) among treatments were observed, rbST treatment tended to increase intensity of L-selectin expression by PMNL. Growth hormone treatment increased number of CD62L+ T cells and expression of CD62L in mice lymph nodes (Smaniotto et al., 2004) and enhanced adhesion of human neutrophils (Ryu et al., 2000). Unexpectedly, percentage of PMNL expressing CD18 during the prepartum period tended to be lower for rbST87.5 cows compared with control cows. It is not clear why treatment did not improve expression of CD18. Culture of human PMNL with GH did not affect expression of CD11b, despite the fact that culture of human PMNL with IGF-1 increased expression intensity of CD11b (Inoue et al., 1998).

Growth hormone can exert direct effects on all major immune cell types (Kelley, 1990) and participates in the development and maintenance of cell-mediated and humoral responses (Meazza et al., 2004). Similarly, IGF-1 plays an important role in the development of immune responses such as T-cell proliferation, chemotaxis, T-cell activation, apoptosis, and natural killer cell cytotoxicity (Weigent, 2013). In the current experiment, intensities of PMNL phagocytosis and oxidative burst were increased during the prepartum period in rbST125 cows compared with control cows. Phagocytosis and oxidative burst were increased when PMNL were incubated with GH or IGF-1 and challenged with *E. coli* (Bjerknes and Aarskog, 1995; Inoue et al., 1998). Furthermore, treatment of malnourished human hemodialysis patients with GH increased PMNL phagocytosis (Kotzmann et al., 2003), and GH replacement therapy of GH-deficient humans increased release of superoxide anion by neutrophils (Reinisch et al., 1996). In the

current experiment, however, differences in GH concentrations between treatments were greatest during the postpartum period but parameters associated with PMNL function were unaltered during the postpartum period. Therefore, it is likely that the main factor affecting prepartum PMNL function was IGF-1.

Cows in the rbST125 treatment had greater IgG anti-ovalbumin concentrations during the prepartum period than control cows. Interestingly, within 7 d of ovalbumin vaccination and start of the rbST treatment, we observed a significant increase in IgG concentration among rbST125 and rbST87.5 cows. On the other hand, there a significant increase in IgG concentration among control cows only 14 d after ovalbumin vaccination. Piglets treated with somatotropin before weaning and transportation had increased IgM concentrations (Kojima et al., 2008) and mice treated with somatotropin had increased IGF-1 and IgG, IgM, and IgA concentrations (Sohmiya et al., 2005). Hattori et al. (2001) demonstrated that expression of GH receptor mRNA is greater in human B cells than in other immune cells types, such as T cells and neutrophils. The rapid increase in IgG concentration within 7 d of the first rbST treatments suggests that the effects of GH, IGF-1, or both, on immune cells are fast.

The improvements in innate and adaptive immune parameters described herein are of great importance for dairy cows during the periparturient period. Several experiments have demonstrated that activity of PMNL from cows diagnosed with metritis is reduced during the prepartum period (Cai et al., 1994; Hammon et al., 2006). In the current experiment, the incidence of metritis among rbST125 cows tended to be reduced compared with that of control cows. Treatment of cows with 142.8 mg of rbST every 14 d from −21 to 42 d relative to calving did not decrease the incidence of metritis but reduced the overall incidence of periparturient diseases (Gulay et al., 2007). More recently, Gohary et al. (2014) did not observe differences in incidence of postpartum diseases when prepartum cows were treated once to thrice with rbST (325 mg every 14 d). Treatment of cows with

rbST at 14-d intervals is known to result in more oscillation of IGF-1 concentration (Gulay et al., 2004a; Ribeiro et al., 2014) compared with shorter intervals (Rivera et al., 2010). Therefore, in the study by Gohary et al. (2014), concentrations of IGF-1 at the time of calving may not have been significantly different between rbST-treated and untreated cows. The half-life of PMNL is approximately 8.9 h (Paape et al., 2003). Therefore, a 7-d interval between rbST treatments was adopted to maintain IGF-1 concentrations constantly higher among rbST treated cows until at least the day of parturition. This is probably a critical factor influencing the maintenance of elevated activity of PMNL from rbST125 cows through the calving period. Another important difference between the current experiment and that described by Gohary et al. (2014) is the dose of rbST. Elevated doses of rbST during the periparturient period may result in greater BCS loss and accentuate negative energy balance (Moallem et al., 1997, 2000), which may result in immunosuppression. Furthermore, treatment of GH-deficient humans with 13 IU of GH/m2 per day (10 to 20 times the normal GH replacement dose) resulted in no improvements in release of proinflammatory cytokines by peripheral immune cells compared with placebo (Zarkesh-Esfahani et al., 2000). Furthermore, signal transducer and activator of transcription 5 (Stat5) response by peripheral blood mononuclear cells was greatest when GH concentration was between 1 and 1,000 ng/mL, whereas GH doses > 1,000 ng/mL suppressed the Stat5 response (Zarkesh-Esfahani et al., 2000). Lymphocyte proliferation after phytohemagglutinin stimulation was increased when peripheral blood mononuclear cells were cultured in the presence of GH < 850 ng/mL, whereas culture in the presence of GH > 850 ng/mL reduced lymphoproliferation (Bozzola et al., 1988–1989). Growth hormone concentration of dairy cows starts to increase approximately 1 wk prepartum, as energy balance starts to decrease (Lucy, 2008). Based on our findings and those of others (Putnam et al., 1999), the decision to use 125 mg of rbST every 7 d in periparturient dairy cows seems correct based on improvements in immune function and minimal effects on metabolism. Although no differences in concentrations of haptoglobin were observed among treatments, rbST125 cows had reduced haptoglobin concentrations

7 d postpartum. Furthermore, among cows with metritis, haptoglobin concentrations 7 d postpartum tended to be lower for rbST125 cows compared with control and rbST87.5 cows (data not shown). The reduced haptoglobin concentration 7 d postpartum among rbST125 cows may have been a consequence of fewer rbST125 cows having metritis or reduced severity of infection or inflammation among rbST125 cows due to greater GH concentrations. Severely burned human patients receiving GH therapy had decreased haptoglobin concentrations (Jeschke et al., 2000). Growth hormone is likely to modulate the acute phase response by increasing IGF-1 and decreasing IL-1 expression, which leads to decreased type 1 acute phase proteins and increased constitutive hepatic proteins (Jarrar et al., 1997; Jeschke et al., 2000). Although rbST treatment did not affect TNF-α concentration in the current experiment, metritic cows treated with rbST had TNF-α concentrations reduced by approximately 17% (data not shown). Pro-monocytic cells engineered by gene transfer to produce human GH secreted less TNF-α in response to challenges with LPS, and GH treatment inhibited TNF-α secretion by human monocytes and macrophages (Haeffner et al., 1997). The effects of treatment of peripartum cows with rbST on severity of inflammatory response and secretion of proinflammatory cytokines should be evaluated because prolonged and uncontrolled inflammatory response might be detrimental to the individual and result in increased morbidity and mortality (Moshage, 1997).

In the current experiment, rbST87.5 and rbST125 cows had reduced BCS compared with control cows starting at approximately 14 d postpartum. Concentrations of NEFA, BHBA, and glucose, however, were not different among treatments. Effects of rbST treatment during the periparturient period on BCS and metabolites are inconclusive and are likely dependent on dose of rbST, frequency of treatment, genetics, DMI, and milk yield. Although Putnam et al. (1999) did not observe effects of rbST treatment on BCS during the peripartum period, cows treated with 142.8 mg of rbST every 14 d had reduced NEFA and BHBA concentrations and increased glucose concentrations around the time of calving

(Putnam et al., 1999). On the other hand, Gohary et al. (2014) observed reduced BCS in wk 3 postpartum, increased NEFA in wk 1 prepartum, increased BHBA in wk 1 postpartum, and increased peripartum glucose concentrations in cows treated with 325 mg of rbST every 14 d during the prepartum period. Although in the current experiment and Gohary et al. (2014), DMI and energy balance were not determined, it is likely that the reduced BCS during the postpartum among rbST-treated cows represents greater fat mobilization to sustain greater milk yield early postpartum. Putnam et al. (1999) also reported greater milk yield among cows treated with rbST but rbST treatment also increased DMI, which may have reduced the extent of negative energy balance in response to greater milk yield. Bovine somatotropin can increase basal lipolysis in cows in negative energy balance and consequently increase NEFA concentrations (Bauman, 1992). Increased adipose tissue mobilization in response to rbST treatment, however, may or may not result in changes in plasma NEFA concentrations because rbST also modulates the utilization of NEFA by several tissues (Bauman, 1992). The uptake and oxidation of NEFA by peripheral tissue can be increased by rbST while glucose uptake is decreased, causing a reduction in NEFA supply to the liver and increased oxidation to ketones (Bauman, 1992; Putnam et al., 1999). Furthermore, rbST regulates gluconeogenesis through the regulation of enzymes such as phosphoenolpyruvate carboxykinase, a key enzyme in the conversion of oxaloacetate to phosphoenolpyruvate (Velez and Donkin, 2004). Although in the current experiment glucose was not increased in rbST-treated cows, this may be a consequence of greater milk yield among rbST-treated cows. Interestingly, on d −7 relative to calving, hepatic content of glycogen was numerically higher (43% higher) in rbST125 cows than in control cows. Treatment of lactating cows and cows under positive energy balance with rbST resulted in decreased liver glycogen content (Pocius and Hertlein, 1986; Knapp et al., 1992). Glycogen degradation is believed to be one of the glucose-sparing mechanisms exerted by rbST to divert nutrients for milk synthesis (Bauman and Vernon, 1993). The role of rbST on glycogen synthesis or breakdown in periparturient cows, however, is not well understood. The numerical increase in hepatic glycogen leads us to speculate that rbST

treatment of periparturient cows may be optimizing energy through different pathways than those observed in cows under positive energy balance. In agreement with others (Gallo and Block, 1990; Putnam et al., 1999), rbST treatment during the peripartum period did not affect hepatic content of total lipids and triglycerides.

CONCLUSIONS

Treatment of peripartum dairy cows with 125 mg of rbST at 7-d intervals improved PMNL expression of L-selectin, PMNL phagocytosis and oxidative burst, and antibody concentrations, and decreased postpartum acute phase protein and incidence of metritis in obese dairy cows. Administration of somatotropin in transition cows could be used as a prepartum strategy to increase IGF-1 concentrations, improve immune function, and decrease postpartum disorders. Although we observed numerical separation on milk yield among treatments, additional research is warranted to evaluate how peripartum rbST treatment may improve milk yield. Further investigations with larger sample sizes and cows of varying BCS should be conducted to confirm the positive effects of rbST treatment on immune function and incidence of diseases.

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CHAPTER III

Effects of treatment of periparturient dairy cows with recombinant bovine somatotropin on health and productive and reproductive parameters

OUTLINE

Objectives of the current experiment were to evaluate the effects of treatment of periparturient dairy cows with recombinant bovine somatotropin (rbST) on incidences of postpartum diseases and performance. Holstein (HO) and Jersey (JS) cows from two herds were enrolled in the experiment at 253 ± 3 d of gestation and assigned to the control (n = 432) and rbST125 (n = 437) treatments. Cows in the rbST125 treatment received 125 mg of rbST, weekly, from -21 to 21 d relative to calving. Blood sampled weekly, from -21 to 21 d relative to calving, from a subsample of cows was used to determine the concentrations of growth hormone (GH, HO = 106) and insulin-like growth factor 1 (IGF-1, HO = 147 and JS = 49). Cows were scored for body condition (BCS) at enrollment and at 1 ± 3, 30 ± 3, and 60 ± 3 days in milk (DIM). Cows were milked thrice daily and energy corrected milk yield was recorded for the first 30 DIM. Treatment of cows with rbST resulted in greater concentrations of GH during the prepartum (log10 back transformed concentrations of GH: HO-control = 7.83 and HO-rbST125 = 10.36 ng/mL) and postpartum (log10 back transformed concentrations of GH: HO-control = 10.45 and HO-rbST125 = 18.47 ng/mL during the postpartum) periods. Similarly, IGF-1 concentrations were higher during the prepartum (HO-control = 115.1 ± 4.9, HO-rbST125 = 137.7 ± 4.7, JS-control = 120.2 ± 8.3, JS-rbST125 = 167.1 ± 8.1 ng/mL) and postpartum (HO-control = 61.3 ± 4.0, HO-rbST125 = 75.2 ± 3.8, JS-control = 35.5 ± 6.9, JS-rbST125 = 54.6 ± 6.9 ng/mL) periods for rbST treated cows. During the prepartum period BCS was not affected by treatment, but BCS during the postpartum period was reduced for rbST treated cows (HO-control = 3.00 ± 0.03, HO-rbST125 = 2.90 ± 0.03, JS-control = 2.64 ± 0.02, JS-rbST125 = 2.61 ± 0.02). Cows from the rbST125 treatment tended to have lower incidence of retained fetal membranes (HO-control = 14.3, HO-rbST125 = 6.1, JS-control = 1.5, JS-rbST125 = 1.2%)

and had reduced incidence of metritis (HO-control = 26.2, HO-rbST125 = 16.6, JS-control = 19.9, JS-rbST125 = 13.3%) than control cows. Ketosis incidence tended to be higher for rbST125 cows (HO-control = 9.4, HO-rbST125 = 11.3, JS-control = 8.5, JS-rbST125 = 13.4%) compared with control cows. The interaction between treatment and herd tended to affect yield of energy corrected milk during the first 30 DIM because among HO cows those treated with rbST during the periparturient period had greater yield (HO-control = 35.5 ± 1.0 vs. HO-rbST125 = 39.4 ± 1.0 kg/d), but among JS cows treatment with rbST did not affect yield of energy corrected milk (JS-control = 26.7 ± 0.6 vs. JS-rbST125 = 27.8 ± 0.6 kg/d). Treatment of periparturient dairy cows with 125 mg of rbST decreased the incidence of uterine disorders in HO and JS cows and increased yield of energy corrected milk during the first 30 DIM among HO cows, despite slightly increasing the incidence of ketosis.

Key words: dairy cow, somatotropin, health, and performance

INTRODUCTION

Feed intake of dairy cows is reduced by approximately 30% from the beginning of the dry period to the last week before calving (Hayirli et al., 1998). The decreased feed intake combined with the increased energy demands from non-lactating to the lactating state, increase of approximately 13 Mcal/d of net energy for lactation (NRC, 2001), results in negative energy balance (**NEB**) from late gestation up to 10 to 12 weeks postpartum (Bertics et al., 1992; Grummer, 1995). In periods of NEB, dairy cows have increased concentrations of growth hormone (**GH**) coupled with insulin resistance that are associated with a coordinated increase in lipolysis and a decrease in glucose uptake and oxidation by peripheral tissues such that nutrients are more readily available for lactogenesis (Bell and Bauman, 1997). Severe NEB, however, leads to excessive lipolysis and elevated concentrations of nonesterified fatty acids (**NEFA**) in the plasma, which may predispose cows to hepatic lipidosis, compromised liver function, and elevated plasma β-hydroxybutyrate (**BHB**) concentrations due to incomplete NEFA oxidation (Grummer et

al., 2004). Under these conditions, periparturient cows may develop metabolic disorders such as fatty liver, ketosis, and displacement of abomasum (Oetzel, 2004). Periparturient dairy cows are also predisposed to infectious diseases (Cai et al., 1994; Kimura et al., 2002; Hammon et al., 2006) because hormonal changes (Burton et al., 1995; Moreira da Silva et al., 1998), shortages in major energetic fuels and minerals (Kehrli et al., 1989a,b; Kimura et al., 2006), and elevated concentrations of NEFA and products of oxidative stress (i.e., reactive oxygen species; Contreras et al., 2012) alter the structure and function of immune cells (Kehrli et al., 1989a,b). Therefore, it is not surprising that approximately 75% of health disorders in dairy cows (e.g. milk fever, ketosis, retained fetal membranes, metritis, mastitis and displacement of abomasum) are diagnosed in the first 30 d postpartum (LeBlanc et al., 2006). Strategies that improve immune function and glucose and lipid metabolism of periparturient dairy cows may reduce the incidences of infectious and metabolic diseases.

Treatment of dairy cows with rbST is associated with increased liver gluconeogenesis, suppression of the insulin's inhibitory effect on gluconeogenesis (Peel and Bauman, 1987), and increased complete oxidation of NEFA to CO_2 through the Kreb's cycle in bovine liver slices (Pocius and Herbein, 1986). Treatment of periparturient cows with rbST may, therefore, increase glucose availability for lactogenesis and other key functions, such as immune competence. Cows treated with 500 mg of rbST, every 14 d, during the prepartum period had increased DMI and glucose concentration and reduced concentrations of NEFA and BHB around the time of calving compared with untreated cows (Putnam et al., 1999). Furthermore, cows treated with 500 mg of rbST, every 14 d, produced 3.3 kg/d more milk in the first 42 d of lactation and 4.6 kg/d more milk during week 6 of lactation compared with control cows (Putnam et al., 1999). In contrast, Jersey cows treated with 500 mg of rbST, every 14 d, from -28 to 14 d postpartum, had reduced fat corrected milk yield (Eppard et al., 1996). Cows treated with 325 mg of rbST, every 14 d, during the prepartum period had an increase in glucose concentration during the peripartum period and a slight increase

in NEFA and BHB concentrations in the first week postpartum, but no differences in DMI, incidence of postpartum diseases, or reproductive performance were observed (Gohary et al., 2014). Treatment of periparturient cows with 142.8 mg of rbST, every 14 d, from -21 to 42 d postpartum, increased peripartum concentrations of GH, IGF-1, glucose, and insulin, increased milk yield during the first 10 weeks of lactation, and decreased the incidences of ketosis, mastitis, and digestive problems (Gulay et al., 2003, 2004, and 2007). The effects of rbST treatment of periparturient dairy cows on incidences of displacement of abomasum, retained fetal membranes and metritis are more controversial (Eppard et al., 1996; Putnam et al., 1999; Gulay et al., 2007; Gohary et al., 2014), perhaps because some of the cited experiments lacked appropriate sample size or because of differences in rbST treatment frequency and dose.

Insulin-like growth factor 1 stimulates growth, differentiation, and functionality of several cell types, including immune cells (Heemserk et al., 1999). In a recent experiment, treatment of Holstein cows with 125 mg of rbST, every 7 d, from -21 to 28 d relative to calving, increased IGF-1 concentrations, increased the intensity of expression of adhesion molecule (L-selectin), the intensity of phagocytosis and oxidative burst by polymorphonuclear leukocyte (**PMNL**), and the concentration of IgG anti-ovalbumin (Silva et al., 2015a). Furthermore, cows treated with 125 mg of rbST had reduced incidence of metritis compared with control cows (23.1% vs. 7.8%), which was associated with reduced haptoglobin concentrations 7 d postpartum, and tended to produce more milk during weeks 3 and 5 of lactation than control cows (Silva et al., 2015a). Inconsistencies in the effects of rbST treatment of periparturient cows on health and performance may result from differences in dose, frequency, and timing of treatment with rbST. The hypotheses of the current experiment were that weekly treatment of periparturient dairy cows from -21 to 21 d relative to calving with 125 mg of rbST would decrease the incidence of infectious (e.g. metritis and mastitis) and metabolic (e.g. ketosis and displacement of abomasum) diseases, improve cow survival and reproductive performance, and increase

yield of energy corrected milk and milk components during the first 30 d of lactation. Objectives of the current experiment were to evaluate the effects of rbST treatment of periparturient dairy cows on postpartum health and reproductive and productive performances.

MATERIALS AND METHODS

The procedures conducted during this experiment were approved by the Institutional Animal Care and Use Committee from the University of Minnesota (protocol #1505-32580A).

Animals, Housing and Nutrition

Pregnant cows (n = 869) from two herds (Holstein - **HO**, nulliparous = 54 and parous = 267; Jersey - **JS,** nulliparous = 210 and parous = 338) were enrolled in the experiment at 253 ± 3 d of gestation (-28 d relative to calving). Holstein cows from a commercial free-stall dairy herd located in Northwest Wisconsin were housed in a naturally ventilated free-stall barn from 253 d of gestation to 21 d postpartum. A TMR was fed once a day at 0900 h from enrollment to calving and at 0400 h from calving to 21 d postpartum. Jersey cows from a commercial free-stall dairy herd located in South Central Minnesota were housed in cross-ventilated barns from 253 d of gestation to 21 d postpartum. A TMR was fed once a day at 0400 h from enrollment to calving and at 0800 h from calving to 21 d postpartum. In both herds, nulliparous and parous animals were fed different diets during the prepartum period and housed in separate pens during the prepartum and postpartum periods. After parturition, primiparous and multiparous cows were fed the same diet in the HO herd and different diets in the JS herd. The composition of the TMR fed in the prepartum (-21 to calving) and postpartum (1 to 21 d postpartum) periods are presented in Table 1.

In both herds, cows that demonstrated signs of calving (discomfort, restlessness, tail twitching, and visualization of the allantoic sac through the vulva) were moved to a box

stall. Within 12 h after calving, cows were moved to a postpartum pen (1 to 21 d postpartum) for daily observation and diagnosis of postpartum diseases. At approximately 21 d postpartum, approximately half of the Jersey cows were moved to another herd that was located 8 miles away from the herd in which cows were housed.

Treatments

At enrollment (-28 d relative to calving), all cows (Holsteins and Jerseys) were scored for body condition (Ferguson et al., 1994) and locomotion (Sprecher et al., 1997). Cows with BCS $\geq$ 2.25 and locomotion score $\leq$ 2 were balanced for parity (nulliparous vs. parous), BCS, previous lactation 305-d mature equivalent milk yield (parous), and previous lactation interval from calving to conception (parous) and were assigned randomly to one of two treatments: untreated control (n = 432) or **rbST125** (n = 437). Cows assigned to the rbST125 treatment received 125 mg of rbST (Posilac, sometribove zinc suspension for injection; Elanco Animal Health, Greenfield, IN), every 7 d, from -21 to 21 d relative to calving. Injections were given subcutaneously in the neck area (HO herd) or in the ischiorectal depression (JS herd), using 1 mL tuberculin syringes with 16 gauge and 5/8 inch long hypodermic needles. Syringes containing 125 mg of rbST were prepared 6 to 12 h before treatment. The contents of commercially available syringes of rbST (Posilac, sometribove zinc suspension for injection; Elanco Animal Health, Greenfield, IN) were dispensed into sterile containers and the appropriate volume of rbST was aspirated into sterile syringes that were kept refrigerated. The syringes were warmed to room temperature (15 to 30 °C) before rbST administration.

Blood Sampling and GH, IGF-1, Glucose, NEFA, BHB, and Calcium Concentrations

Blood was sampled weekly from -28 ± 3 to 24 ± 3 d relative to calving from 106 HO cows to determine the concentrations of GH, and from 147 HO and 49 JS cows to determine the concentrations of IGF-1, glucose, NEFA, and BHB. Insulin concentration was determined in samples collected weekly from -28 ± 3 to 24 ± 3 d relative to calving from 32 HO and

28 JS cows. Within weekly cohort of cows enrolled in the experiment, cows were randomly selected for blood sampling. Cows sampled were balanced for parity (primiparous vs. multiparous) and BCS at enrollment.

Cows were restrained in self-locking stanchions during feeding and blood samples were collected from the coccygeal vein/artery using 22 gauge and 1 inch long needles into empty evacuated tubes and into evacuated tubes that contained K2 EDTA (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ). Tubes were placed in ice until centrifugation (1,200 *g* for 15 min at 4 ^{0}C) for serum or plasma separation within 8 h after blood samples were collected. Serum and plasma were aliquoted into microcentrifuge tubes and stored at -32 ^{0}C until analysis.

Serum GH concentrations were determined in triplicate using a modified radioimmunoassay from an ovine GH assay (Lalman et al., 2000). Serum IGF-1 concentrations were quantified using a commercially available kit (Human IGF-1 Quantikine ELISA; R&D Systems Inc., Minneapolis, MN). The intra- and inter-assay coefficients of variation for the GH and IGF-1 assays were lower than 5%. Plasma concentrations of NEFA were determined using a colorimetric assay (Wako Chemicals USA, Richmond, VA; Ballou et al., 2009) and plasma concentrations of BHB were determined enzymatically (Ranbut, Randox Laboratories, Antrim, UK; Ballou et al., 2009). Control serum (Randox Control Sera, Antrim, UK) was used for the NEFA and BHB assays. The intra-assay CV were 7 and 10% for the NEFA and BHB assays, respectively, and the inter-assay CV were 11 and 8% for the NEFA and BHB assays, respectively. Plasma glucose concentration was determined by enzymatic reaction (Stanbio Laboratory, Boerne, TX). The intra- and inter assay CV were 4 and 3%, respectively. Insulin was analyzed using a commercial ELISA kit for bovine insulin (Mercodia Bovine Insulin ELISA, Mercodia Inc., Uppsala, Sweden). The intra- and interassay CV were 5.2 and 9.4%.

One hundred and six cows (n = 53/treatment) had blood sampled within the first 72 h postpartum. These samples were used to determine serum total calcium concentrations using an automated chemistry analyzer (RX Daytona^{+}, Randox Laboratories Ltd, Antrim, UK).

Body Condition and Locomotion Score

All cows from the HO herd were scored for body condition (1 = emaciated and 5 = obese; 0.25 unit increments; Ferguson et al., 1994) at enrollment and on d 1 ± 3, 30 ± 3, and 60 ± 3 postpartum. In the Jersey herd all cows were scored for body condition at enrollment and on d 1 ± 3 postpartum and 326 and 290 cows were scored for body condition at 30 ± 3 and 60 ± 3 d postpartum, respectively.

In both herds cows were scored for locomotion (1 = normal locomotion and 5 = severely lame; as described by Sprecher et al., 1997) at enrollment. In the JS herd all cows were scored for locomotion at 1 ± 3 d postpartum and 326 and 290 cows were scored for locomotion at 30 ± 3 and 60 ± 3 d postpartum, respectively. Cows with a locomotion score > 2 were considered to be lame. Within herd, body condition and locomotion were scored by the same individual throughout the experiment.

Clinical Examination and Disease Definition

Subclinical hypocalcemia was defined as serum total calcium concentrations between 5.5 and 8.0 mg/dL (Goff, 2008) within 72 h postpartum. Farm personnel were trained to examine cows within 24 h after calving to diagnose and record the occurrence of retained fetal membranes (Kelton et al., 1998). Study personnel palpated cows per rectum on d 4 ± 1, 7 ± 1, 10 ± 1, and 13 ± 1 postpartum for the diagnosis of metritis. Metritis was defined as cows having watery, pink/brown, and fetid uterine discharge (LeBlanc, 2010). In the JS herd, metritic cows were furthered examined and those with rectal temperature > 39.5 °C, anorexia, or depression were considered to have acute metritis (LeBlanc, 2010). The JS

cows were examined for vaginal purulent discharge (vaginal exudate consisting of ≥ 50% of pus) using the Metricheck device (Simcro, Hamilton, New Zealand; McDougall et al., 2007) on d 35 ± 3 postpartum.

Cow-side tests for whole blood BHB concentrations were performed for all cows on d 7 ± 3 and 14 ± 3 postpartum using hand held meters (PX; Precision Xtra, Abbott Diabetes Care Inc., Alameda, CA) to determine incidence of ketosis. Ketosis was defined as at least one sample with BHB concentration ≥ 1,400 μmol/L (Duffield et al., 2009). All cows were observed once daily by farm personnel for displacement of abomasum and thrice daily, during milking, for mastitis. Incidences of displacement of abomasum and mastitis within 60 d postpartum were recorded. Percentage of cows removed from the herd (death/culling) during the first 60 d postpartum was calculated.

Farm personnel responsible for health evaluations were blinded to treatments. Despite the fact that study personnel were not blinded to treatments, at the time of clinical exams and BHB concentration determination, treatments were not known.

Milk yield and Milk Components

Cows were milked thrice daily. The Dairy Herd Improvement Association was hired by the investigators to measure milk yield, milk fat and protein contents, and SCC weekly from 5 ± 3 to 32 ± 3 d postpartum from all HO cows. During the remainder of the lactation milk yield was determined once monthly by the in line milk system. At the onset of the experiment, milk yield, milk fat and protein contents, and SCC were being determined monthly in the JS herd during the official Dairy Herd Improvement Association test. However, approximately 4 months after the start of the experiment the official Dairy Herd Improvement Association test ceased and no milk yield data was recorded. Furthermore, starting at 49 ± 3 DIM all cows were treated with 500 mg of rbST (Posilac, sometribove zinc suspension for injection; Elanco Animal Health, Greenfield, IN) every 11 d in the HO

herd and every 10 d in the JS herd. Therefore, production parameters during the first month of lactation are reported herein. Energy corrected milk was calculated for each cow using the formula (Orth, 1992):

ECM (kg) = [(kg of milk) × 0.327] + [(kg of fat) × 12.95] + [(kg of protein) × 7.2].

Linear SCC was calculated for each cow using the formula (Shook, 1982):

Linear SCC = [In (SCC per ml ÷ 100) ÷ 0.6931] + 3.

Reproductive Management and Parameters

Holstein cows had their estrous cycle presynchronized with two injections of $PGF_{2\alpha}$ (23-36 and 37-50 DIM) and were enrolled in the Ovsynch protocol with a CIDR insert (controlled internal drug release device containing 1.38 g progesterone; Zoetis Animal Health, Florham Park, NJ) at 48-61 DIM (d 0 - GnRH and CIDR, d 7 - $PGF_{2\alpha}$ and CIDR removal, d 9 - GnRH, and, 12 to 14 h later, timed AI). The voluntary waiting period in the HO herd was 50 DIM. Cows were treated with GnRH at 32 ± 3 d after AI and examined for pregnancy 39 ± 3 d after AI by manual palpation of the uterine contents per rectum. Pregnant cows were reexamined for pregnancy at 66 ± 3 and 178 ± 3 d after AI. Cows diagnosed not pregnant at 39 ± 3 d after AI were treated with $PGF_{2\alpha}$ on the same day, GnRH 2 d later, and were re-inseminated at fixed time 12 to 14 h after the GnRH treatment. Conventional semen was used for all inseminations in the HO herd.

Jersey cows were presynchronized with three injections of $PGF_{2\alpha}$ at 39 ± 3, 53 ± 3, and 67 ± 3 DIM. Cows were observed daily for signs of estrus (removal of tail paint) and inseminated if observed in estrus after the voluntary waiting period (40 DIM). Cows not observed in estrus by 79 ± 3 DIM were enrolled in the 5-d Cosynch protocol (d 0 - GnRH, d 5 - $PGF_{2\alpha}$, d 6 - $PGF_{2\alpha}$, d 8 - GnRH and TAI). Cows not re-inseminated in estrus were examined for pregnancy by transrectal ultrasound (5 MHz probe, E.I. Medical, Loveland, CO) at 31 ± 3 d after AI. Pregnant cows were re-examined at 66 ± 3 and 178 ± 3 d after AI by manual palpation of the uterine contents per rectum. Cows diagnosed not pregnant at

31 ± 3 d after AI that had a corpus luteum received a $PGF_{2\alpha}$ injection 2 d later and, if not re-inseminated in estrus within 12 d, were submitted to the 5-d Cosynch protocol for re-insemination at fixed time. Cows diagnosed not pregnant at 31 ± 3 d after AI that did not have a corpus luteum received a GnRH injection on the same day and, if not re-inseminated within 7 d, were submitted to the 5-d Cosynch protocol for re-insemination at fixed time. Sexed semen was used for the first and second inseminations of JS cows observed in estrus, whereas conventional semen was used for insemination of cows at a fixed time.

Percentage of cows receiving first and second postpartum AI at detected estrus, pregnancy per AI (P/AI) after first and second postpartum AI, percentage of cows with pregnancy loss between the first and second pregnancy exams following the first and second postpartum AI, and pregnancy rate up to 150 DIM were calculated.

Statistical Analysis

The experiment had a complete randomized design with animals paired according to parity (nulliparous vs. parous), BCS, previous lactation 305-d mature equivalent milk yield (parous), and previous lactation interval from calving to conception (parous). Sample sizes were calculated based on results from a previous experiment (Silva et al., 2015a) to provide sufficient power to determine statistical significances while preventing type I ($\alpha = 0.05$) and type II ($1 - \beta = 0.80$) errors. Therefore, a sample size of 400 cows/treatment would be sufficient to demonstrate statistical significance when the incidences of metritis and ketosis differ in 8 and 6 percentage units, respectively, between treatments. Furthermore, 400 cows/treatment would be sufficient to demonstrate statistical significance when milk yield differs by 1.6 kg/d between treatments and standard deviation of milk yield is 9 kg/d. Furthermore, a subsample of 50 cows/treatment would be sufficient to demonstrate statistical significance when GH concentration differs by 5 ng/mL between treatments and standard deviation for GH concentration is 12 ng/mL. Similarly, 100 cows/treatment would be sufficient to demonstrate statistical significance when IGF-1 concentration differs by

17.5 ng/mL between treatments and standard deviation of IGF-1 concentration is 51 ng/mL.

Cows assigned to the rbST125 treatment that received < 2 treatments of rbST and control cows that calved within less than two weeks after being moved to the prepartum pen were removed from the experiment and statistical analyses. All statistical analyses were conducted using SAS version 9.4 (SAS/STAT, SAS Inst. Inc., Cary, NC). In all models, cows were used as the experimental unit.

Continuous data were assessed for normality of residuals. Continuous data with residuals not normally distributed were log(10) or square root transformed. Continuous data without repeated measurements (BCS and lactation number at enrollment, previous lactation 305-d mature equivalent milk yield and calving to pregnancy interval, and number of days that cows stayed in the prepartum pen) were analyzed by ANOVA using the GLM procedures of SAS. The models included treatment (control vs. rbST125), herd (HO vs. JS), and the interaction between treatment and herd as fixed effects. Milk yield and milk components in the first 30 d postpartum were analyzed by ANOVA using the GLM procedures of SAS. The models included treatment, herd, parity (nulliparous vs. parous), BCS at enrollment, and their interactions with treatment. Nonsignificant variables ($P > 0.10$) were removed from the models in a backward stepwise elimination procedure.

The MIXED procedure of SAS was used to analyze continuous data with repeated measurements (GH, IGF-1, glucose, insulin, NEFA, BHB, and BCS). The prepartum and postpartum periods were analyzed separately. For analyses of repeated measurements, the repeated statement was used and the model included treatment, time, the interaction between treatment and time, herd, the interaction between treatment and herd, and BCS at enrollment as fixed effects. Cow was included as the random effect and cows were nested within treatment. When concentrations of hormones and metabolites differed between

treatments on d -21 relative to calving, the concentration on d -21 relative to calving was used as a covariate. The structure of covariance (auto-regressive, unstructured, or compound symmetry) was chosen according to the Bayesian information criteria.

Dichotomous data were analyzed by logistic regression using the LOGISTIC procedure of SAS to obtain adjusted odds ratio and their respective confidence intervals. The FREQ procedure of SAS was used to obtain two by two tables of the incidence of events. Variables included in the logistic regression models were treatment, herd, parity, BCS at enrollment, and their interactions with treatment. Nonsignificant ($P > 0.10$) variables were removed by a backward stepwise elimination procedure based on the Wald's statistics criterion.

The speed at which cows became pregnant up to 150 d postpartum was analyzed by the Cox proportional hazards regression using the PHREG procedure of SAS. The model included treatment, herd, parity, BCS at enrollment, and their interaction with treatment. Nonsignificant ($P > 0.10$) variables were removed by a backward stepwise elimination procedure based on the Wald's statistics criterion. The difference between treatments in the interval from calving to establishment of pregnancy was evaluated by the Kaplan-Meier survival analysis using the LIFETEST procedure of SAS. Cows were censored when they did not conceive and left the herd (death/culling) before 150 DIM or when they reached 150 DIM without conceiving. Statistical significance was defined as $P \leq 0.05$ and trends were considered if $0.05 < P \leq 0.10$.

RESULTS

Forty-five cows were removed from the study and statistical analyses (HO-control = 6, HO-rbST125 = 13, JS-control = 11, and JS-rbST125 = 15) for the following reasons: diagnosed not pregnant after enrollment (control = 1, rbST125 = 3), rbST125 cows that calved before receiving at least two treatments of rbST (n = 12), control cows that calved

< 2 weeks after enrollment (n = 7), cows calved > 5 weeks after enrollment (control = 7, rbST125 = 10), abortion (control = 1), injury (rbST125 = 1), and death before calving (control = 1, rbST125 = 2). Eight hundred and twenty four cows remained in the experiment (HO-control = 151, HO-rbST125 = 151, JS-control = 264, and JS-rbST125 = 258).

Average lactation number (P = 0.92) and the percentages of nulliparous (P = 0.92), lactation = 1 (P = 0.99), lactation = 2 (P = 0.63), and lactation $\geq$ 3 (P = 0.78) cows enrolled in the experiment were not different between treatments (Table 2). At enrollment, BCS (P = 0.62), previous lactation 305-d mature equivalent milk yield (P = 0.28), and previous lactation interval from calving to pregnancy (P = 0.90) did not differ between treatments (Table 2). The average number of days that cows stayed in the prepartum pen was not different between treatments (P = 0.28; Table 2). The percentages of cows calving male calves (P = 0.51) and twins (P = 0.17) were not different between treatments (Table 2).

Effects of Treatment on Concentrations of Hormones of the Somatotropic Axis and Metabolites

Holstein cows treated weekly with 125 mg of rbST had greater concentrations of GH prepartum (P < 0.01) and postpartum (P < 0.01) compared with control cows (Figures 1). The log10 back transformed concentrations of GH were 7.83 and 10.36 ng/mL during the prepartum and 10.45 and 18.47 ng/mL during the postpartum for control and rbST125 cows, respectively. Similarly, rbST125 cows had higher prepartum (P < 0.01) and postpartum (P < 0.01) IGF-1 concentrations than control cows (Figure 2). Prepartum (P = 0.56) and postpartum (P = 0.83) glucose concentrations were not affected by treatment (Figure 3). Insulin concentration during the prepartum period was not affected by treatment (P = 0.89; log 10 back transformed insulin concentrations: HO-control = 0.76, HO-rbST125 = 0.76, JS-control = 0.44, JS-rbST125 = 0.42 μU/L; Figure 4). Although treatment did not (P = 0.69; log 10 back transformed insulin concentrations: HO-control = 0.18, HO-rbST125 = 0.19, JS-control = 0.18, JS-rbST125 = 0.16 μU/L) affect insulin concentration

during the postpartum period, the interaction between treatment and day relative to calving tended (P = 0.09) to affect insulin concentration because while on day 4 there was a tendency (P = 0.10) for rbST125 cows to have greater insulin concentration than control cows, on day 25 insulin concentration was (P = 0.05) greater for control cows than rbST125 cows (Figure 4). Treatment did not affect NEFA concentrations during the prepartum (P = 0.14; log 10 back transformed NEFA concentrations: HO-control = 190.2, HO-rbST125 = 206.0, JS-control = 113.3, JS-rbST125 = 135.5 μmol/L) and postpartum (P = 0.20; log 10 back transformed NEFA concentrations: HO-control = 414.0, HO-rbST125 = 439.6, JS-control = 214.4, JS-rbST125 = 257.3 μmol/L) periods (Figure 5). Prepartum (P = 0.39; log 10 back transformed BHB concentrations: HO-control = 344.5, HO-rbST125 = 340.3, JS-control = 499.3, JS-rbST125 = 463.2 μmol/L) and postpartum (P = 0.36; log 10 back transformed BHB concentrations: HO-control = 607.9, HO-rbST125 = 569.0, JS-control = 592.0, JS-rbST125 = 610.9 μmol/L) plasma BHB concentrations were not affected by treatment (Figure 6).

Effects of Treatment on BCS, Incidences of Postpartum Diseases, and Removal from the Herd within 60 DIM

Although treatment did not affect prepartum BCS (P = 0.93), control cows had (P = 0.01) higher BCS on d 30 and 60 postpartum than rbST125 cows (Figure 7). Treatment did not (P = 0.65) affect the incidence of stillbirth, but the interaction between treatment and herd tended to affect (P = 0.09) the incidence of stillbirth because HO-rbST125 cows had reduced incidence of stillbirth compared with HO-control cows, whereas JS-rbST125 cows had greater incidence of stillbirth than JS-control cows (Table 3).

Incidence of subclinical hypocalcemia was not (P = 0.20) affected by treatment (Table 3). Cows from the rbST125 treatment tended (P = 0.08) to have reduced incidence of retained fetal membranes compared with control cows (Table 3). Furthermore, rbST125 cows had (P < 0.01) reduced incidence of metritis compared with control cows (Table 3). Among JS

cows, treatment did not affect the incidences of acute metritis (P = 0.15) and vaginal purulent discharge 35 ± 3 d postpartum (P = 0.91; Table 3).

Incidence of ketosis tended (P = 0.08) to be greater for the rbST125 treatment compared with the control treatment (Table 3). No differences between treatments regarding the incidences of displacement of abomasum (P = 0.80) and mastitis (P = 0.18) within 60 DIM were observed (Table 3). Among JS cows, incidences of lameness at 1 ± 1 (P = 0.76), 30 ± 3 (P = 0.42) and 60 ± 3 (P = 0.33) d postpartum were not affected by treatment (Table 3).

Finally, percentage of cows removed from the herd within 60 d postpartum was not (P = 0.31) different between rbST125 and control treatments (Table 3).

Milk yield and Milk Components

Milk yield in the first 30 d postpartum was (P < 0.01) higher for rbST125 cows than control cows (Table 4). Milk fat percentage was not (P = 0.79) affected by treatment, but there was a tendency (P = 0.08) for the interaction between treatment and herd to affect the milk fat content because while HO-control cows had reduced milk fat percentage than JS-control cows no differences in milk fat percentage were observed between HO-rbST125 and JS-rbST125 cows (Table 4). Milk fat yield was (P < 0.01) greater for rbST125 cows compared with control cows (Table 4). The interaction between treatment and herd affected (P = 0.03) milk fat yield because among HO cows those treated with rbST during the periparturient period had greater milk fat yield, but among JS cows treatment with rbST did not affect yield of fat (Table 4). Milk protein percentage was (P < 0.01) reduced for rbST125 cows compared with control cows, but treatment did not (P = 0.28) affect milk protein yield (Table 4). Energy corrected milk was (P < 0.01) higher for rbST125 cows than control cows (Table 4). The interaction between treatment and herd tended (P = 0.08) to affect yield of ECM because HO-rbST125 cows had greater ECM yield than HO-control

cows, but ECM was not different between JS-rbST125 and JS-control cows (Table 4). Treatment with rbST did not (P = 0.15) affect linear SCC within the first 30 DIM (Table 4).

Reproductive Parameters

Reproductive parameters are depicted in table 5. The likelihood of cows receiving the first postpartum AI at detected estrus was not (P = 0.36) different between treatments. The large difference (P < 0.01) in percentage of cows receiving the first postpartum AI at detected estrus between herds (HO = 2.7% vs. JS = 90.3%) reflects their different management strategies. The interval from calving to first postpartum AI was not (P = 0.60) affected by treatment. The likelihood of pregnancy after the first postpartum AI was not different between treatments (first pregnancy exam - P = 0.23; second pregnancy exam - P = 0.22). The likelihood of pregnancy loss between the first and second pregnancy exams after the first postpartum AI was not (P = 0.83) affected by treatment.

The likelihood of cows receiving the second postpartum AI at detected estrus (P = 0.83) and the DIM at second postpartum AI (P = 0.60) were not affected by treatment. Treatment did not affect the likelihood of pregnancy after the second postpartum AI (first pregnancy exam - P = 0.51; second pregnancy exam - P = 0.73). The likelihood of pregnancy loss between the first and second pregnancy exams after the second postpartum AI did not (P = 0.33) differ between treatments. The rate at which cows became pregnant up to 150 d postpartum was not affected by treatment (P = 0.14; adjusted hazard ratio for pregnancy of control vs. rbST125 cows = 1.14, 95% confidence interval = 0.96, 1.36) or by the interaction between treatment and herd (P = 0.94). The mean (±SEM) intervals from calving to pregnancy were 109.8 ± 3.2, 110.4 ± 3.1, 100.5 ± 2.4, and 105.9 ± 2.5 for HO-control, HO-rbST125, JS-control, and JS-rbST125 cows, respectively. The median intervals from calving to establishment of pregnancy were 112, 112, 96, and 107 d for HO-control, HO-rbST125, JS-control, and JS-rbST125 cows, respectively.

DISCUSSION

Decoupling of the somatotropic axis during the transition from late gestation to early lactation is characterized by a rapid increase in GH concentration and a rapid decrease in IGF-1 concentration, as a consequence of the downregulation of hepatic GH 1-α receptors (GHR1A) expression in the face of NEB (Rhoads et al., 2004; Lucy, 2008). The decreased IGF-1 concentration reduces the IGF-1 negative feedback on GH release by the pituitary gland and maintains elevated GH concentrations (Lucy, 2008; Romero et al., 2012). Although treatment with rbST resulted in greater IGF-1 concentrations during the prepartum and postpartum periods, the differences in IGF-1 concentrations between rbST125 and control cows were greater during the prepartum period, suggesting that from d -17 to -3 relative to calving the GH receptor system was coupled but from d 4 to 25 relative to calving the GH receptor system was decoupled. Downregulation of hepatic GHR1A mRNA starts at approximately 5 d before parturition reaching a nadir 5 d postpartum (Lucy, 2004). Treatment of cows with 87.5 and 125 mg of rbST, weekly, from -21 to 28 d relative to calving, increased the expression of mRNA for GHR1A in the liver at -7 and 7 d relative to calving (Silva et al., 2015b), suggesting that exogenous GH may upregulate GHR1A and possibly expedite the recoupling of the somatotropic axis. Although it is not clear how treatment with rbST may have expedited the recoupling of the somatotropic axis, the recoupling of the somatotropic axis in the postpartum period is dependent on energy balance and insulin concentrations (Butler et al., 2003; Lucy, 2008). Glucose and insulin concentrations were increased in periparturient dairy cows treated with rbST when blood samples were collected daily or every other day around the time of parturition (Putnam et al., 1999; Gulay et al., 2004; Gohary et al., 2014). In the current experiment and in Silva et al. (2015b), glucose and insulin concentrations were not different between cows treated with rbST and control cows but blood samples were collected weekly, which might have limited our ability to detect differences.

In the current experiment, weekly treatment of periparturient dairy cows with 125 mg of rbST, from -21 to 21 d relative to calving, reduced the incidence of uterine disorders. Function of PMNL from cows diagnosed with retained fetal membranes and metritis is impaired during the immediate prepartum and postpartum periods (Cai et al., 1994; Kimura et al., 2002; Hammon et al., 2006). Metabolic challenges and, to a lesser extent, endocrine changes associated with parturition have been implicated in the impairment of PMNL function (Kimura et al., 1999; Hammon et al., 2006). Although less explored, it is also possible that the decoupling of the somatotropic axis during the periparturient period of dairy cows, and the consequent reduced IGF-1 concentration, may impair immune function. Immune cells produce GH and IGF-1 and express receptors for GH and IGF-1 (Merimee et al., 1989), which have been shown to modulate the innate and adaptive immune responses (Kooijman et al., 1996; Weigent, 1996; Ryu et al., 2000; Smaniotto et al., 2004). For example, treatment of malnourished humans with GH increased PMNL phagocytosis (Kotzmann et al., 2003) and GH replacement therapy of GH deficient humans increased release of superoxide anion by neutrophils (Reinisch et al., 1996) and increased the concentrations of granulocyte colony-stimulating factor and blood neutrophil counts (Sohmiya et al., 2005). Insulin-like growth factor 1 modulates immune cell proliferation (Merimee et al., 1989), chemotaxis (Tapson et al., 1988), T-cell activation (Johnson et al., 1992), inhibition of lymphocytes and neutrophils apoptosis (Baserga et al., 1997; Kooijman et al., 2002), and NK cell cytotoxicity (Kooijman et al., 1992). Moreover, IGF-1 increases B-cell differentiation, maturation and proliferation (Gibson et al., 1993; Funk et al., 1994). Insulin growth factor-1 may also modulate the inflammatory response by inhibiting the production of TNF-α, IL-1β, and IL-6 (Guo et al., 2014). In a recent experiment, weekly treatment of periparturient Holstein cows with 125 mg of rbST, from -21 to 28 d relative to calving, increased GH and IGF-1 concentrations, improved PMNL function (e.g. phagocytosis, oxidative burst, expression of L-selectin), increased IgG anti-ovalbumin concentration, and reduced the incidence of metritis (Silva et al., 2015a). The biggest differences in PMNL function and IgG anti-ovalbumin concentration between rbST treated

and control cows were observed from -7 to 0 d relative to calving, when cows had received at least 2 treatments of 125 mg of rbST (Silva et al., 2015a). Thus, it is reasonable to speculate that rbST-induced increase in the prepartum concentration of IGF-1 observed in the current experiment may have improved the immune function during the periparturient period, which may have reduced the incidence of retained fetal membranes and metritis. Despite the fact that the difference in incidence of acute metritis between JS-control and JS-rbST125 was not statistically significant, the numerical reduction in the incidence of acute metritis was 37.9% among JS-rbST125, which is similar to the statistically significant reduction in the incidence of metritis in both herds (HO = 36.6%, JS = 33.2%). This leads us to speculate that the sample size was insufficient to evaluate the effect of rbST treatment on the incidence of acute metritis. No effect of rbST treatment was observed on the incidence of vaginal purulent discharge among JS cows. This might be a consequence of the aggressive antimicrobial and supportive (e.g. anti-inflammatory) therapy provided to the JS cows diagnosed with retained fetal membranes, metritis, and acute metritis in the collaborating herd.

Gohary et al. (2014) did not observe improvements in uterine health of dairy cows treated with 325 mg of rbST every 14 d during the prepartum period. Although concentrations of GH and IGF-1 start to increase shortly after rbST treatment of lactating cows, peak concentrations of GH and IGF-1 are observed approximately 7 d after the first treatment, and a sharp decrease in concentrations of GH and IGF-1 are observed by 8 to 9 d after treatment (Bilby et al., 2006; Rivera et al., 2010). Thus, it is possible that rbST given at 14 d interval (Gohary et al., 2014) resulted in several cows going through the periparturient period while GH and IGF-1 concentrations were low, limiting its benefits to immune cells. Excessive increase in GH concentration resulting from 325 mg rbST dose treatment may have compromised the immune function of cows in the study by Gohary et al. (2014) since cultures of peripheral immune cells (Zarkesh-Esfahani et al., 2000) and lymphocytes (Bozzola et al., 1988-1989) with different concentrations of GH resulted in a quadratic

dose effect on cytokine production, STAT5 activation (Zarkesh-Esfahani et al., 2000) and on lymphocyte proliferation (Bozzola et al., 1988-1989). Finally, in the study by Gohary et al. (2014) cows may have received as few as 1 treatment with rbST during the prepartum period, which may not have exposed cows to a sustained high IGF-1 concentration.

Although the incidence of sub-clinical hypocalcemia was not different between treatments, it is possible that the number of cows used to evaluate this outcome was insufficient to detect a difference. Eppard et al. (1996) demonstrated that cows treated with 500 mg of rbST, every 14 d, from -28 to 14 d relative to calving, and fed cationic diets containing elevated Ca concentration had increased hydroxyproline, a common marker of bone reabsorption. Although the authors suggest that rbST may have increased bone calcium reabsorption, calcium concentrations were not increased by rbST treatment, despite the reduced milk yield among rbST treated cows (Eppard et al., 1996).

Weekly treatment of periparturient cows with 125 mg of rbST tended to increase the incidence of ketosis from 4 to 17 d postpartum. In the subsample of cows from which samples were collected weekly from -21 to 21 d relative calving, plasma NEFA and BHB concentrations were not affected by treatment. Other studies, however, demonstrated that treatment of periparturient dairy cows with rbST reduced the incidence of ketosis (Gulay et al., 2007) and tended to decrease plasma BHB concentration (Silva et al., 2015a) in the postpartum period. In the current experiment, rbST treatment decreased BCS during the postpartum period and increased yield of ECM by approximately 4 and 1 kg/d in the first 30 DIM among Holstein and Jersey cows, respectively. The slight increase in incidence of ketosis in the face of greater body fat depots mobilization and yield of ECM suggests that cows treated with rbST may have been more adapted to greater metabolic challenges. Treatment of dairy cows with rbST increases the uptake and oxidation of NEFA by peripheral tissues, which may lead to a reduction in substrate for hepatic BHB production (Bauman, 1992; Putnam et al., 1999). Silva et al. (2015b) demonstrated that prepartum

hepatic mRNA expression for APOA5 and ApoB100, apolipoproteins components of very-low-density lipoprotein molecules involved in the transport of triglycerides from the liver to the blood stream, were increased among cows treated weekly with 87.5 and 125 mg of rbST from -21 to 28 d relative to calving. Furthermore, postpartum liver mRNA expression for APOA5 and ACOX1, enzymes involved in fatty acid transport and beta-oxidation, were increased among cows treated with 87.5 and 125 mg of rbST, respectively (Silva et al., 2015b). Consequently, rbST treatment during the periparturient period may improve fatty acid oxidation and transport by the liver.

Incidences of displacement of abomasum, mastitis, lameness, and percentage of cows removed from the herd within 60 d postpartum were not affected by treatment. Similarly, Vallimont et al. (2001) and Gohary et al. (2014) did not observed an effect of rbST treatment on incidences of displacement of abomasum and mastitis when cows were treated with 500 or 325 mg of rbST, respectively, during the prepartum period. Although Gulay et al. (2007) did not observe a difference in incidence of displacement of abomasum between rbST treated and control cows, rbST treatment during the periparturient period reduced the incidence of mastitis. As mentioned previously, rbST-induced increases in the concentrations of GH and IGF-1 improved PMNL function and IgG concentration during the periparturient period (Silva et al., 2015b), which may also improve immune response to intra-mammary pathogens.

Yields of milk, fat, and ECM were greater for rbST125 cows than control cows. The rbST-induced increase in GH and IGF-1 concentrations may have improved productive responses through increased lipolysis, nutrient prioritization to the mammary gland, and proliferation and maintenance of epithelial cells of the mammary gland (Bauman, 1992). Milk content of protein was greater for control cows than rbST125 cows, which is likely to be a consequence of dilution effect of increased milk yield from rbST125 cows since total milk protein yield was not different between treatments. In general, treatment of

prepartum and periparturient cows with rbST has been shown to increase yields of milk and ECM (Putnam et al., 1999; Gulay et al., 2003, 2004; Gohary et al., 2014).

Reproductive performance was not improved by rbST treatment during the periparturient period, despite significant improvements in uterine health and higher postpartum IGF-1 concentration of rbST125 cows. Retained fetal membranes and metritis are associated with increased days to first postpartum insemination and decreased pregnancy per AI (Coleman et al., 1985; LeBlanc et al., 2002). Consistent with our results, Gohary et al. (2014) did not demonstrate improvements in reproductive performance of cows treated with rbST during the prepartum period. One may speculate that the improved uterine health during the early postpartum period was counteracted by more severe NEB of rbST125 cows, characterized by reduced BCS postpartum, as a result of the increased yield of ECM (Lucy et al., 1991; Nebel and McGilliard, 1993). It is unlikely, however, that energy balance was significantly lower in rbST treated cows because they had greater IGF-1 concentration during the postpartum period. It is more likely that aggressive diagnosis and treatment of cows with retained fetal membranes and metritis with antimicrobial and anti-inflammatory drugs and the use of ovulation synchronization protocols and fixed time AI may have confounded the results regarding reproductive performance.

CONCLUSIONS

Treatment of periparturient dairy cows with 125 mg of rbST, weekly, from -21 to 21 d relative to calving, may be used to reduce the incidences of retained fetal membranes and metritis. Furthermore, treatment of periparturient dairy cows with 125 mg of rbST may improve yield of ECM with minimal consequences to the concentrations of metabolites. Reduced incidences of retained fetal membranes and metritis may lead to the reduced utilization of antimicrobials and anti-inflammatory drugs, which coupled with increased yield of ECM, may improve profitability of dairy herds. Although the increased yield of ECM and fat depots mobilization among periparturient cows treated with rbST suggests

that they may have had more negative energy balance postpartum, rbST treated cows had greater IGF-1 concentrations, which is associated with less negative energy balance. Additional research is warranted to determine the effects of rbST treatment of prepartum versus periparturient cows with reduced doses at 7 d intervals. This would include carry over effects of rbST on DMI and NEB as well as impacts on health, development and immune and endocrine responses of the calf.

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CHAPTER IV

Hepatic mRNA expression for genes related to somatotropic axis, glucose and lipid metabolisms, and inflammatory responses of dairy cows treated with recombinant bovine somatotropin during the periparturient period

OUTLINE

Objectives of this experiment were to evaluate the effects of recombinant bovine somatotropin (rbST) treatment of periparturient dairy cows on hepatic mRNA expression for genes related to the somatotropic axis and insulin, glucose and lipid metabolism, inflammation, and oxidative stress. Holstein cows were enrolled in the experiment at 253 ± 3 d of gestation and assigned to 1 of 3 treatments: untreated control (n = 53), rbST87.5 (n = 56; 87.5 mg of rbST), and rbST125 (n = 57; 125 mg of rbST). Cows in the rbST87.5 and rbST125 treatments received weekly injections of rbST from -21 to 28 d relative to calving. A sub-sample of cows (control = 20, rbST87.5 = 20, rbST125 = 20) was randomly selected for collection of liver samples according to expected calving date, BCS, and previous lactation 305-d mature equivalent milk yield. Only cows that had liver sampled at -21 ± 3, -7 ± 3, and 7 ± 3 d relative to calving were used in the current experiment. Blood sampled weekly, from -28 to 21 d relative to calving, was used to determine the concentrations of growth hormone, insulin-like growth factor 1, insulin, cortisol, nonesterified fatty acids, β-hydroxybutyrate, glucose, haptoglobin, and tumor necrosis factor-α. Liver samples were used to determine hepatic mRNA expression of 50 genes. Treatment with rbST increased growth hormone concentrations during the postpartum period (control = 9.0 ± 0.7, rbST87.5 = 15.3 ± 1.0, rbST125 = 18.5 ± 1.3 ng/mL) and increased insulin-like growth factor 1 concentrations during the prepartum period (control = 107.4 ± 7.2, rbST87.5 = 126.9 ± 6.6, rbST125 = 139.4 ± 6.9 ng/mL). Control cows had greater postpartum concentrations of β-hydroxybutyrate (control = 776.4 ± 64.0, rbST87.5 = 628.4 ± 59.7, rbST125 = 595.4 ± 60.9 μmol/L) than rbST cows. The rbST87.5 and rbST125 treatments upregulated the hepatic mRNA expression for somatotropic axis genes

(*GHR*, *GHR1A*, *IGF1*, *IGFBP3 and SOCS2*) on d -7 relative to calving and upregulated the mRNA expression for *SOCS2* on d 7. On d -7, rbST87.5 and rbST125 treatments increased mRNA expression for genes involved in hepatic lipid transport (*ANGPTL4*, *APOA5*, *APOB100*, and *SCARB1)* and downregulated mRNA expression for *PPARD*, which is involved in lipid storage. On d 7, rbST tended to upregulate the mRNA expression for genes involved in gluconeogenesis (*PCK1*) and fatty acid beta-oxidation (*ACOX1*) and downregulated the mRNA expression for genes involved in inflammation (*TNFRSF1A*, *ICAM1*, *CXCL1*, *MYD88*, *HIF1A*, *IL1RN*, *NFKBIA*, and *SOCS3*) and oxidative stress (*XBP1*). Administration of rbST during the periparturient period may improve liver function and health by increasing hepatic capacity for gluconeogenesis and lipid transport and by reducing inflammation and oxidative stress.

Key words: dairy cow, hepatic gene expression, somatotropin

INTRODUCTION

The liver performs essential functions in the body by uniquely expressing genes encoding proteins involved in glucose, lipid, and protein metabolism, ketogenesis, immune function, detoxification, hormone catabolism, vitamin and mineral metabolism, and a variety of other functions (Donkin, 2012). Postpartum cow health is closely associated with the capacity of the liver to cope with the changes in nutrient supply and shifting metabolic demands that accompany the initiation of lactation (Drackley et al., 2001). In periparturient dairy cows the liver has an important role in metabolic regulation and control of the somatotropic axis to successfully adapt to the negative energy balance (**NEB**; Grummer et al., 2004). During NEB the expression of growth hormone (**GH**) 1-α receptors (**GHR1A**) by the liver is downregulated to decrease insulin-like growth factor 1 (**IGF-1**) synthesis and its inhibitory effects on synthesis and secretion of GH (Bauman, 2000). Therefore, GH concentrations are increased in periods of NEB, such as the periparturient period, to favor nutrient partitioning for lactogenesis (Bell and Bauman, 1997). This pivotal role of GH in nutrient partitioning includes major alterations in metabolism of carbohydrates and lipids

(Lupu et al., 2001). In adipose tissue, GH acts to facilitate lipolysis, increase insulin resistance to reduce glucose uptake and oxidation, and to decrease lipid synthesis (Boyd and Bauman, 1989). In the liver, GH increases gluconeogenesis (Cohick et al., 1989) through the regulation of enzymes such as phosphoenolpyruvate carboxykinase (**PCK**), suppression of insulin's inhibitory effect on gluconeogenesis (Velez and Donkin, 2004), and increased hepatic fatty acid oxidation to CO_2 (Pocius and Herbein, 1986).

From late gestation to early lactation, insulin resistance and GH-induced lipolysis cause a marked increase in nonesterified fatty acids (**NEFA**) concentration and influx into the liver, which experiences a pronounced metabolic stress to maintain homeostasis (Drackley et al., 2001). The liver takes up approximately one-third of the whole body NEFA flux and this often exceeds its oxidation capacity, increasing the risk of liver lipidosis and ketosis (Drackley et al., 2001). During the peripartum period the liver is exposed to a variety of inflammatory factors such as lipopolysaccharides (**LPS**), pro-inflammatory cytokines, and reactive oxygen species. These inflammatory factors are increased during the periparturient period due to alterations in gastrointestinal physiology, associated with changes in feed intake and composition, and episodes of infectious (e.g. metritis and mastitis) and gastrointestinal (e.g. SARA and displacement of the abomasum) diseases (Plaizier et al., 2008; Vels et al., 2009). Consequently, periparturient cows may present inflammation of the liver, which may induce an acute phase response (Vels et al., 2009), metabolic disorders, and unfold protein response to oxidative stress (Gessner et al., 2014) that collectively result in impaired liver function.

In a recent experiment, treatment of Holstein cows with 125 mg of rbST weekly from -21 to 28 d relative to calving increased IGF-1 concentrations, intensity of expression of L-selectin, intensity of phagocytosis and oxidative burst by polymorphonuclear leukocyte (**PMNL**), concentration of IgG anti-ovalbumin during the prepartum and tended to reduce β-hydroxybutyrate (**BHB**) concentrations during the postpartum (Silva et al., 2015).

Furthermore, cows treated with 125 mg of rbST had reduced incidence of metritis (Silva et al., 2015; Silva et al., 2016), reduced haptoglobin concentration 7 d postpartum (Silva et al., 2015), and increased fat corrected milk yield (Silva et al., 2016). Enhanced knowledge of the processes that regulate liver function and factors that potentiate the capacity of liver to coordinate shifts in nutrient supply and demand by other tissues may lead to significant improvements in peripartum cow management and health. Therefore, the hypotheses of the current experiment were that weekly administration of rbST to peripartum dairy cows would upregulate hepatic mRNA expression for genes related to the somatotropin axis (e.g., *GHR*, *GHR1A*, *IGF1*, and *INSR*), gluconeogenesis (e.g., *G6PC*, *PC1*, and *PCK1*), lipid oxidation and transport (e.g., *ACOX1*, *APOA5*, *APOB100*, and *SCARB1*) and downregulate the expression of mRNA for genes related to ketogenesis (e.g., *HMGCLL1*), lipid synthesis and accumulation (e.g., *DGAT1* and *PPARD*), inflammation (e.g., *MYD88*, *NFKB1*, *HIF1*, and *CXCL1*), and oxidative stress (e.g., *XBP1*). The objectives of the current experiment were to evaluate the effects of peripartum rbST treatment of dairy cows on hepatic mRNA expression for genes related to the somatotropic axis, glucose and lipid metabolisms, inflammation, and oxidative stress.

MATERIALS AND METHODS

Animals, Housing, and Nutrition

All animal procedures conducted during this experiment were approved by the Institutional Animal Care and Use Committee from the University of Minnesota (protocol # 1306-30734A). Cows used in the current experiment are a sub-group of cows used in an experiment that evaluated the effects of rbST treatment during the periparturient period on immune parameters (Silva et al., 2015). Detailed information regarding facilities, management, and nutrition has been previously described (Silva et al., 2015). Briefly, multiparous Holstein cows (lactation = 1 to 6) from a commercial free-stall herd located in Northwest Wisconsin with BCS = 3.75 to 4.75 (Ferguson et al., 1994) and locomotion

score = 1 to 2 (Sprecher et al., 1997) were enrolled in the experiment at 253 ± 3 d of gestation (-28 d relative to calving). From approximately 244 d of gestation to 21 d postpartum cows were housed in a naturally ventilated free-stall barn. On day 22 postpartum, cows were moved from the transition facility to a nearby free-stall dairy, where they were housed for the remainder of their lactation. Composition of TMR fed in the prepartum (far-off and close-up) and immediate postpartum (1 to 21 d postpartum) periods were described in Silva et al. (2015).

Enrollment and Treatments

At enrollment, cows were scored for body condition (1 = emaciated and 5 = obese; 0.25-unit increments; Ferguson et al., 1994) and locomotion (1 = normal locomotion and 5 = severely lame; as described by Sprecher et al., 1997), and BW was estimated using a Holstein dairy cow weigh tape (The Coburn Company Inc., Whitewater, WI). Cows were balanced for lactation number, BCS, BW, previous lactation 305-d mature equivalent milk yield, and previous lactation interval from calving to conception. Cows were assigned randomly to 1 of 3 treatments: untreated control (n = 53), 87.5 mg of rbST (**rbST87.5**; n = 56), and 125 mg of rbST (**rbST125**; n = 57) which were administered every 7 d from -21 to 28 d relative to calving. Six to twelve hours before treatments doses of rbST were prepared. The contents of commercially available syringes of rbST (Posilac, sometribove zinc suspension for injection; Elanco Animal Health, Greenfield, IN) were dispensed into sterile containers and the appropriate weight aspirated into sterile 1 mL tuberculin syringes to provide doses of 87.5 or 125 mg of rbST. Syringes were kept refrigerated until they were warmed to room temperature (15 to 30 °C) for administration. Treatments were administered subcutaneously in the neck area using 16 gauge and 5/8-inch-long hypodermic needles.

A sub-sample of cows (control = 20, rbST87.5 = 20, rbST125 = 20) was randomly selected for collection of liver samples according to expected calving date and balancing for BCS

and previous lactation 305-d mature equivalent milk yield. Only data from cows that had liver samples collected at -21 ± 3, -7 ± 3, and 7 ± 3 d relative to calving are reported herein.

Clinical Examination, Disease Definitions, Milk yield, and BCS

Study personnel palpated cows per rectum daily from d 2 to 21 postpartum for the diagnosis of metritis. Metritis was defined as cows having watery, pink/brown, and fetid uterine discharge (LeBlanc, 2010). Ketosis was defined as cows having circulating BHB concentrations ≥ 1,400 μmol/L (Duffield et al., 2009). Beginning between 55 to 50 d postpartum, all cows received 500 mg of rbST (Posilac, sometribove zinc suspension; Elanco Animal Health, Greenfield, IN) every 11 d through 220 to 225 d of gestation. Therefore, milk data reported herein is the weekly milk yield average from 22 to 30 d of lactation. Cows were milked thrice daily at both facilities but milk yield was not measured in the first 21 DIM because the transition cow facility did not have the capability to do so. Daily milk yield was recorded for individual cows (Afimilk Ltd., Kibbutz Afikim, Israel) starting at approximately 22 DIM and recorded on DairyComp305 software (Valley Ag. Software, Tulare, CA). Body condition was scored (Ferguson et al., 1994) weekly from -28 to 28 d relative to calving.

Hormones, Metabolites, and Indicators of Inflammation Measurements

Cows that had liver samples collected (n = 32) also had blood sampled weekly from -28 to 21 d relative to calving to determine concentrations of GH, IGF-1, insulin, cortisol, NEFA, BHB, glucose, haptoglobin, and tumor necrosis factor α (**TNF-α**). During feeding, when cows were restrained in self-locking headlocks, blood samples were collected from the coccygeal vein/artery using 22 gauge and 1-inch-long needles into evacuated tubes without anticoagulants and evacuated tubes containing K2EDTA (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ). Tubes were placed in ice until centrifugation (1,200 *g* for 15 min at 4 ºC) for serum or plasma separation within 8 h after blood samples were collected.

Serum and plasma were aliquoted into microcentrifuge tubes and stored at -32 ºC until analysis.

Serum GH and total IGF-1 concentrations were determined in triplicate using a modified RIA (Lalman et al., 2000). The intra- and inter-assay coefficients of variation (**CV**) for the GH and IGF-1 assays were lower than 5%. Serum insulin concentrations were determined by a commercial bovine ELISA kit (Mercodia Bovine Insulin ELISA; Mercodia AB, Uppsala, Sweden). The intra- and inter-assay CV were 8 and 5%, respectively. Serum cortisol was measured by a solid phase RIA using a commercially available kit (Coat-A-Count Cortisol; Siemens Medical Solutions Diagnostics, Los Angeles, CA). The intra- and inter-assay CV for cortisol were 7 and 6%, respectively. Plasma concentrations of NEFA were determined using a colorimetric assay (Wako Chemicals USA, Richmond, VA; Ballou et al., 2009) and plasma concentrations of BHB were determined enzymatically (Ranbut, Randox Laboratories, Antrim, UK; Ballou et al., 2009). The intra-assay CV were 7 and 11% for the NEFA and BHB assays, respectively. The inter-assay CV were 4 and 8% for the NEFA and BHB assays, respectively. Plasma glucose concentrations were determined by enzymatic reaction (Stanbio Laboratory, Boerne, TX). The intra- and inter-assay CV were 4 and 3%, respectively. Plasma haptoglobin concentrations were determined by a colorimetric procedure (Hulbert et al., 2011). The intra- and inter-assay CV were 4 and 3%, respectively. Concentrations of plasma TNF-α were determined by ELISA using a protocol described and validated for bovine plasma (Farney et al., 2011). The intra- and inter-assay CV were 5 and 3%, respectively.

Liver Samples and mRNA Extraction and Quality Assessment

Liver tissue samples were collected -21 ± 3, -7 ± 3, and 7 ± 3 d relative to calving to determine mRNA expression of 50 genes. Eight genes were internal control candidates and 42 genes were genes of interest related to the somatotropic axis, metabolism of glucose and lipids, inflammation, and oxidative stress (Table 1).

Percutaneous needle (Bard® Magnum™, Covington, GA) technique was performed to obtain the liver samples (Sigl et al., 2012). Briefly, an area of 15 cm x 15 cm on the right side of the cow was shaved, washed, and degreased with 70% ethanol and subsequently disinfected with an iodine solution (Povidone-Iodine Solution USP 10%, Ricca Chemical Company, Arlington, TX). Local anesthetic (10 mL; Lidocaine Hydrochloride injection 2%, Vedco Inc., Saint Joseph, MO) was used to desensitize skin, subcutaneous tissue, and intercostal muscle. An incision (approximately 2 cm long) was made through the skin at the intersection of an imaginary line running from the tuber coxae to the shoulder joint with the 11^{th} and 12^{th} intercostal space. The trocar was inserted through the incision to collect samples from the caudate lobe of the liver. A skin surgical stapler (APPOSE™ ULC Skin Staplers, New Haven, CT) was used to close the incision. Staples were removed after two weeks before subsequent liver sample collection when incision was healed. Approximately 200 mg of liver tissue were obtained from each sample collection and directly transferred into sterile Eppendorf tubes that were immediately frozen in liquid nitrogen and stored at -80 °C until mRNA extraction.

The mRNA was extracted from 50 mg of liver tissue using RNeasy mini kit columns (Qiagen, Valencia, CA). Total RNA restrained on the columns after extraction protocol was eluted with 50 µL sterile RNase- DNase-free water (Qiagen, Valencia, CA). Quantity and purity of the extracted RNA were estimated using a NanoDrop spectrophotometer (ND-2000, NanoDrop Technologies Inc., Wilmington, DE) at 230, 260, and 280 nm absorbance waves. Purity was accepted when the 280/260 and 260/230 absorbance ratios were $\geq$ 1.90 and 1.80, respectively. The ratios obtained in the samples ranged from 1.97 to 2.08 and 1.77 to 2.28 for the 280/260 and 260/230 ratios, respectively. A portion of each RNA sample was diluted in RNase- DNase-free water (Qiagen, Valencia, CA) to achieve a consistent sample concentration of 100 ng/µL.

The integrity of RNA was assessed with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and the Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's protocol. All samples had an RNA integrity number (RIN) of > 7.

Gene Expression Analysis, Reference Genes Selection, and Data Normalization

Hepatic mRNA expression was assessed by single-molecule imaging of targeted mRNA molecules hybridized with color-coded probe pairs and molecular barcodes using the NanoString nCounter® System (NanoString Technologies, Seattle, WA). The direct digital readout of each mRNA and its relative abundance was measured in 100 ng of total RNA. The nSolver™ 2.0 Analysis Software (NanoString Technologies, Seattle, WA) was used to perform automated quality control (**QC**) metrics to detect flags on imaging, binding density, linearity, and limit detection of control probes. Imaging and binding density QC were set to detect flags when the percentage of fields of view successfully counted by the digital analyzer scan was less than 75 and binding density was outside the 0.05 to 2.25 range. The positive control linearity and limit of detection QC were set to flag lanes when positive control R^2 values were less than 0.95 and when 0.5 fM detection was $\leq$ 2 standard deviations above the mean of the negative controls. No QC flags were detected in the samples.

Eight candidate reference genes *EIF3G*, *GAK*, *HNF4A*, *HPRT*, *SNAPC2*, *SPRYD4*, *UBC*, and *UXT* (Table 1) were evaluated for their ability to fulfill two criteria: to be stably expressed in the samples and to provide mRNA counts that span the range of mRNA counts of the genes of interest. NormFinder software was used to determine the most stable genes (Andersen et al., 2004). Four genes, *HNF4A*, *SPRYD4*, *UBC*, and *UXT*, were the most stable and were used in the data normalization process.

To account for slight differences in hybridization and purification efficiency, data were normalized from each sample to its positive controls and selected reference genes using the nSolver™ 2.0 Analysis Software (NanoString Technologies, Seattle, WA). The positive hybridization controls are RNA control transcripts sequences that are not homologous to any known organism and generate consistent results in gene expression analyses (NanoString Technologies, Seattle, WA). Positive controls allowed for the correction of sample to sample variation due to assay specific factors such as differences in amount of input material or reagents. The negative correction was used to subtract the background noise from the positively corrected data using counts of six negative controls (sequence blank tags) by subtracting the negative controls geometric mean + 2 standard deviations. The normalization procedure consisted of the adjustment of all mRNA counts data to normalization factors generated specifically for each sample based on the geometric means, within and across all samples, of six positive controls and selected reference genes counts.

Statistical Analysis

All statistical analyses were conducted using SAS version 9.4 (SAS/STAT, SAS Inst. Inc., Cary, NC). In all models, cows were used as the experimental unit. Only cows that had a complete set of liver biopsies (n = 32) were included in the statistical analysis.

The assumption of normality of variables and residuals of continuous data were assessed using the UNIVARIATE procedure of SAS and the residual output function of SAS. A natural log or square root transformation was applied to achieve a normal distribution when necessary and data were back transformed to original scale for reporting. Continuous data without repeated measurements (i.e. previous 305-d mature equivalent milk yield, previous interval from calving to conception, lactation number, days in the close-up pens, BCS and BW at enrollment, and milk yield) were analyzed by ANOVA using the MIXED procedure of SAS and treatment included in the model as the fixed effect. The MIXED procedure of SAS was used to analyze continuous data with repeated measurements (i.e. GH, IGF-1,

insulin, cortisol, NEFA, BHB, glucose, haptoglobin, TNF-α, and BCS). For analyses of repeated measurements, the repeated statement was used and treatment, time, and the interaction between treatment and time were included in the model as fixed effects. The structure of covariance (auto-regressive, unstructured, or compound symmetry) was chosen according to the Bayesian information criteria. Cow was included as the random effect and cows were nested within treatment. The prepartum and postpartum periods were analyzed separately because of inherent physiological changes that occur during calving. Dichotomous data (i.e. incidence of postpartum diseases) were analyzed by logistic regression using the LOGISTIC procedure of SAS and two by two tables were generated using the FREQ procedure of SAS.

Analysis of variance was used to analyze the effects of treatment on hepatic mRNA expression at the different time points relative to calving using the GLM procedure of SAS with treatment as a fixed effect. In the model to determine the effect of treatment on hepatic mRNA expression on d -7 relative to calving, hepatic mRNA expression on d -21 relative to calving was used as a covariate. Hepatic mRNA expression on d 7 relative to calving was analyzed separately because of inherent physiological changes that occur during calving.

When the effect of treatment on dependent variables was $P \leq 0.15$, two orthogonal contrasts were used to compare the effects of treatments: control vs. rbST87.5 + rbST125 and rbST87.5 vs. rbST125. Statistical significance of the contrast analysis was defined as $P \leq 0.05$ and tendency was defined as $0.05 < P \leq 0.10$.

RESULTS

Descriptive Parameters at Enrollment and Calving

Twenty-eight cows (control = 10, rbST87.5 = 8, and rbST125 = 10) had collection of liver samples interrupted and were removed from the experiment for the following reasons:

cows calved < 3 weeks after enrollment (n =17) and cows calved > 3 weeks after enrollment (n = 11). Thirty-two cows remained in the experiment and their data were used in the statistical analysis (control = 10, rbST87.5 = 12, and rbST125 = 10).

At enrollment, average lactation number (control = 1.9 ± 0.3, rbST87.5 = 2.1 ± 0.3, rbST125 = 2.1 ± 0.3; *P* = 0.89), BCS (control = 4.0 ± 0.1, rbST87.5 = 4.0 ± 0.1, rbST125 = 4.1 ± 0.1; *P* = 0.60), BW (control = 800.2 ± 21.4, rbST87.5 = 788.9 ± 19.5, rbST125 = 796.3 ± 21.4 kg; *P* = 0.92), previous lactation 305-d mature equivalent milk yield (control = 11,545 ± 640, rbST87.5 = 12,901 ± 585, rbST125 = 12,252 ± 640 kg; *P* = 0.31), previous lactation interval from calving to conception (control = 166.8 ± 22.3, rbST87.5 = 150.2 ± 20.4, rbST125 = 163.8 ± 22.3 d; *P* = 0.84), and days of gestation (control = 253.3 ± 0.2, rbST87.5 = 253.4 ± 0.1, rbST125 = 253.2 ± 0.2 d; *P* = 0.57) were not different among treatments. Number of days cows stayed in the close-up pen before calving (control = 27.0 ± 0.7, rbST87.5 = 28.0 ± 0.7, rbST125 = 27.5 ± 0.7 d; *P* = 0.59) and percentage of cows calving male calves (control = 50.0, rbST87.5 = 41.7, rbST125 = 50.0%; *P* = 0.90) were not different among treatments.

Effects of rbST Treatment on Health Parameters, Milk Yield, and Body Condition Score

The incidence of metritis was not (*P* = 0.78) different among treatments (control = 20.0, rbST87.5 = 9.1, rbST125 = 0.0%), but treatment tended (*P* = 0.13) to affect the incidence of ketosis (control = 44.4, rbST87.5 = 9.1, rbST125 = 10.0%) because control cows had (*P* = 0.04) higher incidence of ketosis than rbST treated cows.

Milk yield from 21 to 30 DIM was not (*P* = 0.28) affected by treatment (control = 38.4 ± 4.2, rbST87.5 = 43.4 ± 4.1, rbST125 = 47.5 ± 3.6 kg/d). Body condition score during the prepartum period was not (*P* = 0.47) affected by treatment (control = 4.0 ± 0.1, rbST87.5 = 3.9 ± 0.1, rbST125 = 4.0 ± 0.1) but there was a tendency (*P* = 0.12) for treatment to affect BCS during the postpartum (control = 3.3 ± 0.1, rbST87.5 = 3.4 ± 0.1, rbST125 = 3.5 ±

0.1). During the postpartum period rbST125 cows had (P = 0.05) higher BCS than rbST87.5 cows. It is important to note that the power provided by the relatively small sample size of the present experiment (control = 10, rbST87.5 = 12, rbST = 10 cows) may be insufficient to detect effects of treatment on parameters such as incidence of postpartum diseases, milk yield, and BCS. Silva et al. (2016) compared the health and milk yield responses of untreated periparturient cows and periparturient cows treated with 125 mg of rbST.

Effects of rbST Treatment on Hepatic Gene Expression

From the 42 genes of interest and 8 internal control genes analyzed (Table 1), only *HMGCLL1* and *GCSF* were not detected. Hepatic mRNA expression on d -21 was not (P > 0.15) different among treatments (Table 2 and 3; Figures 1 to 3) except for the *SOCS3* gene, which had (P = 0.02) greater expression in rbST cows compared with control cows (Table 3 and Figure 3). Twenty-six genes were modulated by treatment on d -7 and/or d 7 relative to calving (Tables 2 and 3; Figures 1 to 3).

Effects of rbST Treatment on Somatotropic Axis

Hepatic expression of mRNA for *GHR* and *GHR1A* on d -7 relative to calving tended (P = 0.07) to be and was (P = 0.03) affected by treatment, respectively. The contrasts demonstrated an upregulation ($P \leq 0.02$) of mRNA expression for *GHR* and *GHR1A* in rbST cows compared with control cows (Table 2 and Figure 1). On d 7 relative to calving, mRNA expression for *GHR1A* tended (P = 0.08) to be affected by treatment because rbST treatment tended (P = 0.06) to upregulate mRNA expression for *GHR1A* compared with control treatment (Table 2 and Figure 1). Although during the prepartum period serum GH concentration was not (P = 0.23) affected by treatment, postpartum serum GH concentrations were (P = 0.01) affected by treatment as rbST cows had (P < 0.01) greater GH concentrations than control cows (Table 4; Figure 1).

Hepatic mRNA expression for *SOCS2* tended to be affected by treatment on d -7 ($P = 0.15$) and 7 ($P = 0.06$). On d -7 rbST treatment tended ($P = 0.09$) to upregulate *SOCS2* expression compared with control, and on d 7 rbST treatment upregulated ($P = 0.02$) mRNA expression for *SOCS2* compared with control cows (Table 2; Figure 1). Although there was a tendency ($P = 0.08$) for treatment to affect mRNA expression for *IGF1R* on d -7 (Table 2), according to the results of the contrasts there were no differences among control and rbST treated cows ($P = 0.15$) and between rbST87.5 and rbST125 cows ($P = 0.15$) regarding mRNA expression for *IGF1R*. The hepatic mRNA expression for *IGF1R* on d 7 was not ($P = 0.27$) affected by treatment (Table 2). The hepatic mRNA expression for *IGF1* on d -7 was ($P = 0.01$) affected by treatment as mRNA expression for *IGF1* was upregulated ($P < 0.01$) among rbST cows compared with control cows (Table 2; Figure 1). On d 7, treatment tended ($P = 0.10$) to affect mRNA expression for *IGF1* because rbST87.5 treatment upregulated ($P = 0.03$) mRNA expression for *IGF1* compared with the rbST125 treatment (Table 2; Figure 1). The mRNA expression for *IGFBP3* on d -7 tended ($P = 0.14$) to be affected by treatment because rbST treatment tended ($P = 0.07$) to upregulate the expression of mRNA for *IGBP3* compared with control. On d 7, however, treatment did not ($P = 0.96$) affect mRNA expression for *IGFBP3* (Table 2; Figure 1). Treatment affected ($P = 0.01$) prepartum serum IGF-1 concentrations (Table 4 and Figure 1) because rbST treated cows had ($P = 0.01$) higher serum IGF-1 concentrations than control cows. Concentrations of IGF-1 during the postpartum period, however, were not ($P = 0.39$) affected by treatment (Table 4 and Figure 1).

There was no effect of treatment on mRNA expression for *INSRB* on d -7 and 7 relative to calving ($P \geq 0.21$; Table 2). Hepatic mRNA expression for *INSR* was not affected by treatment on d -7 ($P = 0.28$). On d 7, treatment tended ($P = 0.07$) to affect mRNA expression for *INSR* as rbST treatment upregulated ($P = 0.05$) mRNA for *INSR* compared with control (Table 2; Figure 1). Serum insulin concentrations, however, were not different among treatments during the prepartum or postpartum periods ($P \geq 0.52$; Table 4).

Effects of rbST Treatment on Glucose and Lipid Metabolism

Treatment did not affect hepatic mRNA expression for the genes *G6PC* and *PC1* on d -7 and 7 relative to calving ($P \geq 0.41$; Table 2). Hepatic mRNA expression for *PCK1* was not affected ($P = 0.98$) by treatment on d -7. On d 7 there was a tendency ($P = 0.15$) for treatment to affect mRNA expression for *PCK1* because rbST treatment tended ($P = 0.06$) to upregulate mRNA expression for *PCK1* compared with control (Table 2). Hepatic mRNA expression for *PCK2* tended ($P = 0.15$) to be affected by treatment on d -7 because rbST treatment tended ($P = 0.07$) to downregulate mRNA expression for *PCK2* compared with control, but treatment did not ($P = 0.38$) affect mRNA expression for *PCK2* on d 7 (Table 2; Figure 2). Surprisingly, treatment did not affect prepartum ($P = 0.73$) and postpartum ($P = 0.57$) glucose plasma concentrations (Table 4).

Hepatic mRNA expression for *ACOX1* was not ($P = 0.47$) affected by treatment on d -7. On d 7, treatment tended ($P = 0.12$) to affect mRNA expression for *ACOX1* because rbST treatment tended ($P = 0.06$) to upregulate its expression compared with control (Table 2; Figure 2). Hepatic mRNA expression for *ANGPTL4*, *APOA5*, *APOB100* ($P \leq 0.05$) and *SCARB1* ($P = 0.02$) were affected by treatment on d -7 as rbST treatment upregulated ($P < 0.03$) mRNA expression for these genes compared with control (Table 2; Figure 2). On d 7, treatment did not ($P \geq 0.25$) affect mRNA expressions for *ANGPTL4*, *APOB100*, and *SCARB1* but tended ($P = 0.10$) to affect mRNA expression for *APOA5*. Treatment with rbST upregulated ($P = 0.05$) mRNA expression for *APOA5* compared with control (Table 2; Figure 2). Hepatic mRNA expression for *PPARD* was not ($P = 0.20$) different among treatments on d -7. Expression of mRNA for *PPARD* on d 7 was ($P = 0.04$) affected by treatment because rbST treatment downregulated ($P = 0.04$) mRNA expression for PPARD compared with control (Table 2; Figure 2). Expression of mRNA for *PPARGC1A* tended ($P = 0.15$) to be affected by treatment on d -7 because rbST treatment tended ($P = 0.06$) to downregulate mRNA expression for *PPARGC1A* compared with control. Expression of mRNA for *PPARGC1A* on d 7, however, was not ($P = 0.43$) affected by treatment (Table

2). Treatment had no effect on mRNA expression for *DGAT1* and *PPARA* on d -7 and 7 relative to calving ($P \geq 0.22$; Table 2). Plasma concentrations of NEFA were not affected by treatment during the prepartum ($P = 0.84$) and postpartum ($P = 0.59$) periods (Table 4 and Figure 2). Although treatment did not ($P = 0.57$) affect BHB concentration during the prepartum period, there was a tendency ($P = 0.11$) for BHB concentration during the postpartum period to be affected by treatment as control cows had ($P = 0.04$) higher plasma BHB concentrations than rbST treated cows (Table 4 and Figure 2).

Effects of rbST Treatment on Inflammation and Oxidative Stress

Hepatic mRNA expression for *TNFRS1A* on d -7 was not ($P = 0.52$) affected by treatment but it tended ($P = 0.11$) to be affected on d 7. Control cows had ($P = 0.04$) upregulated expression of mRNA for *TNFRS1A* on d 7 compared with rbST cows (Table 3 and Figure 3). Treatment, however, did not affect plasma TNF-α concentrations during the prepartum and postpartum periods ($P \geq 0.20$; Table 4). Hepatic mRNA expression for *ICAM1* on d -7 was ($P = 0.02$) affected by treatment as mRNA expression for *ICAM1* was ($P = 0.03$) upregulated by the rbST87.5 treatment compared with the rbST125 treatment (Table 3; Figure 3). Treatment affected ($P = 0.05$) mRNA expression for *ICAM1* on d 7 because control cows had ($P = 0.02$) upregulated expression of mRNA for *ICAM1* compared with rbST cows (Table 3; Figure 3). The mRNA expression for *CXCL1* on d -7 was not ($P = 0.29$) affected by treatment, but on d 7 treatment tended ($P = 0.06$) to affect mRNA expression for *CXCL1* expression because control cows had ($P = 0.02$) upregulated mRNA expression for *CXCL1* expression compared with rbST cows (Table 3; Figure 3). There was no ($P = 0.44$) treatment effect on hepatic mRNA expression for *MYD88* on d -7. On d 7, however, treatment affected ($P = 0.02$) mRNA expression for MYD88 as control cows had ($P = 0.01$) upregulated mRNA expression for *MYD88* compared with rbST cows (Table 3 and Figure 3). Hepatic mRNA expression for *HIF1A* tended ($P = 0.12$) to be affected by treatment on d -7 as control cows tended ($P = 0.09$) to have upregulated mRNA expression for *HIF1A* compared with rbST cows. On d 7, treatment tended ($P = 0.07$) to

affect the expression of mRNA for *HIF1A* as control cows had (P = 0.02) upregulated mRNA expression for *HIF1A* compared with rbST cows (Table 3 and Figure 3). Hepatic mRNA expression for *HP* was not affected by treatment on d -7 (P = 0.20) and on d 7 (P = 0.32) relative to calving (Table 3). Plasma haptoglobin concentrations were not affected by treatment during the prepartum (P = 0.56) and postpartum (P = 0.19) periods (Table 4). Treatment did not (P > 0.15) affect hepatic mRNA expression for *CEBPD*, *JUN*, *MCSF1*, *NFKB1*, *TLR2*, *TNF*, and *TNFRSF5* on d -7 and 7 relative to calving (Table 3).

Hepatic mRNA expression for *NR3C1* was not (P = 0.17) affected by treatment on d -7. On d 7, treatment tended (P = 0.10) to be affected mRNA expression for *NR3C1* because rbST cows had (P = 0.04) upregulated mRNA expression for *NR3C1* than control cows (Table 3). Hepatic mRNA expressions for *IL1RN* on d -7 was not (P = 0.57) affected by treatment but, on d 7, treatment affected (P = 0.05) the expression of mRNA for *IL1RN* as control cows had (P = 0.02) upregulated mRNA expression for *IL1RN* than rbST cows (Table 3; Figure 3). Similarly, treatment did not (P = 0.78) affect the mRNA expression for *NFKBIA* on d -7 but it tended (P = 0.06) to affect the mRNA expression for *NFKBIA* on d 7. Control cows had (P = 0.02) upregulated mRNA expression for *NFKBIA* on d 7 compared with rbST cows (Table 3; Figure 3). Hepatic mRNA expression for *SOCS3* did not (P = 0.23) differ among treatments on d -7 but it differed (P < 0.01) on d 7. Expression of mRNA for *SOCS3* was (P < 0.01) upregulated in control cows compared with rbST treated cows (Table 3 and Figure 3). Serum cortisol concentrations during the prepartum (P = 0.20) and postpartum (P = 0.56) periods were not affected by treatment (Table 4). Hepatic mRNA expression for *XBP1* on d -7 tended (P = 0.07) to be affected by treatment because mRNA expression for *XBP1* tended (P = 0.08) to be upregulated for rbST treated cows compared with control cows (Table 3; Figure 3). On d 7, treatment affected (P = 0.03) mRNA expression for *XBP1* as control cows had (P = 0.01) upregulated expression of mRNA for *XBP1* compared with rbST treated cows (Table 3; Figure 3).

DISCUSSION

Hepatic adaptive responses to NEB during the periparturient period are mediated by the endocrine system through the uncoupling of constituents of the somatotropic axis such as GH, GH receptors, IGF-1, IGF-1 binding proteins, and insulin (Bauman, 2000; Lucy, 2008). During the transition from late gestation to early lactation the increase in GH concentrations enables the shift of nutrients from diet and body stores towards the mammary gland for milk synthesis (Bauman et al., 2000). A rise in IGF-1 concentration is not observed during this period of elevated GH concentrations because the decreased abundance of hepatic GHR, particularly GHR1A, reduces IGF-1 synthesis to prevent a negative feedback of IGF-1 on GH secretion (Rhoads et al., 2004; Lucy, 2008). Although in the sub-sample of cows used in the current experiment GH concentrations prepartum were not affected by rbST treatment, Silva et al. (2015, 2016) demonstrated that weekly treatment of periparturient cows with 125 mg of rbST increased GH concentration prepartum by approximately 30%, similar to the numerical increase observed in the current experiment, suggesting that the current experiment may have been underpowered to detect this difference. Nonetheless, prepartum IGF-1 serum concentrations were increased by the rbST125 treatment in agreement with previous experiments (Silva et al., 2015; Silva et al., 2016). Cows treated with rbST had increased hepatic mRNA expression for *GHR*, *GHR1A*, and *IGF1* on d -7 relative to calving, suggesting that exogenous GH treatment may have increased IGF-1 synthesis through improved hepatic response to GH by increasing GHR1A abundance. Furthermore, treatment of periparturient cows with rbST increased the hepatic mRNA expression for *IGFBP3* on d -7 relative to calving. The *IGFBP3* forms a complex with the acid-labile α-subunit and IGF-1 that prolongs the half-life of IGF-1 and regulates its interaction with cell surface receptors (Rajaram et al., 1997), which may also have increased IGF-1 concentration. Carriquiry et al. (2009) demonstrated increases in hepatic expression of *GHR*, *IGF1*, and *IGFB3* during early lactation in cows treated with 500 mg of rbST, every 10 d, from 12 to 70 d postpartum but, to our knowledge, no data to date reported the effects of rbST on hepatic gene expression during the periparturient period.

The somatotropic axis can be regulated by *SOCS2*, whose mRNA expression in mice's liver is regulated by GH through the signal transducer and activator of transcription 5B (**STAT5B**; Davey et al., 2001). Suppressor of cytokine signaling proteins act as part of a negative feedback system to regulate cytokine signaling pathways to attenuate their signal (Hilton et al., 1998). Even though *STAT5B* expression was not modulated by rbST treatment in the current experiment, cows treated with rbST had increased hepatic mRNA for *SOCS2* 7 d postpartum suggesting that increased postpartum serum GH concentrations in rbST treated cows may have caused GH-STAT5B-induced upregulation of mRNA for *SOCS2* in order to attenuate excessive GH signaling. Over-fed cows challenged intramammarily with LPS between 7 and 14 d postpartum had a marked decrease on hepatic mRNA for *STAT5B* and *SOCS2*, indicative of an exacerbated uncoupling of the somatotropic axis (Graugnard et al., 2013). Taken together, our findings and that of others suggest that treatment of periparturient cows with rbST may have acted directly, modulating SOCS2 hepatic expression, or indirectly, improving the immune function and reducing the incidence of infectious diseases (e.g. metritis; Silva et al., 2015; Silva et al., 2016), to reduce the magnitude of the uncoupling of the somatotropic axis. It is also possible that the rbST treatment of periparturient cows may have attenuated the uncoupling of the somatotropic axis through the upregulation in hepatic mRNA for *INSR*, which could have led to increased liver responsiveness to insulin. Rhoads et al. (2004) demonstrated that cows subjected to hyperinsulinemic-euglycemic clamps during late pregnancy and early lactation had increased hepatic *GHR* and *GHR1A* mRNA expression and plasma IGF-1 concentration, suggesting that insulin may be involved in the recoupling of the somatotropic axis (Lucy, 2008).

Hepatocytes are major targets for GH, which, upon binding to its receptors, activates the Ras-Raf-MEK-MAPK and JAK-STAT signaling pathways resulting in the translocation of homodimers and heterodimers (e.g. *STAT1*, *STAT2*, *STAT3*, *STAT4*, *STAT5A*, *STAT5B* and *STAT6*) to the nucleus to bind to target DNA elements and regulate gene transcription (Zhu

et al., 1998). Among other things, GH regulates the mRNA expression for hepatocyte nuclear factors (**HNF**) through STAT5 signaling (Costa et al., 2003). Hepatocyte nuclear factor 4, for example, is essential for transcription of *PCK1* and *G6PC* genes through the recruitment of the peroxisome proliferator-activated receptor coactivator-1 alpha protein encoded by the gene *PPARGC1A* (Rhee et al., 2003). It is interesting that mRNA expression for *HNF4* and *PPARGC1A* did not differ among treatments, resulting in no changes in *G6PC* mRNA expression. Hepatic mRNA expression for *PCK1*, a key enzyme in the conversion of oxaloacetate to phosphoenolpyruvate (Velez and Donkin, 2004), was increased in rbST125 cows 7 d postpartum, indicating that rbST treatment improved hepatic gluconeogenesis. Furthermore, rbST125 cows had increased hepatic mRNA expression for *ACOX1* 7 d postpartum, an enzyme involved in the fatty acid beta-oxidation that may increase production of ATP to support gluconeogenesis and prevent lipid esterification and accumulation in the liver (Aoyama et al., 1994). Sheep liver slices treated with rbST had increased complete fatty acid oxidation to CO_2 and decreased rates of fatty acid esterification, sparing ATP for increased rates of gluconeogenesis (Emmison et al., 1991). Although the upregulation of mRNA for *PCK1* and *ACOX1* were not associated with increased plasma glucose concentrations we speculate that this was a consequence of the numerical increase in milk yield among rbST treated cows.

Culture of hepatocytes in the presence of GH reduced the accumulation of intracellular TAG (Emmison et al., 1991) and lactating cows treated with rbST were less likely to develop fatty liver in spite of rbST-induced lipolysis and influx of NEFA to the liver (Pocius and Herbein, 1986). On d -7 relative to calving cows treated with rbST had increased hepatic mRNA expression for *SCARB1*, a receptor for high-density lipoprotein, and for *APOA5* and *APOB100*, apolipoproteins involved in the secretion of very-low-density lipoproteins (**VLDL**) from the liver. We speculate that the upregulation of *APOA5* and *APOB100* stimulated the synthesis and/or secretion of VLDL and activated the lipoprotein lipase in peripheral tissues (Kraja et al., 2010), improving transport of

triglycerides from the liver, overall lipid metabolism, and delivery of preformed fatty acids to the mammary gland (Emmison et al., 1991). Furthermore, hepatic mRNA expression for *ANGPTL4*, an angiopoietin-like protein that prevents fat storage and stimulates fat mobilization, was upregulated by rbST treatment on d -7 relative to calving. Upregulation of mRNA expression for *ANGPTL4* in calf hepatocytes has been correlated with NEFA concentrations > 0.6 mmol/L (Wang et al., 2015). In the current experiment, NEFA concentrations did not differ among treatments suggesting that rbST may have acted directly to upregulate mRNA for *ANGPTL4* in periparturient cows as it has in mid lactation cows (Koltes and Spurlock, 2012). The hepatic expression of mRNA for *PPARD*, a nuclear receptor activated by fatty acids and associated with lipid accumulation, was downregulated on d 7 in rbST cows. The rbST-induced changes in hepatic expression of mRNA for *SCARB1, APOA5, APOB100, ANGPTL4* and *PPARD* suggests that treatment of periparturient cows with rbST could alter hepatic lipid metabolism to the point of reducing liver TAG accumulation and increasing liver VLDL secretion, which are expected to improve liver health.

In the current experiment, rbST cows had reduced postpartum BHB concentrations compared with control cows. Ketogenesis is associated with the upregulation of mRNA for *HMGCLL1*, which codifies the 3-hydroxymethyl-3-methylglutaryl-CoA lyase like 1 enzyme and catalyzes the cleavage of *HMGCS2* into acetyl-CoA and acetoacetate (van Dorland et al., 2009). Although we expected that differences in the expression of mRNA for *HMGCLL1* would help explain possible differences in BHB concentrations among treatments, mRNA for *HMGCLL1* was not detected in the current experiment. Decreased ketogenesis postpartum is associated with reduced hepatic accumulation of TAG (Graugnard et al., 2013). Silva et al. (2015) did not observe differences in hepatic TAG and glycogen contents between rbST treated and control cows, but this may have been the consequence of insufficient sample size because hepatic glycogen content of rbST treated cows was 75% greater than control cows. Liver lipidosis develops when the hepatic uptake

of NEFA exceeds its oxidation and secretion as TAG in VLDL by the liver, leading to impaired liver function and incomplete fatty acid oxidation to ketone bodies and causing ketosis (Grummer et al., 2004). In the current experiment, cows treated with rbST had reduced BHB concentration postpartum compared with control cows, which can be a result of improved hepatic NEFA complete oxidation and VLDL secretion as explained previously. It is important to note, however, that the effects of rbST treatment of periparturient cows on BHB concentrations have been mixed with some researchers reporting a positive effect (Gulay et al., 2007; Putnam et al., 1999; Silva et al., 2015) and some reporting a negative effect (Eppard et al., 1996; Gohary et al., 2014; Silva et al., 2016).

In the current experiment hepatic mRNA expression for pro-inflammatory genes were upregulated among control cows 7 d postpartum compared with rbST cows. It is likely that the upregulation of pro-inflammatory genes among control cows was a consequence of numerical difference in incidence of ketosis and metritis compared with rbST treated cows. A bovine microarray platform used to study hepatic gene networks underlying nutrition-induced ketosis revealed an upregulation of genes and nuclear receptors associated with cytokine signaling and inflammation (Loor et al., 2007). Inflammation can induce lipolysis, glycogenolysis, and hepatic lipid accumulation (Chiolero et al., 1997). Cows injected with recombinant bovine TNF-α had increased liver TAG content and decreased liver mRNA for *G6G* and *PCK1* (Bradford et al., 2009). Although plasma TNF-α concentrations were not affected by treatments in the current experiment, control cows had increased hepatic mRNA expression for *TNFRSF1A* 7 d postpartum. Liver proliferative response to injury can be initiated by the release of TNF-α, as observed by a 70% reduction in hepatocyte DNA replication in *TNFRSF1A* knockout mice (Yamada et al., 1997). Thus, the increased hepatic *TNFRSF1A* expression exhibited by control cows compared with rbST treated cows is possibly associated with hepatocyte injury. Treatment of mice with induced hepatic injury with a neutralizing antibody against *CXCL16*, a chemokine that promotes immune

cell infiltration in the liver, reduced hepatic macrophage accumulation and steatosis (Wehr et al., 2014). Control cows had greater mRNA for *CXCL1* 7 d postpartum, a gene that is translated to a chemokine produced by macrophages and hepatocytes that acts similarly to *CXCL16* (Charo and Ransohoff, 2006). In addition, control cows had greater mRNA expression for *ICAM1* 7 d postpartum, a gene that encodes an intercellular adhesion molecule involved in the transmigration of leukocytes into tissues and is upregulated upon cytokine stimulation. These findings suggest that control cows may have been more prone to liver infiltration of immune cells than rbST treated cows. The transcription factor hypoxia-inducible factor 1-alpha encoded by the gene *HIF1A* is involved in the inflammation process increasing angiogenesis, cell proliferation, and tissue regeneration (Cramer et al., 2003). Zhang et al. (2015) demonstrated that drug-induced upregulation of *HIF1A* protein enhances regeneration of lost and damaged tissue in mice after an ear hole punch injury. Among control cows, the hepatic mRNA expression for *HIF1A* tended to be and was upregulated -7 and 7 d relative to calving, respectively, and may be a consequence of increased hepatocyte damage. The expression of the *HIF1A* gene is regulated by the nuclear factor kappa enhancer in B-cells (**NFκB**) encoded by the *NFKB1* gene. Treatment with rbST did not affect the expression of *NFKB1*; however, the mRNA expression for *NFKBIA*, an inhibitor of NFκB signaling pathway, was increased in control cows 7 d postpartum. Taken together, these data suggest that the NFκB signaling cascade was activated in control cows, resulting in greater hepatic *HIF1A* expression, but an anti-inflammatory response, through the upregulation of *NFKBIA*, was also induced perhaps to attenuate the hepatic inflammation process.

Liver macrophages, called Kupffer cells, are able to promote fat accumulation in hepatocytes by expressing IL-1β and by binding to IL-1 receptor type 1 encoded by the gene *IL1R* (Leroux et al., 2012). The mRNA expression for *IL-1 β* and *IL1R* were not evaluated in the current experiment. Nonetheless, the hepatic mRNA expression for *IL1RN*, an inhibitor of the IL-1 receptor type 1, and *SOCS3*, a suppressor of cytokine signaling

pathways, were upregulated in control cows 7 d postpartum. The activation of IL-1 receptor type 1 by IL-1β in response to LPS stimulation dysregulates the hepatic lipid and lipoprotein metabolism and increases lipid peroxidation resulting in the accumulation of oxidized low density lipoproteins in large lipid droplets in the Kupffer cells (Leroux et al., 2012). Consequently, Kupffer cells are activated and produce high amounts of pro-inflammatory cytokines and chemokines, mediating the recruitment of leukocytes to the liver (Leroux et al., 2012). We speculate that the greater incidence of metritis among control cows might have sensitized Kupffer cells to produce pro-inflammatory cytokines and chemokines, which in turn may have stimulated the upregulation of anti-inflammatory genes (e.g. *IL1RN* and *SOCS3*) to contain the pro-inflammatory signals. It is also possible that the greater metabolic challenge of control cows resulted in upregulation of *SOCS3* since hepatic expression of mRNA for *SOCS3* was associated with insulin resistance and steatosis in obese mice (Ueki et al., 2005).

The mRNA expression for *MYD88* was greater in control cows than rbST treated cows 7 d postpartum. Intramammary challenges with LPS 7 d postpartum increased the hepatic expression of mRNA for *TNF*, *MYD88* and *NFKB1* (Graugnard et al., 2013). Therefore, we speculate that the numerical increase in incidence of metritis among control cows may have resulted in greater stimulation of toll-like receptors and upregulation of mRNA for *MYD88* among control cows. It is important to mention, however, that treatment of periparturient cows with rbST increased PMNL expression of adhesion molecules, phagocytosis and oxidative burst, increased antibody concentrations, and decreased the incidence of retained placenta and metritis and the concentration of haptoglobin postpartum (Silva et al., 2015; Silva et al., 2016). Thus, rbST treatment may have directly or indirectly modulated the expression of mRNA for genes associated with inflammation and immune responses.

Metabolic disorders and inflammation are linked to oxidative stress and endoplasmic reticulum (**ER**) stress in periparturient dairy cows (Ringseis et al., 2015). During early lactation the liver is exposed to metabolic and inflammatory challenges such as NEFA, TNF-α, IL-1β, reactive oxygen species, and LPS, which are known inducers of ER stress (Ringseis et al., 2015). Increased mitochondrial lipid flux and exposure to high concentrations of fatty acids disrupt ER membranes and result in the release of stored calcium that contributes to oxidative stress and greatly increase cytoplasmic reactive oxygen species concentrations, resulting in impaired ER protein folding (Wei et al., 2009). Protein misfolding triggers the unfolded protein response, of which the transcription factor X-box binding protein 1 is a critical effector through the regulation of genes involved in ER homeostasis (Yoshida et al., 2001). In addition, the unfolded protein response signaling cascades and inflammatory signaling pathways are tightly linked (Darling and Cook, 2014). The ER stress-induced unfolded protein response is believed to contribute to pathophysiologic conditions of periparturient cows such as fatty liver, ketosis, and hepatic inflammation (Ringseis et al., 2015). In the current experiment, hepatic mRNA expression for *XBP1* was upregulated among control cows, which we speculate to be indicative of a state of oxidative stressed due to inflammation and impaired lipid metabolism as a consequence of the upregulation of mRNA expression for genes related to inflammation and the downregulation of mRNA expression of genes related to lipid oxidation and transport. Graugnard et al. (2013) demonstrated that hepatic mRNA expression for *XBP1* was upregulated during early lactation of over-fed cows challenged intramammarily with LPS supporting the theory of a synergistic effect of impaired lipid metabolism and inflammation on oxidative stress response. The positive effects of rbST treatment on hepatic expression of mRNA for genes related to lipid metabolism, inflammation and oxidative stress during the phase of greatest metabolic and infectious challenges of cows may present an important alternative to reduce the incidence of metabolic diseases.

CONCLUSIONS

Treatment of periparturient cows with 87.5 and 125 mg of rbST, weekly, from -21 to 28 d relative to calving may improve liver health and function and attenuate the decoupling of the somatotropic axis through upregulation of somatotropic axis genes (e.g. *GHR* and *GHR1A*), resulting in increased hepatic *IGF1* mRNA and plasma IGF-1 concentration. Furthermore, treatment with rbST may improve glucose and lipid metabolism through upregulation of transcription of key hepatic enzymes involved in gluconeogenesis and lipid oxidation, and apolipoproteins and lipid receptors responsible for lipid transport and secretion from the liver, resulting in decreased postpartum BHB concentrations among rbST treated cows. Treatment of periparturient cows with rbST may also prevent postpartum hepatic infiltration of immune cells and hepatocyte injury, demonstrated by the downregulation of mRNA expression for genes associated with inflammation compared with control cows. The results of the current experiment demonstrate that rbST treatment during the periparturient period improves hepatic lipid metabolism and inflammatory status, which may have acted synergistically to decrease hepatic oxidative stress.

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CHAPTER V

Effect of recombinant bovine somatotropin on leukocyte mRNA expression for genes related to cell energy metabolism, cytokine production, phagocytosis, oxidative burst, and adaptive immunity

OUTLINE

Objectives of the current experiment were to evaluate the effects of treatment of periparturient dairy cows with recombinant bovine somatotropin (**rbST**) on peripheral leukocytes mRNA expression for genes related to the somatotropic axis, cell energy metabolism, and innate and adaptive immune responses. Cows were enrolled in the experiment at 253 ± 3 d of gestation and assigned to control (n = 98) or to receive 125 mg of rbST, weekly, from -21 to 21 d relative to calving (rbST125; n = 98). Data from a sub-sample of cows (control = 16, rbST125 = 16) are reported herein. Cows were vaccinated with ovalbumin at -21 ± 3, -7 ± 3, and 7 ± 3 d relative to calving. Concentrations of insulin-like growth factor 1 (IGF-1), metabolites, haptoglobin, hemogram, polymorphonuclear leukocyte (PMNL) expression of adhesion molecules, phagocytosis and oxidative burst, and IgG anti-ovalbumin concentrations were determined weekly, from -28 ± 3 to 24 ± 3 relative to calving. Leukocytes were isolated from whole blood on d -21 ± 3, -7 ± 3, and 7 ± 3 relative to calving to determine leukocyte mRNA expression for 72 genes. Cows treated with rbST had higher IGF-1 concentrations than control cows during the prepartum (control = 117.6 ± 11.4 vs. rbST125 = 155.9 ± 11.4 ng/mL) and postpartum (control = 72.5 ± 10.6 vs. rbST125 = 100.0 ± 10.8 ng/mL) periods, respectively. Cows in the rbST125 treatment had greater number of granulocytes 1 wk prepartum (control = 3.7 ± 0.4 vs. rbST125 = 4.6 ± 0.4 x 10^9 cells/L). Expression of CD18 by PMNL during the prepartum (control = 3,262 ± 280 vs. rbST125 = 3,926 ± 260 GMFI) and percentage of PMNL positive for phagocytosis and oxidative burst 1 wk postpartum (control = 59.2 ± 2.8 vs. rbST125 = 67.6 ± 3.1%) were increased in rbST125 cows. Postpartum relative concentration of IgG anti-ovalbumin was higher in rbST125 than control cows (control = 1.5 ± 0.1 vs. rbST125

= 1.9 ± 0.1 optical density). Leukocyte mRNA expression for the glycolytic genes (*INSR*, *LDHA*) on d 7 tended to be greater in rbST125 cows than control cows. Leukocyte mRNA expression for complement receptor (*C5aR*) on d -7 was higher in rbST125 than control cows. Treatment with rbST upregulated mRNA expression for pathogen pattern recognition receptors (*CD14*, *NOD2*, *SCARB1*) on d -7 and *TLR2* on d 7. On d -7, mRNA expression for *Rac1* and *MYD88*, genes associated with internal signaling for phagocytosis and oxidative burst and cytokine production, tended to be greater for rbST125 than control cows. Furthermore, on d -7, rbST treatment upregulated the mRNA expression for antimicrobial peptides (*DEFB3*, *DEFB7*) compared with control. On d 7, leukocyte mRNA expression for *Lyn*, internal signaling protein involved in B-cells activation, was upregulated in rbST125 cows. Treatment of dairy cows with 125 mg of rbST during the periparturient period may improve leukocyte functions by upregulating mRNA expression of genes involved in glycolysis, pathogen recognition, phagocytosis and oxidative burst, antimicrobial peptides, and antibody production.

Key words: dairy cow, leukocyte gene expression, somatotropin

INTRODUCTION

Periparturient dairy cows are immunosuppressed due to the stress of parturition and initiation of lactation. Peripheral blood lymphocytes and neutrophils have declined function from approximately 2 wk before parturition until 2 to 3 wk after parturition (Kehrli et al., 1989a,b). The time line of immunosuppression in periparturient cows coincides with decreased concentrations of insulin-like growth factor 1 (**IGF-1**), a cell growth and differentiation factor involved in the regulation of innate and adaptive immune functions (Heemskerk et al.,1999). Leukocytes can produce IGF-1 and express IGF-1 receptors (Merimee et al., 1989). Insulin-like growth factor-1 has been demonstrated to modulate a variety of immune cell functions in human and mice models including immune cell proliferation (Merimee et al., 1989), chemotaxis (Tapson et al., 1988), T-cell activation (Johnson et al., 1992), inhibition of neutrophils apoptosis (Kooijman et al., 2002),

polymorphonuclear leukocytes (**PMNL**) phagocytosis and oxidative burst (Inoue et al., 1998), and B-cell differentiation, maturation and proliferation (Funk et al., 1994; Gibson et al., 1993).

Treatment of healthy lactating cows with recombinant bovine somatotropin (**rbST**) induced an increase in PMNL and total leukocyte count in circulation and increased PMNL oxidative burst activity and NADPH-oxidase activity (Burvenich et al., 1999). Furthermore, addition of rbST or IGF-1 in cultured peripheral blood lymphocytes from lactating dairy cows enhanced lymphocyte proliferation (Chang et al., 1995). Although treatment of periparturient dairy cows with rbST increases serum IGF-1 concentrations during the peripartum, despite the uncoupling of the somatotropic axis (Vicini et al., 1991; Gulay et al., 2004a,b), little is known about its effects on immune response. In recent experiments conducted by our group, treatment of periparturient Holstein cows with 125 mg of rbST increased IGF-1 concentrations (Silva et al., 2015; Silva et al. 2016a; Silva et al., 2016b) and resulted in increased PMNL expression of the adhesion molecule (CD62L), phagocytosis and oxidative burst, and serum concentrations of ovalbumin-specific IgG (Silva et al., 2015). In addition, cows treated with 125 mg of rbST had reduced incidences of retained fetal membranes and metritis (Silva et al., 2015; Silva et al., 2016a) and reduced haptoglobin concentrations 7 d postpartum (Silva et al., 2015).

Enhanced knowledge of the processes that regulate and potentiate the capacity of immune cells to fight and eliminate infections may lead to significant improvements in peripartum cow management and health. Administration of rbST to enhance leukocyte function might be an important strategy to prevent immunosuppression and infectious diseases in periparturient cows (Silva et al., 2015; Silva et al., 2016a; Silva et al., 2016b). Thus, there is reason to explore the potential for this endocrine pathway as a regulator of immunity and the mechanisms involved on its actions. Therefore, the hypotheses of the current experiment were that weekly administration of rbST to periparturient dairy cows would

upregulate peripheral leukocyte mRNA expression for genes related to: somatotropic axis (e.g. *GHR*, *IGF1R*, *STATs*, *JAKs*, and *SOCS2*), cell energy metabolism (e.g. *INSR*, *LDHA*, *PC1*, *GSK3B*, *ACOX1*, and *PPARs*), pathogen pattern recognition receptors (e.g. *CD14*, *TLRs*, and *NOD2*), antimicrobial enzymes and peptides (e.g. *CATHLs* and *DEFBs*), phagocytosis and oxidative burst (e.g., *Rac1*, *Rac2*, *RhoA*, and *MPO*), cytokine production (e.g. *MYD88*, *NFKB1*, *IL1B*, and *TNFa*) and antibody production (e.g. *Blk* and *Lyn*). The objectives of the current experiment were to evaluate the effects of peripartum rbST treatment of dairy cows on peripheral leukocyte mRNA expression for genes related to the somatotropic axis, immune cells energy metabolism, and innate and adaptive immune responses.

MATERIALS AND METHODS

The procedures conducted during this experiment were approved by the Institutional Animal Care and Use Committee from the University of Minnesota (protocol # 1408-31746A).

Animals, Housing, and Nutrition

Cows used in the current experiment are a sub-sample of cows used by Silva et al. (2016a). Holstein cows (lactation $\geq$ 1) from a commercial free-stall dairy herd located in Northwest Wisconsin were enrolled in the experiment at 253 ± 3 d of gestation (-28 d relative to calving). Cows were housed in the same naturally ventilated free-stall barn from approximately 34 d prepartum to 21 d postpartum. As cows demonstrated signs of calving (discomfort, restlessness, tail twitching, and visualization of the allantoic sac through the vulva) they were moved from the freestall pen to a box stall. Within 12 h after calving cows were moved to a postpartum freestall pen (1 to 21 d postpartum) for daily observation and examination for diagnosis of postpartum diseases. Composition of TMR fed during prepartum (far-off and close-up) and immediate postpartum (1 to 21 d postpartum) periods were described in Silva et al. (2016a).

Enrollment and Treatments

At enrollment, cows were scored for body condition (1 = emaciated and 5 = obese; 0.25-unit increments; Ferguson et al., 1994) and locomotion (1 = normal locomotion and 5 = severely lame; as described by Sprecher et al., 1997). Healthy cows with BCS $\geq$ 2 (Ferguson et al., 1994) and locomotion score $\leq$ 2 (Sprecher et al., 1997) were balanced for lactation number, BCS, and previous lactation 305-d mature equivalent milk yield and assigned randomly to one of two treatments: untreated control (n = 98) or **rbST125** (n = 98). In the course of the experiment, a sub-group of cows (16 per treatment) was selected based on predicted calving date and BCS for evaluation of leukocyte mRNA expression for genes of interest. Only data from these cows are reported herein. Cows enrolled in the rbST125 treatment received 125 mg of rbST (Posilac, sometribove zinc suspension for injection; Elanco Animal Health, Greenfield, IN), every 7 d, from -21 to 21 d relative to calving. Injections of rbST were given subcutaneously in the neck area using 1 mL tuberculin syringes with 16 gauge and 5/8 inch long hypodermic needles. Syringes containing 125 mg of rbST were prepared 6 to 12 h before treatment. The contents of commercially available syringes of rbST (Posilac, sometribove zinc suspension for injection; Elanco Animal Health, Greenfield, IN) were dispensed into sterile containers and the appropriate volume of rbST was aspirated into sterile syringes that were kept refrigerated. The syringes were warmed to room temperature (15 to 30 °C) before rbST administration.

Health, BCS, and Milk yield

Subclinical hypocalcemia was defined as serum total calcium concentrations between 5.5 and 8.0 mg/dL (Goff, 2008) within 72 h postpartum. Farm personnel were trained to examine cows within 24 h after calving to diagnose and record the occurrence of retained fetal membranes (Kelton et al., 1998). Study personnel palpated cows per rectum on d 4 ± 1, 7 ± 1, 10 ± 1, and 13 ± 1 postpartum for the diagnosis of metritis. Metritis was defined as cows having watery, pink/brown, and fetid uterine discharge (LeBlanc, 2010). Ketosis

was defined as cows having circulating β-hydroxybutyrate (**BHB**) concentrations ≥ 1,400 µmol/L (Duffield et al., 2009).

Cows were milked thrice daily. The Dairy Herd Improvement Association was hired to measure milk yield weekly from 5 ± 3 to 21 ± 3 d postpartum from all cows and milk data was recorded on DairyComp305 software (Valley Ag. Software, Tulare, CA). Milk data reported herein are weekly milk yield averages of the first 21 ± 3 d of lactation. Cows' body condition was scored (Ferguson et al., 1994) at d -28 ± 3, 0 ± 3, 21 ± 3 and 60 ± 3 relative to calving.

Blood Sampling, Calcium, IGF-1, Metabolites, and Haptoglobin Assays

Blood samples were collected weekly from -28 ± 3 to 24 ± 3 d relative to calving to determine concentrations of IGF-1, nonesterified fatty acids (**NEFA**), BHB, glucose, and haptoglobin. During feeding, when cows were restrained in self-locking headlocks, blood samples were collected from the coccygeal vein/artery using 22 gauge and 1-inch-long needles into 10-mL empty evacuated tubes without anticoagulants and into evacuated tubes containing K2 EDTA (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ). Tubes were placed in ice until centrifugation (1,200 *g* for 15 min at 4 ºC) for serum and plasma separation within 4 h after blood samples were collected. Serum and plasma were aliquoted into microcentrifuge tubes and stored at -32 ºC until analysis.

Blood sampled within the first 72 h postpartum were used to determine serum total calcium concentrations using an automated chemistry analyzer (RX Daytona^{+}, Randox Laboratories Ltd, Antrim, UK). Serum total IGF-1 concentrations were determined using a commercially available kit (Human IGF-1 Quantikine ELISA; R&D Systems Inc., Minneapolis, MN). The intra- and inter-assay coefficients of variation for the IGF-1 assay were 5%. Plasma concentrations of NEFA were determined using a colorimetric assay (Wako Chemicals USA, Richmond, VA; Ballou et al., 2009) and plasma concentrations of

BHB were determined enzymatically (Ranbut, Randox Laboratories, Antrim, UK; Ballou et al., 2009). The intra-assay CV were 7 and 10% for the NEFA and BHB assays, respectively, and the inter-assay CV were 11 and 8% for the NEFA and BHB assays, respectively. Plasma glucose concentration was determined by enzymatic reaction (Stanbio Laboratory, Boerne, TX). The intra- and inter assay CV were 4 and 3%, respectively. Haptoglobin concentrations were determined by a colorimetric procedure (Hulbert et al., 2011). The intra- and interassay CV were 4 and 3%, respectively.

Hemogram and PMNL Immune Response Assays

Blood samples collected from the coccygeal vein or artery into 3-mL evacuated tubes containing K2 EDTA (Kendall Monoject, Mansfield, MA) were used for the hemogram. Samples were collected twice weekly from -28 ± 1 to 24 ± 1 d relative to calving. Blood samples were analyzed within 4 h of sample collection using a Vet Scan HM2 (Abaxis, Union City, CA) hemogram machine.

Blood samples collected twice weekly from -28 ± 1 to 24 ± 1 d relative to calving into 10-mL heparinized evacuated tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) were used for determination of *ex vivo* innate immune responses by flow cytometry as described before (Hulbert et al., 2011). Detailed information of the assays used to determine *ex vivo* innate immune responses were described in our previous experiment (Silva et al., 2015). Briefly, indirect immunofluorescence staining was used to determine PMNL expression of adhesion molecules L-selectin (also known as CD62L) and β2 integrins (also known as CD18). Phagocytic and oxidative burst activities of peripheral PMNL were determined upon challenge with fluorescently labeled enteropathogenic bacteria (Escherichia coli 0118:H8) and oxidation of dihydrorhodamine 123 (Molecular Probes/Invitrogen, Eugene, OR). Flow cytometry was carried out on a BD FACSCanto II (BD Biosciences, Franklin Lakes, NJ) and analyzed using FlowJo 7.6.4 software (Tree Star Inc., San Carlos, CA). The PMNL population was identified on basis of forward- and side-

scattered properties. Data reported herein is referent to percentages of PMNL positive for phagocytosis and oxidative burst and intensity of expression of CD18 and CD62L molecules and phagocytosis and oxidative burst as geometric mean fluorescence intensity (GMFI).

Ovalbumin Immunization and IgG Anti-Ovalbumin Assay

Cows were immunized with 0.5 mg of chicken egg ovalbumin (Type VII; Sigma-Aldrich) diluted in 0.5 mL of PBS and emulsified in Quil A adjuvant (0.5 mg of Quil A/0.5 mL of PBS; Accurate Chemical & Scientific Corp., Westbury, NY) by subcutaneous injections in the neck area on d -21 ± 3, -7 ± 3, and 7 ± 3 relative to calving. Blood sampled weekly from d -28 ± 3 to 24 ± 3 into 10-mL evacuated tubes without anticoagulant (Becton Dickinson Vacutainer Systems) was used to determine IgG anti-ovalbumin concentration. Blood tubes were placed on ice until centrifugation (1,200 × g for 15 min at 4°C) and serum was stored at -32 °C until analysis. Immunoglobulin G anti-ovalbumin concentration in serum was determined by ELISA and compared based on optical density (Mallard et al., 1997). Detailed assay procedure was described in a previous experiment (Silva et al., 2015).

Leukocyte Isolation and mRNA Extraction and Quality Assessment

Blood samples were collected at -21 ± 3, -7 ± 3, and 7 ± 3 d relative to calving from the coccygeal vein or artery into 10-mL heparinized evacuated tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) and placed on ice until leukocyte isolation and mRNA extraction to determine the expression of mRNA for 72 genes related to somatotropic axis and cell energy metabolism (Tables 1) and immune responses (Table 2).

Peripheral blood leukocytes were isolated from 1 mL of whole blood and mRNA was extracted using the QIAamp® RNA Blood mini columns kit procedure (Qiagen, Valencia, CA). Total RNA restrained in the columns after extraction protocol were dissolved in 50

µL sterile RNase- DNase-free water (Qiagen, Valencia, CA). Quantity and purity of the extracted RNA were measured using a NanoDrop spectrophotometer (ND-2000, NanoDrop Technologies Inc., Wilmington, DE) at 230, 260, and 280 nm absorbance waves. A portion of RNA sample solution, based on each sample's RNA yield, was diluted in RNase- DNase-free water (Qiagen, Valencia, CA) to achieve a single concentration of 50 ng/µL across all samples. Diluted samples were reassessed using NanoDrop spectrophotometer to verify and readjust, if necessary, all samples to the final single concentration of 50 ng/µL. Purity was accepted when the 280/260 and 260/230 absorbance ratios were $\geq$ 1.90 and 1.80, respectively.

For RNA integrity assessment, the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) was used. The RNA integrity number (**RIN**) was determined using the Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's protocol. The RIN acceptable values for high RNA integrity and low degradation range from 7 to 10. All experimental samples RIN values were above 8.

Gene Expression Analysis, Reference Genes Selection, and Data Normalization

Leukocyte mRNA expression was assessed by direct digital quantification of hybridization of multiplexed target mRNA molecules with color-coded probe pairs, molecular barcodes, and single-molecule imaging using the NanoString nCounter® System (NanoString Technologies, Seattle, WA). The direct digital readout of each mRNA and its relative abundance was measured in 100 ng of total RNA. The nSolver™ 2.0 Analysis Software (NanoString Technologies, Seattle, WA) was used to perform automated quality controls (**QC**) metrics to detect flags on imaging, binding density, linearity, and limit detection of control probes. Imaging and binding density QC were set to detect flags when the percentage of fields of view successfully counted by the digital analyzer scan was less than 75 and binding density was outside the 0.05 to 2.25 range. The positive control linearity and limit of detection QC were set to flag lanes when positive control R^2 values were less

than 0.95 and when 0.5fM detection was $\leq$ 2 standard deviations above the mean of the negative controls. No QC flags were detected in the experimental samples.

The genes *PPIE*, *SDHA*, *SPRYD4*, *UXT*, *XBP1*, and *YWHAZ* (Table 1) were chosen as candidates for reference genes because they were stably expressed in bovine lymphocytes (Spalenza et al., 2011), neutrophils (Stevens et al., 2011), and in liver tissue of periparturient Holstein cows treated with rbST (Silva et al., 2016b). Stability analysis to determine the most stable genes were performed using the NormFinder software (Andersen et al., 2004). Five genes, *PPIE*, *SDHA*, *UXT*, *XBP1*, and *YWHAZ*, were the most stable according to the NormFinder analysis and selected to be used in the data normalization process.

To account for slight differences in hybridization and purification efficiency, data were normalized from each sample to its positive controls and selected reference genes using the nSolver™ 2.0 Analysis Software (NanoString Technologies, Seattle, WA). The positive hybridization controls are RNA control transcripts sequences that are not homologous to any known organism and generate consistent results in gene expression analyses (NanoString Technologies, Seattle, WA) ranging from 10 to 10,000 counts. Positive controls allowed for the correction of sample to sample variation due to assay specific factors such as differences in amount of input material or reagents. The negative correction was used to subtract the background noise from the positively corrected data using counts of six negative controls, sequence blank tags, by subtracting the negative controls geometric mean + 2 standard deviations. The normalization procedure consisted of the adjustment of all mRNA counts data to normalization factors generated specifically for each sample based on the geometric means, within and across all samples, of six positive controls and selected reference genes counts.

Statistical Analysis

All statistical analyses were conducted using SAS version 9.4 (SAS/STAT, SAS Inst. Inc., Cary, NC). In all models, cows were used as experimental unit. Dichotomous data (i.e. male calf and incidence of postpartum diseases) were analyzed by logistic regression using the LOGISTIC procedure of SAS and two by two tables were generated using the FREQ procedure of SAS. Continuous data were analyzed by ANOVA and the assumption of normality of variables and residuals of continuous data were assessed using the UNIVARIATE procedure of SAS and the residual output function. A log or square root transformation of dependent variables was applied to achieve a normal distribution when necessary. The MIXED procedure was performed to determine the effect of treatment on continuous data without repeated measurements (i.e. lactation number, 305-d mature equivalent milk yield, and BCS at enrollment). The MIXED procedure was used to analyze continuous data with repeated measurements (i.e. IGF-1, NEFA, BHB, glucose, haptoglobin, BCS, and milk yield) including treatment, time, and the interaction between treatment and time as fixed effects. The structure of covariance (auto-regressive, unstructured, or compound symmetry) was chosen according to the Bayesian information criteria. The prepartum and postpartum periods were analyzed separately because of inherent physiological changes that occur during calving.

Effect of treatment on leukocyte mRNA expression at the different time points relative to calving was determined by analysis of variance using the MIXED procedure of SAS. In all models, treatment was included as a fixed effect. The model to evaluate differences between treatments on leukocyte gene expression relative to d -21 of calving included concentration of lymphocyte and granulocyte on d -21 as covariates. In the model to determine the effect of treatment on leukocyte mRNA expression on d -7 relative to calving, leukocyte mRNA expression on d -21 relative to calving and lymphocyte and granulocyte concentrations on d -7 were included as covariates. In the model to evaluate the effect of treatment on leukocyte mRNA expression on d 7 relative to calving

lymphocyte and granulocyte concentrations on d 7 were include as covariates. Leukocyte mRNA expression on d -7 relative to calving was not included in the model to evaluate the effect of treatment on leukocyte mRNA expression on d 7 postpartum because of inherent physiological changes associated with calving. When the effects of covariates were not significant ($P > 0.10$) they were removed from the model in a backward stepwise elimination procedure. Statistical significance was defined as $P \leq 0.05$ and tendency was considered if $0.05 < P \leq 0.10$.

RESULTS

Descriptive Parameters at Enrollment and Calving

At enrollment, average lactation number (control = 1.9 ± 0.2 vs. rbST125 = 1.7 ± 0.2; $P = 0.35$), BCS (control = 3.5 ± 0.1 vs. rbST125 = 3.5 ± 0.1; $P = 0.55$), previous lactation 305-d mature equivalent milk yield (control = 12,037 ± 675 vs. rbST125 = 12,409 ± 675 kg; $P = 0.70$), and days of gestation (control = 252.9 ± 0.3 vs. rbST125 = 252.3 ± 0.3; $P = 0.21$) were not different between treatments. Number of days cows stayed in the prepartum pen during the trial tended ($P = 0.07$) to differ between treatments (control = 26.4 ± 0.8 vs. rbST125 = 24.3 ± 0.8 d). The percentage of cows calving male calves did not differ between treatments (control = 62.5 vs. rbST125 = 37.5%; $P = 0.16$).

Effects of rbST Treatment on Health Parameters, BCS, and Milk Yield

Incidences of subclinical hypocalcemia (control = 9.1 vs. rbST125 = 12.5%; $P = 0.81$) and serum total calcium concentrations in the first 72 h postpartum (control = 9.1 ± 0.3 vs. rbST125 = 8.6 ± 0.3 mg/dL; $P = 0.21$) were not different between treatments. Treatment had no effect on incidences of retained fetal membranes (control = 9.4 vs. rbST125 = 0.0%; $P = 0.95$) and metritis (control = 25.0 vs. rbST125 = 0.0%; $P = 0.95$). Ketosis incidences were not affected by treatments (control = 18.8 vs. rbST125 = 18.8%; $P = 1.00$).

Body condition score from 0 to 60 DIM was not affected by treatment (control = 3.1 ± 0.1 vs. rbST125 = 3.2 ± 0.1; $P = 0.63$). Milk yield during the first 21 DIM was not affected by treatment (control = 32.7 ± 2.3 vs. rbST125 = 36.3 ± 2.3 kg/d; $P = 0.27$).

It is important to note that the power provided by the relatively small sample size of the present experiment (control = 16 and rbST125 = 16 cows) may be insufficient to detect effects of treatment on parameters such as incidence of postpartum diseases and milk yield. Silva et al. (2016a) evaluated health and milk yield responses of periparturient cows treated with 125 mg of rbST.

Effects of rbST Treatment on IGF-1, NEFA, BHB, Glucose, and Haptoglobin Concentrations

Cows treated with 125 mg of rbST had ($P = 0.02$) and tended to have ($P = 0.08$) greater prepartum and postpartum concentrations of IGF-1, respectively (Table 3). Treatment had no effect on prepartum concentrations of NEFA ($P = 0.98$), BHB ($P = 0.79$), and glucose ($P = 0.72$; Table 3). Similarly, postpartum NEFA ($P = 0.88$), BHB ($P = 0.68$), and glucose ($P = 0.51$) concentrations were not affected by treatment (Table 3). Haptoglobin concentration was not affected by treatment during the prepartum period ($P = 0.97$), but during the postpartum period control cows had ($P = 0.04$) higher haptoglobin concentrations than rbST125 cows (Table 3).

Effects of rbST Treatment on Hemogram Parameters

Concentrations of total white blood cells during the prepartum ($P = 0.26$) and postpartum ($P = 0.25$) periods were not affected by treatment, but an interaction ($P = 0.03$) between treatment and day was observed during the prepartum as control cows tended (control = 12.0 ± 1.2 vs. rbST125 = 8.9 ± 1.2 x 10^9 cells/L; $P = 0.08$) to have higher white blood cells concentrations on d -21 than rbST125 cows (Table 4). No effect of treatment was observed on concentration of lymphocytes and percentage of leukocytes classified as lymphocytes

during the prepartum (P = 0.21 and P = 0.16, respectively) and postpartum (P = 0.15 and P = 0.22, respectively) periods (Table 4). Concentration of granulocytes (P = 0.93) and percentage of leukocytes classified and granulocytes (P = 0.19) during the prepartum period were not affected by treatment (Table 4). The interaction between treatment and day relative to calving affected (P = 0.01) granulocyte concentration on the prepartum because on d -7 relative to calving rbST125 cows tended (P = 0.09) to have greater granulocyte concentration than control cows (control = 3.7 ± 0.4 vs. rbST125 = 4.6 ± 0.4 x 10^9 cells/L). During the postpartum period, concentration of granulocytes (P = 0.92) and percentage of leukocytes classified as granulocytes (P = 0.23) were not affected by treatment (Table 4). The granulocyte to lymphocyte ratio was not affected by treatment during the prepartum (P = 0.17) and postpartum (P = 0.17) periods (Table 4).

Effects of rbST Treatment on PMNL Expression of CD62L and CD18, PMNL Phagocytosis and Oxidative Burst Activity, and Anti-Ovalbumin IgG Concentration

Expression of the adhesion molecule CD62L by PMNL during the prepartum (P = 0.44) and postpartum (P = 0.59) periods did not differ between treatments (Table 4). Cows treated with rbST tended (P = 0.09) to have greater CD18 expression by PMNL during the prepartum than control cows, but no (P = 0.83) difference was observed during the postpartum period (Table 4).

Percentages of PMNL positive for phagocytosis and oxidative burst were not affected by treatment during the prepartum (P = 0.18) and postpartum (P = 0.87) periods (Table 4). The interaction between treatment and day relative to calving tended (P = 0.10) to affect the percentage of PMNL positive for phagocytosis and oxidative burst during the postpartum period. On d 7 postpartum, rbS125 cows had (P = 0.05) greater percentage of PMNL positive for phagocytosis and oxidative burst than control cows (control = 59.2 ± 2.8 vs. rbST125 = 67.6 ± 3.1%). During the prepartum and postpartum periods the intensity

of fluorescence for phagocytosis ($P = 0.22$ and $P = 0.81$, respectively) and oxidative burst ($P = 0.22$ and $P = 0.79$, respectively) were not affected by treatment (Table 4).

Prepartum concentrations of serum anti-ovalbumin IgG were not ($P = 0.30$) different between treatments, but in the postpartum period rbST125 cows had ($P = 0.01$) greater anti-ovalbumin IgG concentrations than control cows (Figure 2).

Effects of rbST Treatment on Peripheral Leukocyte Gene Expression

Nine genes (*APOA5*, *APOB100*, *GCSF3*, *GHR1A*, *G6PC*, *IGF1*, *IGFBP3*, *IL6*, and *Src*) were not detected in leukocytes in the current experiment. At baseline (d -21), nine genes (*IGF1R*, *ACOX1*, *PC1*, *PCK1*, *IL1B*, *SOD2*, *C5aR*, *MYD88*, and *Blk*) were ($P \leq 0.05$) and eight genes (*STAT2*, *STAT3*, *PCK2*, *PPARd*, *PRKAA1*, *DEFB7*, *Rac1*, and *RELA*) tended to be ($0.05 < P \leq 0.10$) expressed differently between treatments. From the 57 genes of interest detected in the leukocytes, 17 were expressed differently between treatments on d -7 and/or 7 relative to calving.

Genes Associated with the Somatotropic Axis. Leukocyte mRNA expression for *GHR* on d -7 ($P = 0.16$) and 7 ($P = 0.62$) relative to calving were not affected by treatment (Table 5). Expression of mRNA for *IGF1R* and *JAK1* on d -7 were reduced ($P \leq 0.04$) in rbST125 cows, but on d 7 there were no ($P \geq 0.29$) differences between treatments regarding mRNA expression for *IGF1R* and *JAK1* (Table 5). Leukocyte mRNA expression for *JAK2* and *JAK3* were not ($P > 0.10$) affected by treatment on d -7 and 7 relative to calving (Table 5). Expression of mRNA for *STAT4* on d -7 tended ($P = 0.06$) to be reduced by rbST125 treatment compared with control, but no ($P = 0.14$) effect of treatment was observed on d 7 (Table 5). Treatment had no ($P > 0.10$) effect on leukocyte mRNA expression for *SOCS2*, *STAT1*, *STAT2*, *STAT3*, *STAT5a*, and *STAT5b* on d -7 and 7 relative to calving (Table 5).

Genes Associated with Cell Energy Metabolism. Leukocyte mRNA expression for *INSR* on d -7 was not ($P = 0.38$) affected by treatment, but on d 7, leukocyte from rbST125 cows tended ($P = 0.06$) to have greater mRNA for *INSR* than leukocytes from control cows (Table 5). Treatment had no ($P > 0.10$) effect on leukocyte mRNA expression of genes involved in the glucose metabolism *GSK3B*, *PC1*, *PCK1*, and *PCK2* on d -7 and 7 relative to calving. Leukocyte mRNA expression for *LDHA* on d -7 was not ($P = 0.15$) affected by treatment, but on d 7 *LDHA* mRNA expression tended ($P = 0.06$) to be upregulated in rbST125 cows (Table 5). No effect of treatment ($P > 0.10$) was observed on mRNA for genes involved in lipid metabolism *ACOX1*, *PPARa*, *PPARd*, *PPARg*, and *PRKAA1* on d -7 and 7 relative to calving (Table 5).

Genes Associated with Complement and Innate Pathogen Receptors. Leukocyte mRNA expressions for *C5aR* and *CD14* on d -7 were ($P \leq 0.05$) upregulated in rbST125 cows compared with control cows, but on d 7 relative to calving no ($P > 0.10$) differences were observed (Table 6 and Figure 1). Treatment with rbST tended to upregulate mRNA expression for *NOD2* ($P = 0.06$) and *SCARB1* ($P = 0.08$) on d -7 relative to calving, but no ($P > 0.10$) effect of treatment was observed on mRNA expression for *NOD2* and *SCARB1* on d 7 (Table 6; Figure 1). Expression of mRNA for *TLR2* on d -7 relative to calving was not ($P = 0.65$) affected by treatment, but rbST treatment tended ($P = 0.09$) to upregulate mRNA expression for *TLR2* on d 7 (Table 6 and Figure 1). Treatment did not ($P > 0.10$) affect mRNA for *TLR4* on d -7 and 7 relative to calving (Table 6).

Genes Associated with Antimicrobial Enzymes and Peptides. Treatment had no ($P > 0.10$) effect on mRNA expression for *CATHL4* and *CATHL5* on d -7 and 7 relative to calving (Table 6). However, on d -7, cows treated with rbST had ($P = 0.02$) and tended ($P = 0.08$) to have greater leukocyte mRNA expression for *DEFB3* and *DEFB7*, respectively, than control cows (Table 6 and Figure 1). On d 7, no ($P > 0.10$) effects of treatment on mRNA expression for *DEFB3* and *DEFB7* were observed (Table 6 and Figure 1). Treatment had

no ($P > 0.10$) effect on mRNA expression for *DEFB4*, *MPO*, *NOX1*, *NOX2*, and *SOD2* on d -7 and 7 relative to calving (Table 6).

Genes Associated with Cytokines and Intracellular Signaling. Treatment had no effect ($P > 0.10$) on leukocyte mRNA expression for cytokines *IL1B*, *IL8*, and *TNFa* on d -7 and 7 relative to calving (Table 6). Similarly, mRNA expression for *ATF3* was not ($P > 0.10$) affect by treatment on d -7 and 7 relative to calving (Table 6). Cows treated with rbST tended to have upregulation of mRNA expression for *MYD88* ($P = 0.08$) and *Rac1* ($P = 0.10$) on d -7 compared with control cows, but on d 7 no ($P > 0.10$) treatment effect was observed (Table 6 and Figure 1). Expression of mRNA for *NFAT* on d -7 was ($P = 0.03$) downregulated in rbST125 cows compared with control cows, but on d 7 it was not ($P = 0.30$) affected by treatment (Table 6). Leukocyte mRNA expression for *Blk* tended ($P = 0.06$) to be and was ($P = 0.05$) downregulated in rbST125 cows on d -7 and 7, respectively, compared with control (Table 6 and Figure 2). Treatment did not ($P = 0.24$) affect mRNA expression for *Lyn* on d -7, but on d 7 rbST125 treatment tended ($P = 0.06$) to upregulate mRNA for *Lyn* compared with control treatment (Table 6 and Figure 2). Treatment had no ($P > 0.10$) effect on mRNA for *ERK1*, *ERK2*, *MAP3K7*, *NFKB1*, *PIK3CD*, *Rac2*, *RELA*, *RhoA*, *Syk*, and *TRAF6* on d -7 and 7 relative to calving (Table 6).

DISCUSSION

Leukocytes can produce GH and IGF-1 and express their receptors, GHR and IGF-1R (Merimee et al., 1989; Rosenfeld et al., 1979). Detection of IGF-1 and IGF-1R mRNA and the proteins they encode in peripheral blood leukocytes suggest that IGF-1/IGF-1R signaling pathways might exert regulatory functions on the immune system (Smith, 2010). In the current experiment, treatment with rbST increased prepartum serum IGF-1 concentrations compared with control cows, in agreement with our previous experiments (Silva et al., 2015, 2016a,b). In hepatic tissue, cows treated with 125 mg of rbST, weekly, from d -21 to 28 relative to calving had upregulated mRNA expression for *GHR*, *GHR1A*,

and *IGF1* -7 d relative to calving, suggesting that exogenous GH treatments upregulate hepatic mRNA expression for *GHR1A* and therefore IGF-1 synthesis (Silva et al, 2016b). In the current experiment, leukocyte mRNA expression for *GHR* was not affected by rbST125 treatment and the genes *GHR1A* and *IGF1* were not detected, suggesting that exogenous GH was not able to induce IGF-1 synthesis by leukocytes. Moreover, leukocyte mRNA expression for *IGF1R* was upregulated on d -7 in control cows. Because of the inherent uncoupling of the somatotropic axis during the periparturient period, control cows had a 50% reduction on serum IGF-1 concentrations from d -24 to -3 relative to calving whereas rbST125 cows had a 30% reduction. Upregulation of leukocyte mRNA for *IGF1R* expression on d -7 in control cows may be a cell mechanism response to maintain IGF-1R internal signaling during a drastic decrease in systemic IGF-1 concentrations. Growth hormone binding to its receptors initiates the activation of JAK-STAT signaling pathways (Zhu et al., 1998). Surprisingly, *JAK1*, and *STAT4* were reduced on d -7 in cows treated with rbST. The reason for the rbST-induced downregulation of mRNA expression for *JAK1* and *STAT4* in leukocytes is unknown, but may indicate a cell response mechanism to excess stimuli by exogenous GH in rbST125 cows.

During inflammation, extracellular glucose and intracellular glycogen stores are the preferred metabolic sources of energy used for activation, proliferation, survival, differentiation, phagocytosis, and chemotaxis of leukocytes (Pithon-Curi et al., 2004; Weisdorf et al., 2015). In the current experiment, rbST treatment did not affect leukocyte mRNA expression for genes related to gluconeogenesis (i.e. *PC1*, *PCK1*, *PCK2*), glycogen synthesis (i.e. *GSK3B*), and lipid oxidation (i.e. *ACOX1*, *PPARs*). On the other hand, hepatic mRNA expressions for *PCK1* and *ACOX1* were upregulated by treatment of periparturient cows with rbST (Silva et al., 2016b). It is possible that the lack of differences between treatments regarding leukocyte expression of mRNA for genes involved in gluconeogenesis and lipid metabolism is a consequence of the lack of differences between treatments regarding plasma glucose, NEFA, and BHB concentrations in the current

experiment. Expression of mRNA for *LDHA* and *INSR* on d 7 postpartum tended to be upregulated in rbST125 cows. Expression of mRNA for *LDHA*, which catalyzes lactate production from pyruvate, is induced by the hypoxia-inducible factor 1-α (**HIF-1α**; Semenza et al., 1996). Leukocytes activation by pathogens is dependent on glycolysis, as inhibition of glycolysis with 2-deoxyglucose decreases the inflammatory response, whereas inhibitors of mitochondrial respiration have no effect on inflammatory response (Kellet, 1996). A key mechanism for HIF-1α activation by pathogens involves the mammalian target of rapamycin (**mTOR**). After *TLR* stimulation, mTOR helps immune cells meet high metabolic demands by increasing HIF-1α expression, which in turn upregulates *LDHA* expression (Land and Tee, 2007). Dendritic cells lacking tuberous sclerosis complex 1, a mTOR inhibitor, exhibit increased glycolysis and upregulation of mRNA expression of *LDHA* (Wang et al., 2013). In addition, the mTOR-HIF-1α axis also has a role in the memory function of the innate immune system, known as trained immunity (Saeed, et al., 2014). The mTOR pathway is upregulated by growth factors such as IGF-1 and insulin and the *INSR* can be activated by mTOR signaling (Yin et al., 2016). Therefore, increased IGF-1 concentrations and upregulation of mRNA for *LDHA* and *INSR* in rbST treated cows might result in increased glycolytic response and lactate production during inflammatory responses and might be a characteristic of trained innate immunity stimulated by rbST125 treatment.

Humoral innate immune responses are fundamental to the development of powerful and effector functions of cell mediated immune responses in host defense. In the current experiment, cows treated with rbST had upregulated leukocyte mRNA expression for complement receptor *Ca5R* on d -7 prepartum. The activation of the complement C5a induces neutrophil chemotaxis, adhesion and transmigration, phagocytosis, oxidative burst, and delays neutrophil apoptosis (Guo and Ward, 2005). Furthermore, innate immune responses are initiated by cell surface receptors that detect pathogen-associated molecular patterns (**PAMPs**) and trigger internal signaling pathways that lead to the production of

cytokines and chemokines, upregulation of adhesion molecules, and phagocytosis (Schiff et al., 1997; Tak and Firestein, 2001). Leukocyte mRNA expression for the PAMPs receptors *TLR2*, *CD14*, *NOD2*, and *SCARB1* were upregulated in rbST treated cows. Pathogen binding to PAMPs recognition receptors induces receptor dimerization and recruitment of *MYD88* that initiates the recruitment of *TRAF6*, which then activates the IKKα complex allowing the translocation of nuclear factor-κB to the nucleus for transcription of proinflammatory genes (Akira and Hoshino, 2003). Cows treated with rbST had upregulation of leukocyte mRNA for *MYD88* on d -7 relative to calving, which may be associated with the upregulated mRNA expression of PAMPs recognition receptors. Stevens et al. (2011) compared neutrophils from early lactation and mid-lactation cows and observed that during early lactation neutrophils were not able to induce an adequate *TLR*4-mediated innate response and had reduced mRNA expression for *TRAF6*, *IL8*, and *C5aR*. Moreover, IGF-1 has been demonstrated to facilitate the interactions between promoter regions with transcription factors, including activator protein-1 and nuclear factor-κB (Giuliani et al., 2006). The rbST-induced increase in peripartum IGF-1 concentrations and upregulation of leukocyte mRNA expression for PAMPs receptors (*TLR2*, *CD14*, *NOD2*), *SCARB1*, and *MYD88* on d -7 may be associated with immune cells' increased capacity to recognize pathogens and activate an inflammatory response.

The gene *Rac1* encodes a protein member of the Rho-family GTPases that plays a key role in actin reorganization of cytoskeleton to promote membrane ruffling during the phagocytosis process induced by binding of microbes to specific cell surface receptors (Hall, 1994; Machesky and Hall, 1996). In addition, *Rac1* is a key activator of the superoxide generating NADPH oxidase in phagocytes, which plays an important role on oxidative burst microbial killing (Ridley, 1995). Expression of mRNA for *Rac1* on d -7 relative to calving tended to be upregulated by rbST treatment. One week prepartum the fluorescence intensity for phagocytosis and oxidative burst were 20% and 33%,

respectively, higher for rbST125 cows than control cows, albeit not statistically significant. In a previous experiment, Silva et al., (2015) demonstrated that rbST treatment significantly increased the intensity of phagocytosis and oxidative burst during the prepartum. In the current experiment, percentage of PMNL positive for phagocytosis and oxidative burst on the first week postpartum was greater for rbST125 cows than control cows, but no differences in the expression of mRNA for *Rac1* 7 d after calving were observed. Our data suggests, not conclusively, that rbST treatment may have improved PMNL phagocytic and oxidative burst capabilities through modulation of *Rac1* expression. Ridley et al. (1992), using an *in vitro* model, demonstrated that signal transduction *Rac1* is required for organization of polymerized actin in membrane ruffling induced by growth factors. The process of *Rac1* induced phagocytosis of opsonized and non-opsonized targets may also be mediated by integrin αMβ2, a cell adhesion molecule comprised of one α-(*Mac1*) and one β-(CD18) subunit (Taborda and Casadevall, 2002; Le Cabec et al., 2002). Leukocyte mRNA expression for CD18 receptor was not evaluated in the current experiment, but expression of CD18 subunit by peripheral PMNL was increased in rbST125 cows during the prepartum period. Therefore, the combination of upregulated leukocyte mRNA expressions for phagocytic receptors and internal signaling *Rac1* and increased PMNL expression of integrin CD18 might explain improved phagocytosis and oxidative burst responses in rbST125 cows.

Cows treated with rbST had upregulated leukocyte mRNA expression for encoding genes *DEFB3* and *DEFB7* on d -7 relative to calving. Beta-defensins are antimicrobial peptides that contribute to innate host defense via direct bactericidal activity, as well as to adaptive immunity through effector and regulatory functions (Selsted and Ouellette, 2005). The mechanism of action of beta defensins involves membrane permeabilization and chemotactic behavior for cells of the adaptive immune system (Selsted and Ouellette, 2005). It has also been demonstrated that beta-defensin 3 can activate antigen-presenting cells via *TLR2* in a NFκB dependent manner (Funderburg et al., 2007). Insulin-like growth

factor 1 has the potential to regulate the expression of antimicrobial peptides (Sorensen et al., 2003). Human keratinocytes expressed human beta-defensin 3 in response to IGF-1 (Sorensen et al., 2003). Thus, it is likely that the rbST125-induced increase in IGF-1 concentrations during the prepartum upregulated the mRNA expression for *DEFB3* and *DEFB7*.

Serum concentrations of anti-ovalbumin IgG were significantly increased in rbST125 cows compared with control cows, in agreement with our previous findings (Silva et al., 2015). B-cells play diverse roles in immune function by further differentiating into immunoglobulin-secreting plasma cells. Although total lymphocyte concentration was not different between control and rbST125 cows, IGF-1 enhances immunoglobulin production in a process independent of B-cell proliferation (Baudler et al., 2005). Signaling components lying downstream from IGF-1R may condition B-cell function. Activation of B-cells through B-cell receptor triggers the src-family of protein tyrosine kinases (**PTK**) Blk, Fyn, and Lyn (Sefton and Taddie, 1994). Interestingly, control cows had upregulation of leukocyte mRNA expression for *Blk* on d -7 and 7 relative to calving, but this upregulation in expression of mRNA for *Blk* was not translated into increased IgG production. Texido et al. (2000) demonstrated that homozygous mutant mice free of *Blk* mRNA and Blk protein maintained B-cells development, *in vitro* activation, and antibody production. Thus, neither B-cell development nor B-cell responses *in vitro* and *in vivo* were altered by the lack of Blk. This suggests that other src-family PTKs can compensate for the lack of Blk. Furthermore, in mice with *Blk*/*Fyn* doubly deficient B-cells a key role of Lyn on src-family PTKs mediated B-cell functions has been observed (Texido et al., 2000). Importantly, rbST125 treatment upregulated leukocyte mRNA expression for *Lyn* on d 7 relative to calving, which coincided with the increased IgG responses to ovalbumin immunization. This suggests that activation of src-family PTK Lyn signaling is a possible mechanism for increasing B-cell IgG production in rbST treated cows. In addition, upregulation of mRNA for *TLR2* in rbST125 cows on d 7 may have contributed to increase

the antibody response to ovalbumin vaccinations. Combinations of *TLR* ligands synergistically enhance the magnitude and quality of immune responses, including the generation of follicular helper T-cells, the survival of antigen-presenting cells, and the affinity of antibodies (Zhu et al., 2010; Orr et al., 2014). Activation of *TLR2* is reported to stimulate T helper 2 responses *in vivo* (Dillion et al., 2004). Furthermore, when *TLR2* agonist is used to activate *TLR2* as an adjuvant in a protein immunization system, it contributes to the maintenance of antigen-specific CD4+ T-cell responses after booster immunization, leading to the generation of class-switched antibodies correlating to the expansion and activation of B-cells (Lee et al., 2016). Therefore, the amplified increase in IgG against ovalbumin upon successive boosting in rbST125 cows may also be a result of improved innate response which coordinates an improved adaptive response.

Modulation of mRNA expressions in peripheral leukocytes observed in the current experiment suggests that it may be possible to improve immune responses of periparturient cows with rbST treatment. The upregulation of genes involved in innate and adaptive immune responses, especially on d -7, by rbST treatment were translated into improvements in phagocytosis, oxidative burst, and IgG production observed in the present and previous (Silva et al., 2015) experiments. The current findings indicate that rbST improved *in vivo* leukocytes capability to recognize pathogens, to trigger proinflammatory and phagocytic and oxidative burst internal signaling, and to produce antimicrobial peptides and antibodies. These regulations resulted in a more powerful immune response to eliminate postpartum uterine contaminations at onset of parturition and reduce incidences of metritis and, consequently, postpartum haptoglobin concentrations (Silva et al., 2015; Silva et al., 2016a).

Messenger RNA originates from active genes and is used as a template for proteins that determine the immune cells phenotype; thus, mRNA expression can be used to investigate intracellular events in leukocytes. However, it should be pointed out that mRNA undergoes

posttranscriptional modifications that may or may not result in translation to active proteins. Therefore, measuring mRNA alone may have limitations to describe leukocytes mechanism of actions. Furthermore, *in vivo* experiments that evaluate peripheral leukocytes function work with the uncertain assumption that circulating leukocytes were in a resting stage or not being activated before the start of treatments, as leukocytes were not in a controlled environment as provided by *in vitro* experiments.

CONCLUSIONS

Treatment with 125 mg of rbST, weekly, from -21 to 21 d relative to calving, improve innate and adaptive immune functions by upregulating leukocyte mRNA expression of genes involved in glycolysis, PAMPs recognition receptors, phagocytosis, and production of antimicrobial peptides and antibodies. These changes are expected to facilitate the activation of mechanisms that recognize and eliminate pathogens. Additional experiments are warranted to evaluate mRNA expression of isolated immune cell lineages such as monocyte-macrophages, neutrophils, and B- and T-lymphocytes to determine which cell lineage specific mechanisms are being modulated by rbST. In addition, measurements of phosphorylation and activation of internal signaling molecules involved in innate (e.g. macrophages and neutrophils phagocytosis and oxidative burst capacities) and adaptive (e.g. T-lymphocytes cytotoxicity capacity and B-lymphocytes antibody production) immune responses associated with measurements of protein yields as a result of upregulated mRNA transcription and translation are warranted to confirm the mechanisms that truly exert a biological impact on immune cells function in cows treated with rbST.

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CHAPTER VI

Prepartum Stocking Density: Effects on metabolic, health, reproductive, and productive parameters

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OUTLINE

The objectives of the current experiment were to determine the effects of two prepartum stocking densities on milk yield, concentration of metabolites during the peripartum, and health and reproductive parameters of dairy cows. Jersey animals enrolled in the experiment at 254 ± 3 d of gestation were balanced for parity (nulliparous vs parous) and previous lactation projected 305-d mature equivalent milk yield (parous) and assigned to one of two treatments: 80SD = 80% headlock stocking density (38 animals/48 headlocks) and 100SD = 100% headlock stocking density (48 animals/48 headlocks). The number of experimental units was 8 (4 replicates and 2 pens/treatment/replicate). In total, 154 nulliparous and 184 parous animals were enrolled in the 80SD treatment and 186 nulliparous and 232 parous animals were enrolled in the 100SD treatment. At the start of each replicate, treatments were switched within pen. Cows were milked thrice daily and monthly milk yield, fat and protein content, and somatic cell count data were recorded up to 155 d postpartum. Plasma non-esterified fatty acid concentration was measured weekly from -18 ± 3 to 17 ± 3 d relative to calving and plasma beta-hydroxy butyrate was measured weekly from 1 ± 2 to 17 ± 3 d relative to calving. Cows were examined 1, 4 ± 1, 7 ± 1, 10 ± 1 and 13 ± 1 d relative to calving for diagnosis of uterine diseases. Blood was sampled for determination of progesterone concentration and resumption of ovarian cycles 35 ± 3 and 45 ± 3 d relative to calving. Average headlock (74.1 ± 0.4% vs 94.5 ± 0.3%) and stall (80.8 ± 0.4% vs 103.1 ± 0.4%) stocking density were reduced for the 80SD treatment compared with the 100SD treatment. Treatment did not affect incidence of retained fetal

membranes (80SD = 5.1, 100SD = 7.8%), metritis (80SD = 21.2, 100SD = 16.7%), acute metritis (80SD = 9.9, 100SD = 9.4%), and VPD (80SD = 5.8, 100SD = 7.9%). Concentrations of NEFA (80SD = 251.5 ± 6.1, 100SD = 245.9 ± 5.6 μmol/L) and BHBA (80SD = 508.2 ± 14.3, 100SD = 490.9 ± 13.6 μmol/L) were not different between treatments. Treatment had no effect on percentage of cows removed from the herd on the first 60 d postpartum (80SD = 6.1, 100SD = 5.1%) and on rate of removal from the herd up to 305 d postpartum {80SD = referent, 100SD [(adjusted hazard ratio (95% confidence interval)] = 1.02 (0.75, 1.38)}. Percentages of cows pregnant to first (80SD = 41.9, 100SD = 48.4%) and second (80SD = 49.3, 100SD = 42.0%) postpartum AI were not different between treatments. Finally, treatment did not affect energy corrected milk yield up to 155 d postpartum (80SD = 33.8 ± 0.5, 100SD = 33.4 ± 0.5 kg/d). In herds with weekly or twice weekly movement of new cows to the prepartum pen and separate housing of nulliparous and parous animals, a target stocking density of 100% of headlocks on the day of movement does not affect health, metabolic, reproductive, and productive parameters.
Key words: stocking density, prepartum cow

INTRODUCTION

Stocking density is an important topic for the dairy industry for several reasons. Changes in behavior are observed when stocking density is increased (Hosseinkhani et al., 2008; Proudfoot et al., 2009), raising issues regarding animal well-being. Two small but elegant studies conducted in research facilities of the University of British Columbia in Canada demonstrated the effects of overstocking of prepartum cows on behavior and feed intake. Elevated stocking density, cow to headlock ratio 2:1 vs 1:1 (Hosseinkhani et al., 2008) and feed bunk space 30 cm/cow vs 60 cm/cow (Proudfoot et al., 2009), resulted in increased rate of feed intake, fewer meals per day, increased feed sorting, decreased overall feed intake, increased standing time, and increased rate of displacement from the feeding area (Hosseinkhani et al., 2008; Proudfoot et al., 2009). Furthermore, it has been suggested that increased stocking density limits normal variation in individual cow behavior (Ito et al.,

2014). Because of the limited number of experimental units used in most of these experiments, little is known about the effects of stocking density on health and performance.

The consequences of stocking density for dominant and submissive cows are likely to be distinct. Dominant cows may be predisposed to ruminal acidosis as a consequence of increased rate of feed intake, fewer meals per day, and increased feed sorting (Hosseinkhani et al., 2008). Conversely, submissive cows are more likely to have metabolic diseases such as hepatic lipidosis and ketosis because of reduced feed intake and to develop lameness because of increased standing time and displacement rate (Hosseinkhani et al., 2008; Proudfoot et al., 2009). This is particularly important when nulliparous and parous animals are housed together because nulliparous animals, which have smaller frame and body weight, are often submissive. In a study designed to evaluate the effects of a dietary supplement on productive and health parameters of prepartum nulliparous and parous animals housed together it was observed that for every 10% increase in stocking density above 80% of headlocks there was a 0.7 kg/d decrease in milk yield among first lactation cows (Oetzel et al., 2007). Even though this was not a study designed to evaluate the impact of stocking density on productive parameters, many have used this study as the basis for recommending that stocking density during the prepartum period should not exceed 80%, particularly when heifers and cows are commingled.

The experiments cited previously were conducted with Holstein cows. To our knowledge, no experiments comparing the behavior of transition Holstein and Jersey cows under different managerial circumstances exists. A difficulty in commingling Holstein and Jersey cows to compare their behavior is the difference in body size, which is more likely to play a role in submissive and dominant behaviors than genetic composition of the cows. Nonetheless, differences in behavioral, immune, and neuroendocrine responses to stress

may exist according to the genetic composition of the cows; therefore, the external validity of studies conducted with one breed to other breeds is questionable.

We hypothesized that increasing prepartum stocking density affects metabolic parameters and consequently affects health and productive parameters of peripartum dairy cows. The objectives of the current experiment were to determine the effect of increasing prepartum stocking density from 80 to 100% of headlocks on the day of regrouping on metabolic, health, reproductive, and productive parameters of first lactation and mature cows housed separately during the prepartum period.

MATERIALS AND METHODS

Cows, Facilities, Management, and Nutrition

The experiment was conducted from October 2012 to December 2013 with cows enrolled from October 2012 to February 2013 and calving occurring from October 2012 to March 2013. Prepartum Jersey animals were enrolled in the experiment at 254 ± 3 d of gestation. During the prepartum period animals were separated by parity (nulliparous vs parous) and housed in one of four free-stall pens with 44 stalls and 48 headlocks that were identical in size (31.7 x 11 m, 347.8 m^2) and design. Stalls measured 230 cm (length) x 107 cm (width) x 114 cm (neck rail height) and headlocks measured 61cm/headlock. Pens had two water troughs at each end of the pen that measured 366 cm by 56 cm. The barn was cross-ventilated and had artificial lighting.

As animals demonstrated signs of calving (discomfort, restlessness, tail twitching, and visualization of the allantoic sac through the vulva) they were moved to a boxstall. During the immediate postpartum period (1 to 21 ± 3 DIM) cows were grouped by parity (primiparous vs multiparous) and housed in free-stall pens with 240 stalls and 260 headlocks. These pens had five water troughs per pen that measured 366 cm by 56 cm. The

barn was cross-ventilated and had artificial lighting (16 h of light and 8 h of dark). After calving, animals from different treatments were commingled in the same pen, but first lactation cows and mature cows were kept separate throughout lactation. From 1 to 21 ± 3 DIM, pens were stocked at 91.6 and 100% of headlocks and stalls, respectively. From 21 ± 3 DIM until diagnosis of pregnancy, at 66 ± 3 d after AI, cows were housed in cross-ventilated free-stall barns with 260 headlocks and 240 stalls. From the second pregnancy diagnosis to the end of the lactation cows were housed in a nearby naturally ventilated free-stall dairy (10 miles distance) with 260 stalls and 280 headlocks. Stocking density from 21 DIM to the end of lactation varied between 110 and 120% of headlocks and between 119% and 130% of stalls.

A TMR was fed once a day from enrollment to calving. Primiparous and multiparous cows were fed the same TMR from 1 DIM until the time of the second pregnancy diagnosis, and primiparous and multiparous cows were fed the same TMR from the time of the second pregnancy diagnosis to drying off. Feed was delivered once a day during the winter and twice a day (70 and 30% for morning and afternoon feedings, respectively) during the summer for lactating cows. Composition of TMR fed in the prepartum and immediate postpartum (1 to 21 ± 3 DIM) periods are described in Table 1.

Treatments

At enrollment, animals were balanced for parity (nulliparous or parous) and previous lactation 305-d mature equivalent milk yield (parous) and were assigned to one of the four study pens. Treatment applied to the study pens in the first replicate was determined by a coin toss. Animals were assigned to the 80% headlock stocking density (**80SD**, n = 2 pens and 4 replicates) or 100% headlock stocking density (**100SD**, n = 2 pens and 4 replicates). Twice a week, thereafter, groups of 2 to 15 cows (median = 9 cows) were moved to the 80SD and 100SD pens to re-establish the desired stocking density. At the start of each replicate and on the days of movement of new cows to the study pens, the desired stocking

densities were 80% of headlocks, 86.3% of stalls, and 9.2 m^2/cow for the 80SD treatment and 100% of headlocks, 109% of stalls, and 7.2 m^2/cow for the 100SD treatment.

At the end of each replicate, a new 80SD and 100SD group started but pens were switched to avoid location bias, even though pens were identical in design and had similar temperature and humidity throughout the study (data not shown). A total of 8 replicates (4 replicates and 2 pens/treatment/replicate). Thus, each pen had the 80SD and 100SD treatments twice during the experiment. The number of cows in each pen was counted twice daily during the prepartum period by study personnel, and daily stocking density was calculated by the number of cows in the pen divided by the number of headlocks or stalls.

Body Condition and Locomotion score

At enrollment and 1 ± 1, 35 ± 3, and 56 ± 3 DIM all cows were scored for body condition (1 = emaciated and 5 = obese; 0.25-unit increment as described by Ferguson et al., 1994) and locomotion (1 = normal locomotion and 5 = severely lame; as described by Sprecher et al., 1997). Cows with locomotion score $\geq$ 3 were considered lame.

Blood Sampling and Analysis of Metabolites in Plasma

Blood samples were collected from all cows -17 ± 3, -11 ± 3, -4 ± 3, 1 ± 1, 10 ± 3, and 17 ± 3 d relative to calving from the coccygeal vein/artery immediately after feeding while cows were restrained in self-locking headlocks. Needles used were 22 gauge and 1 inch long, and samples were collected into evacuated tubes containing K2 EDTA (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ). The utmost care was taken to minimize stress during sample collection. Tubes were placed in ice until centrifugation for plasma separation (1,200 x *g* for 15 min at 4^0C). Plasma was aliquoted into microcentrifuge tubes and stored at -32 ^{0}C until analysis.

Samples collected weekly from -17 ± 3 to 17 ± 3 d relative to calving were analyzed for concentrations of NEFA using a colorimetric assay (Wako Chemicals USA, Richmond, VA; Ballou et al., 2009). Concentrations of BHBA were determined enzymatically (Ranbut, Randox Laboratories, Antrim, UK; Ballou et al., 2009) from samples collected weekly from 1 ± 1 to 17 ± 3 DIM. A plate reader (Spectramax 340; Molecular Devices, Sunnyvale, CA) was used to measure the absorbance for the colorimetric and enzymatic assays. Control serum (Randox Control Sera, Antrim, UK) was used for the NEFA and BHBA assays. The intraassay CV were 6.1% and 9.8% for the NEFA and BHBA assays, respectively. The interassay CV were 8.9% and 11.8% for the NEFA and BHBA assays, respectively.

Clinical Examination and Definitions of Diseases

All cows were examined 1, 4 ± 1, 7 ± 1, 10 ± 1 and 13 ± 1 DIM for diagnosis of retained fetal membrane, metritis, and acute metritis. Retained fetal membrane was defined as retention of fetal membrane past 24 h postpartum. Metritis was defined as cows with watery, pink/brown, and fetid uterine discharge. Cows with symptoms of metritis and rectal temperature > 39.5 ºC, or anorectic, or depressed were considered to have acute metritis (LeBlanc, 2010). All cows were examined for vaginal purulent discharge (**VPD**), formerly known as clinical endometritis, using the Metricheck device (Simcro, New Zealand; McDougall et al., 2007) 35 ± 3 DIM and cows with $\geq$ 50% of pus in the exudate were considered positive for VPD.

All cows were observed once daily for displacement of abomasum and thrice daily for mastitis throughout their lactation. Data regarding incidence of displacement of abomasum and mastitis from 0 to 60 DIM are reported herein. Cows were followed from 0 to 305 DIM to determine percentage of cows removed (sold or dead) from the herd and the speed at which cows were removed from the herd.

Resumption of Ovarian Cycles Postpartum

Blood was sampled on 35 ± 3 and 45 ± 3 DIM for determination of progesterone concentration by RIA using a commercial kit (Coat-a-Count Progesterone, Siemens Medical Solutions Diagnostics, Los Angeles, CA). Cows with at least one sample with progesterone concentration $\geq$ 1 ng/mL were considered to have resumed their estrous cycle.

Production Parameters

Cows were milked thrice daily. Monthly, milk yield, milk fat and protein content, and SCC were recorded for individual cows during the official DHIA test. Data regarding milk yield, milk fat and protein content, and somatic cell count were collected from 0 to 155 DIM. The DHIA testing ceased in August of 2013; therefore, it was not possible to collect production data for the entire lactation. Energy corrected milk was calculated for each cow using the formula (Orth, 1992):

ECM (kg) = [(kg milk) x 0.327] + [(kg fat) x 12.95] + [(kg protein) x 7.2].

All cows received recombinant bST (500 mg of Posilac; Elanco Animal Health, Greenfield, IN) every 10 d starting 57 ± 3 d postpartum.

Reproductive Management, Reproductive Parameters and Removal from the Herd

All cows were subjected to the same reproductive program. Cows were presynchronized with three injections of prostaglandin $F_{2\alpha}$ given on 39 ± 3, 53 ± 3, and 67 ± 3 DIM. Throughout the experiment, cows were observed daily for signs of estrus (removal of tail paint) and inseminated in the same morning if observed in estrus after 50 DIM. Cows not observed in estrus were enrolled in the 5d Cosynch protocol (GnRH, 5 d later $PGF_{2\alpha}$, 24 h later $PGF_{2\alpha}$, and 72 h later GnRH and timed AI) 79 ± 3 DIM and were inseminated at fixed time 87 ± 3 DIM.

Cows not reinseminated in estrus were presented for pregnancy diagnosis by ultrasound (5 MHz probe, E.I. Medical, Loveland, CO) 31 ± 3 d after AI and pregnant cows were re-examined 66 ± 3 and 178 ± 3 d after AI by manual palpation of the uterine contents per rectum. Cows diagnosed open were submitted to one of two resynchronization protocols. Nonpregnant cows with a corpus luteum received a $PGF_{2\alpha}$ injection 33 ± 3 d after AI and, if not re-inseminated in estrus by 45 ± 3 d after AI, were submitted to the 5d Cosynch protocol and re-inseminated at fixed time 53 ± 3 d after AI. Nonpregnant cows without a corpus luteum received a GnRH injection 31 ± 3 d after AI and, if not re-inseminated in estrus by 38 ± 3 d after AI, were submitted to the 5d Cosynch protocol and re-inseminated at fixed time 46 ± 3 d after AI.

From October 2012 to April 2013, only non-sexed semen was used for all inseminations of all cows. Starting in May of 2013, sexed semen was used for insemination of cows observed in estrus (first and second postpartum AI), whereas non-sexed semen was used for insemination of cows at fixed time (first and second postpartum AI). Throughout the experiment, semen from 23 different sires (15 non-sexed and 8 sexed) were used for first and second postpartum AI. Farm personnel responsible for estrus detection and AI were blinded to treatments.

Percentage of cows receiving first AI in estrus, percentage of cows pregnant to first and second AI, and percentage of cows with pregnancy loss from 31 ± 3 to 66 ± 3 d after first and second AI were calculated. Interval from calving to conception and interval from calving to removal from the herd were calculated for individual cows. For purposes of statistical analysis of interval from calving to conception, cows were right censored when they did not conceive and left the herd (died or sold) before 305 d of lactation or when they reached 305 d of lactation without conceiving. Conversely, for purposes of statistical analysis of interval from calving to removal from the herd, cows were right censored when

they were dried off (approximately 220 d of gestation) and when they reached 305 d of lactation.

Statistical Analysis

The experiment had a randomized switch back design with pen as the experimental unit. Four replicates were used, and within each replicate 2 pens were used per treatment, resulting in 8 experimental units per treatment. In the first replicate, pens were blocked by parity (nulliparous and parous), a coin was tossed to determine the treatment of each of the four study pens. In each of the replicates cows were balanced for parity (nulliparous or parous) and previous lactation 305-d mature equivalent milk yield (parous) and, within parity, were assigned to one of the four study pens. Based on data from a previous experiment conducted in the same herd, the expected average and standard deviation of monthly ECM yield from calving up to 155 d postpartum were 33.5 kg/d and 2.0 kg/d, respectively. Thus, a total of 8 replicates were necessary to detect statistical significance ($\alpha = 0.05$, $ß = 0.20$) when the difference in average ECM yield in the first 155 d postpartum is 2.4 kg/d. Similarly, based on previous experiments conducted in the same herd, the average and standard deviation NEFA concentrations in the first 21 d postpartum were 320 and 9.5 µmol/L, respectively. Therefore, a total of 8 replicates were necessary to detect statistical significance ($\alpha = 0.05$, $ß = 0.20$) when the difference in average NEFA concentration in the first 21 d postpartum is 11 µmol/L.

All statistical analyses were conducted using SAS version 9.2 (SAS/STAT®, SAS Inst. Inc., Cary, NC). To ensure that cows subjected to different treatments and used in different replicates did not differ, data regarding age at enrollment, lactation number at enrollment, days of gestations (at enrollment and calving), BCS at enrollment, interval from enrollment to calving, previous lactation 305-d mature equivalent milk yield, and previous lactation interval from calving to conception were analyzed by ANOVA (PROC GLIMMIX) with a model that included treatment (80SD vs. 100SD), replicate (1 to 4) and, when appropriate,

parity (nulliparous vs. parous). Age at enrollment, days of gestation at enrollment, and previous lactation interval from calving to conception did not have a normal distribution; therefore, statistical analysis was performed on log values and results are presented as back-transformed log values. Incidence of male calves and twin calving were analyzed by logistic regression (PROC LOGISTIC) with a model that included treatment (80SD vs. 100SD), replicate (1 to 4), and parity (nulliparous vs. parous).

Binomial dependent variables were analyzed by logistic regression using the GLIMMIX procedure with a binary distribution. Continuous data were analyzed by ANOVA using the GLIMMIX procedure with a normal distribution. Repeated measurements were analyzed by ANOVA for repeated measures using the MIXED procedure. The structure of covariance (auto-regressive, unstructured, or compound symmetry) was chosen according to the Bayesian Akaike information criteria. In all models, treatment (80SD vs. 100SD), replicate (1 to 4), and parity (nulliparous vs parous) were included as fixed effects, pen was included as the random effect, cows were nested within replicate and pen, and treatment was nested within pen and replicate. For analysis of repeated measurements variables, time and the interaction between treatment and time were included in the model as fixed effects. Only the independent variables with $P < 0.10$ were retained in the model.

Additional independent variables offered to the model to evaluate the effects of treatment on percentage of cows receiving first postpartum AI in estrus and DIM at first postpartum AI were parity, calf sex (male vs. female), twin calving (singleton vs. twin), and stillbirth (alive vs. dead). Additional independent variables offered to the model to evaluate the effect of treatment on pregnancy to first and second postpartum AI were parity, calf sex (male vs. female), twin calving (singleton vs. twin), stillbirth (alive vs. dead), AI protocol (estrus vs. timed AI), and semen (conventional vs. sexed). Interval from first to second postpartum AI and DIM at second postpartum AI did not have a normal distribution; therefore, statistical analysis was performed on log values and results are presented as

back-transformed log values. Only the independent variables with $P < 0.10$ were retained in the model.

The rate at which cows were removed from the herd was analyzed by Cox proportional hazard ratio using the PHREG procedure with removal of variables by a stepwise backward elimination process based on the Wald's statistics criterion when $P > 0.10$. The model included treatment (80SD vs. 100SD), replicate (1 to 4), parity (nulliparous vs. parous), calf sex (male vs. female), twin calving (singleton vs. twin), and stillbirth (alive vs. dead) as fixed effects. Cow was used as the experimental unit in the Cox proportional hazard ratio analysis. Kaplan-Meyer survival analysis, using the LIFETEST procedure, was used to compare the interval from enrollment to removal from the herd between treatments. Because the Kaplan-Meyer survival analysis is a univariable analysis, only the calculated means to occurrence of the event are presented herein. Statistical significance was defined as $P \leq 0.05$ and statistical tendencies as $0.05 < P \leq 0.10$.

RESULTS

Thirteen 80SD heifers (8.4%), 1 80SD cow (0.6%), 13 100SD heifers (6.6%), and 1 100SD cow (0.4%) initially enrolled in the experiment did not calve within 35 d after enrollment, were subsequently diagnosed not pregnant and sold. Therefore, data presented herein is referent to 324 80SD animals (141 nulliparous, 183 parous) and 404 100SD animals (173 nulliparous, 231 parous).

At enrollment, days of gestation tended ($P = 0.07$) to be shorter for 80SD cows compared with 100SD cows (253.9 ± 1.0 vs 254.2 ± 1.0 d). At enrollment, age (80SD = 31.2 ± 1.0 mo, 100SD = 31.0 ± 1.0 mo; $P = 0.66$), lactation number (80SD = 1.04 ± 0.07 lactation, 100SD = 1.01 ± 0.06 lactation; $P = 0.75$), and BCS (80SD = 3.06 ± 0.02, 100SD = 3.06 ± 0.01; $P = 0.90$) were not different between treatments. Among cows, treatments did not differ regarding previous lactation 305-d mature equivalent milk yield (80SD = 10,252 ±

127.3 kg, 100SD = 10,246 ± 110.3 kg; *P* = 0.97) and previous lactation interval from calving to conception (80SD = 82.9 ± 1.0 d, 100SD = 79.6 ± 1.0 d; *P* = 0.31).

Interval from enrollment to calving was not (P = 0.27) different between treatments (80SD = 27.6 ± 0.4 d, 100SD = 27.0 ± 0.4 d). Upon calving, gestation length was not (*P* = 0.60) different between treatments (80SD = 281.5 ± 0.4 d, 100SD = 281.2 ± 0.4 d). Incidence of male calves was not (*P* = 0.49) different among heifers (80SD = 32.6%, 100SD = 36.1%) and cows (80SD = 54.9%, 100SD = 53.7%) assigned to the different treatments. Incidence of twin births also was not (*P* = 0.93) different among heifers (80SD = 0.7%, 100SD = 0%) and cows (80SD = 5.2%, 100SD = 4.4%) assigned to the 80SD and 100SD treatments.

Daily average stocking densities based on number of headlocks (80SD = 74.1 ± 0.4%, 100SD = 94.5 ± 0.3%; P < 0.01) and stalls (80SD = 80.8 ± 0.4%, 100SD = 103.1 ± 0.4%; P < 0.01) were different between treatments (Figure 1). Average temperature humidity index did not differ across the 4 study pens (average ± SD = 17.1 ± 14.9, range = 3.3–59.4).

Incidence of Diseases Postpartum

Incidences of peripartum diseases were not different between 80SD and 100SD treatments (Table 2). Similarly, incidences of displacement of abomasum and mastitis in the first 60 d postpartum were not affected by treatment (Table 2). Percentages of cows with locomotion score > 2 at 1 ± 1, 35 ± 3, and 56 ± 3 d postpartum were not different between treatments (Table 2). Similarly, treatment did not affect the likelihood of cows being removed from the herd within 60 d postpartum (Table 2). The rate at which cows in the 100SD treatment were removed from the herd [adjusted hazard ratio (AHR) (95% CI) = 1.02 (0.75, 1.38)] did not (*P* = 0.90) differ from that of cows in the 80SD treatment. The mean (± SEM) intervals from calving to removal from the herd were 258.3 ± 4.5 d for the 80SD treatment and 262.5 ± 3.9 d for the 100SD treatment (Figure 2).

Body Condition Score and Metabolic Parameters

Body condition score was not affected by treatment (Figure 3). Concentrations of NEFA (80SD = 251.5 ± 6.1, 100SD = 245.9 ± 5.6 μmol/L) was not different between treatments (Figure 4). Similarly, concentration of BHB (80SD = 508.2 ± 14.3, 100SD = 490.9 ± 13.6 μmol/L) was not different between treatments (Figure 5).

Resumption of Ovarian Cycles Postpartum and Reproductive Parameters

The percentages of cows characterized as cyclic by 35 ± 3 and 45 ± 3 DIM were not different between treatments (Table 3). Similarly, the likelihood of cows being inseminated in estrus and the DIM at first postpartum AI were not different between treatments (Table 3). The percentages of cows diagnosed pregnant 31 ± 3 and 66 ± 3 d after first postpartum AI were not different between treatments (Table 3). Incidence of pregnancy loss between 31 ± 3 and 66 ± 3 d after first postpartum AI was not different between treatments (Table 3). The interval from first to second postpartum AI and the DIM at second postpartum AI were not different between 80SD and 100SD treatments (Table 3). The percentages of cows diagnosed pregnant 31 ± 3 and 66 ± 3 d after the second postpartum AI were not different between 80SD and 100SD treatments (Table 3). Similarly, incidence of pregnancy loss between 31 ± 3 and 66 ± 3 d after the second postpartum AI was not affected by treatment (Table 3).

Milk Yield and Milk Components

Average daily milk yield from calving to 155 DIM was not different between treatments (Table 4). Similarly, fat and protein yield were not different between 80SD and 100SD cows (Table 4). Consequently, yield of 3.5% fat corrected milk and energy corrected milk were not different between treatments (Table 4; Figure 6).

DISCUSSION

The stocking densities on the day of regrouping of the current experiment were based on the commonly recommended stocking density of 80% of headlocks. Because stocking density of prepartum pens is dynamic and dependent on number of cows calving per day, the average daily stocking densities were 74.1 and 94.5% of headlocks for 80SD and 100SD treatments, respectively, despite the fact that new cows were moved into the prepartum pen twice weekly. The stocking densities based on number of stalls, however, were 80.8 and 103.1% for 80SD and 100SD treatments, respectively. Industry standards suggest that headlock stocking density > 80% may affect performance of postpartum cows. No controlled experiments demonstrating the effects of stocking density on productive or health parameters had been conducted up to date. Thus, the recommendation of 80% headlock stocking density may have been adopted based on the findings of Oetzel et al. (2007). In that experiment, which was designed to evaluate the effects of a prepartum feed additive on performance of lactating dairy cows, the authors determined, retrospectively, that for every 10% increase in prepartum stocking density above 80% a 0.7 kg/d decrease in milk yield of primiparous cows between 5 and 85 DIM was expected (Oetzel et al., 2007). Because this was not a controlled experiment to evaluate the effects of prepartum stocking density on performance, it is likely that differences in prepartum stocking density were associated with seasonal variation in number of calving, confounding the results of the experiment (Oetzel et al., 2007). Furthermore, nulliparous and parous animals were housed together (Oetzel et al., 2007). Therefore, it is not surprising that stocking density was associated with milk yield of nulliparous animals, which are smaller and more likely to be submissive that parous animals. In the current experiment, nulliparous and parous animals were housed separately. Although many herds commingle nulliparous and parous animals, our objective was to evaluate the effect of prepartum stocking density under adequate management conditions (e.g. separation of nulliparous and parous animals, sufficient water availability, deep bedded stalls, etc.). Therefore, the findings of the current experiment may not be easily extrapolated to herds with inadequate management.

Treatment did not affect metabolic status before calving as observed by the similar NEFA concentrations from -18 to 17 d relative to calving and the similar BCS from -28 to 0 d relative to calving. Similarly, there were no differences between treatments regarding postpartum NEFA and BHB concentrations and BCS. Although we could speculate that DMI was not different between treatments because no differences were noted for metabolic parameters, ECM yield, and BCS, DMI data was not collected because the experiment was conducted in a commercial herd. Therefore, it is not possible to rule out differences in feed efficiency between treatments. Although we conclude that increasing prepartum headlock stocking density from 80 to 100% may not affect metabolic parameters of peripartum animals, it is not clear what the maximum acceptable stocking density during the prepartum period is. In a recent experiment, Huzzey et al. (2012) demonstrated that when non-lactating pregnant (214 d of gestation) nulliparous and parous animals housed together were exposed to 200% stocking density (1 vs 2 cow/stall) behavioral and metabolic changes were observed. However, the effects of stocking density on behavior and metabolism were dependent on parity. Changes in stocking density from 100 to 200% increased the interval to approach the feed bunk among nulliparous animals, reduced the percentage of feeding time within 3 h of feed delivery among nulliparous and parous animals, and increased displacement from the feed bunk (Huzzey et al., 2012). Although, greater stocking density increased group DMI, elevated stocking density was associated with greater plasma NEFA and glucose concentrations and greater fecal cortisol concentration among nulliparous animals but not parous animals (Huzzey et al., 2012). Based on glucose tolerance test, the authors demonstrated that nulliparous and parous animals exposed to 200% stocking density were more likely to be resistant/desensitized to insulin (Huzzey et al., 2012). These are important metabolic alterations in prepartum animals that may have a profound effect on immune response and health parameters during the peripartum period.

In the current experiment, stocking density had no effect on incidence of uterine diseases. Actually, there was a large numerical difference in incidence of metritis in favor of the 100SD treatment. This is surprising because one of the hypotheses of the current experiment was that reduced prepartum stocking density would result in reduced incidence of immune related (e.g. retained fetal membranes) and infectious (e.g. metritis) diseases because of improved energy status during the peripartum period. Randomly selected sub-groups of 80SD and 100SD animals were used to evaluate the effects of prepartum stocking density on immune parameters (Dresch et al., 2013). Stocking density did not affect PMNL count, PMNL phagocytic activity or oxidative burst (Dresch et al., 2013). Therefore, despite our initial hypotheses, it is not surprising that stocking density had no effect on incidence of retained fetal membranes, metritis, and VPD. The lack of difference in incidence of displacement of abomasum and the lack of differences on NEFA and BHB concentration between treatments suggest that 100% prepartum stocking density did not impact metabolism of peripartum cows compared with 80% prepartum stocking density. One of the initial hypotheses of the current experiment was that increased stocking density would increase incidence of lameness as a consequence of increased displacement from the feed bunk and decreased lying time (Proudfoot et al., 2009). Surprisingly, there was no effect of treatment on percentage of cows with locomotion score > 2 at 0, 35 and 56 DIM despite the fact that during 47.8% of the prepartum period there were at least 2 more cows than stalls in the 100SD treatment and during 100% of the prepartum period there were 4 fewer cows than stalls in the 80SD treatment. The lack of effect of stocking density on percentage cows removed from the herd within 60 DIM and the lack of effect of stocking density on rate at which cows were removed from the herd up to 305 DIM reinforce the conclusion that stocking density had no effect on health status of peripartum cows in a situation in which heifers and cows were housed separately.

Because of the negative association between peripartum health and metabolic disorders and reproductive and productive performances, we hypothesized that animals in the 80SD

treatment would have improved reproductive and productive performances. The close relationship between metabolic status and resumption of ovarian cycles postpartum (Butler, 2003) and the fact that treatment did not affect metabolic status peripartum explain why incidence of cyclic cows at 35 and 45 DIM were similar between treatments. The lack of differences in reproductive parameters between treatments is likely a consequence of the lack of differences between treatments regarding the incidence of peripartum disorders and cyclicity at 35 and 45 DIM. Similarly, the lack of difference between treatments regarding productive performance is likely associated with the lack of difference between treatments regarding metabolic and health parameters. Furthermore, the nearly identical yield of energy corrected milk and metabolic parameters pre and postpartum allow us to speculate that prepartum 100% stocking density of headlocks on the day of regrouping does not affect energy status and feed intake. It is interesting that several studies have demonstrated that increasing stocking density affects feeding and lying behavior (Olofsson, 1999; DeVries and von Keyserlingk, 2006; Proudfoot et al., 2009) but because these experiments had limited number of experimental units, effects of stocking density on health, reproductive, and productive parameters were not evaluated. Nonetheless, researchers continue to affirm that behavioral changes due to managerial changes warrant adoption of dramatically different management of dairy cows. In the current experiment, feeding and lying behaviors of 80SD and 100SD animals were evaluated (Lobeck et al., 2013; K. M. Lobeck-Luchterhand, P. R. B. Silva, R. C. Chebel, and M. I. Endres, unpublished data). The 100SD treatment resulted in greater displacement from the feed-bunk than the 80SD treatment independent of parity. Feeding time was reduced for nulliparous animals in the 80SD treatment compared with the 100SD treatment but feeding time was greater for parous animals in the 80SD treatment than those in the 100SD treatment (K. M. Lobeck-Luchterhand, P. R. B. Silva, R. C. Chebel, and M. I. Endres, unpublished data). Interestingly, stocking density had no effect on lying time of prepartum cows but nulliparous animals in the 80SD treatment had more lying bouts per day than nulliparous animals in the 100SD treatment. These are important considerations because

they demonstrate that increasing average daily stocking density in 20 percentage units (from 80 to 100%) affected behavior of prepartum animals. On the other hand, changes in behavior associated with elevated stocking density had no impact on metabolic status or health, reproductive, and productive parameters. In a recent experiment, we demonstrated that despite the fact that a stable prepartum pen (no entrance of new cows) reduces competition in the feed bunk it had no positive effects on immune, metabolic, health, reproductive or productive performances (Silva et al., 2013a; Silva et al., 2013b). Thus, recommendations regarding management of dairy cows may not be based solely on their effects on behavior of cows.

CONCLUSIONS

The current experiment is important because it is a controlled experiment that compared stocking densities in a large dairy operation with a large number of animals. In situations in which management is adequate (e.g. separation of nulliparous and parous animals, adequate water availability, proper heat abatement, deep bedding), increasing prepartum stocking density on the day of regrouping from 80 to 100% of headlocks did not affect metabolic status and incidence of peripartum diseases. Consequently, reproductive and productive performances of animals exposed to 100% stocking density were not compromised compared with animals exposed to 80% stocking density. Recommendations such as reduced prepartum stocking density have a profound economic impact on dairy operations because of increased fixed cost for building of facilities. Because minute behavioral alterations are not necessarily associated with changes in immune, metabolic, health, reproductive and productive parameters, one may not base managerial recommendations solely on behavioral responses of dairy cows.

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CHAPTER VII

Effects of prepartum stocking density on innate and adaptive leukocyte responses and serum and hair cortisol concentrations

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OUTLINE

Objectives were to evaluate the effects of prepartum stocking density on innate and adaptive leukocyte responses, serum cortisol and haptoglobin concentrations and hair cortisol concentration of Jersey cows. The cows (254 ± 3 d of gestation) were balanced for parity (nulliparous vs. parous) and previous lactation projected 305-d mature equivalent milk yield and assigned to one of two treatments: 80SD = 80% stocking density (38 animals/48 headlocks) and 100SD = 100% stocking density (48 animals/48 headlocks). Pens (n = 4) were identical in size and design and each pen received each treatment a total of 2 times (4 replicates; 80SD: n = 338; 100SD: n = 418). A sub-group of cows (n = 48/treatment per parity) was randomly selected on week 1 of each replicate from which blood was sampled weekly from d -14 to 14 (d 0 = calving) to determine polymorphonuclear leukocyte (PMNL) phagocytosis, oxidative burst, and expression of CD18 and L-selectin, and hemogram. The same sub-group of cows was treated with chicken egg ovalbumin on d -21, -7, and 7 and had blood sampled weekly from d -21 to 21 for determination of serum IgG anti-ovalbumin concentration. Blood was sampled weekly from d -21 to 21 to determine glucose, cortisol, and haptoglobin concentrations in serum. Hair samples collected at enrollment and within 24 h of calving were analyzed for cortisol concentration. The percentage of leukocytes classified as granulocyte and the granulocyte to the lymphocyte ratio were not affected by treatment. Treatment did not affect the percentage of PMNL positive for phagocytosis and oxidative burst or the intensity of phagocytosis and oxidative burst. Similarly, treatment did not affect the percentage of

PMNL expressing CD18 and L-selectin or the intensity of expression of CD18 and L-selectin. Concentration of IgG anti-ovalbumin was not affected by treatment. Serum concentrations of haptoglobin and cortisol were not affected by treatment. Similarly, hair cortisol concentration at calving was not affected by treatment. According to the current experiment, a target stocking density of 80% did not improve leukocyte responses compared with 100% target stocking density.

INTRODUCTION

Overstocking prepartum dairy animals is believed to be a threat to the animals' health and performance because of its effects on feeding and standing behaviors (Hosseinkhani et al., 2008; Huzzey et al., 2006). Recently, our group demonstrated a negative effect of elevated stocking density (100% vs. 80% of headlocks) in the prepartum period on competitive behavior at the feed bunk and lying time (Lobeck-Luchterhand et al., 2015). Furthermore, recent experiments suggest that overstocking pens of commingled nulliparous and parous non-lactating animals affects energy metabolism (Huzzey et al., 2012). Oetzel et al. (2007) suggested that, when prepartum nulliparous and parous animals are commingled, an increase in 10 percentage points in stocking density (headlocks) above 80% results in reduced milk yield among nulliparous animals (0.7 kg/d less milk from 3 to 85 DIM). Despite the mounting evidence, a large percentage of dairy herds still have insufficient feeding and lying space for prepartum dairy cows.

Dairy ewes housed in high (1.5 m^2/ewe) stocking density from late gestation to mid-lactation had reduced anti-ovalbumin IgG concentration compared with ewes housed in low (3 m^2/ewe) stocking density (Carporese et al., 2009). Furthermore, ewes that were housed in high stocking density conditions tended to have greater number of aggressive interactions, had reduced milk yield, and had increased milk somatic cell count (Carporese et al., 2009). The consequences of elevated stocking density during the prepartum period

on leukocyte responses, serum and hair cortisol concentrations, and markers of systemic inflammation of Jersey nulliparous and parous animals are unknown.

The hypotheses of the current experiment were that a reduced stocking density (80 vs. 100% of headlocks) would result in reduced serum and hair cortisol concentrations, improved PMNL activity (phagocytosis and oxidative burst), increased expression of adhesion molecules by PMNL (L-selectin and CD18), increased IgG concentration in response to an ovalbumin challenge, and reduced serum concentrations of haptoglobin. Therefore, the objectives of the current experiment were to evaluate whether reduced stocking density (80 vs. 100%) during the prepartum period would improve innate and adaptive leukocyte responses and reduce concentrations of markers of stress and inflammation of periparturient Jersey animals.

MATERIALS AND METHODS

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Minnesota (#1105B99113).

Cows, Housing, and Feeding

Cows used in the current experiment (n = 96) are a sub-group of animals used in a larger experiment (n = 756; Silva et al., 2014). Detailed description regarding facilities, management, and nutrition may be found in Silva et al. (2014). Briefly, the experiment was conducted from October 2012 to December 2013 in a commercial dairy located in Southern Minnesota. Throughout the experiment, Jersey cows were housed in cross-ventilated free-stall barns. During the prepartum period (d -28 to 0; d 0 = calving) nulliparous and parous animals were housed separately in one of four free-stall pens that were identical in size and design. All experimental pens had 44 deep sand bedded free-stalls (230 cm length × 107 cm width × 114 cm height) with a head-to-head double row configuration. The width of stall space for each cow or stall divider was determined by side-lunging pipe loops with

wide opening between the top and bottom pipes. Stalls were located between two main alleys (feed alley and stall alley) in the central of each pen occupying approximately 32% of total pen area. Pens feed bunk area had 48 self-locking headlocks (61 cm width/headlock) trough where cows had access to the feed bunk and were free to withdraw at any time. As cows demonstrated signs of calving (discomfort, restlessness, tail twitching, and visualization of the allantoic sac through the vulva) they were moved to a box stall. During the immediate postpartum period (1 to 21 ± 3 DIM) cows were grouped by parity (primiparous vs multiparous) and housed in free-stall pens with 240 stalls and 260 headlocks. After calving, cows from different treatments were commingled in the same pen but first lactation cows and mature cows were kept separate throughout the lactation. From 1 to 21 ± 3 DIM, pens were stocked at 91.6% and 100% of headlocks and stalls, respectively. Stocking density from 21 DIM to the end of the lactation varied between 110 and 120% of headlocks and between 119% and 130% of stalls. Artificial lighting was provided during the prepartum (24 h of light) and postpartum (16 h of light and 8 h of dark) period. From enrollment to calving a different TMR was fed to nulliparous and parous animals once a day. From 1 DIM until drying-off primiparous and multiparous cows were fed the same TMR. Composition of TMR fed in the prepartum and immediate postpartum (1 to 21 ± 3 DIM) periods are described in Silva et al. (2014).

Treatments

At enrollment, cows were balanced for parity (nulliparous = 340 or parous = 416) and previous lactation 305-d mature equivalent milk yield (parous) and were assigned to one of the four study pens. Treatment applied to the study pens in the first replicate was determined by a coin toss. Animals were assigned to the 80% stocking density (**80SD**, n = 2 pens and 4 replicates) or 100% stocking density (**100SD**, n = 2 pens and 4 replicates) based on headlocks. Twice weekly, thereafter, groups of 2 to 15 cows (median = 9 cows) were moved to the 80SD and 100SD pens to re-establish the desired stocking density. At the start of each replicate and on the days of movement of new cows to the study pens, the

desired stocking densities were 80% of headlocks, 86.3% of stalls, and 9.2 m^2/cow for the 80SD treatment and 100% of headlocks, 109% of stalls, and 7.2 m^2/cow for the 100SD treatment. At the end of each replicate, a new 80SD and 100SD group started but pens were switched to avoid location bias. There were a total of 8 replicates (4 replicates and 2 pens/treatment per replicate). Thus, each pen had the 80SD and 100SD treatments twice during the experiment. Numbers of cows in each pen were counted twice daily during the prepartum period by study personnel and daily stocking densities were calculated as the number of cows in the pen divided by the number of headlocks or stalls.

On the first day of each replicate a sub-group of cows was chosen for determination of innate leukocyte responses and concentration of antibodies (n = 5 to 7 cows per treatment per replicate). The selection of these cows was based on BCS and gestation length, in order to assure that sampling occurred on the same date for all cows. Ninety six cows (n = 48/treatment) were used for evaluation of innate and adaptive leukocyte responses, hemogram, serum concentrations of glucose, haptoglobin, and cortisol, and hair cortisol concentration.

Body Condition and Locomotion Scores

At enrollment and 1 ± 1, 28 ± 3, and 56 ± 3 d postpartum animals were scored for body condition (1 = emaciated and 5 = obese; 0.25 unit increment as described by Ferguson et al., 1994; Fig. 1) and locomotion (1 = normal locomotion and 5 = severely lame; as described by Sprecher et al., 1997; Fig 1).

Hemogram and Innate Immune Responses and Antibody Concentration Assays

Blood sampled on d -14 ± 3, -7 ± 3, 0 ± 3, 7 ± 3, and 14 ± 3 were used for hemogram (Fig. 1). Samples collected into evacuated tubes with EDTA (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) were analysed using a Vet Scan HM2 (Abaxis, Union City, CA). Complete blood count was performed but only data referent to concentration of

granulocytes relative to total leukocytes and the granulocytes to lymphocytes ratio are reported.

Ex vivo innate leukocyte response was evaluated on d -14 ± 3, -7 ± 3, 0 ± 3, 7 ± 3, and 14 ± 3 (Fig. 1) as described by Hulbert et al. (2011). Samples were collected into heparinized evacuated tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA). Expression of L-selectin and CD18 by peripheral PMNL was determined by indirect immunofluorescence staining. Briefly, the assay consisted of incubating 200 µL of whole blood at 4^0 C for 30 minutes with 5 µg/mL of anti-bovine CD62L (DU1-29, VMRD Inc., Pullman, WA) monoclonal antibody produced in mouse or 2.5 µg/mL of anti-bovine CD18 (BAQ30A, VMRD Inc., Pullman, WA, USA) monoclonal antibody produced in mouse. Prior to the incubation of cells with an anti-mouse IgG-FITC secondary polyclonal antibody (AbD Serotec, Raleigh, NC, USA) diluted 1:400 in PBS solution (Sigma-Aldrich, St. Louis, MO, USA), erythrocytes were hypotonically lysed. After washing the cells with PBS solution, samples were analyzed by flow cytometry. Blood from non-diseased cows were used as positive and negative controls in all assays. Negative controls consisted of incubating 200 µL of PBS solution instead of the monoclonal antibody. Phagocytic and oxidative burst activity of peripheral PMNL were determined upon challenge with enteropathogenic bacteria (*Escherichia coli* 0118:H8) as previously described by Hulbert et al. (2011). Briefly, the assay to determine phagocytosis and oxidative burst consisted of incubating 200 µL of whole blood with 100 µM of dihydrorhodamine 123 (Molecular Probes, Invitrogen, USA), an oxidative-sensitive indicator, and 40 µL of fluorescently labeled bacteria (10^9 cfu/mL) at 38.5 ^{0}C for 15 minutes, with surface bacteria fluorescence removed using 0.4% Trypan Blue Solution (Sigma-Aldrich, St. Louis, MO, USA). After washing with milliQ water to remove excess dye, erythrocytes were lysed with hyperconcentrated PBS solution. Lastly, the cells were resuspended in PBS solution for immediate flow cytometry analyses. Blood from non-diseased cows were used as positive and negative controls. Unlabeled bacteria were used as negative controls for the

phagocytosis assay and samples that received no dihydrorhodamine 123 served as negative controls for the oxidative burst assay. All flow cytometry data were collected on a BD FACSCANTO II (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed using FlowJo 7.6.4 software (Tree Star Inc., San Carlos, CA, USA). The PMNL population was identified on basis of forward and side scattered properties. After strictly gating the PMNL population, data from 3 parameters were collected for analysis: forward scatter, side scatter, and log fluorescence. Data are reported as PMNL intensity of phagocytosis, oxidative burst, and expression of CD18 and L-selectin molecules expressed as geometric mean fluorescence intensity (**GMFI**). Phagocytic and adhesion molecule intensity was an indirect indication of the number of phagocytised bacteria and adhered molecules by PMNL, respectively. Oxidative burst intensity was an indirect indication of the amount of reactive oxygen species produced via oxidation of dihydrorhodamine 123. Furthermore, percentages of PMNL positive for phagocytosis, oxidative burst, and percentages of PMNL positive for expression of CD18 and L-selectin molecules were calculated.

Animals received injections of 0.5 mg of chicken egg ovalbumin (Sigma-Aldrich, St. Louis, MO) diluted in Quil A adjuvant (0.5 mg of Quil A/ml of PBS; Accurate Chemical & Scientific Corp., Westbury, NY) on d -21 ± 3, -7 ± 3, and 7 ± 3. Blood was sampled weekly from d -21 ± 3 to 21 ± 3 into evacuated tubes without anticoagulant (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) for determination of IgG anti-ovalbumin in serum (Fig. 1) by ELISA as described by Mallard et al. (1997). The intra-assay and inter-assay CV were 6.8% and 8.4%, respectively.

Metabolites, Cortisol, and Haptoglobin Assays

Blood samples collected into evacuated tubes without anticoagulant (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) on d -21± 3, -14 ± 3, -7 ± 3, 0 ± 3, 7 ± 3, 14 ± 3, and 21 ± 3 were used for determination of serum glucose, haptoglobin and cortisol concentrations (Fig. 1). Glucose concentrations were determined by enzymatic reaction (Stanbio Laboratory, Boerne, TX). The intra-assay and inter-assay CV were 4.9% and

9.7%, respectively. Haptoglobin concentrations were determined by a colorimetric procedure (Hulbert et al., 2011) using a plate reader (Spectramax 340; Molecular Devices, Sunnyvale, CA). The intra-assay and inter-assay CV were 1.5 and 1.8%, respectively. Serum cortisol concentrations were determined using a solid-phase radioimmunoassay kit (Coat-a-Count, Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA). The intra- and inter-assay coefficients of variation were 9.2 and 4.2%, respectively.

Blood samples collected into evacuated tubes containing EDTA (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) on d -18 ± 3, -11 ± 3, -4 ± 3, 3 ± 3, 10 ± 3, and 17 ± 3 were used to determine plasma concentrations of NEFA and BHBA (Fig. 1). Concentrations of NEFA were determined using a colorimetric assay (Wako Chemicals USA, Richmond, VA; Ballou et al., 2009) and concentrations of BHBA were determined enzymatically (Ranbut, Randox Laboratories, Antrim, UK; Ballou et al., 2009). Control serum (Randox Control Sera, Antrim, UK) was used for the NEFA and BHBA assays. The intra-assay CV were 6.1 and 9.8% for the NEFA and BHBA assays, respectively. The inter-assay CV were 8.9 and 11.8% for the NEFA and BHBA assays, respectively.

Hair samples were collected at enrollment and on the day after calving (Fig. 1) from the same area, at the top of the head. Samples were stored in hermetically closed plastic bags, in the dark and at room temperature. Preparation of samples before the assay and details of the assay to determine cortisol concentration are described by Burnett et al. (2014). Briefly, hair samples were washed with warm water and let dry for 1 d then 250 mg of hair samples were washed with 5 mL of isopropanol and let dry for 5 d at room temperature. Samples were minced using a 10 mL milling cup with 12 mm balls in a Retsch Mixer Mill MM400 ball mill (Retsch, Hannover, Germany). Extraction of hair cortisol was performed by sonicating 20 mg of ground hair with 1 mL of HPLC-grade methanol (EMD Chemicals, Darmstadt, Germany) for 30 minutes followed by an overnight incubation on an incubator shaker (New Brunswick Scientific Incubator Shaker, Edison, NJ) at 100 rpm and 50 °C.

The supernatant was collected and evaporated at 45 °C under nitrogen gas stream. Dried samples were reconstituted in 100 µL of PBS. Hair cortisol was analyzed using a commercially available assay kit designed for salivary cortisol (Salimetrics Expanded Range, High Sensitivity 1-E3002, State College, PA). The minimal concentration of cortisol that can be distinguished from the 0 standard using this kit is 0.003 µg/dL. Samples were aliquoted into wells in duplicate (25 µl), and absorbance measured using a wavelength of 450 nm in a microplate plate reader (Biorad xMark, Hercules, CA). The average inter- and intra-assay CV was 7.7% and 3.5%, respectively.

Clinical Examination, Disease Definitions, and Production Responses

All cows enrolled in the experiment were examined on d 1, 4 ± 1, 7 ± 1, 10 ± 1 and 13 ± 1 for the diagnosis of retained fetal membrane, metritis, and acute metritis (Fig. 1). Retained fetal membrane was defined as retention of fetal membrane past 24 h postpartum. Metritis was defined as cows with watery, pink/brown, and fetid uterine discharge. Cows with symptoms of metritis and rectal temperature > 39.5 ºC, or anorectic, or depressed were considered to have acute metritis (LeBlanc, 2010). All cows were examined for vaginal purulent discharge, formerly known as clinical endometritis, using the Metricheck device (Simcro, New Zealand; McDougall et al., 2007) 35 ± 3 DIM (Fig. 1). Cows with $\geq$ 50% of pus in the vaginal exudate were considered positive for vaginal purulent discharge. All cows were observed thrice daily for mastitis throughout their lactation but only data regarding incidence of mastitis from study d 0 to 60 are reported herein (Fig. 1).

Cows were milked thrice daily. Monthly, milk yield, milk fat and protein content, and somatic cell count were recorded for individual cows during the official DHIA test. Data regarding milk yield, milk fat and protein content, and somatic cell count were collected from 0 to 155 DIM (Fig. 1). The DHIA testing ceased in August of 2013; therefore, it was not possible to collect production data for the entire lactation. Energy corrected milk was calculated for each cow using the formula (Orth, 1992):

ECM (kg) = [(kg milk) x 0.327] + [(kg fat) x 12.95] + [(kg protein) x 7.2].

All cows received recombinant bST (500 mg of Posilac; Elanco Animal Health, Greenfield, IN) every 10 d starting 57 ± 3 d postpartum.

Statistical analysis

The experiment had a randomized design with pen as the experimental unit. There were 4 replicates and within each replicate there were 2 pens per treatment, resulting in 8 experimental units per treatment. In the first replicate, pens were blocked by parity (nulliparous and parous) and a coin was tossed to determine the treatment of each of the four study pens. In each of the replicates cows were balanced for lactation number and previous lactation 305-d mature equivalent milk yield (parous) and, within parity, were assigned to one of the four study pens.

The sample size was calculated based on the expected reduction in mean intensity of oxidative burst and reduction in mean intensity of phagocytosis on the day of calving. Thus, a sample size of 8 would be enough to demonstrate statistical difference when intensity of phagocytosis and oxidative burst were increased by 28% and 38%, respectively, for cows in the 80SD compared with cows in the 100SD treatment. Expected standard deviation for intensity of phagocytosis and oxidative burst are 880 and 3,000 GMFI, respectively (Silva et al., 2013).

Statistical analyses were conducted using SAS version 9.2 (SAS/STAT®, SAS Inst. Inc., Cary, NC). To ensure that cows subjected to different treatments and used in different replicates did not differ, data regarding age at enrollment, previous lactation 305-d mature equivalent milk yield (parous), days of gestations at enrollment, BCS at enrollment, and interval from enrollment to calving were analyzed by ANOVA using the GLIMMIX procedure. Incidence of male calves, twin calving, stillbirth, retained fetal membrane,

metritis, acute metritis, endometritis, and mastitis and removal from the herd within 60 DIM were analyzed by logistic regression using the GLIMMIX procedure with the binary distribution and logit function.

Binomial outcomes were analyzed by logistic regression using the GLIMMIX procedure with the logit function for binary distribution. Continuous data with a normal distribution were analyzed by ANOVA using the GLIMMIX procedure. Repeated measurements were analyzed by ANOVA for repeated measures using the MIXED procedure. The structure of covariance (auto-regressive, unstructured, or compound symmetry) was chosen according to the Bayesian information criteria. In all models, treatment (80SD vs. 100SD), replicate (1 to 4), and parity (nulliparous vs. parous) were included as fixed effects, pen was included as the random effect, and cows were nested within replicate and pen. For analysis of repeated measurements variables, time and the interaction between treatment and time were included in the model as fixed effects. Only the independent variables with $P < 0.10$ were retained in the model.

Statistical significance was defined as $P \leq 0.05$ and statistical tendencies as $0.05 < P \leq 0.10$.

RESULTS

Only data referent to the 96 cows used to evaluate leukocyte and metabolic responses and concentrations of haptoglobin and cortisol are presented herein. There were no differences between treatments regarding age at enrollment (nulliparous: 80SD = 22.04 ± 1.75, 100SD = 22.58 ± 1.75 mo; parous: 80SD = 44.83 ±1.75, 100SD = 41.63 ± 1.75 mo; treatment – $P = 0.46$, parity – $P < 0.01$, treatment by parity – $P = 0.29$), previous lactation 305-d mature equivalent milk yield (80SD = 10,171.00 ± 281.13, 100SD = 10,324 ± 281.13 kg; $P = 0.71$), days of gestation at enrollment (80SD = 253.98 ± 0.49, 100SD = 254.00 ± 0.49 d; $P = 0.98$), BCS at enrollment (80SD = 3.06 ± 0.06, 100SD = 3.08 ± 0.06; $P = 0.80$), and interval

from enrollment to calving (80SD = 28.33 ± 0.99, 100SD = 27.52 ± 0.99 d; P = 0.57). Percentage of cows calving male calves was not different (P = 0.78; Table 1). None of the nulliparous animals delivered twins or had stillborn calves. Among parous animals, there were no differences between treatments regarding percentage of animals delivering twins (P = 0.60) and stillborn (P = 0.60) calves (Table 1). Similarly, the incidences of retained fetal membranes (P = 0.54), metritis (P = 0.22), acute metritis (P = 0.59), vaginal purulent discharge (P = 0.58), mastitis within 60 DIM (P = 0.66) and removal from the herd within 60 DIM (P = 1.00) were not different between treatments (Table 1). There was no (P = 0.38) difference between treatments regarding ECM yield (80SD = 32.72 ± 0.97, 100SD = 33.99 ± 0.99 kg/d).

Hemogram, Innate Immunity and Antibody Concentration

Treatment did not (P = 0.69) affect the percentage of leukocytes classified as granulocytes (Table 2). The interaction between treatment and day tended (P = 0.08) to affect percentage of leukocytes classified as granulocytes. On d 0 ± 3, 80SD cows tended (P = 0.08) to have fewer leukocytes classified as granulocytes than 100SD cows (Fig. 2). Treatment (P = 0.91) and the interaction between treatment and day (P = 0.28), however, did not affect the ratio of granulocytes:lymphocytes (Table 2).

Treatment (P = 0.65) and the interaction between treatment and day (P = 0.57) did not affect the percentage of PMNL that were positive for phagocytosis and oxidative burst (Table 2). Among PMNL positive for phagocytosis and oxidative burst, treatment (P = 0.91) and the interaction between treatment and day did not (P = 0.84) affect the intensity of phagocytosis (Table 2), however intensity of phagocytosis was affected by parity (nulliparous = 3,447.85 ± 87.61 vs. parous = 3,154.24 ± 86.99 GMFI; P = 0.02). Intensity of oxidative burst of PMNL positive for phagocytosis and oxidative burst was not affected by treatment (P = 0.84) or the interaction between treatment and day (P = 0.29; Table 2), but was affected by parity (nulliparous = 10,154.00 ± 785.78 vs. parous = 7,628.62 ±

782.46 GMFI; $P = 0.02$). The percentage of PMNL expressing CD18 was not affected by treatment ($P = 0.13$) or by the interaction between treatment and day ($P = 0.15$; Table 2). Similarly, intensity of expression of CD18 was not affected by treatment ($P = 0.29$) or by the interaction between treatment and day ($P = 0.30$; Table 2). The percentage of PMNL expressing L-selectin was not affected by treatment ($P = 0.56$) or by the interaction between treatment and day ($P = 0.61$; Table 2). Intensity of L-selectin expression was not affected by treatment ($P = 0.71$) or by the interaction between treatment and day ($P = 0.78$; Table 2).

Concentrations of serum IgG anti-ovalbumin were not affected by treatment ($P = 0.50$) or by the interaction between treatment and day ($P = 0.56$; Table 2). Concentrations of IgG in the colostrum were not ($P = 0.83$) different between treatments (80SD = 91.77 ± 4.98 vs. 100SD = 93.30 ± 4.88 g/dL). Parity affected ($P < 0.01$) colostrum IgG concentrations (nulliparous = 80.41 ± 6.89 vs. parous = 104.67 ± 7.04 g/dL).

Metabolic Parameters, Haptoglobin, and Serum and Hair Cortisol Concentrations

Concentrations of glucose were not affected by treatment ($P = 0.57$) or by the interaction between treatment and day relative to calving ($P = 0.84$; Table 3) but were ($P < 0.01$) affected by parity (nulliparous = 71.59 ± 1.10 vs. parous = 66.57 ± 1.10 mg/dL). Similarly, treatment ($P = 0.66$) and the interaction between treatment and day did not ($P = 0.52$) affect the concentration of NEFA (Table 3). Parity affected ($P < 0.01$) NEFA concentration (nulliparous = 289.41 ± 10.65 vs. parous = 210.94 ± 11.34 µmol/L). Beta-hydroxybutyrate concentration was not affected by treatment ($P = 0.18$) or by the interaction between treatment and day ($P = 0.23$; Table 3) and was affected by parity (nulliparous = 526.50 ± 25.96 vs. parous = 431.14 ± 13.26 µmol/L; $P = 0.02$). Concentration of haptoglobin was not affected by treatment ($P = 0.27$) or by the interaction between treatment and days relative to calving ($P = 0.83$.; Table 3) and was affected by parity (nulliparous = 7.62 ± 0.23 vs. parous = 6.91 ± 0.23 OD; $P = 0.03$).

Serum concentration of cortisol was not (P = 0.64) affected by treatment (Table 3). Similarly, the interaction between treatment and days relative to calving did not (P = 0.81) affect serum cortisol concentration (Fig. 3a). Parity affected (P = 0.03) serum cortisol concentration (nulliparous = 5.75 ± 0.46 vs. parous = 7.21 ± 0.46 ng/mL). Concentrations of cortisol in hair at enrollment (P = 0.36) and at calving (P = 0.84) were not affected by treatment (Fig. 3b).

DISCUSSION

The hypothesis of the current experiment was that increased stocking density would alter behavior and feed intake sufficiently to compromise the innate and adaptive leukocyte responses of periparturient Jersey cows. In the current experiment, increasing prepartum stocking density from 80 to 100% had no effect on responses associated with immune function, metabolism, stress, and systemic inflammation. This is surprising in light of previously published data demonstrating an effect of elevated stocking density on behavior and metabolism. Lobeck-Luchterhand et al. (2015) demonstrated that in pens in which Jersey cows were housed at 100% stocking density there was a greater number of displacements from the feed bunk compared with pens in which stocking density was 80%. Effects of stocking density on daily feeding time, however, were contradictory (Lobeck-Luchterhand et al., 2015). Daily feeding time of nulliparous animals housed at 80% stocking density was reduced compared with nulliparous animals housed at 100% stocking density. On the other hand, parous animals housed at 80% stocking density had greater daily feeding time than those housed at 100% stocking density. In the last 8 d before calving, animals housed at 80% stocking density had greater daily lying time than animals housed at 100% stocking density. Controlled experiments with dairy cows evaluating the effects of stocking density during the prepartum period on leukocyte function do not exist and controlled experiments evaluating the effects of stocking density during the prepartum period on health of periparturient dairy cows are usually underpowered. The lack of differences in leukocyte function of cows housed at 80 and 100% stocking density is in

agreement with the lack of differences in incidences of postpartum diseases reported by Silva et al. (2014). Importantly, the results of the current experiment, in addition to the findings reported by Silva et al. (2014) and Lobeck-Lutcherhand et al. (2015), cast doubt regarding the importance of some behavioral responses to leukocyte function, metabolism, and health of periparturient dairy cows.

In the current experiment, percentage of leukocytes classified as granulocytes on the day of calving was greater for animals housed at 100% stocking density compared with animals housed at 80% stocking density. Castration and hot-iron dehorning induced pain and stress have been associated with neutrophilia (Doherty et al., 2007; Ting et al., 2003). Housing of prepartum Jersey cows at 100% stocking density increased displacement at the feed bunk and reduced lying time during the last week prepartum (Lobeck-Lutcherhand et al., 2015), which are behaviors associated with stress. Nonetheless, there were no differences in serum and hair cortisol concentration between treatments during the prepartum period, suggesting that other factors than stress may have caused the rise in granulocyte on the day of calving among animals housed at 100% stocking density. Huzzey et al. (2012) demonstrated that heifers commingled with cows and housed in pens with 200% stocking density (cows per headlocks) had increased plasma NEFA, plasma glucose, and fecal cortisol concentrations, and were more resistant to insulin than heifers commingled with cows and housed at 100% stocking density (Huzzey et al., 2012). Stress induced rise in cortisol concentration (Nanda et al., 1990) may affect leukocyte responses because cortisol down regulates expression of L-selectin and β2-integrins by PMNL (Burton and Kehrli, 1995; Burton et al., 1995; Burton et al., 2005), inhibits synthesis of pro-inflammatory cytokines (IL-4, IL-6, IL-12, IFN-γ), favors the secretion of anti-inflammatory and immunosuppressive (IL-10) cytokines (Wiegers et al., 2005), and affects production of antibodies through the blockage of cytokines secreted by CD4+ Thelper1 or CD4+ Thelper2 (Salak-Johnson and McGlone, 2007). In the current experiment, the high stocking density (100%) may not have been dramatic enough to produce a significant increase in

cortisol secretion. Unfortunately, Huzzey et al. (2012) did not evaluate leukocyte responses of non-lactating dairy animals exposed to 100% vs. 200% stocking density. Carporese et al. (2009) demonstrated that serum IgG anti-ovalbumin concentrations were increased in dairy ewes housed at 3 m^2/ewe compared with ewes housed at 1.5 m^2/ewe. Surprisingly, cell-mediated immune response (e.g. skinfold thickness in response to phytohaemagglutinin) was only improved in ewes housed at 3 m^2/ewe and offered an external paddock (Carporese et al., 2009).

As mentioned previously, 100% stocking density resulted in greater competition at the feed bunk (Lobeck-Lutcherhand et al., 2015), but it may not have been intense enough to elicit stress-related neuroendocrine and immune responses. Exposure of male rats to adult fight-experienced male rats for 24 h per day (continuous) caused a reduction in the concentration of CD4 T helper and CD8 T cytotoxic cells compared with male rats exposed to adult fight-experienced male rats for 4 h per day (transient; Stefanski et al., 2013). Interestingly, concentrations of monocytes and granulocytes were increased on d 3 and 7 after onset of challenge in rats continuously and transiently exposed to adult fight-experienced male rats (Stefanski et al., 2013). The intensity of the stressor and the frequency at which animals are exposed to the stressor determine the extent of the neuroendocrine response to the stressors and the consequences to the immune function (Moberg, 2000). Another important factor modulating the response to stressors is genetics. Temperamental bulls had greater increase in cortisol concentration 24 h after transportation compared with calm bulls (Hulbert et al., 2011). Regardless of temperament, however, transportation increased the ratio of neutrophil to monocytes by 24 h after transportation, which returned to pre-transportation levels within 48 h (Hulbert et al., 2011). Although calm and temperamental bulls had decreased PMNL activity by 48 h after transportation, PMNL activity of calm bulls was greater at 96 h after transportation than during the pre-transportation period, whereas temperamental bulls had slight increases in PMNL activity returning to pre-transportation levels by 96 h (Hulbert et al., 2011).

It was not possible to evaluate feed intake because the current experiment was conducted in a commercial herd, but stocking density did not affect metabolic parameters of periparturient Jersey animals. This leads to the speculation that increasing stocking density from 80 to 100% may not have affected energy status of the animals during the periparturient period, which is important for leukocyte responses. As cows approach calving, feed intake decreases (Hayirli et al., 2002) and energy demands increase because of colostrogenesis and lactogenesis. Thus, dairy cows undergo negative energy balance in the first 6 to 8 weeks postpartum (Grummer et al., 2004). Reduced feed intake during the periparturient period has been associated with reduced neutrophil activity (Hammon et al., 2006). Furthermore, cows diagnosed with metritis had reduced feed intake during the prepartum and postpartum periods (Huzzey et al., 2007). It is possible that in the current experiment, the differences in stocking densities were not dramatic enough to result in significant reduction in feed intake and negative energy balance. Holstein cows housed in pens with two cows per feeding bin had increased rate of feed intake, reduced number of meals per day, and more sorting of the TMR compared with Holstein cows housed in pens with one cow per feeding bin (Hosseinkhani et al., 2008). Similarly, Holstein cows housed in pens with 30 cm of feed bunk space had reduced visit feed time during the periparturient period, and reduced DMI and greater displacement rate during the prepartum compared with Holstein cows housed in pens with 60 cm of feed bunk space (Proudfoot et al., 2009). These findings suggest that greater stocking pressure may have a greater effect on feeding behavior, possibly leading to compromised metabolic and immune functions. Huzzey et al. (2012) demonstrated that Holstein cows (nulliparous and parous commingled) housed at 200% stocking density took longer to approach the feed bunk when TMR was offered, spent less time eating immediately after TMR was offered, and had greater number of displacements at the feed bunk, but DMI was greater for cows housed at 200% stocking density than cows housed at 100% stocking density. The findings of the current experiment

and that of others suggest that behavioral responses to stressors may have limited effects on DMI and metabolic responses.

In summary**,** reducing prepartum stocking density from 100% to 80% did not improve innate and adaptive leukocyte function of periparturient dairy cows. Although it is possible that the treatment applied (100%) in the current experiment was insufficient to cause enough stress to elicit a strong neuroendocrine response that would affect immune and metabolic responses, cows housed at 100% stocking density had altered behavior at the feed bunk and stalls. This suggests that mild behavioral changes may not be associated with immune and metabolic functions.

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Table 1. Composition (% of DM unless otherwise noted) of TMR offered to far-off (-37 to -23 d prepartum), close-up (-22 to 0 d prepartum) and postpartum (d 1 to 21 postpartum) cows

Item	Far-off	Close-up	Postpartum
Dry matter, %	50.20	49.53	48.44
NE_L, Mcal/kg	1.50	1.46	1.62
CP	14.73	14.49	16.75
ADF	28.94	29.01	24.79
NDF	41.02	43.29	35.79
Ether extract	3.91	3.68	4.03
Ash	10.60	11.41	8.11
Calcium	1.68	1.76	0.82
Phosphorus	0.36	0.36	0.34
Magnesium	0.38	0.39	0.31
Potassium	1.25	1.28	1.65
Sulfur	0.37	0.36	0.26
Sodium	0.06	0.07	0.51
Chlorine	0.96	0.79	0.61

Table 2. Effect of treatment[1] on calving and health parameters

tem	Control[1]	rbST87.5[1]	rbST125[1]	*P*-value
Male Calf, %	41.5[A]	54.7[AB]	60.4[B]	0.14
Twins, %	7.5	3.8	1.9	0.39
Metritis, %	23.1[A]	18.0[AB]	7.8[B]	0.12
Ketosis, %	13.2	18.9	11.3	0.52
Removed from the herd, %	9.4	20.8	18.9	0.26

[A,B] Within the same row, means with different superscripts tended to differ ($0.015 < P \leq 0.05$).

[1]Control = cows received no treatment; rbST87.5 = cows received 87.5 mg of recombinant (r)bST every 7 d from -21 to 28 d relative to calving; and, rbST125 = cows received 125 mg of rbST every 7 d from -21 to 28 d relative to calving.

Table 3. Effect of treatment (Trt) on hemogram and polymorphonuclear leukocytes (PMNL) adhesion molecules

	Treatment[1]			*P*-value		
Item	Control[1]	rbST87.5[1]	rbST125[1]	Trt	Day	Trt x Day
Prepartum						
Granulocytes, %	39.7 ± 1.8	37.4 ± 1.8	39.8 ± 1.9	0.57	<0.01	0.95
Lymphocytes, %	52.8 ± 1.9	55.8 ± 1.9	53.1 ± 2.0	0.49	<0.01	0.79
Granulocyte:Lymphocyte	0.86 ± 0.07	0.77 ± 0.07	0.83 ± 0.07	0.52	<0.01	0.97
PMNL L-selectin+, %	97.9 ± 1.8	92.9 ± 1.8	95.4 ± 1.9	0.16	0.25	0.31
PMNL CD 18+, %	98.8 ± 0.9[A]	96.0 ± 1.0[B]	98.6 ± 1.0[AB]	0.08	0.65	0.49
Postpartum						
Granulocytes, %	34.7 ± 2.3	36.7 ± 2.5	40.3 ± 2.4	0.25	0.05	0.47
Lymphocytes, %	59.2 ± 2.5	58.6 ± 2.6	54.4 ± 2.5	0.34	0.08	0.14
Granulocyte:Lymphocyte	0.72 ± 0.09	0.79 ± 0.09	0.96 ± 0.09	0.25	0.09	0.41
PMNL L-selectin+, %	98.5 ± 0.7	98.1 ± 0.8	96.5 ± 0.7	0.15	0.05	0.40
PMNL CD 18+, %	98.1 ± 0.6	97.8 ± 0.6	98.7 ± 0.6	0.59	0.08	0.19

[A,B]Within the same row, means with different superscripts tended to differ ($0.015 < P \leq 0.05$).
[1]Control = cows received no treatment; rbST87.5 = cows received 87.5 mg of recombinant (r)bST every 7 d from -21 to 28 d relative to calving; and, rbST125 = cows received 125 mg of rbST every 7 d from -21 to 28 d relative to calving.

Table 4. Effect of treatment (Trt) on metabolites and cortisol

	Treatment[1]			*P*-value		
Item	Control[1]	rbST87.5[1]	rbST125[1]	Trt	Day	Trt x Day
Prepartum						
NEFA, µmol/L	313.7 ± 24.5	332.8 ± 24.1	328.3 ± 24.3	0.85	<0.01	0.17
BHBA, µmol/L	380.7 ± 13.7	377.1 ± 13.4	369.7 ± 13.5	0.84	<0.01	0.60
Glucose, mg/dL	75.5 ± 1.2	75.5 ± 1.1	75.5 ± 1.2	0.99	0.91	0.34
Cortisol, ng/mL	6.6 ± 0.6	7.3 ± 0.6	7.4 ± 0.6	0.66	<0.01	0.23
Postpartum						
NEFA, µmol/L	527.5 ± 36.8	592.9 ± 36.8	544.8 ± 36.5	0.43	<0.01	0.37
BHBA, µmol/L	747.5 ± 40.2	753.2 ± 40.1	648.8 ± 39.7	0.12	<0.01	0.97
Glucose, mg/dL	68.0 ± 1.1	67.2 ± 1.1	68.8 ± 1.1	0.58	0.04	0.28
Cortisol, ng/mL	4.4 ± 0.5	4.1 ± 0.5	3.9 ± 0.5	0.71	0.77	0.62

[1]Control = cows received no treatment; rbST87.5 = cows received 87.5 mg of recombinant (r)bST every 7 d from -21 to 28 d relative to calving; and, rbST125 = cows received 125 mg of rbST every 7 d from -21 to 28 d relative to calving.

Table 1. Composition (% of DM unless otherwise noted) of TMR offered to prepartum (-21 ± 3 to 0 d relative to calving) and postpartum (1 to 21 ± 3 d postpartum) cows

Item	Holstein Herd				Jersey Herd			
	Parous		Nulliparous		Parous		Nulliparous	
	Prepartum	Postpartum	Prepartum	Postpartum	Prepartum	Postpartum	Prepartum	Postpartum
DM, %	50.36	49.93	47.75	49.93	47.07	48.11	46.76	46.69
NE_L, Mcal/kg	1.44	1.64	1.42	1.64	1.47	1.64	1.36	1.60
CP	14.12	15.46	13.79	15.46	15.89	18.66	14.72	16.01
ADF	29.83	19.35	32.01	19.35	27.75	18.32	32.00	20.20
NDF	43.76	30.96	46.45	30.96	38.92	26.71	44.52	32.51
Ether extract	2.62	3.59	2.60	3.59	3.5	4.40	2.96	3.50
Ash	9.24	7.87	8.72	7.87	9.79	8.71	11.08	8.13
Calcium	1.35	0.91	0.99	0.91	1.48	1.26	1.29	1.13
Phosphorus	0.35	0.47	0.37	0.47	0.39	0.37	0.38	0.47
Magnesium	0.32	0.30	0.31	0.30	0.43	0.29	0.40	0.32
Potassium	1.09	1.33	1.13	1.33	1.25	1.85	1.18	1.38
Sulfur	0.29	0.34	0.22	0.34	0.44	0.34	0.43	0.35
Sodium	0.16	0.47	0.15	0.47	0.15	0.58	0.17	0.50
Chloride	0.94	0.36	0.33	0.36	0.38	0.61	0.38	0.38

Table 2. Descriptive data for periparturient Holstein and Jersey dairy cows at enrollment (-28 d relative to calving) and number of days cows spent in the prepartum pens according to treatment (Trt)[1].

Item	Holstein Herd		Jersey Herd		*P*-value		
	Control[1] (n = 151)	rbST125[1] (n = 151)	Control (n = 264)	rbST125 (n = 258)	Trt	Herd	Trt x Herd
Number of lactations (± SEM)	1.7 ± 0.1	1.6 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	0.92	<0.01	0.60
Nulliparous, %	15.2	17.2	39.8	37.2	0.92	<0.01	0.48
Lactation = 1, %	31.1	31.1	28.0	27.9	0.99	0.34	0.99
Lactation = 2, %	31.1	31.8	17.4	19.4	0.63	<0.01	0.77
Lactation $\geq$ 3, %	22.5	19.9	14.8	15.5	0.78	0.03	0.56
Body condition score (± SEM)	3.51 ± 0.04	3.53 ± 0.03	3.24 ± 0.03	3.25 ± 0.03	0.62	<0.01	0.76
305-d mature equivalent milk, kg (± SEM)	12,147.6 ± 255.1	12,810.3 ± 258.1	7,575.7 ± 408.1	7,645.9 ± 412.3	0.28	<0.01	0.39
Interval from calving to pregnancy, d (± SEM)	160.2 ± 6.8	153.3 ± 6.8	99.2 ± 8.4	108.0 ± 8.2	0.90	<0.01	0.30
Days in the prepartum pen (± SEM)	26.1 ± 0.4	26.1 ± 0.4	23.6 ± 0.3	22.8 ± 0.3	0.28	<0.01	0.33
Male calf	54.7	55.6	25.5	28.6	0.51	<0.01	0.69
Twins	5.3	2.7	3.0	2.0	0.17	0.29	0.74

[1]Control = cows received no treatment (n = 415); rbST125 = cows received 125 mg of rbST every 7 d from -21 to 21 d relative to calving (n = 409).

Table 3. Effects of treatment (Trt)[1] of periparturient Holstein and Jersey cows with 125 mg of recombinant bovine somatotropin (rbST) on calving and postpartum health parameters

Item, %	Holstein Herd		Jersey Herd		AOR (95% CI)[2]	*P*-value		
	Control[1] (n = 151)	rbST125[1] (n = 151)	Control (n = 264)	rbST125 (n = 258)		Trt	Herd	Trt x Herd
Stillbirth	5.3	2.7	3.4	7.5	1.40 (0.74, 2.70)	0.65	0.24	0.09
Subclinical hypocalcemia[3]	13.0	18.2	0.0	22.2	2.14 (0.68, 6.77)	0.20	0.95	0.96
Retained fetal membranes	14.3	6.1	1.5	1.2	0.48 (0.21, 1.09)	0.08	<0.01	0.49
Metritis	26.2	16.6	19.9	13.3	0.60 (0.41, 0.86)	<0.01	<0.01	0.95
Acute metritis	-	-	8.7	5.4	0.60 (0.30, 1.20)	0.15	-	-
Vaginal purulent discharge at 35 ± 3 DIM	-	-	15.4	16.0	1.04 (0.56, 1.91)	0.91	-	-
Ketosis[4]	9.4	11.3	8.5	13.4	1.52 (0.96, 2.41)	0.08	0.02	0.64
Displacement of abomasum within 60 DIM	4.0	6.0	2.3	0.8	0.90 (0.39, 2.07)	0.80	0.03	0.12
Mastitis within 60 DIM	22.5	27.8	7.2	9.3	1.32 (0.88, 1.98)	0.18	<0.01	0.73
Locomotion score >2 at 1 ± 1 DIM	-	-	16.8	17.7	1.07 (0.68, 1.70)	0.76	-	-
Locomotion score >2 at 30 ± 3 DIM	-	-	12.9	16.0	1.29 (0.69, 2.41)	0.42	-	-
Locomotion score >2 at 60 ± 3 DIM	-	-	12.1	8.7	0.68 (0.32, 1.47)	0.33	-	-
Removed from the herd within 60 DIM	7.3	11.3	6.8	7.0	1.32 (0.78, 2.22)	0.31	0.81	0.22

[1]Control = cows received no treatment (n = 415); rbST125 = cows received 125 mg of rbST every 7 d from -21 to 21 d relative to calving (n = 409).

[2]Adjusted odds ratio and 95% confidence interval for the effect of treatment. Control treatment was set as referent.

[3]Serum total calcium concentration between 5.5 and 8.0 mg/dL (Goff, 2008) within the first 72 h postpartum from a sub-sample of cows (Holstein herd: Control = 46, rbST125 = 44; Jersey herd: Control = 7, rbST125 = 9).

[4]BHB blood concentration $\geq$ 1,400 µmol/L (Duffield et al., 2009) at least once at 7 ± 3 and 14 ± 3 d postpartum.

Table 4. Effects of treatment (Trt)[1] of periparturient Holstein and Jersey cows with 125 mg of recombinant bovine somatotropin (rbST) on milk yield and quality in the first 30 d postpartum (LSM ± SEM)

Item	Holstein Herd		Jersey Herd		*P*-value		
	Control[1] (n = 151)	rbST125[1] (n = 151)	Control (n = 264)	rbST125 (n = 258)	Trt	Herd	Trt x Herd
Milk yield, kg/d	31.8 ± 0.8	34.9 ± 0.8	22.3 ± 0.6	23.4 ± 0.6	<0.01	<0.01	0.13
Fat, %	4.42^{x} ± 0.08	4.56^{x} ± 0.08	4.69^{y} ± 0.05	4.59^{x} ± 0.06	0.79	0.03	0.08
Fat, kg/d	1.33^{a} ± 0.04	1.51^{b} ± 0.04	1.02^{c} ± 0.03	1.06^{c} ± 0.03	<0.01	<0.01	0.03
Protein, %	3.72 ± 0.05	3.54 ± 0.05	3.74 ± 0. 04	3.61 ± 0.04	<0.01	0.35	0.52
Protein, kg/d	1.11 ± 0.03	1.17 ± 0.03	0.81 ± 0.02	0.83 ± 0.02	0.28	<0.01	0.43
ECM, kg/d	35.5^{a} ± 1.0	39.4^{b} ± 1.0	26.7^{c} ± 0.6	27.8^{c} ± 0.6	<0.01	<0.01	0.08
Linear SCC	4.43 ± 0.19	4.37 ± 0.19	3.53 ± 0.12	3.26 ± 0.12	0.15	<0.01	0.49

[1]Control = cows received no treatment (n = 415); rbST125 = cows received 125 mg of rbST every 7 d from -21 to 21 d relative to calving (n = 409).

[a,b,c]Within herd and within row, numbers with different lowercase letters differ ($P \leq 0.01$).

[x,y]Between herds and within row, numbers with different lowercase letters differ ($P < 0.05$).

Table 5. Effect of treatment (Trt)[1] of periparturient Holstein and Jersey cows with 125 mg of recombinant bovine somatotropin (rbST) on reproductive parameters

Item	Holstein Herd		Jersey Herd		AOR (95% CI)[2]	*P*-value		
	Control[1] (n = 151)	rbST125[1] (n = 151)	Control (n = 264)	rbST125 (n = 258)		Trt	Herd	Trt x Herd
First postpartum AI								
AI upon detected estrus, %	4.4	3.1	91.2	89.4	1.30 (0.74, 2.29)	0.36	<0.01	0.92
DIM at AI (±SEM)	70.1 ± 1.1	69.7 ± 1.1	59.3 ± 0.8	60.0 ± 0.9	NA	0.60	< 0.01	0.54
Pregnant at 31 or 39 ± 3 d, %	43.6	43.4	34.9	27.7	1.21 (0.89, 1.65)	0.23	0.12	0.42
Pregnant at 66 ± 3 d, %	39.9	41.0	32.4	24.6	1.22 (0.89, 1.68)	0.22	0.99	0.46
Pregnancy loss, %	8.6	5.7	7.2	11.3	0.90 (0.35, 2.31)	0.83	0.31	0.94
Second postpartum AI								
AI upon detected estrus, %	20.3	20.0	75.5	76.2	1.05 (0.68, 1.62)	0.83	< 0.01	0.81
DIM at AI (±SEM)	112.0 ± 3.0	114.4 ± 3.0	89.7 ± 2.1	90.6 ± 2.1	NA	0.60	< 0.01	0.76
Pregnant at 31 or 39 ± 3 d, %	30.7	30.3	42.8	39.6	1.14 (0.77, 1.67)	0.51	0.04	0.60
Pregnant at 66 ± 3 d, %	25.3	28.8	38.2	35.4	1.07 (0.72, 1.59)	0.73	0.05	0.79
Pregnancy loss, %	17.4	5.0	10.8	10.8	1.65 (0.61, 4.52)	0.33	0.88	0.31

[1]Control = cows received no treatment (n = 415); rbST125 = cows received 125 mg of rbST every 7 d from -21 to 21 d relative to calving (n = 409).

[2]Adjusted odds ratio and 95% confidence interval for the effect of treatment. Control treatment was set as referent.

Table 1. Genes and accession numbers according to National Center for Biotechnology Information (NCBI)

Gene symbol	Gene description	Accession number
Somatotropic axis and insulin		
GHR	Total growth hormone receptor	NM_176608.1
GHR1A	Growth hormone receptor 1 alpha	AY748827.1
IGF1R	Insulin like growth factor 1 receptor	NM_001244612.1
IGF1	Insulin like growth factor 1	NM_001077828.1
IGFBP3	Insulin-like growth factor-binding protein 3	NM_174556.1
INSR	Insulin receptor	XM_002688832.2
INSRB	Insulin receptor beta	XM_002688832.2
SOCS2	Suppressor of cytokine signaling 2	NM_177523.2
STAT5B	Signal transducer and activator of transcription 5B	NM_174617.3
Glucose and lipid metabolism		
ACOX1	Peroxisomal acyl-coenzyme A oxidase 1	NM_001035289.2
ANGPTL4	Angiopoietin-like 4	NM_001046043.2
APOA5	Apolipoprotein A-V	NM_001083492.1
APOB100	Apolipoprotein 100 B	AJ399505.1
DGAT1	Diglyceride acyltransferase	NM_174693.2
G6PC	Glucose-6-phosphatase catalytic subunit	NM_001076124.1
HMGCLL1	3-hydroxymethil-3-methylglutaryl-CoA lyase-like1	NM_001191193.1
PC1	Pyruvate carboxylase	NM_177946.3
PCK1	Phosphoenolpyruvate carboxykinase 1	NM_174737.2
PCK2	Phosphoenolpyruvate carboxykinase 2	NM_001205594.1
PPARA	Peroxisome proliferator-activated receptor alpha	NM_001034036.1
PPARD	Peroxisome proliferator-activated receptor delta	NM_001083636.1
PPARGC1A	PPAR gamma coactivator 1 alpha	NM_177945.3
SCARB1	Scavenger receptor class B member 1	NM_174597.2
Inflammation and oxidative stress		
CEBPD	CCAAT/enhancer binding protein delta	NM_174267.2
CXCL1	Chemokine (C-X-X motif) ligand 1	NM_175700.1
GCSF	Granulocyte colony stimulating factor	NM_174028.1
HIF1A	Hypoxia-inducible factor 1 alpha	NM_174339.3
HP	Haptoglobin	NM_001040470.1
ICAM1	Intercellular adhesion molecule 1	NM_174348.2
IL1RN	Interleukin 1 receptor antagonist	NM_174357.2
JUN	Activating protein-1 AP1	NM_001077827.1
MCSF1	Macrophage colony stimulating factor 1	NM_174026.1
MYD88	Myeloid differentiation primary response gene 88	NM_001014382.2
NFKB1	Nuclear factor kappa enhancer in B-cells 1	NM_001076409.1
NFKBIA	NF-kappa B inhibitor alpha	NM_001045868.1
NR3C1	Nuclear receptor subfamily 3 group C member 1	NM_001206634.1
SOCS3	Suppressor of cytokine signaling 3	NM_174466.2
TLR2	Toll-like receptor 2	NM_174197.2
TNF	Tumor necrosis factor alpha	NM_173966.2
TNFRSF1A	TNF receptor superfamily member 1A	NM_174674.2
TNFRSF5	TNF receptor superfamily member 5	NM_001105611.1
XBP1	X-box binding protein 1	NM_001034727.1
Candidate reference genes		
EIF3G	Eukaryotic translation initiation factor 3, subunit G	NM_001079623.1
GAK	Cyclin G associated kinase	NM_001046084.1
HNF4A	Hepatocyte nuclear factor 4 alpha	NM_001015557.1
HPRT	Hypoxanthine phosphoribosyltransferase	NM_001034035.1
SNAPC2	Small nuclear RNA activating complex 2	NM_001034516.1
SPRYD4	SPRY domain containing 4	NM_001076173.1
UBC	Polyubiquitin	NM_001206307.1
UXT	Ubiquitously-expressed transcript	NM_001037471.1

Table 2. Effect of treatment[1] of periparturient cows with recombinant bovine somatotropin (**rbST**) on hepatic gene expression (mRNA sqrt counts LSM ± SEM) of genes related to somatotropic axis, insulin, and metabolisms of glucose and lipid

Gene	Day -21			*P*	Day -7			*P*	Day 7			*P*
	Control[1] (n = 10)	rbST87.5[1] (n = 12)	rbST125[1] (n = 10)		Control (n = 10)	rbST87.5 (n = 12)	rbST125 (n = 10)		Control (n = 10)	rbST87.5 (n = 12)	rbST125 (n = 10)	
Somatotropic axis and insulin												
GHR	41.2 ± 3	42.8 ± 3	41.6 ± 3	0.91	36.8^{a} ± 3	45.0^{b} ± 3	45.2^{b} ± 3	0.07	27.6 ± 2	34.6 ± 2	29.5 ± 2	0.13
GHR1A	33.2 ± 3	34.9 ± 2	33.9 ± 3	0.88	28.0^{a} ± 3	37.0^{b} ± 2	36.7^{b} ± 2	0.03	21.0^{A} ± 2	27.8^{B} ± 2	24.0^{B} ± 2	0.08
IGF1R	6.2 ± 0.5	6.2 ± 0.4	6.5 ± 0.5	0.88	5.10 ± 0.4	6.2 ± 0.3	5.4 ± 0.4	0.08	6.6 ± 0.3	5.8 ± 0.3	6.0 ± 0.3	0.27
IGF1	22.8 ± 3	26.9 ± 2	24.1 ± 3	0.48	19.9^{a} ± 3	27.9^{b} ± 2	32.7^{b} ± 3	0.01	17.1^{ab} ± 2	20.8^{a} ± 2	14.1^{b} ± 2	0.10
IGFBP3	34.5 ± 2	34.5 ± 2	34.2 ± 2	0.99	33.4^{A} ± 2	39.1^{B} ± 2	37.1^{B} ± 2	0.14	26.7 ± 2	27.3 ± 2	27.3 ± 2	0.96
SOCS2	18.2 ± 2	20.3 ± 1	19.3 ± 2	0.63	19.4^{A} ± 2	24.6^{B} ± 2	22.1^{B} ± 2	0.15	16.3^{a} ± 2	22.1^{b} ± 2	21.0^{b} ± 2	0.06
INSR	14.8 ± 1	15.4 ± 0.5	15.2 ± 1	0.62	14.3 ± 1	15.0 ± 0.5	13.8 ± 1	0.28	13.6^{a} ± 1	15.4^{b} ± 1	14.5^{b} ± 1	0.07
INSRB	16.7 ± 2	14.5 ± 1	16.0 ± 1	0.41	17.1 ± 1	15.4 ± 1	14.7 ± 1	0.21	16.7 ± 1	14.5 ± 1	16.1 ± 1	0.41
Glucose metabolism												
G6PC	61.2 ± 3	65.3 ± 2	68.9 ± 3	0.12	61.6 ± 3	67.4 ± 3	64.0 ± 3	0.41	65.5 ± 6	71.4 ± 6	74.8 ± 6	0.52
PC1	28.7 ± 2	28.7 ± 2	27.9 ± 2	0.95	31.6 ± 3	30.6 ± 2	31.0 ± 3	0.96	43.8 ± 3	47.8 ± 3	41.9 ± 3	0.45
PCK1	84.2 ± 5	75.6 ± 4	79.9 ± 5	0.42	82.7 ± 6	83.6 ± 5	84.3 ± 5	0.98	76.0^{A} ± 9	98.0^{B} ± 9	99.9^{B} ± 9	0.15
PCK2	25.5 ± 1	23.3 ± 1	24.1 ± 1	0.43	28.2^{A} ± 2	24.0^{B} ± 1	25.4^{B} ± 1	0.15	23.9 ± 2	24.8 ± 2	28.4 ± 2	0.38
Lipid metabolism												
ACOX1	61.7 ± 2	57.1 ± 2	60.7 ± 2	0.26	64.3 ± 3	60.2 ± 2	60.1 ± 3	0.47	51.8^{A} ± 4	50.6^{B} ± 4	65.1^{B} ± 4	0.12
ANGPTL4	9.0 ± 1	9.9 ± 1	7.9 ± 1	0.57	6.7^{a} ± 1	11.8^{b} ± 1	9.7^{b} ± 1	< 0.01	19.2 ± 2	18.9 ± 2	16.9 ± 2	0.65
APOA5	31.5 ± 4	34.4 ± 3	30.9 ± 4	0.75	24.5^{a} ± 4	38.3^{b} ± 3	37.0^{b} ± 3	0.02	25.3^{a} ± 6	42.7^{b} ± 6	35.1^{b} ± 6	0.10
APOB100	70.5 ± 8	76.5 ± 8	75.1 ± 8	0.86	53.1^{a} ± 8	78.5^{b} ± 7	71.2^{b} ± 7	0.05	49.9 ± 9	72.4 ± 9	65.2 ± 9	0.25
DGAT1	18.1 ± 1	18.2 ± 1	17.9 ± 1	0.97	16.5 ± 1	17.4 ± 1	17.1 ± 1	0.61	20.3 ± 1	19.3 ± 1	19.3 ± 1	0.70
PPARA	29.3^{A} ± 1	27.1^{B} ± 1	28.1^{B} ± 1	0.12	28.5 ± 1	27.8 ± 1	27.9 ± 1	0.84	24.6 ± 1	25.8 ± 1	27.7 ± 1	0.22
PPARD	5.1 ± 1	4.4 ± 1	4.7 ± 1	0.69	6.3 ± 1	5.4 ± 0.5	5.0 ± 1	0.20	7.1^{a} ± 1	4.7^{b} ± 1	6.1^{b} ± 1	0.04
PPARGC1A	10.6 ± 2	7.2 ± 1	8.8 ± 2	0.26	11.9^{A} ± 2	7.5^{B} ± 1	8.7^{B} ± 2	0.15	10.6 ± 2	9.5 ± 2	12.3 ± 2	0.43
SCARB1	9.8 ± 1	11.0 ± 1	10.2 ± 1	0.48	7.9^{a} ± 1	10.5^{b} ± 1	11.0^{b} ± 1	0.02	12.1 ± 1	12.8 ± 1	12.1 ± 1	0.87

[a,b]Within a row, means of contrast control vs. rbST87.5 + rbST125 and rbST87.5 vs. rbST125 with different superscripts differed ($P \leq 0.05$).

[A,B]Within a row, means of contrast control vs. rbST87.5 + rbST125 and rbST87.5 vs. rbST125 with different superscripts tended to differ ($0.05 < P \leq 0.10$).

[1]Control = cows received no treatment; rbST87.5 = cows received 87.5 mg of recombinant bovine somatotropin (rbST) every 7 d from -21 to 28 d relative to calving; rbST125 = cows received 125 mg of rbST every 7 d from -21 to 28 d relative to calving.

Table 3. Effect of treatment[1] of periparturient cows with recombinant bovine somatotropin (**rbST**) on hepatic gene expression (mRNA sqrt counts LSM ± SEM) of genes related to inflammation and oxidative stress

Gene	Day -21			*P*	Day -7			*P*	Day 7			*P*
	Control[1] (n = 10)	rbST87.5[1] (n = 12)	rbST125[1] (n = 10)		Control (n = 10)	rbST87.5 (n = 12)	rbST125 (n = 10)		Control (n = 10)	rbST87.5 (n = 12)	rbST125 (n = 10)	
Inflammation												
CEBPD	18.7 ± 1	19.0 ± 1	18.4 ± 1	0.88	20.1 ± 1	21.1 ± 1	20.0 ± 1	0.71	23.1 ± 2	19.0 ± 2	18.4 ± 2	0.18
CXCL1	2.9 ± 1	3.5 ± 0.5	3.0 ± 1	0.66	3.4 ± 1	2.8 ± 0.4	2.3 ± 0.5	0.29	5.9^{a} ± 1	3.6^{b} ± 1	3.3^{b} ± 1	0.06
HIF1A	15.4 ± 1	15.1 ± 0.5	15.6 ± 1	0.76	17.8^{A} ± 1	16.6^{B} ± 1	14.8^{B} ± 1	0.12	22.3^{a} ± 2	16.0^{b} ± 2	16.7^{b} ± 2	0.07
HP	247 ± 62	307 ± 51	274 ± 59	0.75	92 ± 54	214 ± 38	157 ± 44	0.20	272 ± 63	287 ± 60	176 ± 63	0.32
ICAM1	14.6 ± 1	15.3 ± 1	14.3 ± 1	0.44	14.4^{ab} ± 1	16.4^{a} ± 1	14.6^{b} ± 1	0.02	16.2^{a} ± 1	14.4^{b} ± 1	13.6^{b} ± 1	0.05
IL1RN	5.4 ± 1	6.4 ± 0.5	5.7 ± 1	0.32	5.4 ± 1	6.2 ± 1	5.3 ± 1	0.57	10.8^{a} ± 1	7.3^{b} ± 1	6.4^{b} ± 1	0.05
JUN	13.0 ± 1	12.9 ± 1	12.2 ± 1	0.51	14.0 ± 1	15.1 ± 1	13.4 ± 1	0.38	17.3 ± 2	13.5 ± 2	15.5 ± 2	0.21
MCSF1	10.3 ± 1	9.9 ± 1	9.7 ± 1	0.79	11.3 ± 0.5	11.1 ± 0.4	10.6 ± 0.5	0.52	11.6 ± 1	10.1 ± 1	10.1 ± 1	0.33
MYD88	15.6 ± 1	15.5 ± 0.5	16.1 ± 1	0.61	16.4 ± 1	16.7 ± 1	15.6 ± 1	0.44	18.8^{a} ± 1	15.9^{b} ± 1	16.1^{b} ± 1	0.02
NFKB1	9.9 ± 0.3	9.8 ± 0.3	9.3 ± 0.3	0.46	10.1 ± 0.5	10.8 ± 0.4	10.5 ± 0.4	0.47	11.1 ± 1	9.8 ± 1	10.4 ± 1	0.46
NFKBIA	21.3 ± 1	21.3 ± 1	20.5 ± 1	0.75	21.5 ± 1	22.3 ± 1	21.3 ± 1	0.78	22.4^{a} ± 1	19.9^{b} ± 1	19.5^{b} ± 1	0.06
NR3C1	21.8 ± 1	22.3 ± 1	22.2 ± 1	0.90	20.4 ± 1	22.0 ± 0.5	21.0 ± 1	0.17	19.1^{a} ± 1	20.7^{b} ± 1	21.1^{b} ± 1	0.10
SOCS3	9.6^{a} ± 1	14.2^{b} ± 1	12.6^{b} ± 1	0.05	9.9 ± 1	12.1 ± 1	9.9 ± 1	0.23	17.9^{a} ± 2	11.9^{b} ± 2	8.5^{b} ± 2	<0.01
STAT5B	10.5 ± 0.4	10.7 ± 0.3	10.6 ± 0.3	0.86	10.0 ± 1	10.1 ± 1	10.2 ± 1	0.98	10.7 ± 1	10.1 ± 1	9.7 ± 1	0.34
TLR2	6.6 ± 1	7.5 ± 1	6.9 ± 1	0.52	6.8 ± 1	8.0 ± 1	7.1 ± 1	0.35	7.6 ± 1	7.3 ± 1	6.4 ± 1	0.33
TNF	4.6 ± 0.5	4.4 ± 0.4	4.4 ± 0.5	0.96	4.5 ± 0.4	5.5 ± 0.4	5.0 ± 0.4	0.22	3.9 ± 0.5	4.6 ± 0.5	5.2 ± 0.5	0.19
TNFRSF1A	18.9 ± 1	17.1 ± 1	18.3 ± 1	0.15	18.0 ± 1	18.9 ± 1	18.3 ± 1	0.52	22.7^{a} ± 2	18.1^{b} ± 2	18.7^{b} ± 2	0.11
TNFRSF5	4.3 ± 0.5	5.0 ± 0.5	4.3 ± 0.5	0.51	5.0 ± 1	6.0 ± 1	5.6 ± 1	0.55	5.6 ± 1	4.6 ± 1	5.3 ± 1	0.62
Oxidative stress												
XBP1	40.1 ± 2	43.6 ± 2	42.3 ± 2	0.57	38.4^{A} ± 2	45.8^{B} ± 2	41.3^{B} ± 2	0.07	59.1^{a} ± 5	47.2^{b} ± 5	40.1^{b} ± 5	0.03

[a,b]Within a row, means of contrast control vs. rbST87.5 + rbST125 and rbST87.5 vs. rbST125 with different superscripts differed ($P \leq 0.05$).

[A,B]Within a row, means of contrast control vs. rbST87.5 + rbST125 and rbST87.5 vs. rbST125 with different superscripts tended to differ ($0.05 < P \leq 0.10$).

[1]Control = cows received no treatment; rbST87.5 = cows received 87.5 mg of recombinant bovine somatotropin (rbST) every 7 d from -21 to 28 d relative to calving; rbST125 = cows received 125 mg of rbST every 7 d from -21 to 28 d relative to calving.

Table 4. Effect of treatment (Trt) of periparturient cows with recombinant bovine somatotropin (**rbST**) on body condition score and concentrations of cortisol, glucose, haptoglobin, and TNF-α (LSM ± SEM)

Item	Treatment[1]			*P*		
	Control (n = 10)	rbST87.5 (n = 12)	rbST125 (n = 10)	Trt	Day	Trt x Day
Prepartum						
GH, ng/mL	8.6 ± 0.6	11.0 ± 0.7	12.3 ± 0.8	0.23	0.29	0.33
IGF-1, ng/mL	107.4[a] ± 7.2	127.0[b] ± 6.6	139.4[b] ± 6.9	0.01	0.16	0.11
Insulin, ng/mL	0.95 ± 0.1	0.98 ± 0.1	1.03 ± 0.1	0.89	<0.01	0.38
Glucose, mg/dL	70.4 ± 3.0	73.5 ± 2.7	72.7 ± 2.9	0.73	0.26	0.11
NEFA, µmol/L	226.0 ± 15.4	221.2 ± 13.6	200.5 ± 13.2	0.84	<0.01	0.80
BHB, µmol/L	347.5 ± 27.3	339.1 ± 24.3	309.8 ± 25.7	0.57	<0.01	0.60
Cortisol, ng/mL	3.0 ± 0.1	5.4 ± 0.1	5.2 ± 0.6	0.20	0.01	0.86
Haptoglobin, OD[2] x 100	2.5 ± 0.3	2.1 ± 0.2	2.1 ± 0.2	0.56	<0.01	0.57
TNF-α, log	3.0 ± 0.1	3.2 ± 0.1	2.8 ± 0.1	0.21	0.14	0.96
Postpartum						
GH, ng/mL	9.0[a] ± 0.7	15.3[b] ± 1.0	18.5[b] ± 1.3	0.01	0.03	0.52
IGF-1, ng/mL	52.5 ± 8.7	51.4 ± 7.5	65.5 ± 8.0	0.39	<0.01	0.15
Insulin, ng/mL	0.21 ± 0.05	0.27 ± 0.04	0.22 ± 0.04	0.52	0.21	0.53
Glucose, mg/dL	63.9 ± 2.6	67.0 ± 2.4	63.7 ± 2.5	0.57	0.91	0.63
NEFA, µmol/L	522.3 ± 27.8	609.0 ± 30.7	527.7 ± 26.9	0.59	0.02	0.46
BHB, µmol/L	776.4[a] ± 64.0	628.4[b] ± 59.7	595.4[b] ± 60.9	0.11	0.02	0.45
Cortisol, ng/mL	2.7 ± 0.1	3.8 ± 0.1	2.6 ± 0.1	0.56	<0.01	0.49
Haptoglobin, OD x 100	1.8 ± 0.4	2.5 ± 0.4	1.3 ± 0.4	0.19	0.03	0.74
TNF-α, log	2.8 ± 0.1	2.9 ± 0.1	2.7 ± 0.1	0.36	0.49	0.57

[a,b]Within a row, means of contrast control vs. rbST87.5 + rbST125 and rbST87.5 vs. rbST125 with different superscripts differed ($P \leq 0.05$) when prepartum and postpartum periods were analyzed separately.

[1]Control = cows received no treatment; rbST87.5 = cows received 87.5 mg of recombinant bovine somatotropin (rbST) every 7 d from -21 to 28 d relative to calving; rbST125 = cows received 125 mg of rbST every 7 d from -21 to 28 d relative to calving.

[2]OD = optical density.

Table 1. Somatotropic axis and cell energy metabolism related genes and accession numbers according to National Center for Biotechnology Information (NCBI)

Gene symbol	Gene description	Accession number
Somatotropic axis and insulin		
GHR	Total growth hormone receptor	NM_176608.1
GHR1A	Growth hormone receptor 1 alpha	AY748827.1
IGF1	Insulin like growth factor 1	NM_001077828.1
IGF1R	Insulin like growth factor 1 receptor	NM_001244612.1
IGFBP3	Insulin-like growth factor-binding protein 3	NM_174556.1
JAK1	Janus kinase 1	NM_001206534.1
JAK2	Janus kinase 2	XM_865133.5
JAK3	Janus kinase 3	XM_010806603.1
SOCS2	Suppressor of cytokine signaling 2	NM_177523
STAT1	Signal Transducer and Activator of Transcription 1	NM_001077900.1
STAT2	Signal Transducer and Activator of Transcription 2	NM_001205689.1
STAT3	Signal Transducer and Activator of Transcription 3	NM_001012671.2
STAT4	Signal Transducer and Activator of Transcription 4	NM_001083692.2
STAT5a	Signal Transducer and Activator of Transcription 5 alpha	NM_001012673.1
STAT5b	Signal Transducer and Activator of Transcription 5 beta	NM_174617.4
INSR	Insulin receptor	XM_002688832.2
Glucose and lipid metabolism		
ACOX1	Peroxisomal acyl-coenzyme A oxidase 1	NM_001035289.2
APOA5	Apolipoprotein A-V	NM_001083492.1
APOB100	Apolipoprotein 100 B	AJ399505.1
G6PC	Glucose-6-phosphatase catalytic subunit	NM_001076124.1
GSK3B	Glycogen synthase kinase 3 beta	NM_001101310
LDHA	Lactate dehydrogenase alpha	NM_174099
PC1	Pyruvate carboxylase	NM_177946.3
PCK1	Phosphoenolpyruvate carboxykinase 1	NM_174737.2
PCK2	Phosphoenolpyruvate carboxykinase 2	NM_001205594.1
PPARA	Peroxisome proliferator-activated receptor alpha	NM_001034036.1
PPARD	Peroxisome proliferator-activated receptor delta	NM_001083636.1
PPARG	Peroxisome proliferator-activated receptor gamma	NM_181024
PRKAA1	Protein kinase AMP-activated alpha 1	NM_001109802
Candidates reference genes		
PPIE	Peptidylprolyl isomerase E (cyclophilin E)	NM_001098161.1
SDHA	Succinate dehydrogenase complex subunit A	NM_174178
SPRYD4	SPRY domain containing 4	NM_001076173.1
UXT	Ubiquitously-expressed transcript	NM_001037471.1
XBP1	X-box binding protein 1	NM_001034727
YWHAZ	Tyrosine 3,5-monooxygenase activation protein zeta	BM446307

Table 2. Immune function related genes and accession numbers according to National Center for Biotechnology Information (NCBI)

Gene symbol	Gene description	Accession number
ATF3	Activating transcriptor factor 3	NM_001046193
Blk	B lymphocyte kinase	NM_001075968.2
C5aR	Complement component 5a receptor	NM_001007810.3
CATHL4	Cathelicidin 4	NM_174827.2
CATHL5	Cathelicidin 5	NM_174510.3
CD14	Cluster of differentiation 14	NM_174008.1
CSF3	Granulocyte-colony stimulating factor 3	NM_174028.1
DEFB3	Defensin beta 3	NM_001282581.1
DEFB4	Defensin beta 4	NM_174775.1
DEFB7	Defensin beta 7	NM_001102362.2
ERK1	Extracellular signal-regulated kinase 1	NM_001110018.1
ERK2	Extracellular signal-regulated kinase 2	NM_175793.2
IL1B	Interleukin 1 beta	NM_174093.1
IL6	Interleukin 6	NM_173923.2
IL8	Interleukin 8	NM_173925
Lyn	Tyrosine-protein kinase Lyn	AB562971.1
MAP3K7	Mitogen-activated protein kinase kinase kinase 7	NM_001081595.1
MPO	Myeloperoxidase	NM_001113298.2
MYD88	Myeloid differentiation primary response gene 88	NM_001014382.2
NFAT	Nuclear factor of activated T-Cells	NM_001166615.1
NFKB1	Nuclear factor of kappa light polypeptide enhancer in B-cells 1	NM_001076409.1
NOD2	Nucleotide-binding oligomerization domain-containing protein 2	NM_001002889.1
NOX1	NADPH oxidase 1	NM_001191340.1
NOX2	NADPH oxidase 2	NM_174035.4
PIK3CD	Phosphatidylinositol bisphosphate 3-kinase, catalytic subunit D	NM_001205548.1
Rac1	Ras-related C3 botulinum toxin substrate 1	NM_174163.2
Rac2	Ras-related C3 botulinum toxin substrate 2	NM_175792.2
RELA	v-rel avian reticuloendotheliosis viral oncogene homolog A	NM_001080242.2
RhoA	Ras homolog gene family, member A	NM_176645.3
SCARB1	Scavenger receptor class B member 1	NM_174597
SOD2	Superoxide dismutase 2	NM_201527.2
Src	Proto-oncogene tyrosine-protein kinase	NM_001110804.1
Syk	Spleen tyrosine kinase	NM_001037465.2
TLR2	Toll-like receptor 2	NM_174197.2
TLR4	Toll-like receptor 4	NM_174198.6
TNFa	Tumor necrosis factor alpha	NM_173966.2
TRAF6	TNF receptor associated factor 6	NM_001034661.2

Table 3. Effect of treatment (Trt) on metabolic parameters and haptoglobin (LSM ± SEM)

Item	Treatment[1]		*P*		
	Control (n = 16)	rbST125 (n = 16)	Trt	Day	Trt x Day
Prepartum					
IGF-1, ng/mL	117.6 ± 11.4	155.9 ± 11.4	0.02	<0.01	0.71
NEFA, µmol/L	238.0 ± 36.2	239.1 ± 36.3	0.98	0.03	0.82
BHB, µmol/L	446.4 ± 31.6	434.7 ± 31.4	0.79	0.28	0.91
Glucose, mg/dL	79.9 ± 1.2	80.5 ± 1.2	0.72	0.05	0.42
Haptoglobin, OD[2] x 100	1.5 ± 0.2	1.6 ± 0.2	0.97	0.20	0.67
Postpartum					
IGF-1, ng/mL	72.5 ± 10.6	100.0 ± 10.8	0.08	<0.01	0.76
NEFA, µmol/L	427.1 ± 73.4	411.5 ± 75.2	0.88	<0.01	0.74
BHB, µmol/L	539.2 ± 38.8	562.6 ± 39.4	0.68	0.41	0.28
Glucose, mg/dL	78.1 ± 1.8	76.4 ± 1.8	0.51	0.08	0.15
Haptoglobin, OD[2] x 100	4.2 ± 0.5	2.9 ± 0.6	0.04	<0.01	0.19

[1]Control = cows received no treatment; rbST125 = cows received 125 mg of recombinant (r) bST every 7 d from -21 to 21 d relative to calving.
[2]OD = optical density.

Table 4. Effect of treatment (Trt) on hemogram parameters and polymorphonuclear leukocyte (PMNL) expression of adhesion molecules, phagocytosis and oxidative burst (LSM ± SEM)

Item	Treatment[1]		*P*		
	Control (n = 16)	rbST125 (n = 16)	Trt	Day	Trt x Day
Prepartum					
White blood cells 10^9 x cells/L	11.5 ± 1.1	9.7 ± 1.1	0.26	0.11	0.03
Lymphocytes 10^9 x cells/L	7.4 ± 0.9	5.8 ± 0.9	0.21	0.60	0.56
Lymphocytes, %	61.8 ± 2.6	56.6 ± 2.6	0.16	<0.01	0.57
Granulocytes 10^9 x cells/L	3.3 ± 0.3	3.3 ± 0.3	0.93	<0.01	0.01
Granulocytes, %	30.5 ± 2.4	35.2 ± 2.4	0.19	<0.01	0.67
Granulocyte:lymphocyte	0.6 ± 0.1	0.7 ± 0.1	0.17	<0.01	0.74
PMNL+					
CD62L, GMFI[3]	4,458 ± 593	5,093 ± 561	0.44	0.62	0.57
CD18, GMFI	3,262 ± 280	3,926 ± 260	0.09	0.02	0.15
Phagocytosis and oxidative burst, %	65.2 ± 1.7	68.5 ± 1.7	0.18	0.01	0.55
Phagocytosis intensity, GMFI	4,476 ± 344	5,098 ± 355	0.22	0.55	0.80
Oxidative burst intensity, GMFI	13,511 ± 1,137	15,553 ± 1,173	0.22	0.23	0.41
Postpartum					
White blood cells 10^9 x cells/L	10.7 ± 0.9	9.2 ± 0.9	0.25	0.69	0.57
Lymphocytes 10^9 x cells/L	6.4 ± 0.7	4.9 ± 0.7	0.15	0.27	0.92
Lymphocytes, %	60.8 ± 3.8	54.1 ± 3.9	0.22	0.11	0.38
Granulocytes 10^9 x cells/L	3.7 ± 0.5	3.6 ± 0.5	0.92	0.28	0.24
Granulocytes, %	33.3 ± 3.7	39.8 ± 3.8	0.23	0.02	0.40
Granulocyte:lymphocyte	0.7 ± 0.1	0.9 ± 0.1	0.17	0.08	0.51
PMNL+					
CD62L, GMFI[3]	5,666 ± 921	6,408 ± 1,021	0.59	0.81	0.25
CD18, GMFI	3,033 ± 269	2,943 ± 312	0.83	0.17	0.80
Phagocytosis and oxidative burst, %	61.2 ± 2.1	60.7 ± 2.4	0.87	0.23	0.10
Phagocytosis intensity, GMFI	4,743 ± 399	4,892 ± 460	0.81	0.54	0.18
Oxidative burst intensity, GMFI	12,896 ± 1,446	13,497 ± 1,690	0.79	0.14	0.69

[1]Control = cows received no treatment; rbST125 = cows received 125 mg of recombinant (r) bST every 7 d from -21 to 21 d relative to calving.
[3]Geometric mean fluorescence intensity.

Table 5. Effect of treatment[1] on leukocyte mRNA expression for genes related to somatotropic axis and energy metabolism (mRNA counts/100 ng of RNA; LSM ± SEM)

Gene	Day -21		*P*	Day -7		*P*	Day 7		*P*
	Control[1] (n = 16)	rbST125[1] (n = 16)		Control (n = 16)	rbST125 (n = 16)		Control (n = 16)	rbST125 (n = 16)	
Somatotropic axis									
GHR	13 ± 4	17 ± 5	0.21	18 ± 4	15 ± 4	0.16	15 ± 4	14 ± 3	0.62
IGF1R	114 ± 10	160 ± 14	0.01	170 ± 10	140 ± 8	0.03	146 ± 14	169 ± 16	0.29
JAK1	3,246 ± 76	3,179 ± 76	0.55	3,291 ± 74	3,056 ± 74	0.04	3,037 ± 114	3,016 ± 114	0.90
JAK2	310 ± 19	351 ± 19	0.14	393 ± 11	382 ± 11	0.47	344 ± 21	366 ± 21	0.47
JAK3	670 ± 24	666 ± 24	0.90	676 ± 26	621 ± 26	0.15	720 ± 45	735 ± 45	0.81
SOCS2	59 ± 9	59 ± 9	0.97	46 ± 5	39 ± 5	0.38	45 ± 6	55 ± 6	0.27
STAT1	1,284 ± 81	1,465 ± 81	0.12	1,492 ± 61	1,578 ± 61	0.33	1,565 ± 159	1,543 ± 159	0.93
STAT2	418 ± 28	497 ± 28	0.06	502 ± 22	534 ± 22	0.34	592 ± 54	541 ± 54	0.52
STAT3	708 ± 24	772 ± 24	0.08	796 ± 18	820 ± 18	0.38	821 ± 51	847 ± 51	0.74
STAT4	208 ± 18	186 ± 18	0.41	201 ± 12	167 ± 12	0.06	188 ± 14	157 ± 14	0.14
STAT5a	740 ± 27	746 ± 27	0.88	797 ± 28	754 ± 26	0.27	737 ± 38	720 ± 37	0.76
STAT5b	332 ± 21	370 ± 21	0.22	379 ± 12	369 ± 12	0.59	397 ± 26	383 ± 26	0.72
Cell energy metabolism									
INSR	87 ± 10	93 ± 11	0.52	97 ± 8	93 ± 8	0.38	82 ± 12	104 ± 13	0.06
ACOX1	429 ± 21	494 ± 24	0.05	511 ± 21	513 ± 21	0.95	518 ± 34	579 ± 37	0.24
GSK3B	419 ± 34	398 ± 33	0.61	453 ± 26	443 ± 26	0.66	480 ± 37	504 ± 38	0.60
LDHA	2,880 ± 163	3,322 ± 175	0.24	3,288 ± 157	3,769 ± 168	0.15	3,914 ± 193	4,773 ± 213	0.06
PC1	19 ± 2	26 ± 3	0.02	18 ± 2	22 ± 2	0.17	24 ± 4	33 ± 5	0.15
PCK1	4 ± 0.8	8 ± 2	0.01	7 ± 1	6 ± 1	0.90	6 ± 1	7 ± 1	0.26
PCK2	88 ± 11	106 ± 12	0.08	109 ± 11	118 ± 11	0.32	106 ± 14	129 ± 16	0.14
PPARa	39 ± 3	43 ± 4	0.47	48 ± 3	47 ± 3	0.79	40 ± 5	45 ± 5	0.51
PPARd	136 ± 11	165 ± 13	0.10	165 ± 8	183 ± 9	0.17	192 ± 14	194 ± 14	0.91
PPARg	30 ± 5	33 ± 5	0.70	40 ± 5	47 ± 5	0.33	37 ± 6	39 ± 6	0.84
PRKAA1	442 ± 24	505 ± 28	0.10	499 ± 15	494 ± 15	0.82	546 ± 32	548 ± 32	0.95

[1]Control = cows received no treatment; rbST125 = cows received 125 mg of recombinant (r) bST every 7 d from -21 to 21 d relative to calving.

Table 6. Effect of treatment[1] on leukocyte mRNA expression for genes related to innate and adaptive immune responses (mRNA counts/ 100 ng of RNA; LSM ± SEM)

Gene	Day -21		*P*	Day -7		*P*	Day 7		*P*
	Control[1] (n = 16)	rbST125[1] (n = 16)		Control (n = 16)	rbST125 (n = 16)		Control (n = 16)	rbST125 (n = 16)	
ATF3	7 ± 2	8 ± 2	0.80	12 ± 2	9 ± 2	0.39	6 ± 1	5 ± 1	0.84
Blk	136 ± 9	105 ± 9	0.02	123 ± 7	104 ± 7	0.06	81 ± 6	62 ± 6	0.05
CATHL4	1 ± 0.5	1 ± 0.6	0.75	2 ± 0.7	1 ± 0.7	0.36	6 ± 3	4 ± 2	0.60
CATHL5	1 ± 0.1	1 ± 0.1	0.43	1 ± 0.1	1 ± 0.1	0.90	2 ± 0.7	2 ± 0.6	0.80
CD14	185 ± 22	215 ± 2	0.35	218 ± 24	291 ± 24	0.05	309 ± 40	348 ± 40	0.51
C5aR	99 ± 9	136 ± 9	<0.01	110 ± 6	137 ± 6	<0.01	100 ± 13	118 ± 13	0.34
DEFB3	146 ± 22	192 ± 29	0.23	217 ± 22	152 ± 15	0.02	513 ± 115	415 ± 93	0.51
DEFB4	35 ± 7	49 ± 10	0.25	42 ± 7	57 ± 9	0.18	141 ± 37	122 ± 32	0.71
DEFB7	33 ± 7	52 ± 7	0.08	34 ± 5	48 ± 5	0.08	97 ± 24	81 ± 20	0.61
ERK1	103 ± 5	111 ± 5	0.24	112 ± 5	102 ± 5	0.18	114 ± 6	114 ± 6	0.98
ERK2	1,162 ± 25	1,180 ± 25	0.62	1,197 ± 26	1,216 ± 26	0.60	1,459 ± 127	1,522 ± 127	0.73
IL1B	979 ± 204	1,583 ± 204	0.05	1,372 ± 104	1,383 ± 104	0.95	1,124 ± 122	1,141 ± 122	0.93
IL8	202 ± 107	325 ± 107	0.43	238 ± 36	207 ± 36	0.55	254 ± 41	252 ± 41	0.98
Lyn	5,588 ± 136	5,798 ± 136	0.29	5,999 ± 127	5,785 ± 127	0.24	4,687 ± 172	5,319 ± 183	0.06
MAP3K7	256 ± 8	243 ± 8	0.28	240 ± 7	237 ± 7	0.73	231 ± 10	233 ± 10	0.88
MPO	15 ± 3	14 ± 3	0.82	14 ± 2	11 ± 2	0.35	8 ± 3	9 ± 3	0.87
MYD88	2,392 ± 111	2,795 ± 111	0.02	3,037 ± 96	3,299 ± 96	0.08	3,200 ± 200	3,209 ± 200	0.97
NFAT	302 ± 14	301 ± 14	0.96	300 ± 15	250 ± 15	0.03	216 ± 13	197 ± 13	0.30
NFKB1	795 ± 23	803 ± 23	0.81	808 ± 18	777 ± 18	0.24	735 ± 27	776 ± 27	0.29
NOD2	43 ± 5	49 ± 5	0.38	50 ± 4	62 ± 4	0.06	56 ± 6	65 ± 6	0.28
NOX1	3 ± 0.7	3 ± 0.7	0.96	2 ± 0.5	2 ± 0.5	0.99	2 ± 0.4	3 ± 0.5	0.19
NOX2	1,498 ± 63	1,484 ± 63	0.88	1,508 ± 38	1,524 ± 38	0.76	1,602 ± 107	1,610 ± 107	0.96
PIK3CD	1,271 ± 59	1,379 ± 59	0.22	1,419 ± 34	1,464 ± 34	0.39	1,562 ± 127	1,573 ± 127	0.95
Rac1	2,269 ± 55	2,409 ± 55	0.09	2,398 ± 43	2,503 ± 43	0.10	2,399 ± 77	2,573 ± 77	0.13
Rac2	4,124 ± 113	4,133 ± 113	0.95	4,135 ± 120	3,912 ± 116	0.16	4,613 ± 226	4,747 ± 230	0.80
RELA	220 ± 9	243 ± 9	0.06	236 ± 10	232 ± 10	0.81	209 ± 8	217 ± 8	0.52
RhoA	3,228 ± 48	3,212 ± 48	0.82	3,295 ± 41	3,252 ± 41	0.46	3,201 ± 92	3,223 ± 92	0.87
SCARB1	193 ± 16	214 ± 16	0.39	254 ± 14	290 ± 14	0.08	314 ± 33	359 ± 34	0.37
SOD2	2,014 ± 217	2,835 ± 217	0.01	3,465 ± 281	3,985 ± 281	0.25	3,423 ± 594	4,522 ± 594	0.22
Syk	1,604 ± 97	1,685 ± 97	0.57	1,536 ± 52	1,473 ± 52	0.41	1,355 ± 101	1,430 ± 101	0.60
TLR2	505 ± 20	516 ± 20	0.70	548 ± 20	562 ± 20	0.65	551 ± 41	645 ± 45	0.09
TLR4	395 ± 30	464 ± 30	0.12	484 ± 22	490 ± 22	0.86	515 ± 40	549 ± 40	0.57
TNFa	97 ± 9	103 ± 9	0.63	97 ± 8	90 ± 8	0.58	107 ± 8	102 ± 8	0.63
TRAF6	141 ± 4	142 ± 4	0.80	147 ± 5	139 ± 5	0.28	130 ± 10	139 ± 11	0.17

[1]Control = cows received no treatment; rbST125 = cows received 125 mg of recombinant (r) bST every 7 d from -21 to 21 d relative to calving.

Table 1. Composition of TMR offered to far-off (d -55 ± 3 to -31 ± 3 relative to calving), close-up (d -31 ± 3 to 0 relative to calving) and postpartum (d 1 to 21 ± 3 relative to calving) cows

Item, % of DM	Parous			Nulliparous		
(unless otherwise indicated)	Far-off	Close-up	Postpartum	Far-off	Close-up	Postpartum
DM, %	42.33	40.44	46.08	42.92	43.01	45.35
NE_L, Mcal/kg	1.45	1.39	1.64	1.37	1.43	1.65
CP	15.05	14.62	19.49	14.38	14.98	17.64
ADF	33.33	32.79	21.44	34.55	32.24	20.66
NDF	47.05	47.38	32.40	49.83	47.66	32.93
Ether extract	4.82	3.36	5.10	3.85	3.88	5.35
Ash	9.24	9.80	8.52	9.96	9.09	8.21
Calcium	0.91	1.24	1.03	0.99	0.81	1.08
Phosphorus	0.33	0.33	0.42	0.34	0.36	0.48
Magnesium	0.42	0.35	0.35	0.40	0.36	0.35
Potassium	1.46	1.09	1.40	1.47	1.19	1.25
Sulfur	0.25	0.31	0.30	0.25	0.26	0.31
Sodium	0.23	0.15	0.47	0.23	0.13	0.37
Chlorine	0.60	0.97	0.48	0.62	0.47	0.33

Table 2. Effects of prepartum stocking density on incidence of postpartum health disorders, lameness, and removal from the herd within 60 d postpartum

Item	80SD[1], %	100SD[1], %	AOR[2] (95% CI)	*P* – value
Retained fetal membranes	5.1	7.8	1.55 (0.78, 3.07)	0.19
Metritis	21.2	16.7	0.71 (0.46, 1.09)	0.11
Acute metritis	9.9	9.4	0.87 (0.45, 1.66)	0.64
Vaginal purulent discharge at 35 ± 3 DIM	5.8	7.9	1.41 (0.65, 3.05)	0.35
Mastitis up to 60 DIM	2.9	4.6	1.94 (0.70, 5.39)	0.18
Displacement of abomasum up to 60 DIM	1.0	0.7	0.76 (0.10, 5.80)	0.78
Locomotion score > 2 at 1 ± 1 DIM	0.6	0.0	0.26 (0.02, 3.19)	0.27
Locomotion score > 2 at 35 ± 3 DIM	3.8	2.6	0.66 (0.25, 1.75)	0.37
Locomotion score > 2 at 56 ± 3 DIM	3.5	2.1	0.56 (0.12, 2.69)	0.44
Removed within 60 DIM	6.1	5.1	0.84 (0.38, 1.83)	0.63

[1]80SD = cows housed in prepartum pens with 80% target headlock stocking density (38/48); 100SD = cows housed in prepartum pens with 100% target headlock stocking density (48/48).
[2]AOR = Adjusted odds ratio. The 80SD treatment was set as referent.

Table 3. Effects of prepartum stocking density on resumption of ovarian cycles postpartum, estrus expression, percentage of cows pregnant after first and second postpartum AI, and number of inseminations

Item	80SD[1]	100SD[1]	AOR[2] (95% CI)	*P* – value
Cows cyclic by 35 DIM[3], %	45.5	48.2	1.19 (0.72, 1.95)	0.47
Cows cyclic by 45DIM[3], %	60.0	66.3	1.30 (0.67, 2.55)	0.40
First postpartum AI				
Cows inseminated in estrus, %	85.0	88.4	1.24 (0.70, 2.21)	0.43
DIM (±SEM)	57.4 ± 2.5	56.7 ± 2.4	NA	0.73
Pregnant at 31 ± 3 d, %	46.6	52.6	1.18 (0.83, 1.67)	0.33
Pregnant at 66 ± 3 d, %	41.9	48.4	1.23 (0.87, 1.74)	0.23
Pregnancy loss from 31 ± 3 to 66 ± 3 d, %	8.7	6.8	0.82 (0.26, 2.57)	0.72
Interval from first to second AI (±SEM)[3]	30.2 ± 1.1	29.5 ± 1.0	NA	0.69
Second postpartum AI				
DIM (±SEM)[4]	100.0 ± 1.0	98.0 ± 1.0	NA	0.47
Pregnant at 31 ± 3 d, %	52.9	46.3	0.74 (0.44, 1.26)	0.25
Pregnant at 66 ± 3 d, %	49.3	42.0	0.72 (0.43, 1.21)	0.20
Pregnancy loss from 31 ± 3 to 66 ± 3 d, %	5.1	7.6	1.47 (0.31, 6.98)	0.61

[1]80SD = cows housed in prepartum pens with 80% target headlock stocking density (38/48); 100SD = cows housed in prepartum pens with 100% target headlock stocking density (48/48).
[2]AOR = Adjusted odds ratio. The 80SD treatment was set as referent.
[3]Cows cyclic by 35 DIM = cows with progesterone concentration > 1 ng/mL at 39 DIM; Cows cyclic by 45 DIM = cows with progesterone concentration > 1 ng/mL at 35 or 45 DIM, or both.
[4]Values are back-transformed logarithmic values.

Table 4. Monthly productive parameters and milk quality up to 155 d postpartum of cows submitted to different prepartum stocking densities

Item, kg/d	80SD[1]	100SD[1]	*P* – value
Milk yield	28.49 ± 0.53	28.17 ± 0.50	0.68
Fat yield	1.33 ± 0.02	1.31 ± 0.02	0.47
Protein yield	1.01 ± 0.02	1.00 ± 0.01	0.85
3.5% FCM	33.90 ± 0.52	33.39 ± 0.48	0.48
ECM yield	33.84 ± 0.52	33.39 ± 0.48	0.54

[1]80SD = cows housed in prepartum pens with 80% target headlock stocking density (38/48); 100SD = cows housed in prepartum pens with 100% target headlock stocking density (48/48).

Table 1. Percentage (mean ± SEM) of animals delivering males, twins, stillborn calves and incidence of peripartum diseases of the sub-groups of 80SD[1] and 100SD[1] animals used to evaluate immune parameters and hair cortisol concentration

Items	80SD[1], %	100SD[1], %	*P* – value
Male calf	47.92	51.06	0.78
Twins*	4.17	0.00	0.60
Stillborn calves*	4.17	0.00	0.60
Retained fetal membranes	4.17	8.33	0.54
Metritis	25.00	14.89	0.22
Acute metritis	12.50	8.33	0.59
Vaginal purulent discharge	0.00	9.30	0.58
Mastitis within 60 DIM**	6.25	4.17	0.66
Removed from the herd within 60 DIM	8.33	8.33	1.00

[1]80SD – pre-partum pens' target stocking density = 80% of headlocks; and, 100SD – pre-partum pens' target stocking density = 100% of headlocks.

*Data referent to parous animals because no nulliparous animals had twins or stillborn calves.

**Data referent to nulliparous animals because no parous animals had mastitis within 60 DIM.

Table 2. Effects of prepartum stocking density[1] on hemogram, immune and adaptive parameters

	Treatment		*P* – value		
Item	80SD[1]	100SD[1]	TRT	Day	TRT x Day
Granulocytes, %*	35.58 ± 1.30	36.35 ± 1.33	0.69	< 0.01	0.08
Granulocyte:lymphocyte	0.70 ± 0.04	0.71 ± 0.04	0.91	< 0.01	0.28
PMNL leukocytes					
Phagocytosis and oxidative burst positive, %	58.98 ± 1.69	57.85 ± 1.71	0.65	< 0.01	0.57
Intensity of phagocytosis, GMFI	3,308.38 ± 87.78	3,293.70 ± 89.50	0.91	< 0.01	0.84
Intensity of oxidative burst, GMFI	8,774.35 ± 784.56	9,007.82 ± 791.47	0.84	< 0.01	0.29
CD18 expression positive, %	98.87 ± 0.24	99.43 ± 0.25	0.13	0.18	0.15
CD18 expression intensity, GMFI	3,593.00 ± 279.40	4,028.44 ± 282.29	0.29	< 0.01	0.30
L-Selectin expression positive, %	97.79 ± 0.50	97.36 ± 0.51	0.56	< 0.01	0.61
L-Selectin expression intensity, GMFI	4,122.53 ± 211.31	4,330.90 ± 217.81	0.71	< 0.01	0.78
IgG anti-ovalbumin, OD x 10^3	1.42 ± 0.08	1.50 ± 0.08	0.50	< 0.01	0.56

[1]80SD – pre-partum pens' target stocking density = 80% of headlocks; and, 100SD – pre-partum pens' target stocking density = 100% of headlocks.

*Percentage of leukocytes classified as granulocytes.

Table 3. Effects of prepartum stocking density[1] on glucose, NEFA, BHBA, haptoglobin and cortisol concentrations

Items	Treatments		*P* – value		
	80SD[1]	100SD[1]	TRT	Day	TRT x Day
Glucose, mg/dL	68.62 ± 1.11	69.55 ± 1.12	0.57	< 0.01	0.84
NEFA, µmol/L	242.30 ± 11.66	249.82 ± 11.64	0.66	< 0.01	0.52
BHBA, µmol/L	503.96 ± 28.02	448.25 ± 27.94	0.18	< 0.01	0.23
Haptoglobin, OD x 10^2	7.46 ± 0.23	7.08 ± 0.23	0.27	< 0.01	0.83
Serum cortisol, ng/mL	6.79 ± 0.46	6.16 ± 0.47	0.64	< 0.01	0.81

[1]80SD – pre-partum pens' target stocking density = 80% of headlocks; and, 100SD – pre-partum pens' target stocking density = 100% of headlocks.

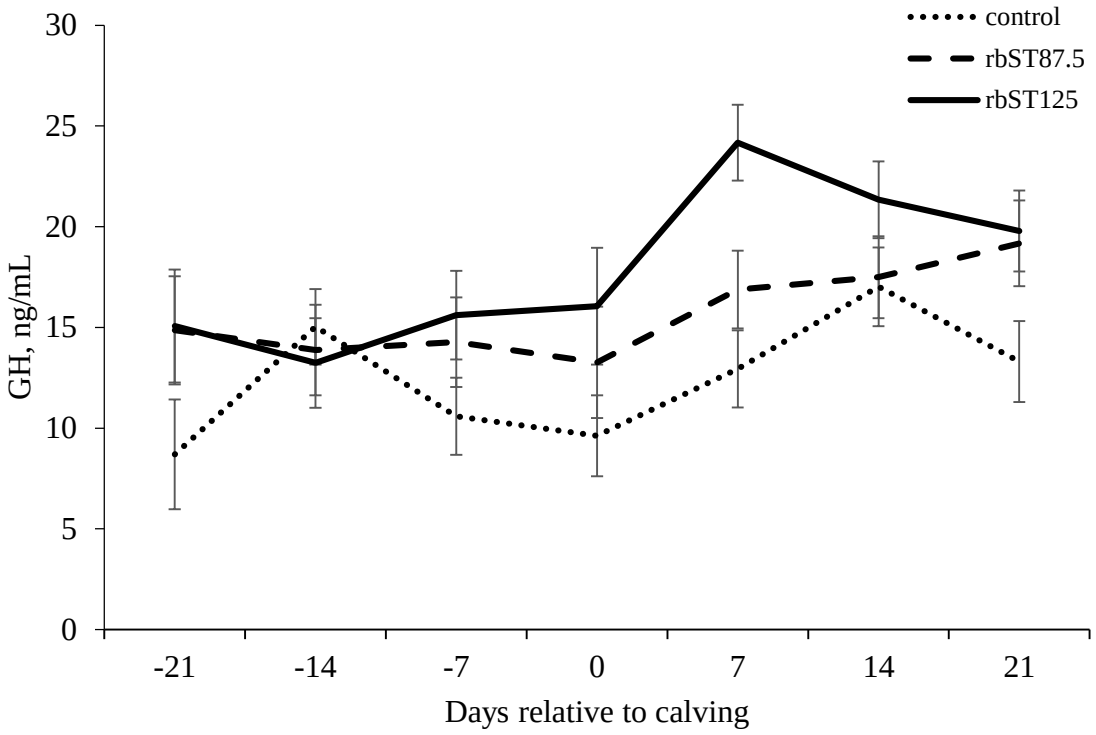


Figure 1A. Growth hormone concentration according to treatment. Treatments: control = cows received no treatment; rbST87.5 = cows received 87.5 mg of rbST every 7 d from -21 to 28 d relative to calving; and, rbST125 = cows received 125 mg of rbST every 7 d from -21 to 28 d relative to calving. *Prepartum period* - Treatment (P = 0.06), day (P = 0.94), and interaction between treatment and day (P = 0.61). Contrasts: control vs. rbST87.5 - P = 0.08, control vs. rbST125 - P = 0.02, rbST87.5 vs. rbST125 - P = 0.60, linear - P = 0.18, and quadratic - P = 0.05. *Postpartum period* - Treatment (P < 0.01), day (P = 0.76), and interaction between treatment and day (P = 0.25). Contrasts: control vs. rbST87.5 - P = 0.04, control vs. rbST125 - P < 0.01, rbST87.5 vs. rbST125 - P = 0.19, linear - P = 0.26, and quadratic - P < 0.01.

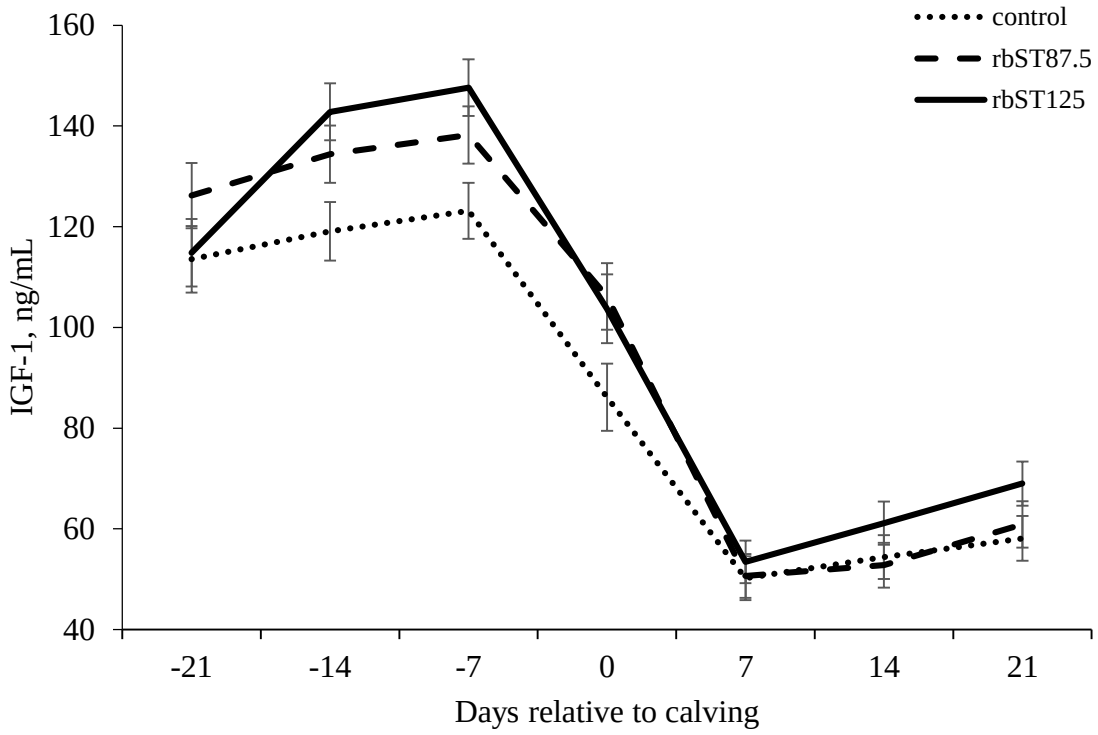


Figure 1B. Insulin-like growth factor 1 concentration according to treatment. Treatments: control = cows received no treatment; rbST87.5 = cows received 87.5 mg of rbST every 7 d from -21 to 28 d relative to calving; and, rbST125 = cows received 125 mg of rbST every 7 d from -21 to 28 d relative to calving. *Prepartum period* - Treatment ($P = 0.01$), day ($P < 0.01$), and interaction between treatment and day ($P = 0.16$). Contrasts: control vs. rbST87.5 - $P = 0.01$, control vs. rbST125 - $P = 0.01$, rbST87.5 vs. rbST125 - $P = 0.88$, linear - $P = 0.04$, and quadratic - $P = 0.04$. *Postpartum period* - Treatment ($P = 0.38$), day ($P < 0.01$), and interaction between treatment and day ($P = 0.49$).

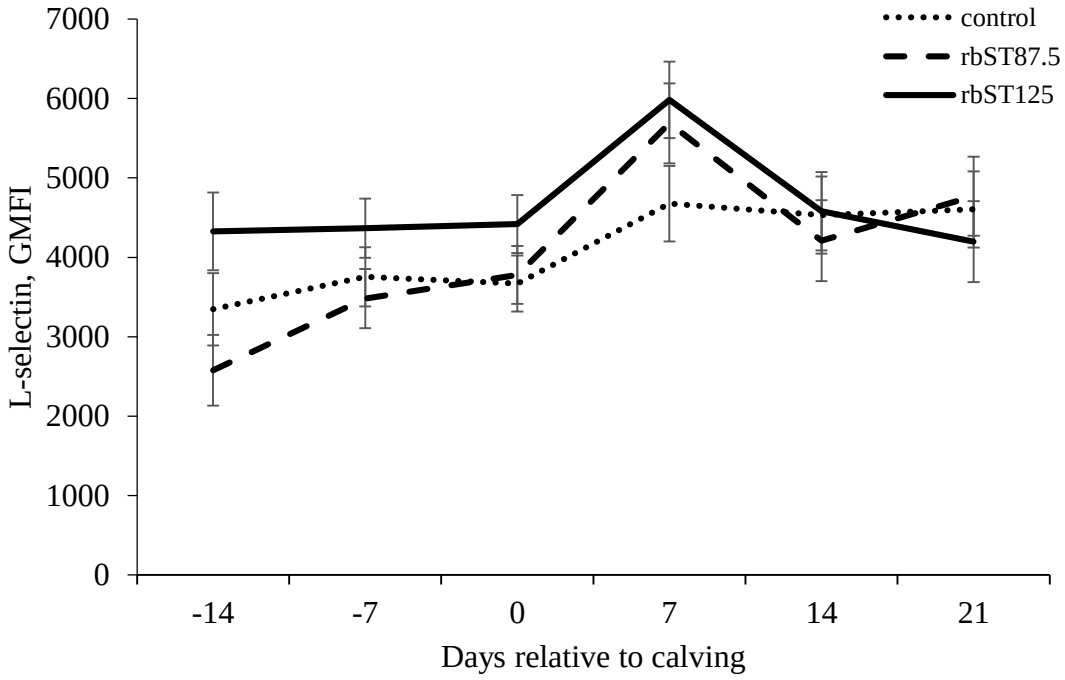


Figure 2A. Geometric mean fluorescence intensity (GMFI) of PMNL L-selectin expression according to treatment. Treatments: control = cows received no treatment; rbST87.5 = cows received 87.5 mg of rbST every 7 d from -21 to 28 d relative to calving; and, rbST125 = cows received 125 mg of rbST every 7 d from -21 to 28 d relative to calving. *Prepartum period* - Treatment ($P = 0.02$), day ($P = 0.23$), and interaction between treatment and day ($P = 0.68$). Contrasts: control vs. rbST87.5 - $P = 0.42$, control vs. rbST125 - $P = 0.05$, rbST87.5 vs. rbST125 - $P < 0.01$, linear - $P = 0.16$, and quadratic - $P = 0.01$. *Postpartum period* - Treatment ($P = 0.77$), day ($P < 0.01$), and interaction between treatment and day ($P = 0.27$).

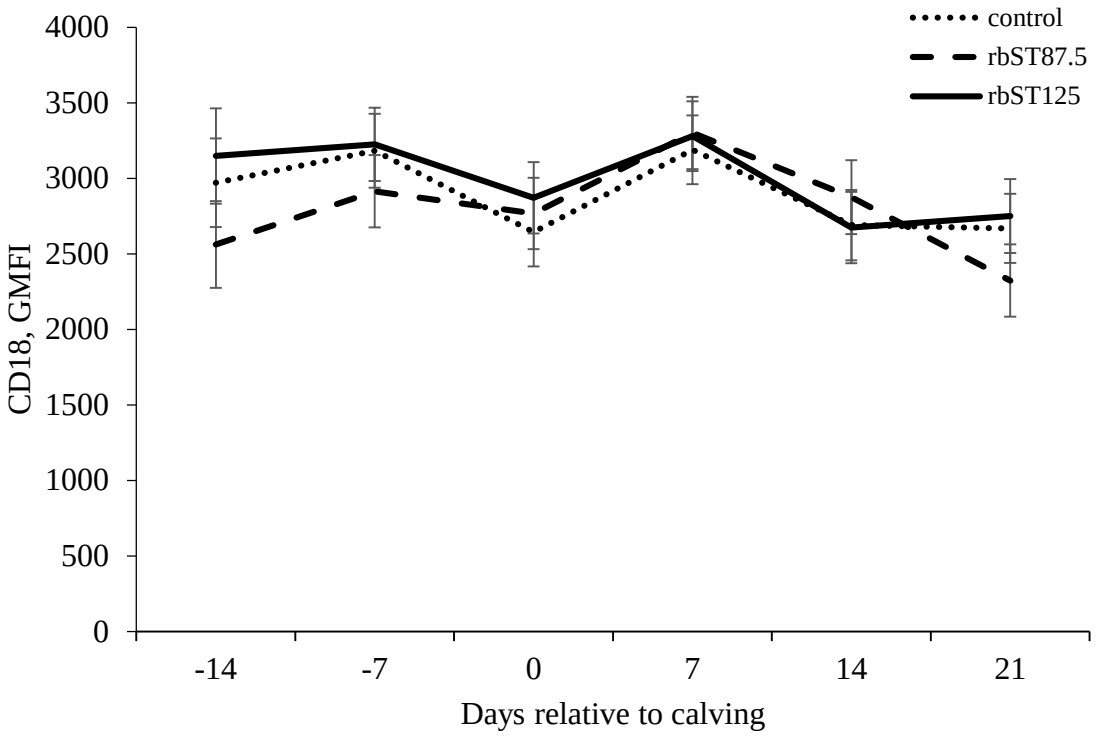


Figure 2B. Geometric mean fluorescence intensity (GMFI) of PMNL CD18 expression according to treatment. Treatments: control = cows received no treatment; rbST87.5 = cows received 87.5 mg of rbST every 7 d from -21 to 28 d relative to calving; and, rbST125 = cows received 125 mg of rbST every 7 d from -21 to 28 d relative to calving. *Prepartum period* - Treatment ($P = 0.43$), day ($P = 0.10$), and interaction between treatment and day ($P = 0.78$). *Postpartum period* - Treatment ($P = 0.96$), day ($P < 0.01$), and interaction between treatment and day ($P = 0.50$).

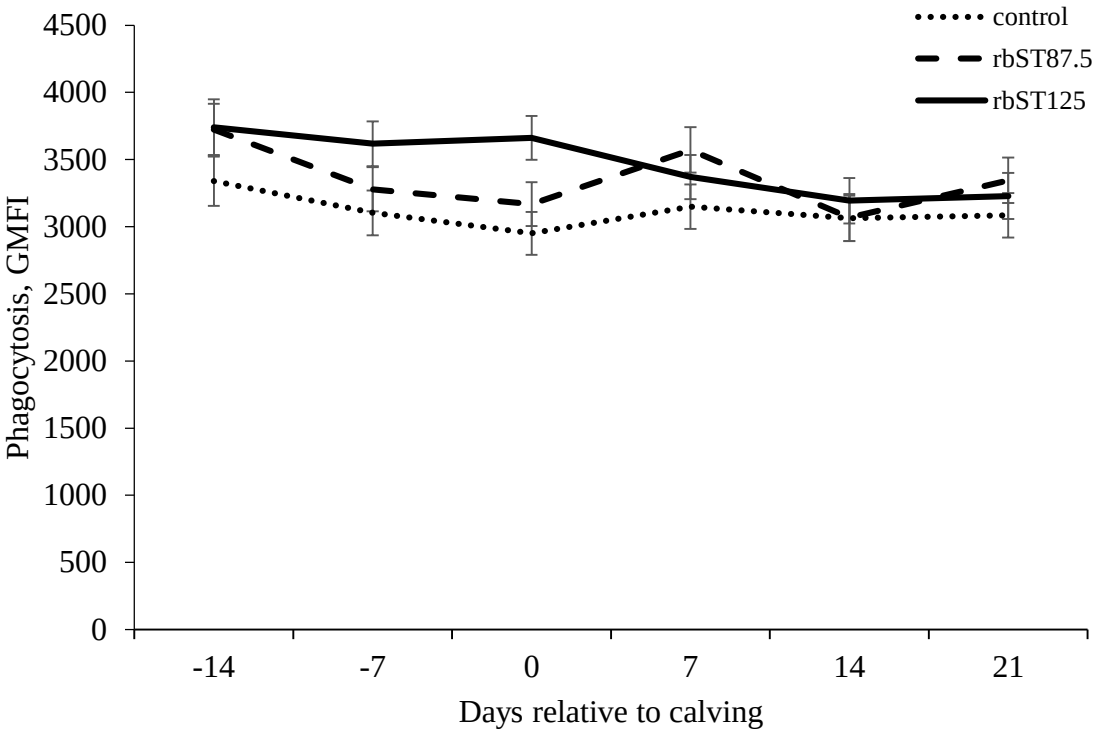


Figure 3A. Geometric mean fluorescence intensity (GMFI) of PMNL positive for phagocytosis according to treatment. Treatments: control = cows received no treatment; rbST87.5 = cows received 87.5 mg of rbST every 7 d from -21 to 28 d relative to calving; and, rbST125 = cows received 125 mg of rbST every 7 d from -21 to 28 d relative to calving. *Prepartum period* - Treatment ($P = 0.02$), day ($P = 0.02$), and interaction between treatment and day ($P = 0.57$). Contrasts: control vs. rbST87.5 - $P = 0.17$, control vs. rbST125 - $P < 0.01$, rbST87.5 vs. rbST125 - $P = 0.14$, linear - $P = 0.44$, and quadratic - $P < 0.01$. *Postpartum period* - Treatment ($P = 0.35$), day ($P = 0.12$), and interaction between treatment and day ($P = 0.70$).

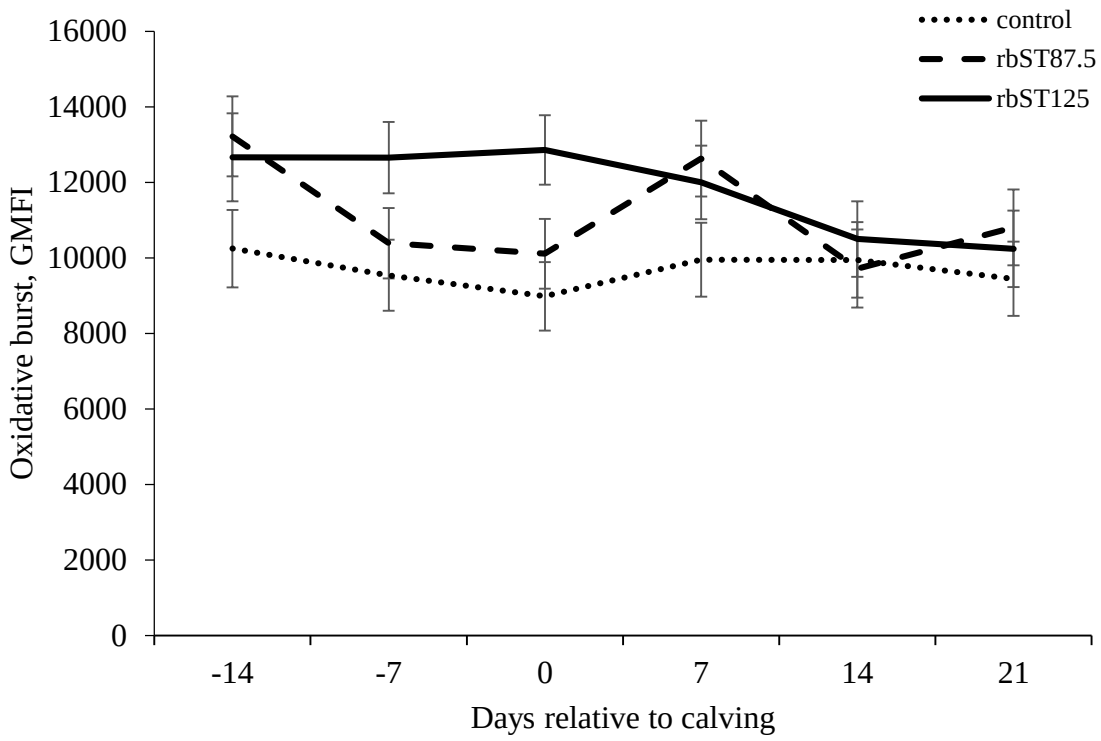


Figure 3B. Geometric mean fluorescence intensity (GMFI) of PMNL positive for oxidative burst according to treatment. Treatments: control = cows received no treatment; rbST87.5 = cows received 87.5 mg of rbST every 7 d from -21 to 28 d relative to calving; and, rbST125 = cows received 125 mg of rbST every 7 d from -21 to 28 d relative to calving. *Prepartum period* - Treatment ($P = 0.02$), day ($P = 0.07$), and interaction between treatment and day ($P = 0.28$). Contrasts: control vs. rbST87.5 - $P = 0.13$, control vs. rbST125 - $P < 0.01$, rbST87.5 vs. rbST125 - $P = 0.18$, linear - $P = 0.35$, and quadratic - $P < 0.01$. *Postpartum period* - Treatment ($P = 0.43$), day ($P = 0.03$), and interaction between treatment and day ($P = 0.50$).

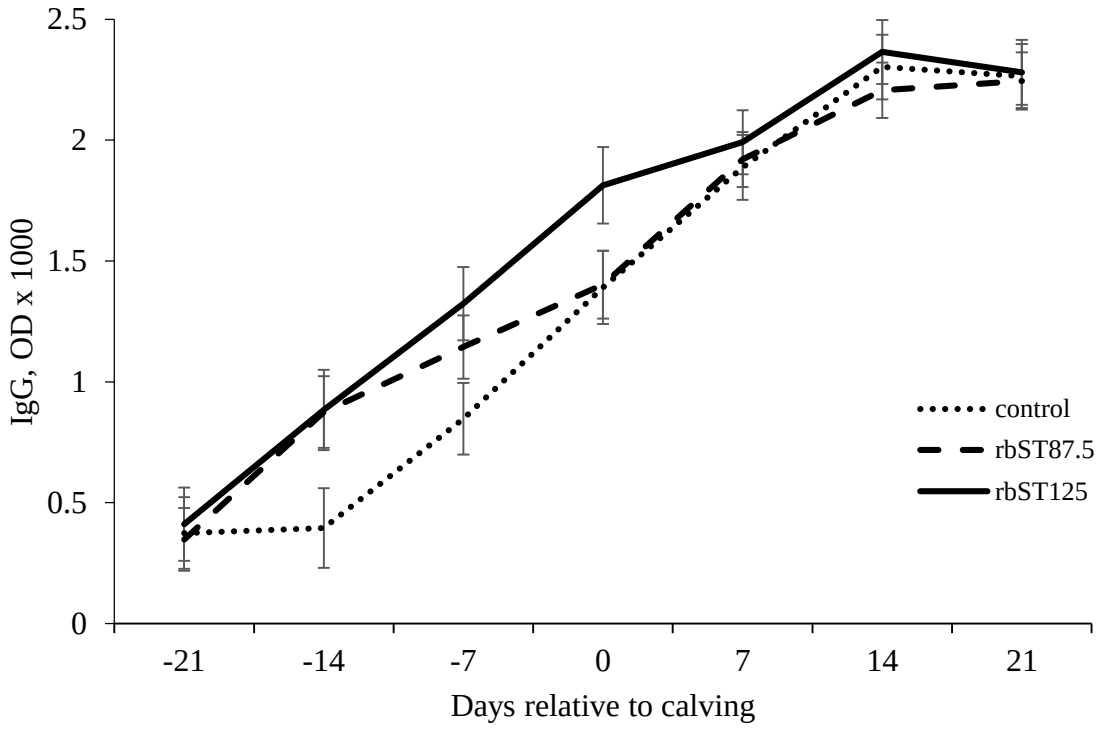


Figure 4. Immunoglobulin G anti-ovalbumin optical density (OD) according to treatment. Ovalbumin immunizations were performed on d -21, -7, and 7 relative to calving. Treatments: control = cows received no treatment; rbST87.5 = cows received 87.5 mg of rbST every 7 d from -21 to 28 d relative to calving; and, rbST125 = cows received 125 mg of rbST every 7 d from -21 to 28 d relative to calving. *Prepartum period* - Treatment (P = 0.09), day ($P < 0.01$), and interaction between treatment and day (P = 0.16). Contrasts: control vs. rbST87.5 - P = 0.20, control vs. rbST125 - P = 0.03, rbST87.5 vs. rbST125 - P = 0.28, linear - P = 0.43, and quadratic - P = 0.04. *Postpartum period* - Treatment (P = 0.83), day ($P < 0.01$), and interaction between treatment and day (P = 0.88).

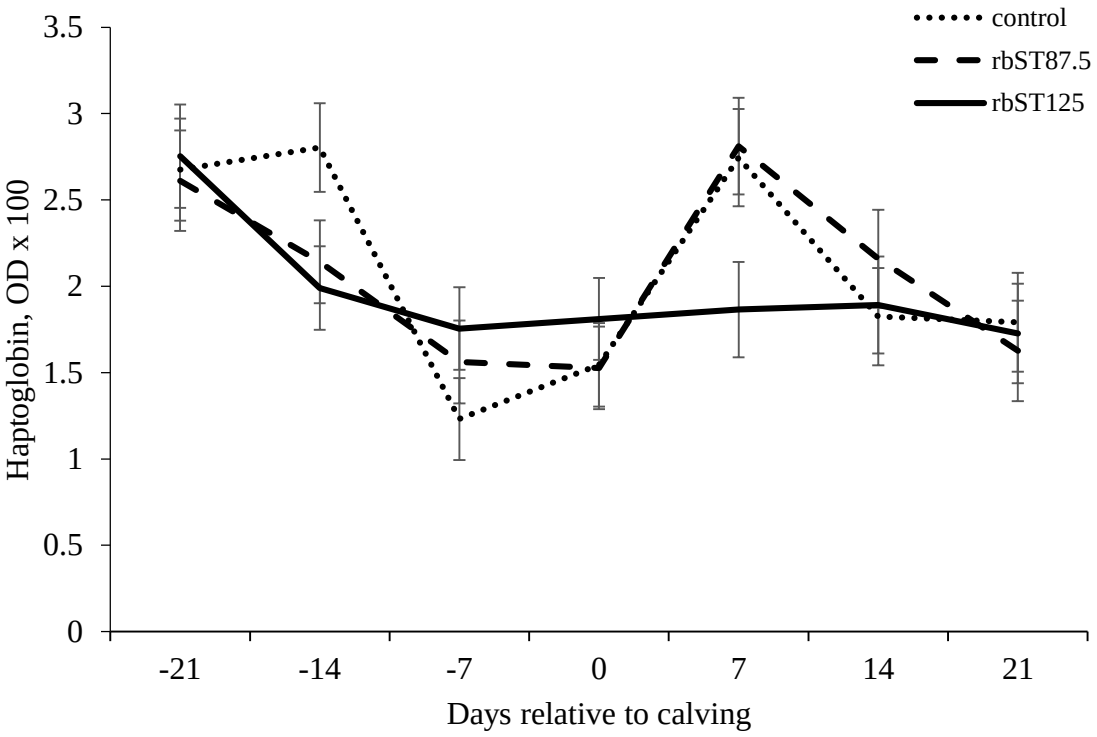


Figure 5A. Haptoglobin optical density (OD) according to treatment. Treatments: control = cows received no treatment; rbST87.5 = cows received 87.5 mg of rbST every 7 d from -21 to 28 d relative to calving; and, rbST125 = cows received 125 mg of rbST every 7 d from -21 to 28 d relative to calving. *Prepartum period* - Treatment ($P = 0.74$), day ($P < 0.01$), and interaction between treatment and day ($P = 0.21$). *Postpartum period* - Treatment ($P = 0.45$), day ($P < 0.01$), and interaction between treatment and day ($P = 0.08$). Contrasts on d 7 postpartum: control vs. rbST87.5 - $P = 0.87$, control vs. rbST125 - $P = 0.03$, and rbST87.5 vs. rbST125 - $P = 0.02$.

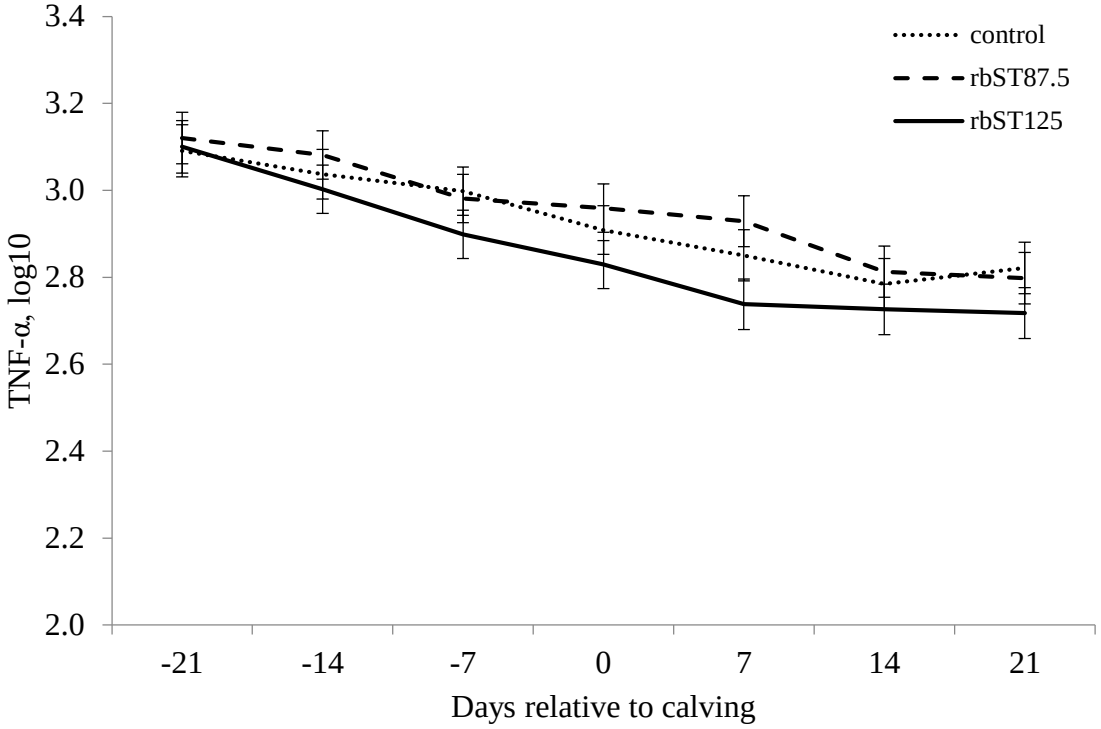


Figure 5B. Tumor necrosis factor-alpha (TNF-α) concentration (pg/mL) according to treatment. Treatments: control = cows received no treatment; rbST87.5 = cows received 87.5 mg of rbST every 7 d from -21 to 28 d relative to calving; and, rbST125 = cows received 125 mg of rbST every 7 d from -21 to 28 d relative to calving. *Prepartum period* - Treatment ($P = 0.56$), day ($P < 0.01$), and interaction between treatment and day ($P = 0.38$). *Postpartum period* - Treatment ($P = 0.27$), day ($P < 0.01$), and interaction between treatment and day ($P = 0.17$).

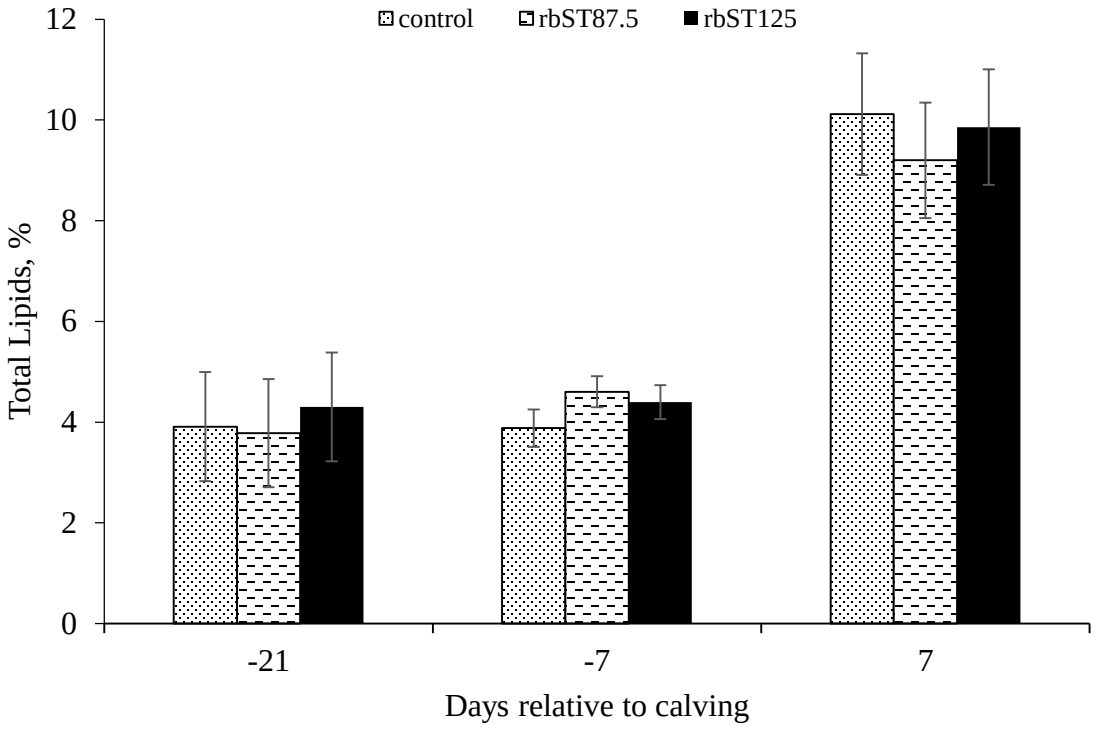


Figure 6A. Liver total lipids percentage according to treatment. Treatments: control = cows received no treatment; rbST87.5 = cows received 87.5 mg of rbST every 7 d from -21 to 28 d relative to calving; and, rbST125 = cows received 125 mg of rbST every 7 d from -21 to 28 d relative to calving. *Day -21* - Treatment (P = 0.45). *Day -7* - Treatment (P = 0.33). *Day 7*- Treatment (P = 0.85).

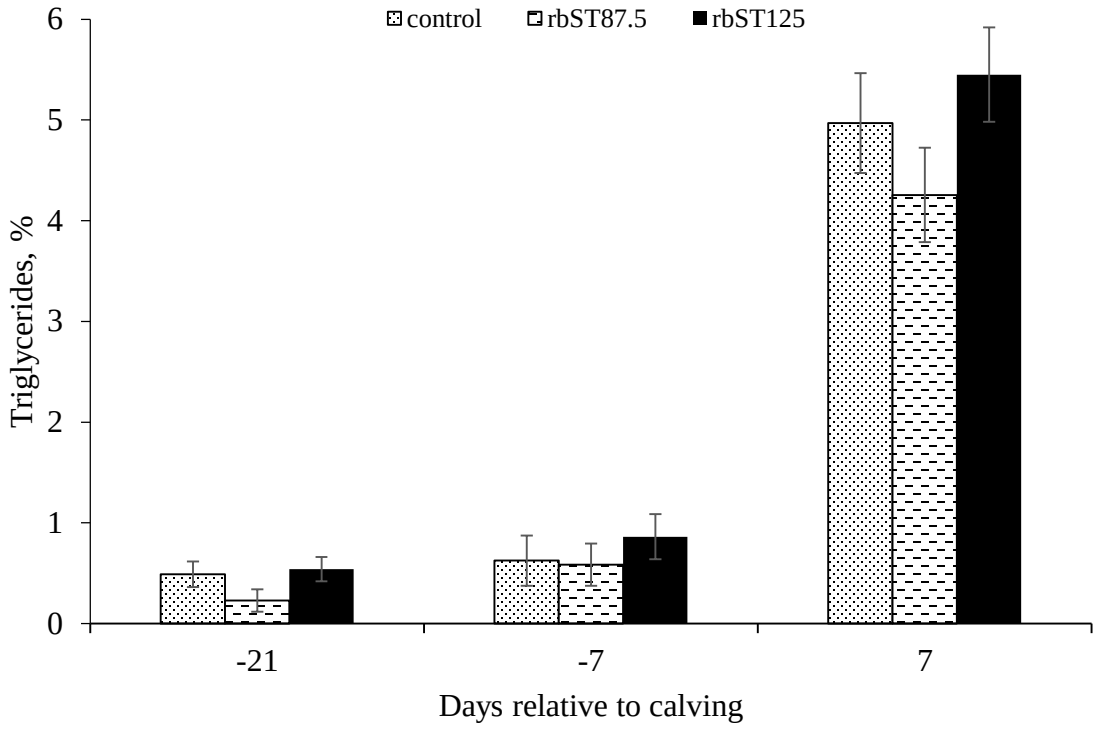


Figure 6B. Liver triglycerides percentage according to treatment. Treatments: control = cows received no treatment; rbST87.5 = cows received 87.5 mg of rbST every 7 d from -21 to 28 d relative to calving; and, rbST125 = cows received 125 mg of rbST every 7 d from -21 to 28 d relative to calving. *Day -21* - Treatment (P = 0.14). *Day -7* - Treatment (P = 0.65). *Day 7*- Treatment (P = 0.21).

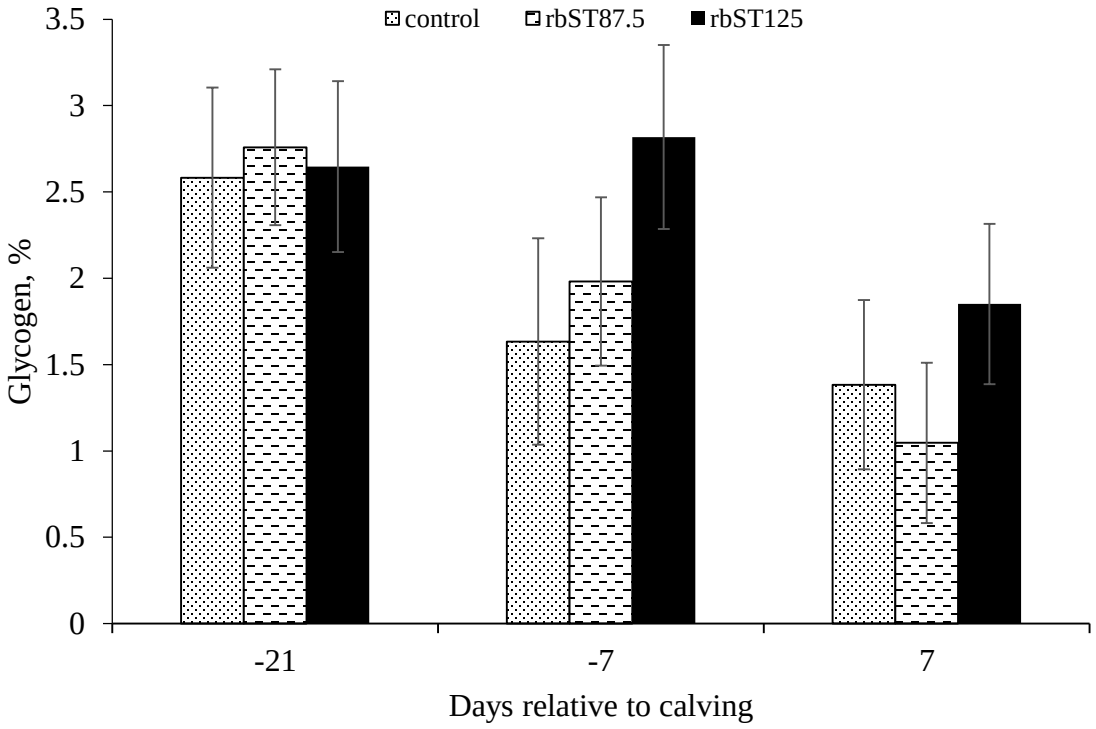


Figure 6C. Liver glycogen percentage according to treatment. Treatments: control = cows received no treatment; rbST87.5 = cows received 87.5 mg of rbST every 7 d from -21 to 28 d relative to calving; and, rbST125 = cows received 125 mg of rbST every 7 d from -21 to 28 d relative to calving. *Day -21* - Treatment (P = 0.97). *Day -7* - Treatment (P = 0.31). *Day 7*- Treatment (P = 0.48).

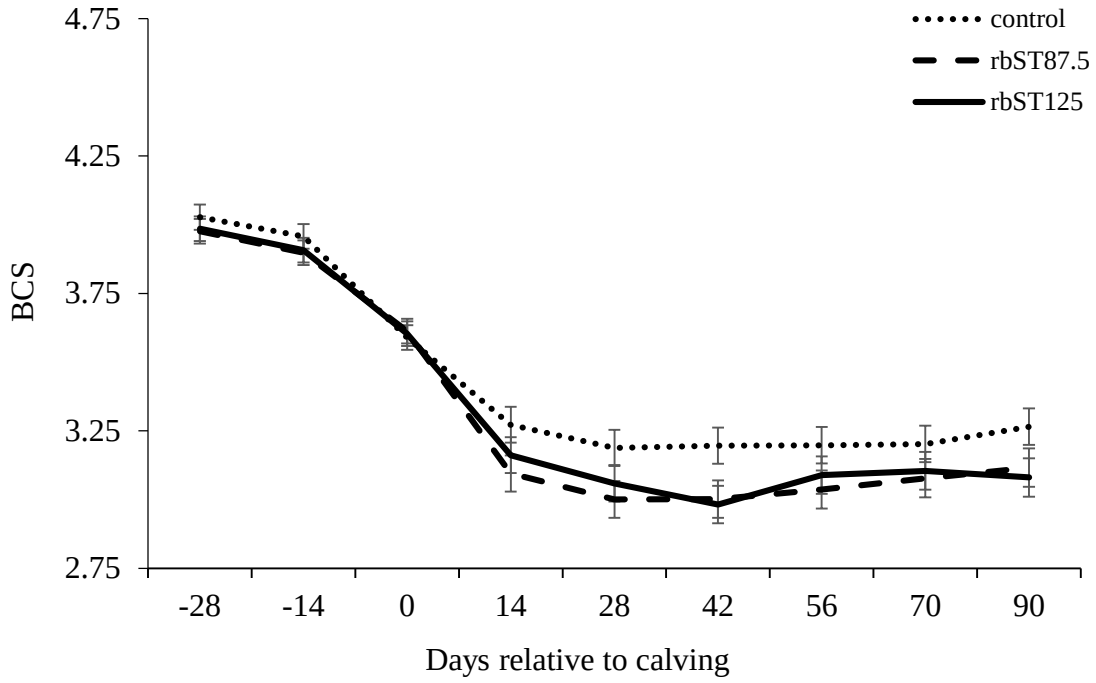


Figure 7. Body condition score according to treatment. Treatments: control = cows received no treatment; rbST87.5 = cows received 87.5 mg of rbST every 7 d from -21 to 28 d relative to calving; and, rbST125 = cows received 125 mg of rbST every 7 d from -21 to 28 d relative to calving. *Prepartum period* - Treatment ($P = 0.83$), day ($P < 0.01$), and interaction between treatment and day ($P = 0.69$). *Postpartum period* - Treatment ($P = 0.10$), day ($P = 0.01$), and interaction between treatment and day ($P = 0.79$). Contrasts: control vs. rbST87.5 - $P = 0.05$, control vs. rbST125 - $P = 0.09$, rbST87.5 vs. rbST125 - $P = 0.76$, linear - $P = 0.08$, and quadratic - $P = 0.21$.

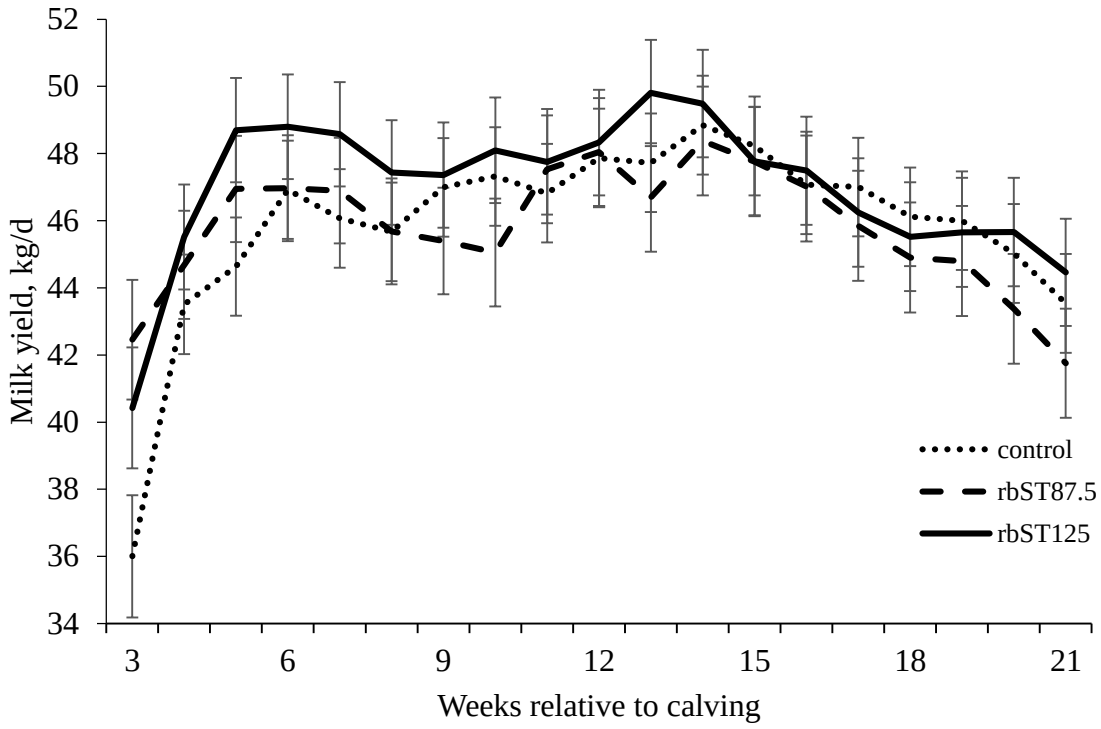


Figure 8. Milk yield (Kg/d) according to treatment. Treatments: control = cows received no treatment; rbST87.5 = cows received 87.5 mg of rbST every 7 d from -21 to 28 d relative to calving; and, rbST125 = cows received 125 mg of rbST every 7 d from -21 to 28 d relative to calving. Treatment (P = 0.77), week (P < 0.01), and interaction between treatment and week (P = 0.02).

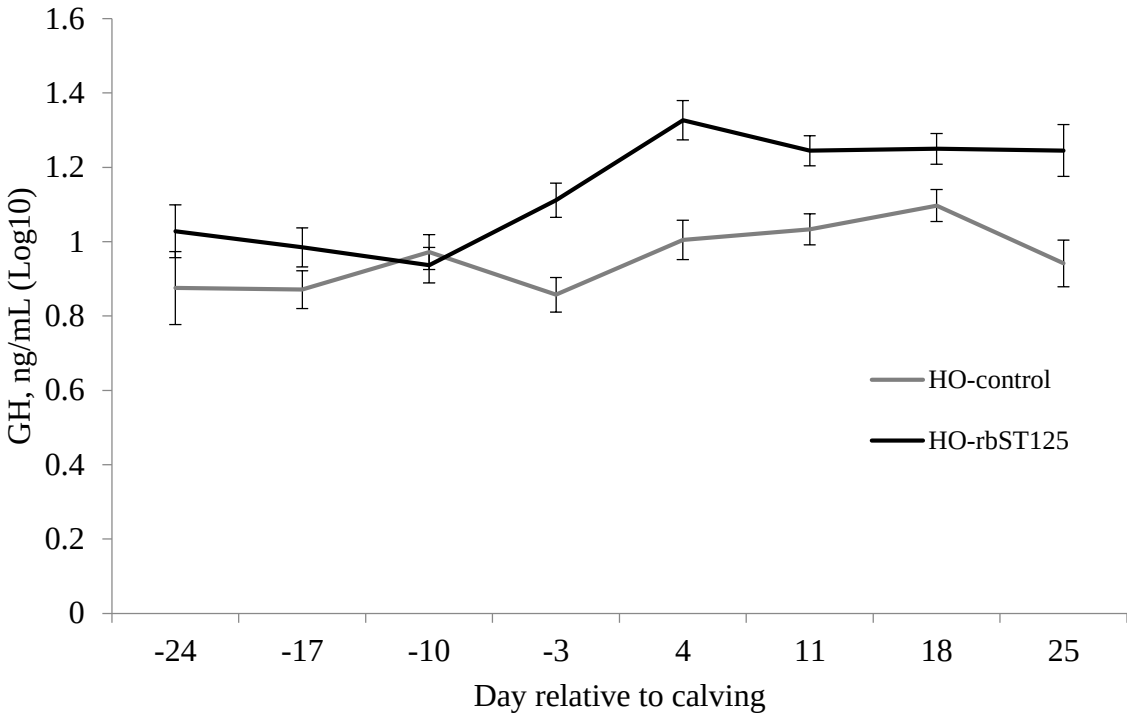


Figure 1. Growth hormone (GH) concentration of Holstein cows according to treatment and day relative to calving. Treatments: control = cows received no treatment; rbST125 = cows received 125 mg of rbST, every 7 d, from -21 to 21 d relative to calving. Prepartum period: Treatment - $P < 0.01$, day relative to calving - $P = 0.72$, and the interaction between treatment and day relative to calving - $P = 0.02$. Postpartum period: Treatment - $P < 0.01$, day relative to calving - $P = 0.76$, and the interaction between treatment and day relative to calving - $P = 0.25$. Results are reported as LSM (± SEM) of the Log10 analysis.

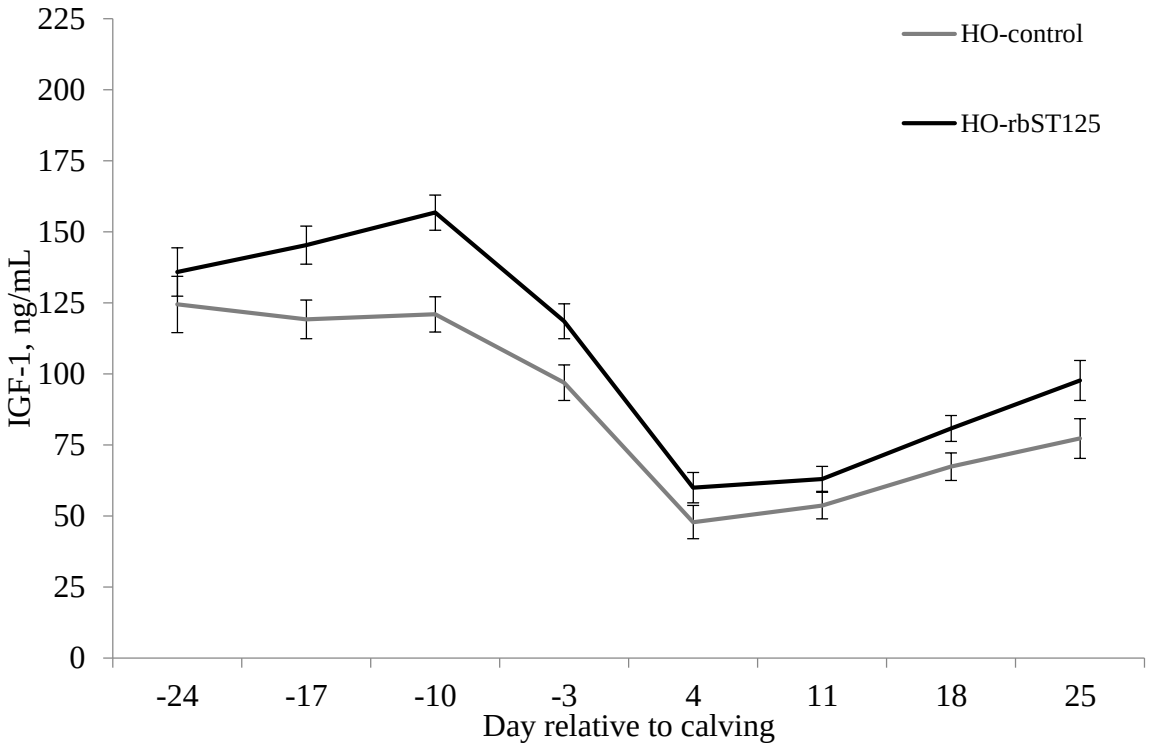


Figure 2 (Panel A). Insulin-like growth factor 1 (IGF-1) concentration of Holstein (A) and Jersey (B) cows according to treatment and day relative to calving. Treatments: control = cows received no treatment; rbST125 = cows received 125 mg of rbST, every 7 d, from -21 to 21 d relative to calving. Herds: HO = Holstein; JS = Jersey. Prepartum period: Treatment - $P < 0.01$, day relative to calving - $P < 0.01$, interaction between treatment and day relative to calving - $P < 0.01$, herd - $P < 0.01$, and interaction between treatment and herd - $P = 0.14$. Postpartum period: Treatment - $P < 0.01$, day relative to calving - $P < 0.01$, interaction between treatment and day relative to calving - $P = 0.98$, herd - $P < 0.01$, and interaction between treatment and herd - $P = 0.58$. Results are reported as LSM ± SEM.

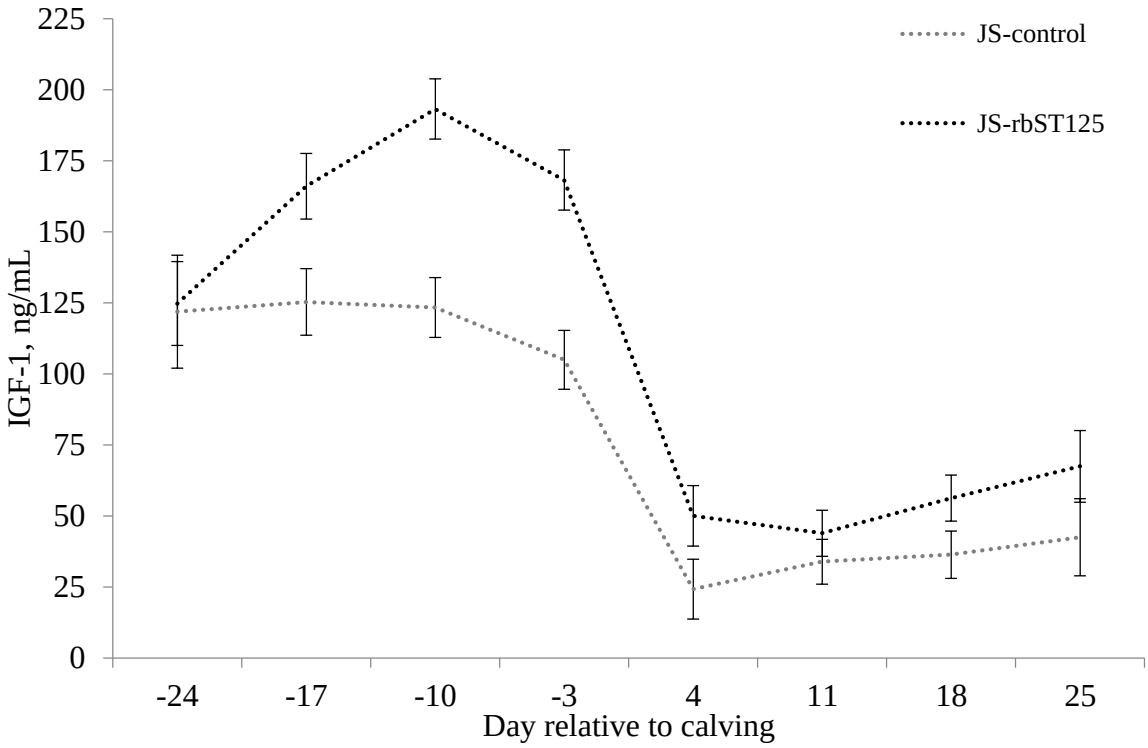


Figure 2 (Panel B). Insulin-like growth factor 1 (IGF-1) concentration of Holstein (A) and Jersey (B) cows according to treatment and day relative to calving. Treatments: control = cows received no treatment; rbST125 = cows received 125 mg of rbST, every 7 d, from -21 to 21 d relative to calving. Herds: HO = Holstein; JS = Jersey. Prepartum period: Treatment - $P < 0.01$, day relative to calving - $P < 0.01$, interaction between treatment and day relative to calving - $P < 0.01$, herd - $P < 0.01$, and interaction between treatment and herd - $P = 0.14$. Postpartum period: Treatment - $P < 0.01$, day relative to calving - $P < 0.01$, interaction between treatment and day relative to calving - $P = 0.98$, herd - $P < 0.01$, and interaction between treatment and herd - $P = 0.58$. Results are reported as LSM ± SEM.

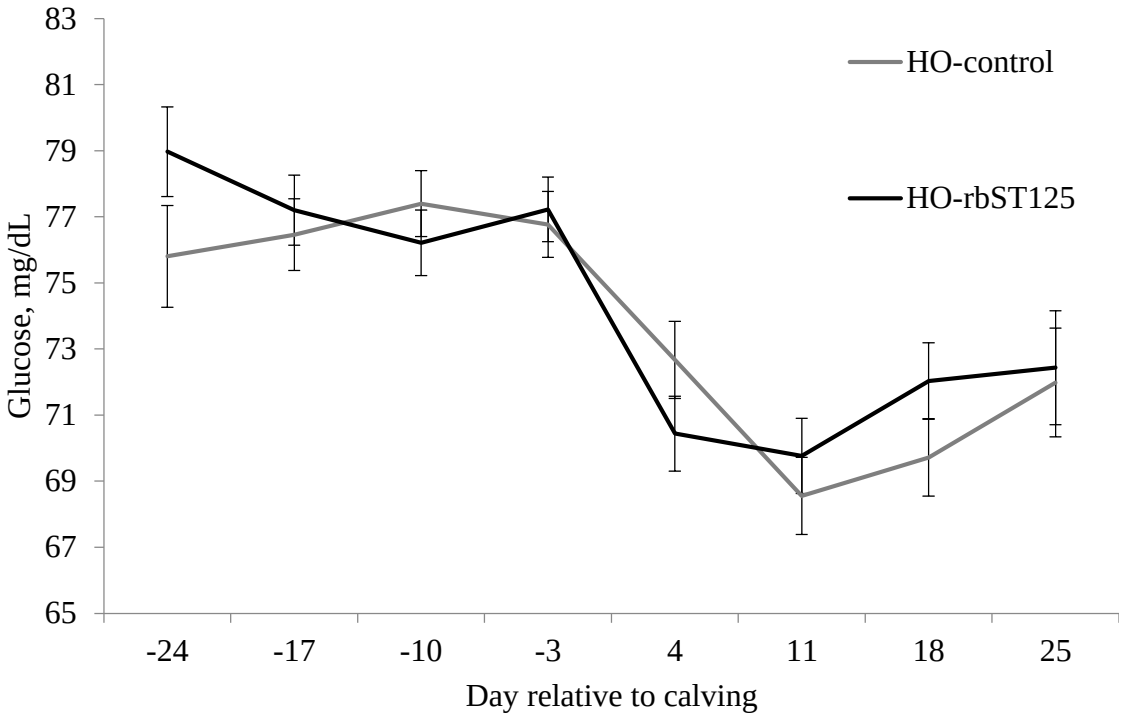


Figure 3 (Panel A). Glucose concentration of Holstein (A) and Jersey (B) cows according to treatment and day relative to calving. Treatments: control = cows received no treatment; rbST125 = cows received 125 mg of rbST, every 7 d, from -21 to 21 d relative to calving. Herds: HO = Holstein; JS = Jersey. Prepartum period: Treatment - $P = 0.56$, day relative to calving - $P = 1.00$, interaction between treatment and day relative to calving - $P = 0.17$, herd - $P < 0.01$, and interaction between treatment and herd - $P = 0.71$. Postpartum period: Treatment - $P = 0.81$, day relative to calving - $P < 0.01$, interaction between treatment and day relative to calving - $P = 0.02$, herd - $P = 0.18$, and interaction between treatment and herd - $P = 0.86$. Results are reported as LSM ± SEM.

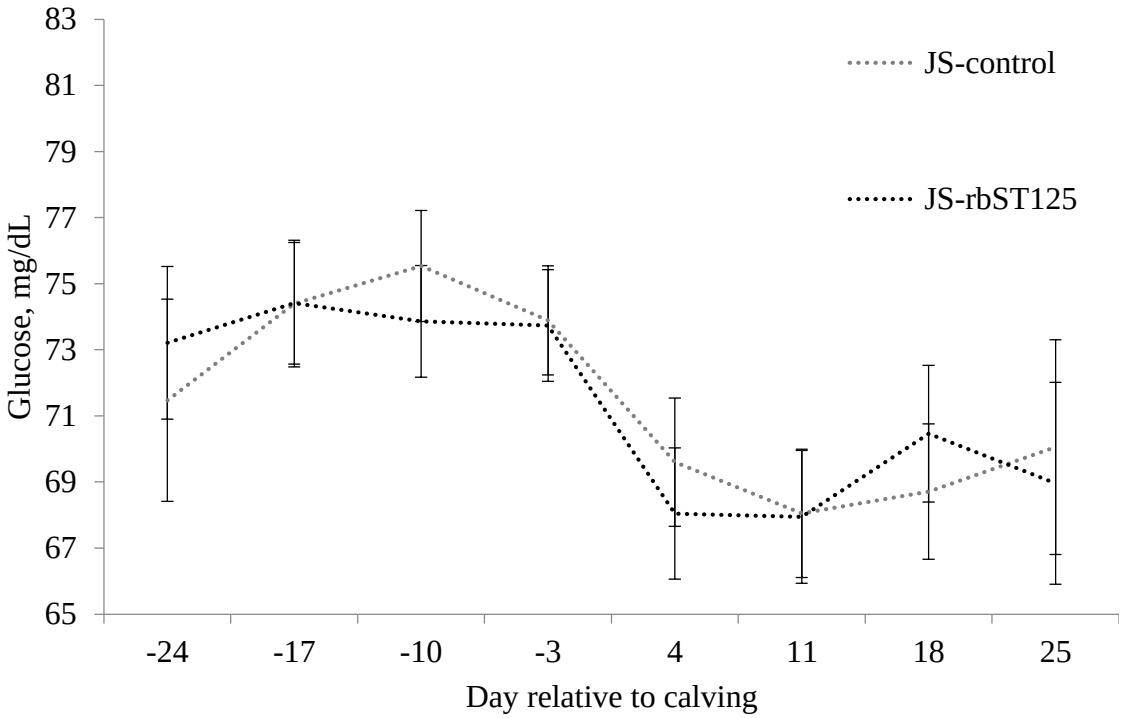


Figure 3 (Panel B). Glucose concentration of Holstein (A) and Jersey (B) cows according to treatment and day relative to calving. Treatments: control = cows received no treatment; rbST125 = cows received 125 mg of rbST, every 7 d, from -21 to 21 d relative to calving. Herds: HO = Holstein; JS = Jersey. Prepartum period: Treatment - $P = 0.56$, day relative to calving - $P = 1.00$, interaction between treatment and day relative to calving - $P = 0.17$, herd - $P < 0.01$, and interaction between treatment and herd - $P = 0.71$. Postpartum period: Treatment - $P = 0.81$, day relative to calving - $P < 0.01$, interaction between treatment and day relative to calving - $P = 0.02$, herd - $P = 0.18$, and interaction between treatment and herd - $P = 0.86$. Results are reported as LSM ± SEM.

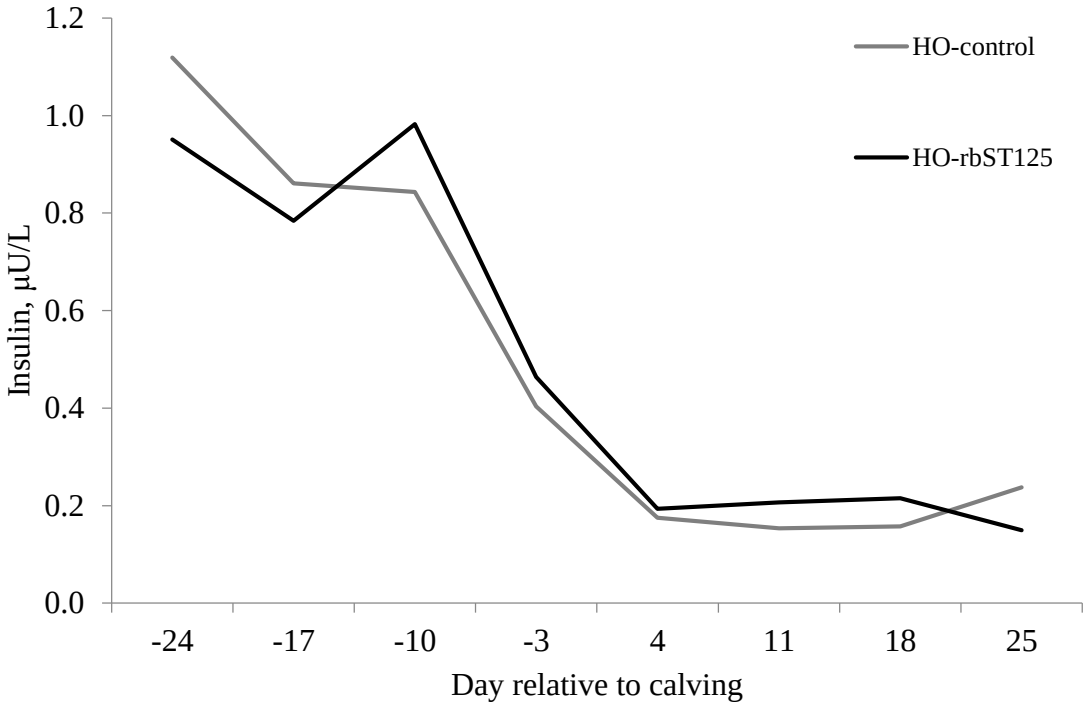


Figure 4 (Panel A). Insulin concentration of Holstein (A) and Jersey (B) cows according to treatment and day relative to calving. Treatments: control = cows received no treatment; rbST125 = cows received 125 mg of rbST, every 7 d, from -21 to 21 d relative to calving. Herds: HO = Holstein; JS = Jersey. Prepartum period: Treatment - $P = 0.89$, day relative to calving - $P < 0.01$, interaction between treatment and day relative to calving - $P = 0.32$, herd - $P < 0.01$, and interaction between treatment and herd - $P = 0.81$. Postpartum period: Treatment - $P = 0.69$, day relative to calving - $P = 0.79$, interaction between treatment and day relative to calving - $P = 0.09$, herd - $P = 0.39$, and interaction between treatment and herd - $P = 0.54$. Results reported are the back transformed values of the LSM of the Log10 analysis.

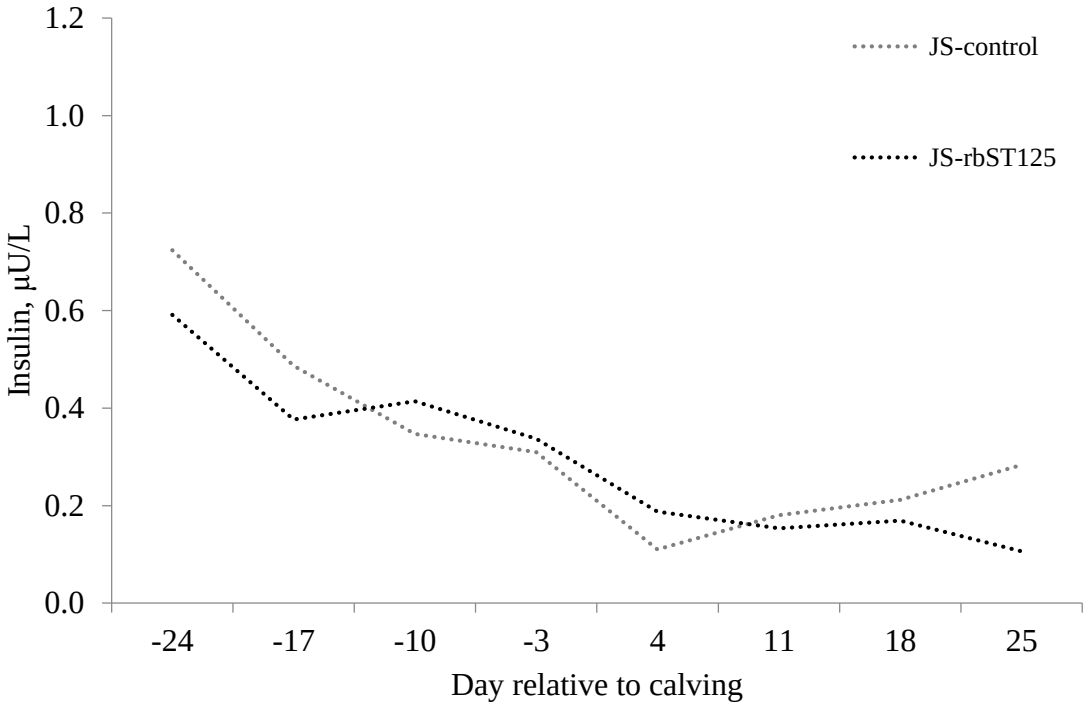


Figure 4 (Panel B). Insulin concentration of Holstein (A) and Jersey (B) cows according to treatment and day relative to calving. Treatments: control = cows received no treatment; rbST125 = cows received 125 mg of rbST, every 7 d, from -21 to 21 d relative to calving. Herds: HO = Holstein; JS = Jersey. Prepartum period: Treatment - $P = 0.89$, day relative to calving - $P < 0.01$, interaction between treatment and day relative to calving - $P = 0.32$, herd - $P < 0.01$, and interaction between treatment and herd - $P = 0.81$. Postpartum period: Treatment - $P = 0.69$, day relative to calving - $P = 0.79$, interaction between treatment and day relative to calving - $P = 0.09$, herd - $P = 0.39$, and interaction between treatment and herd - $P = 0.54$. Results reported are the back transformed values of the LSM of the Log10 analysis.

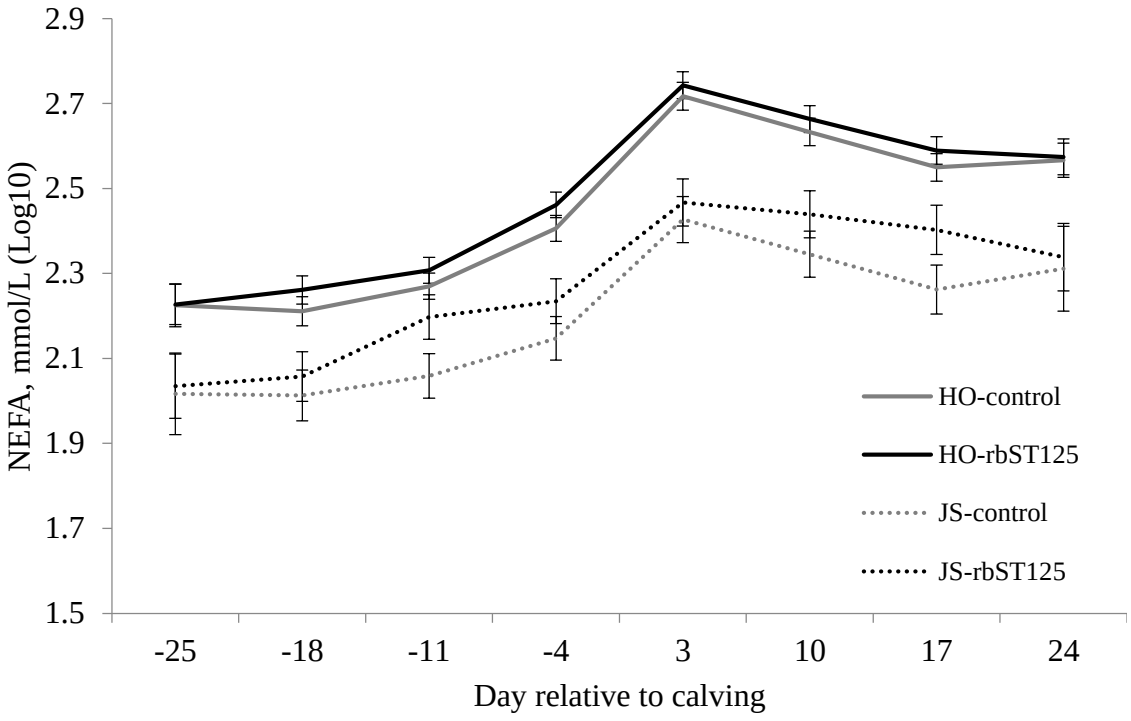


Figure 5. Nonesterified fatty acid (NEFA) concentration of Holstein and Jersey cows according to treatment, day relative to calving, and herd. Treatments: control = cows received no treatment; rbST125 = cows received 125 mg of rbST, every 7 d, from -21 to 21 d relative to calving. Herds: HO = Holstein; JS = Jersey. Prepartum period: Treatment - $P = 0.14$, day relative to calving - $P < 0.01$, interaction between treatment and day relative to calving - $P = 0.81$, herd - $P < 0.01$, and interaction between treatment and herd - $P = 0.52$. Postpartum period: Treatment - $P = 0.20$, day relative to calving - $P < 0.01$, interaction between treatment and day relative to calving - $P = 0.80$, herd - $P < 0.01$, and interaction between treatment and herd - $P = 0.45$. Results are reported as LSM (± SEM) of the Log10 analysis.

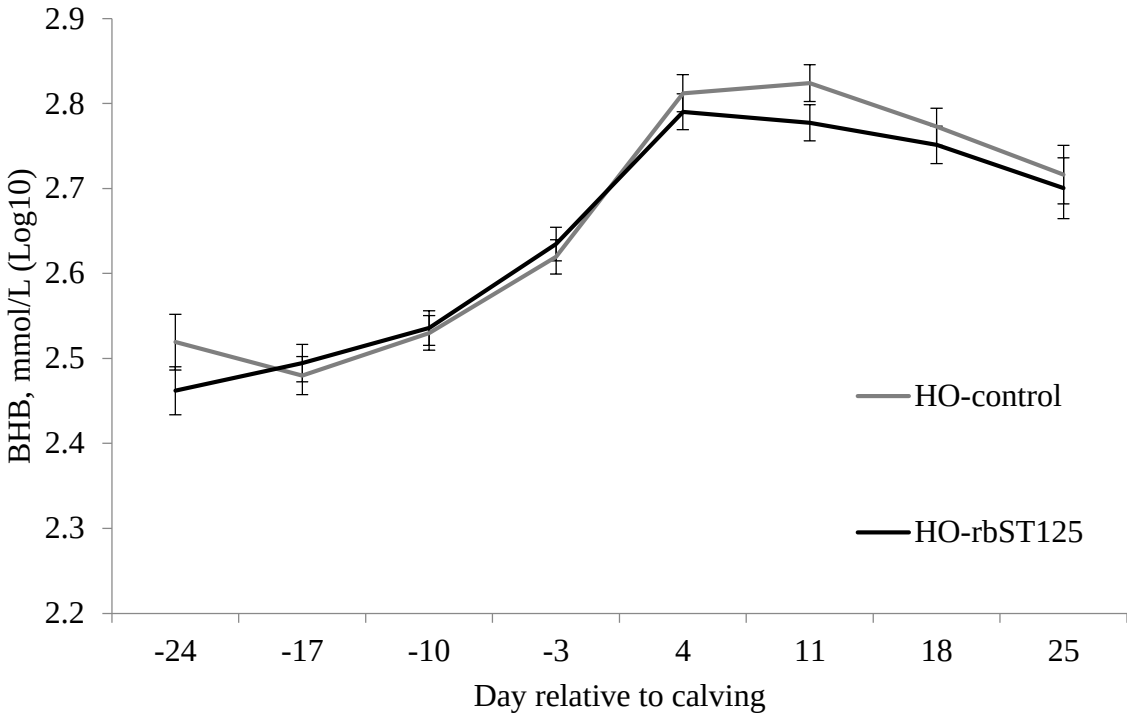


Figure 6 (Panel A). Concentration of β-hydroxybutyrate (BHB) of Holstein (A) and Jersey (B) cows according to treatment and day relative to calving. Treatments: control = cows received no treatment; rbST125 = cows received 125 mg of rbST, every 7 d, from -21 to 21 d relative to calving. Herds: HO = Holstein; JS = Jersey. Prepartum period: Treatment - $P = 0.39$, day relative to calving - $P = 0.33$, interaction between treatment and day relative to calving - $P = 0.74$, herd - $P < 0.01$, and interaction between treatment and herd - $P = 0.54$. Postpartum period: Treatment - $P = 0.36$, day relative to calving - $P < 0.01$, interaction between treatment and day relative to calving - $P = 0.68$, herd - $P = 0.66$, and interaction between treatment and herd - $P = 0.33$. Results are reported as LSM (± SEM) of the Log10 analysis.

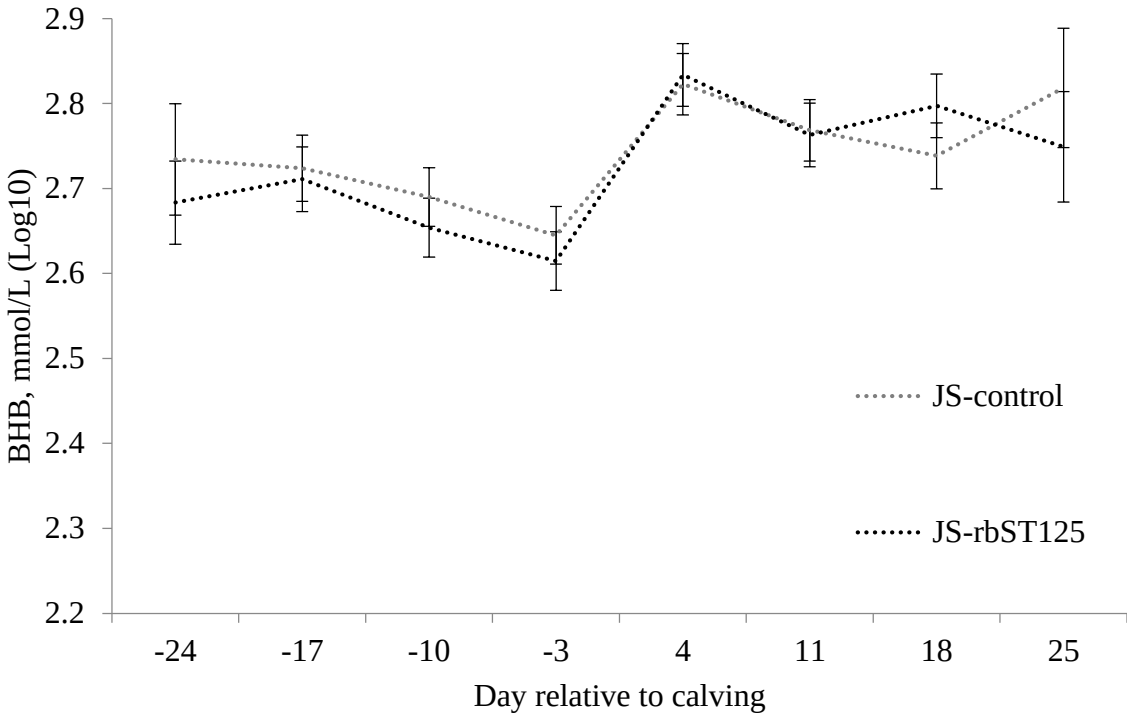


Figure 6 (Panel B). Concentration of β-hydroxybutyrate (BHB) of Holstein (A) and Jersey (B) cows according to treatment and day relative to calving. Treatments: control = cows received no treatment; rbST125 = cows received 125 mg of rbST, every 7 d, from -21 to 21 d relative to calving. Herds: HO = Holstein; JS = Jersey. Prepartum period: Treatment - $P = 0.39$, day relative to calving - $P = 0.33$, interaction between treatment and day relative to calving - $P = 0.74$, herd - $P < 0.01$, and interaction between treatment and herd - $P = 0.54$. Postpartum period: Treatment - $P = 0.36$, day relative to calving - $P < 0.01$, interaction between treatment and day relative to calving - $P = 0.68$, herd - $P = 0.66$, and interaction between treatment and herd - $P = 0.33$. Results are reported as LSM (± SEM) of the Log10 analysis.

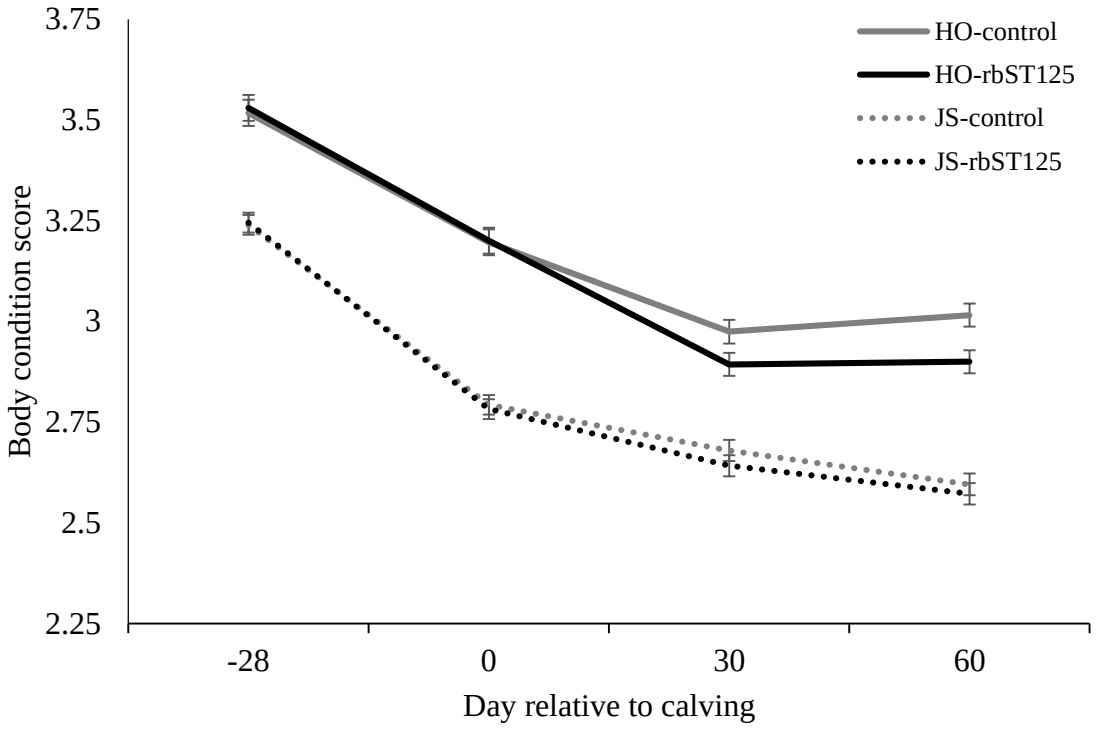


Figure 7. Body condition score of Holstein and Jersey cows according to treatment, day relative to calving, and herd. Treatments: control = cows received no treatment; rbST125 = cows received 125 mg of rbST, every 7 d, from -21 to 21 d relative to calving. Herds: HO = Holstein; JS = Jersey. Prepartum period: Treatment - $P = 0.93$, day relative to calving - $P < 0.01$, interaction between treatment and day relative to calving - $P = 0.62$, herd - $P < 0.01$, and interaction between treatment and herd - $P = 0.85$. Postpartum period: Treatment - $P = 0.01$, day relative to calving - $P = 0.01$, interaction between treatment and day relative to calving - $P = 0.74$, herd - $P < 0.01$, and interaction between treatment and herd - $P = 0.17$. Results are reported as LSM ± SEM.

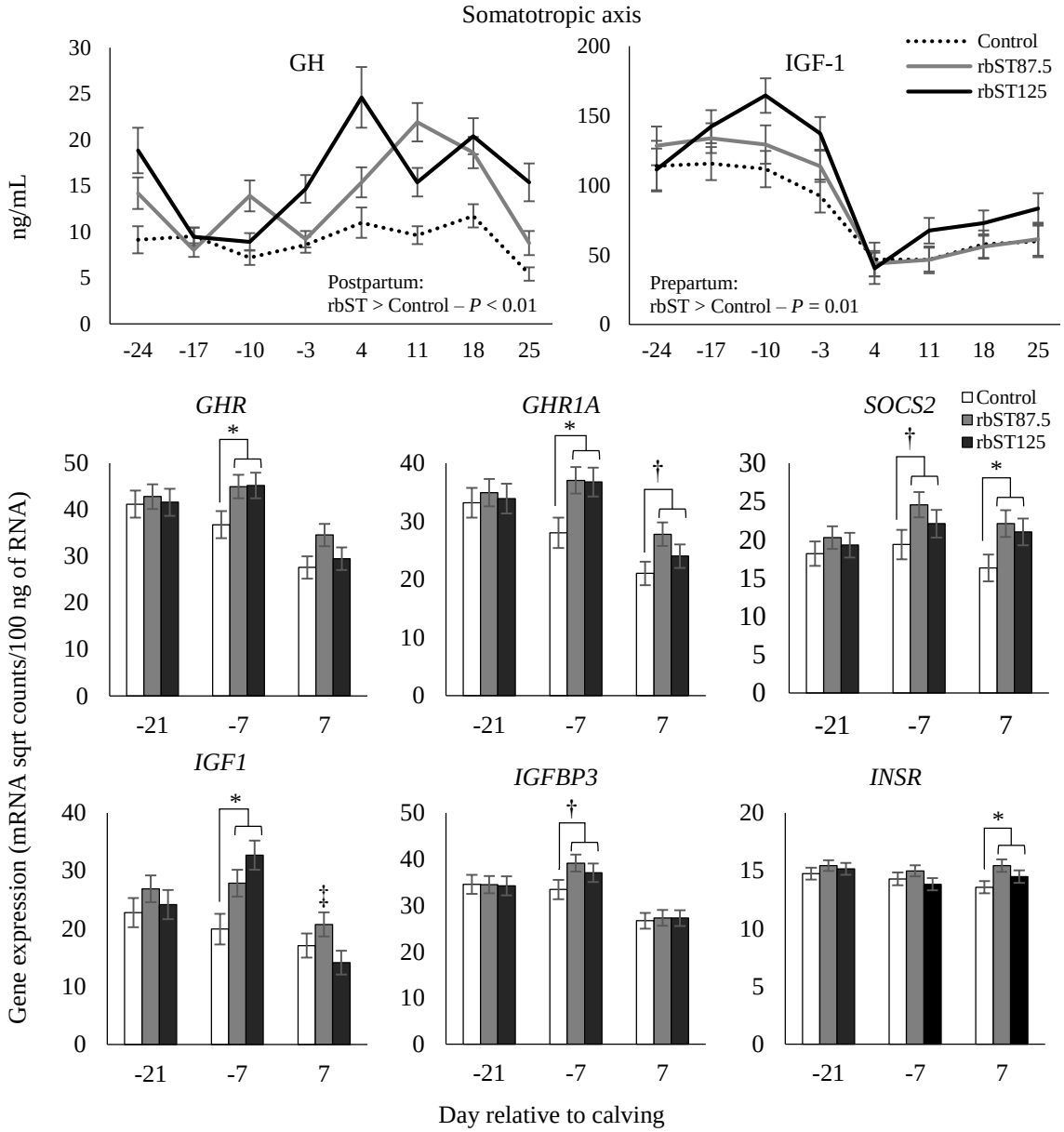


Figure 1. Effect of treatment of periparturient cows with recombinant bovine somatotropin (**rbST**) on serum GH and IGF-1 concentrations and hepatic mRNA expression for genes related to somatotropic axis. Treatments: control = cows received no treatment; rbST87.5 = cows received 87.5 mg of rbST every 7 d from -21 to 28 d relative to calving; and, rbST125 = cows received 125 mg of rbST every 7 d from -21 to 28 d relative to calving. Prepartum GH: treatment ($P = 0.23$), day ($P = 0.29$), and interaction between treatment and day ($P = 0.33$); Postpartum GH: treatment ($P = 0.01$), day ($P = 0.03$), and interaction between treatment and day ($P = 0.52$). Prepartum IGF-1: treatment ($P = 0.01$), day ($P = 0.16$), and interaction between treatment and day ($P = 0.11$). Postpartum IGF-1: treatment ($P = 0.39$), day ($P < 0.01$), and interaction between treatment and day ($P = 0.15$). See Table 2 for statistical analyses of hepatic gene expression: * = control vs. rbST87.5 + rbST125 differed ($P \leq 0.05$); † = contrasts control vs. rbST87.5 + rbST125 tended to differ ($0.05 < P \leq 0.10$); ‡ = contrast rbST87.5 vs. rbST125 differed ($P \leq 0.05$).

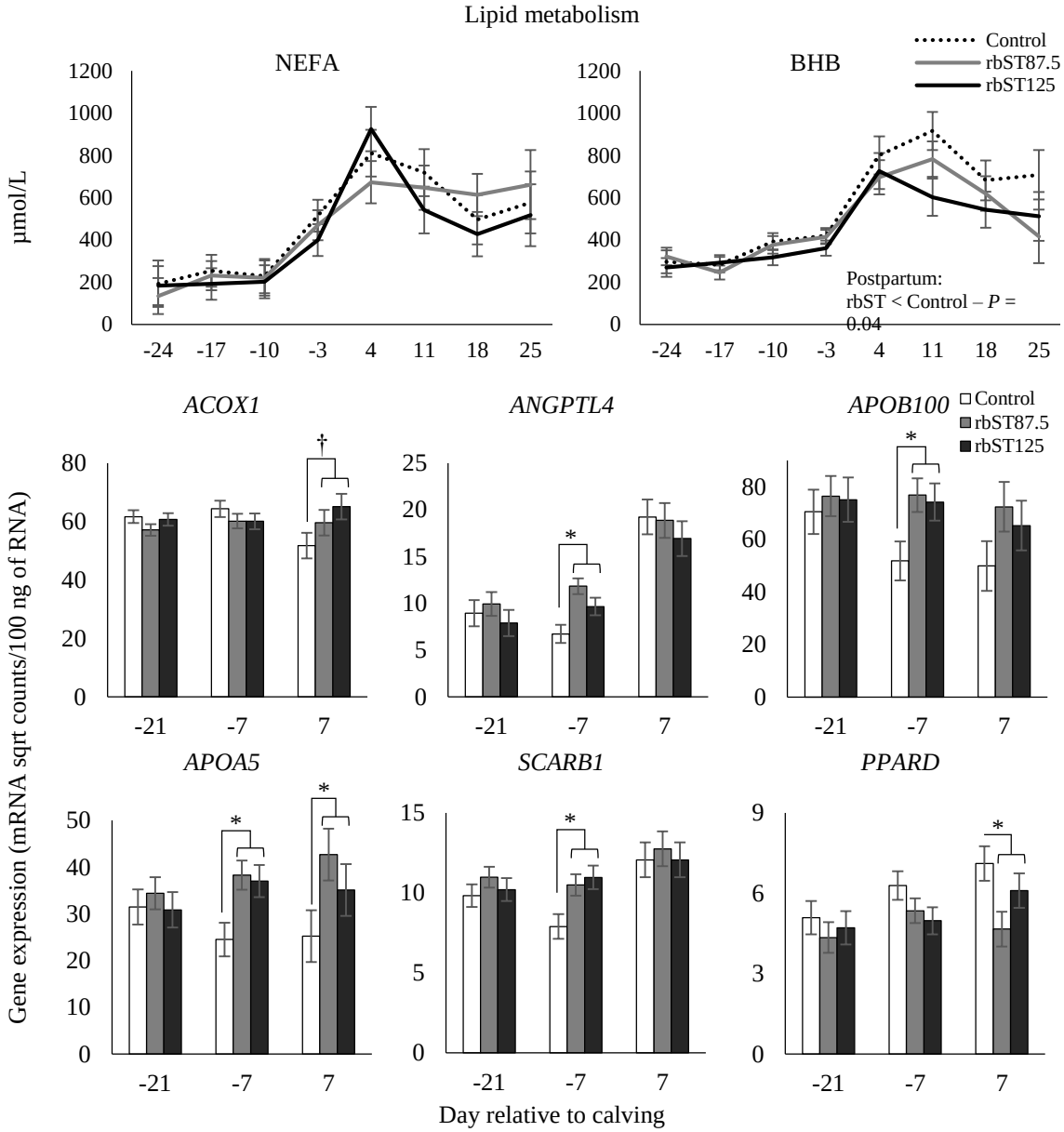


Figure 2. Effect of treatment of periparturient cows with recombinant bovine somatotropin (**rbST**) on serum NEFA and BHB concentrations and hepatic mRNA expression for genes related to lipid metabolism. Treatments: control = cows received no treatment; rbST87.5 = cows received 87.5 mg of rbST every 7 d from -21 to 28 d relative to calving; and, rbST125 = cows received 125 mg of rbST every 7 d from -21 to 28 d relative to calving. Prepartum NEFA: treatment ($P = 0.84$), day ($P < 0.01$), and interaction between treatment and day ($P = 0.80$); Postpartum NEFA: treatment ($P = 0.59$), day ($P = 0.02$), and interaction between treatment and day ($P = 0.46$); Prepartum BHB: treatment ($P = 0.57$), day ($P < 0.01$), and interaction between treatment and day ($P = 0.60$); Postpartum BHB: treatment ($P = 0.11$), day ($P = 0.02$), and interaction between treatment and day ($P = 0.45$). See Table 2 for statistical analyses of hepatic gene expression: * = contrasts control vs. rbST87.5 + rbST125 differed ($P \leq 0.05$); † = contrast control vs. rbST87.5 + rbST125 tended to differ ($0.05 < P \leq 0.10$).

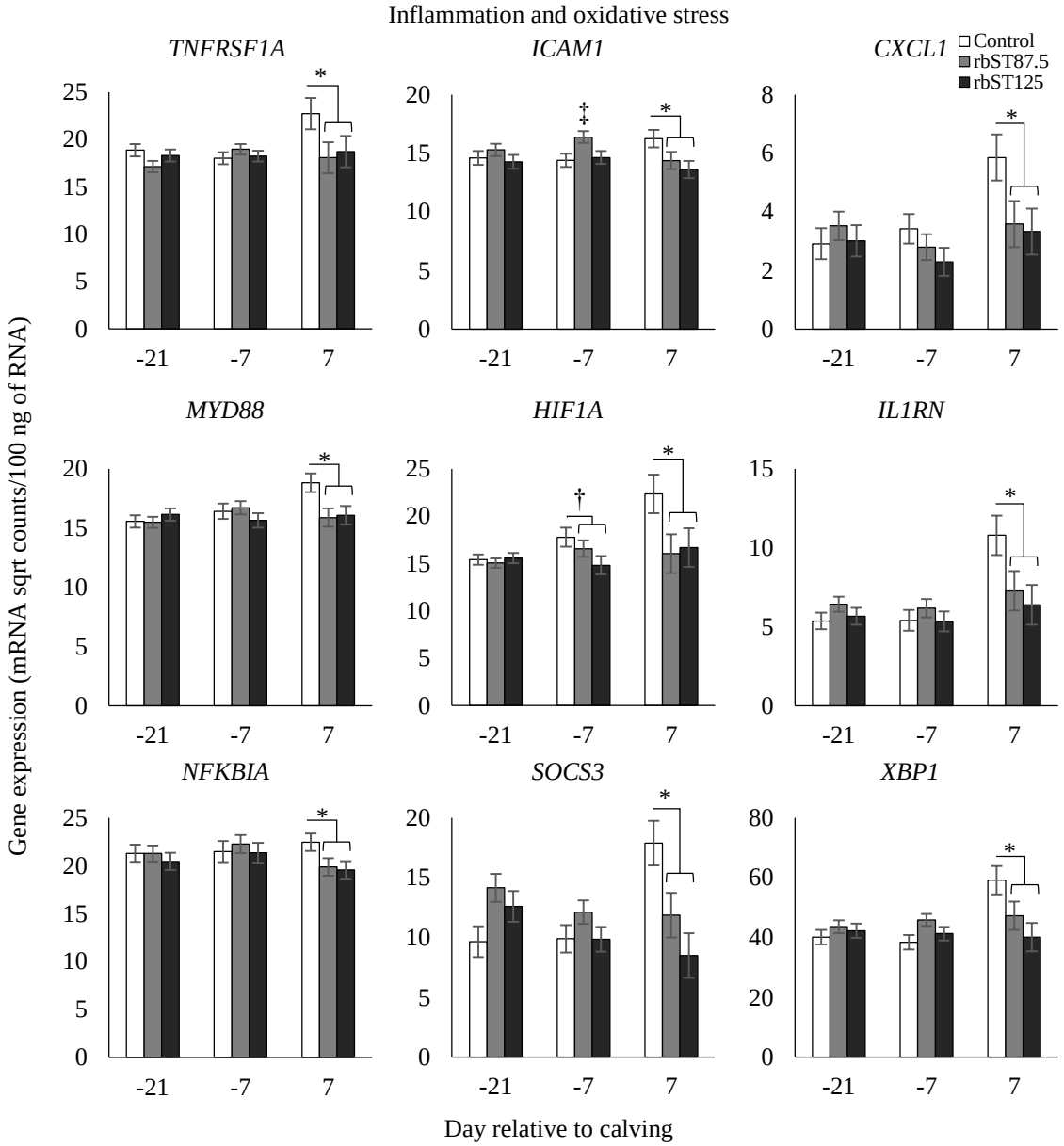


Figure 3. Effect of treatment of periparturient cows with recombinant bovine somatotropin (**rbST**) on hepatic mRNA expression of genes related to inflammatory responses and oxidative stress. Treatments: control = cows received no treatment; rbST87.5 = cows received 87.5 mg of rbST every 7 d from -21 to 28 d relative to calving; and, rbST125 = cows received 125 mg of rbST every 7 d from -21 to 28 d relative to calving. See Table 3 for statistical analyses of hepatic gene expression: * = contrast control vs. rbST87.5 + rbST125 differed ($P \leq 0.05$); † = contrast control vs. rbST87.5 + rbST125 tended to differ ($0.05 < P \leq 0.10$); ‡ = contrast rbST87.5 vs. rbST125 differed ($P \leq 0.05$).

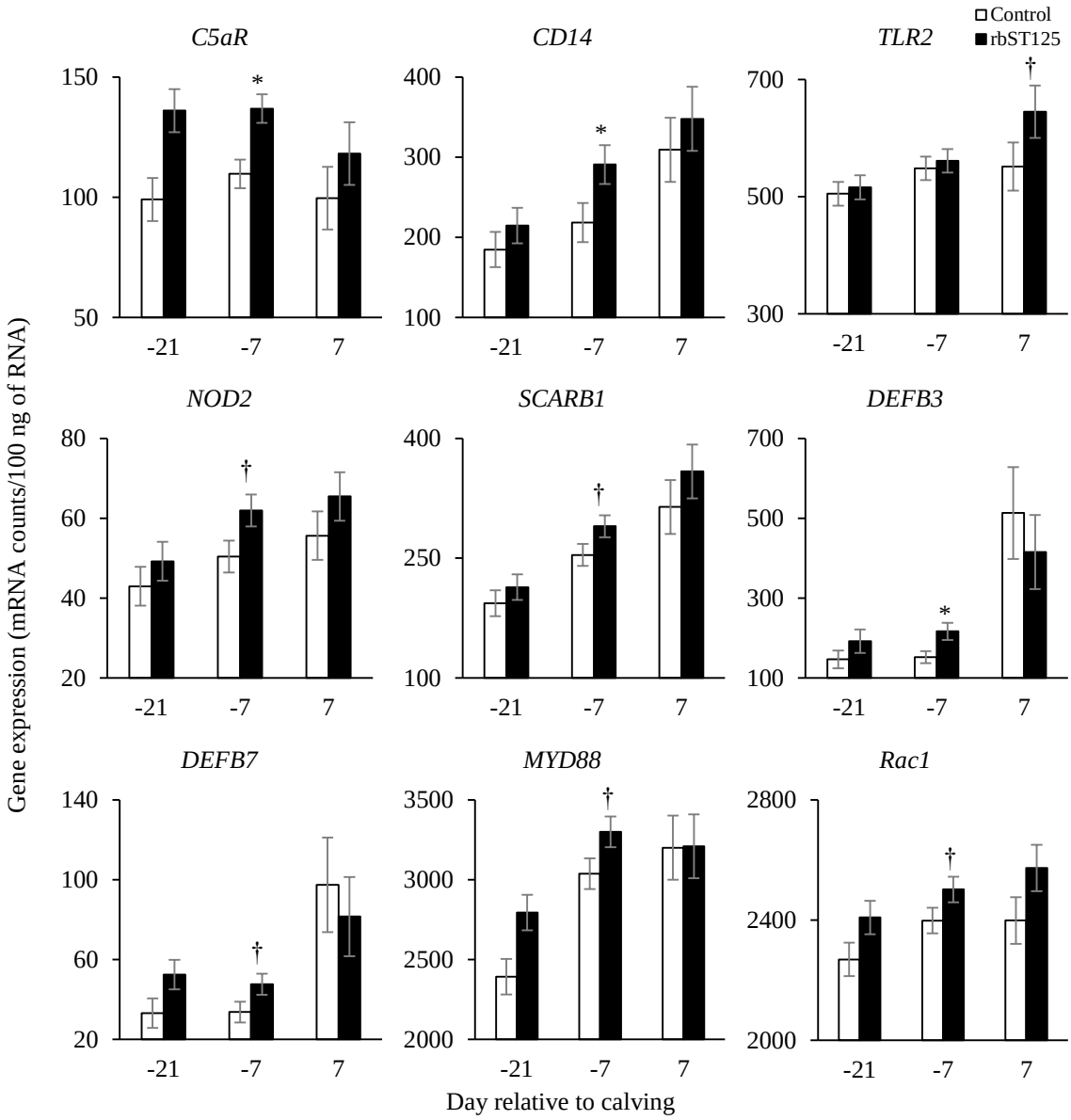


Figure 1. Effect of treatment on leukocyte mRNA expression for genes related to innate immune responses. Treatments: control = cows received no treatment; rbST125 = cows received 125 mg of rbST every 7 d from -21 to 21 d relative to calving. See Tables 6 for statistical analyses of leukocyte gene expression: * = control vs. rbST125 differed ($P < 0.05$); † = control vs. rbST125 tended to differ ($0.05 < P \leq 0.10$).

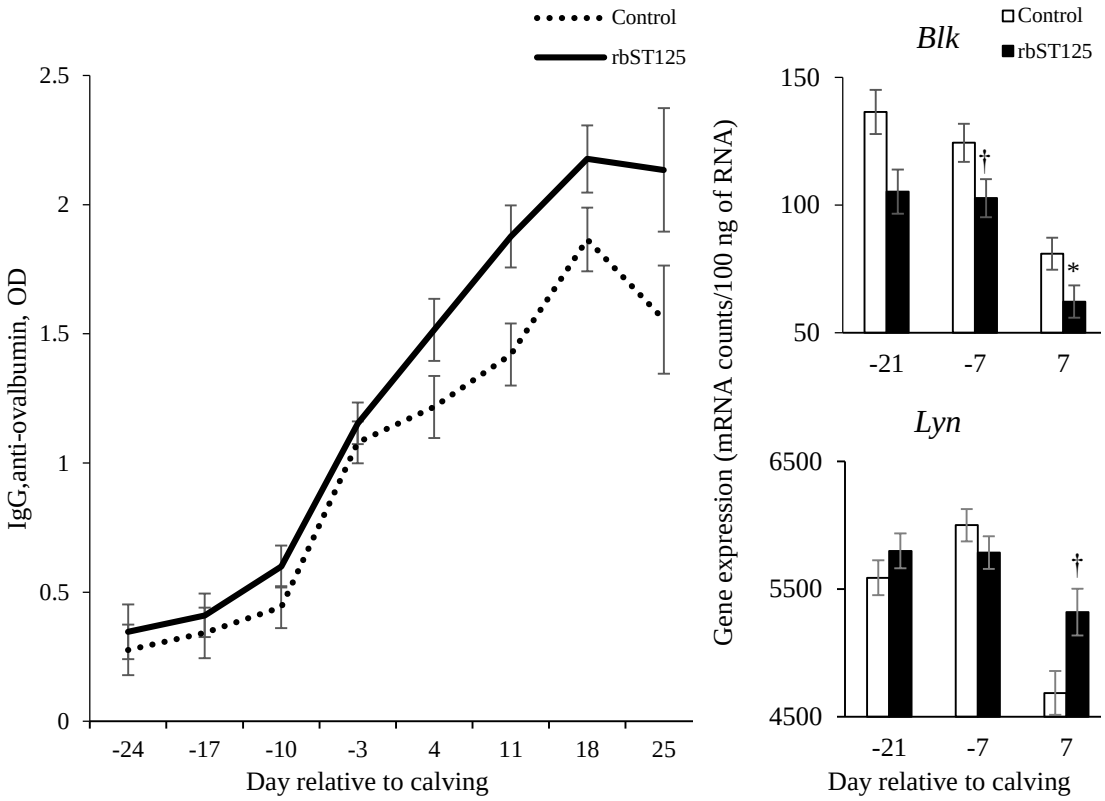


Figure 2. Effect of treatment on IgG anti-ovalbumin optical density (OD) and leukocyte mRNA expression for genes related to adaptive immune response. Treatments: control = cows received no treatment; rbST125 = cows received 125 mg of rbST every 7 d from -21 to 21 d relative to calving. Prepartum IgG: treatment (P = 0.30), day (P < 0.01), and interaction between treatment and day (P = 0.81); Postpartum IgG: treatment (P = 0.01), day (P < 0.01), and interaction between treatment and day (P = 0.56). See Table 6 for statistical analyses of leukocyte gene expression: * = control vs. rbST125 differed ($P \leq 0.05$); † = control vs. rbST125 tended to differ ($0.05 < P \leq 0.10$).

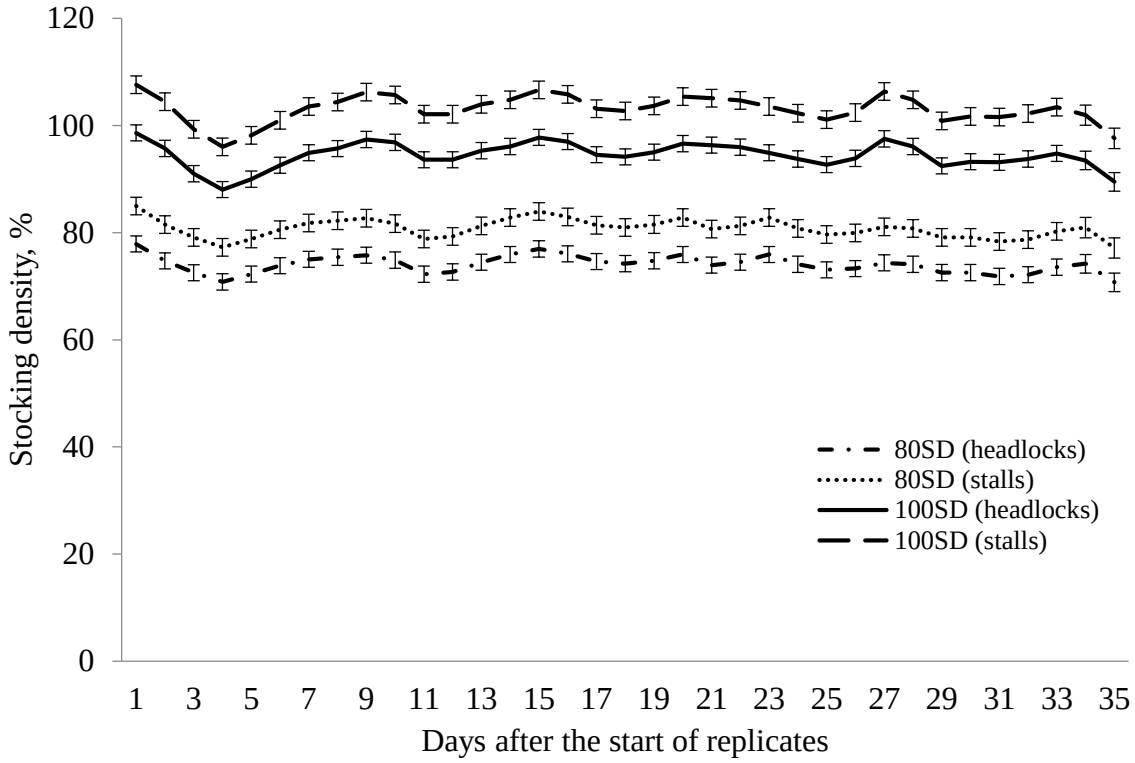


Figure 1. Stocking density based on headlocks and stalls according to treatments. 80SD = cows housed in prepartum pens with 80% target headlock stocking density (38/48); 100SD = cows housed in prepartum pens with 100% target headlock stocking density (48/48). Treatment affected the mean (± SEM) stocking densities based on number of headlocks (80SD = 74.1 ± 0.4%, 100SD = 94.5 ± 0.3%; $P < 0.01$) and stalls (80SD = 80.8 ± 0.4%, 100SD = 103.1 ± 0.4%; $P < 0.01$).

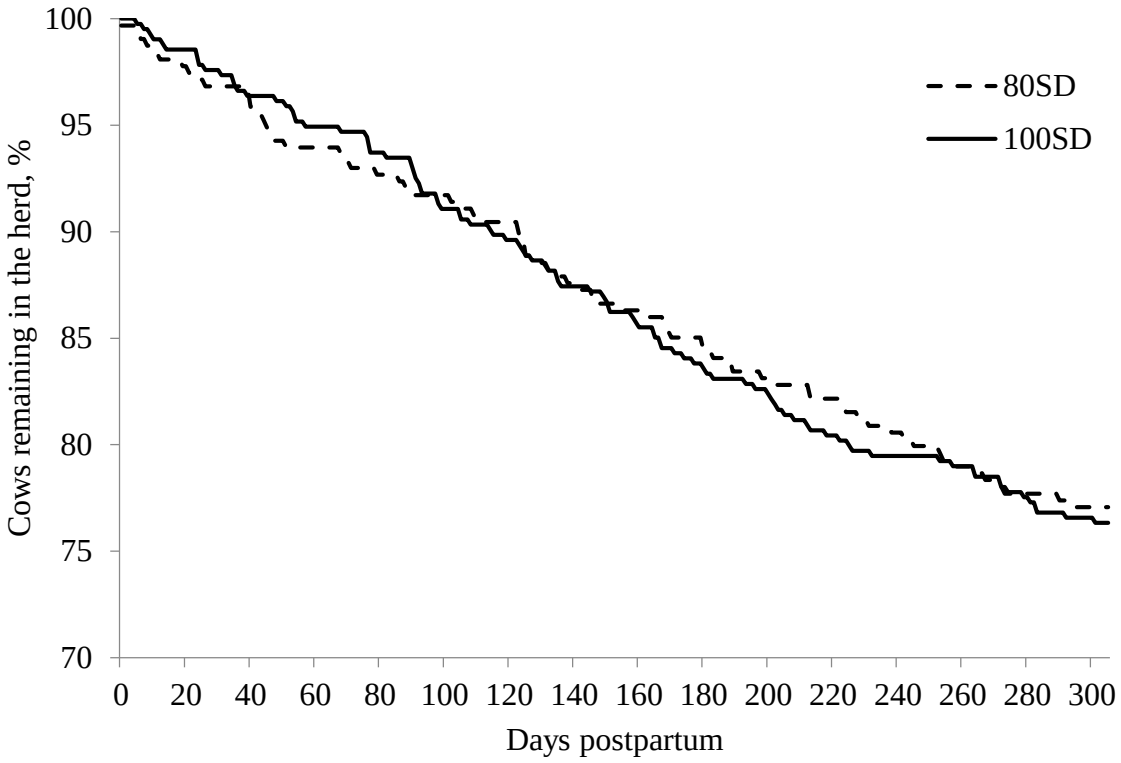


Figure 2. Survival analysis of interval from calving to removal from the herd according to prepartum stocking density. The adjusted hazard ratio (AHR) for removal from the herd was not (P = 0.90) different between the cows housed in prepartum pens with 100% target headlock stocking density [100SD; AHR (95% CI) = 1.02 (0.75, 1.38) 48/48] and cows housed in prepartum pens with 80% target headlock stocking density (80SD; 38/48). The mean (± SEM) intervals from calving to removal from the herd were 258.3 ± 4.5 d for the 80SD treatment and 262.5 ± 3.9 d for the 100SD treatment.

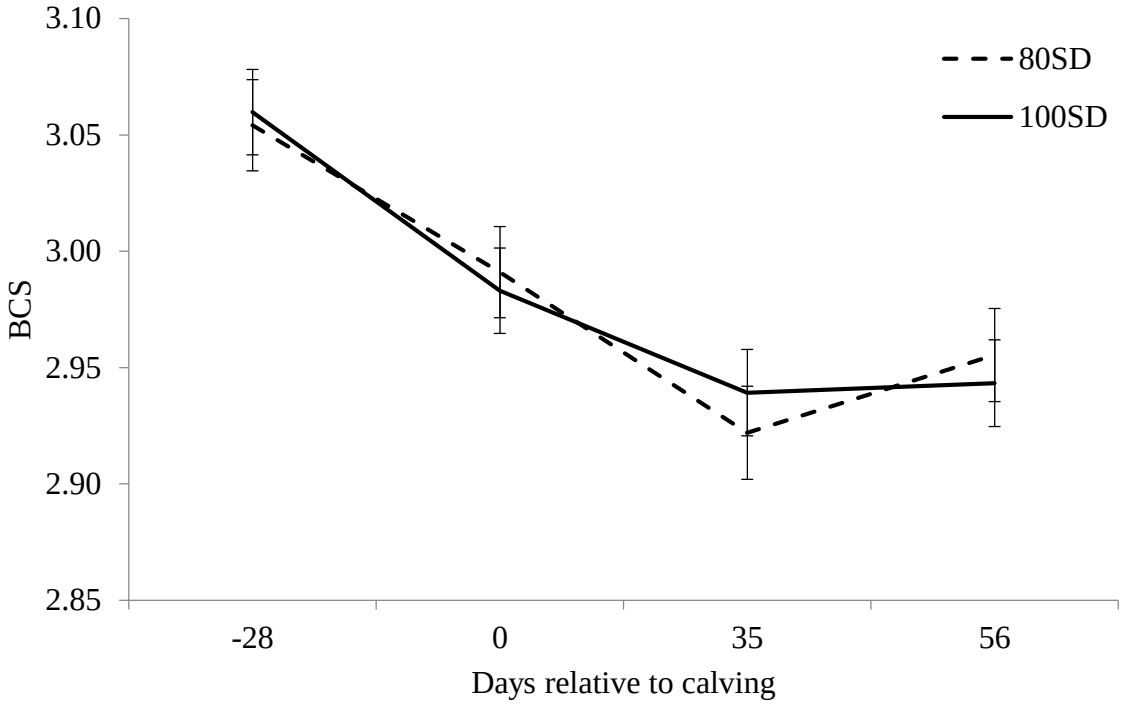


Figure 3. Body condition score of cows housed in prepartum pens with 80% target headlock stocking density (80SD; 38/48) and cows housed in prepartum pens with 100% target headlock stocking density (100SD; 48/48). Treatment ($P = 0.97$) and the interaction between treatment and days relative to calving ($P = 0.66$) did not affect BCS throughout the study.

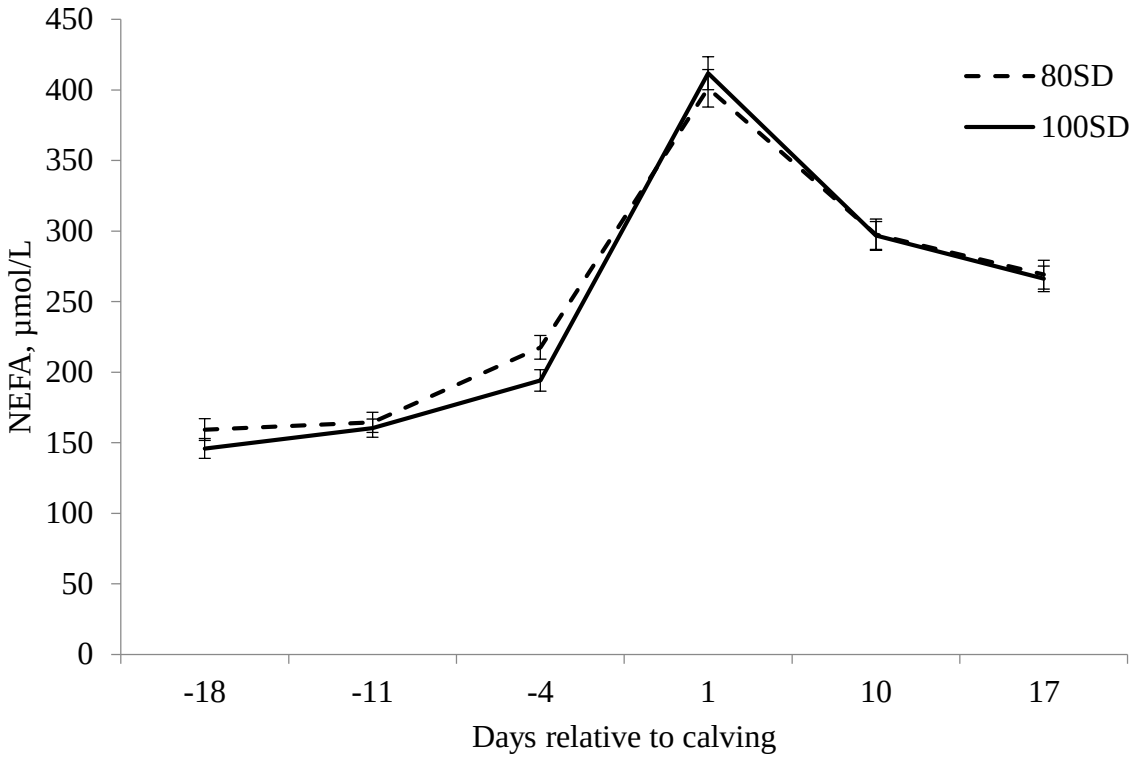


Figure 4. Mean (±SEM) plasma concentrations of NEFA of cows submitted to different prepartum stocking density. 80SD = cows housed in prepartum pens with 80% target headlock stocking density (38/48); 100SD = cows housed in prepartum pens with 100% target headlock stocking density (48/48). No effects of treatment (P = 0.51) and of the interaction between treatment and days relative to calving (P = 0.32) on NEFA concentrations were observed.

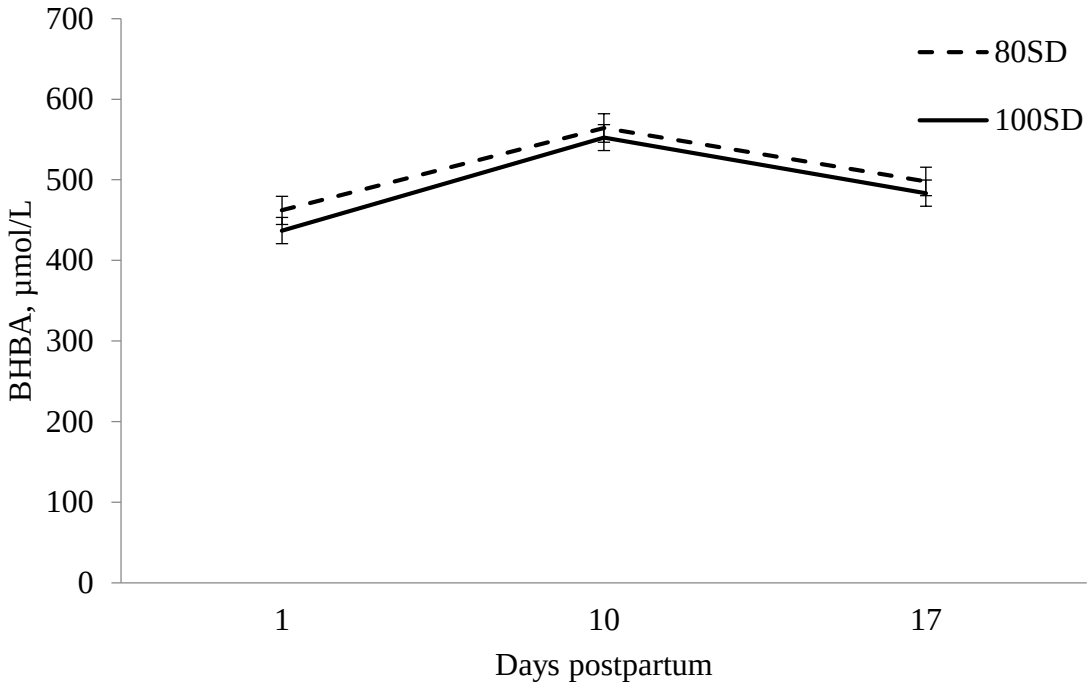


Figure 5. Mean (±SEM) plasma concentrations of BHBA of cows submitted to different prepartum stocking density. 80SD = cows housed in prepartum pens with 80% target headlock stocking density (38/48); 100SD = cows housed in prepartum pens with 100% target headlock stocking density (48/48). No effects of treatment (P = 0.40) and of the interaction between treatment and days relative to calving (P = 0.83) on BHBA concentrations were observed.

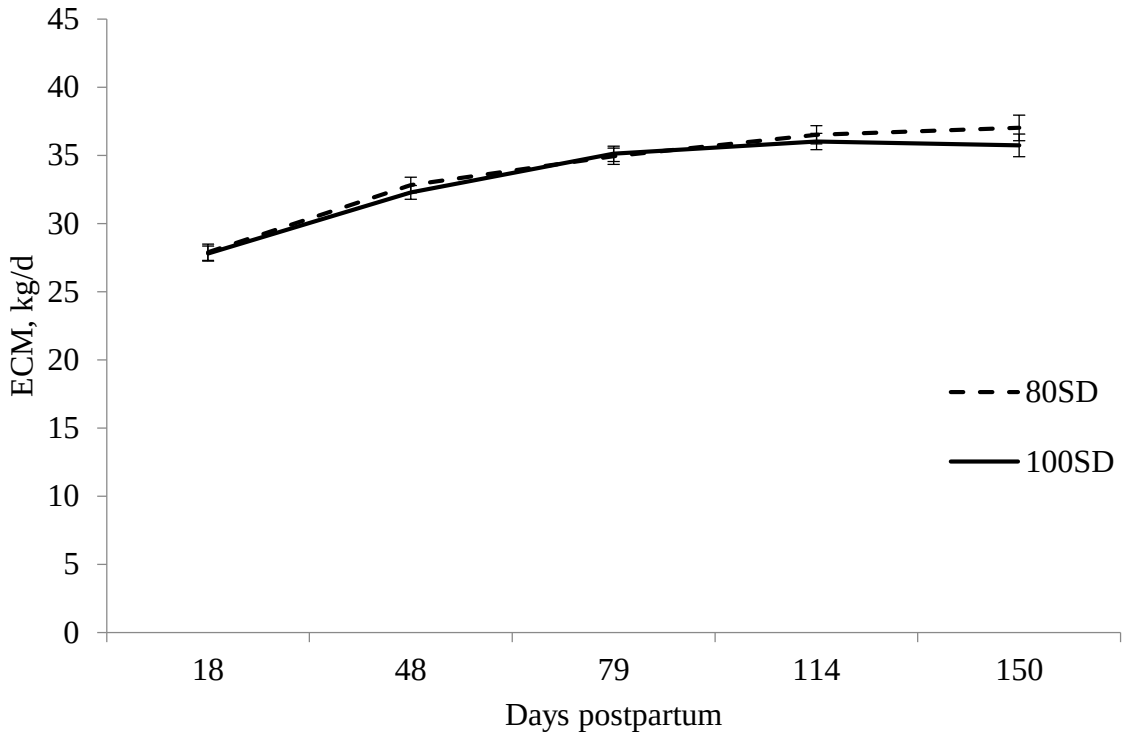


Figure 6. Yield of ECM according to prepartum grouping strategy. 80SD = cows housed in prepartum pens with 80% target headlock stocking density (38/48); 100SD = cows housed in prepartum pens with 100% target headlock stocking density (48/48). No effect of treatment ($P = 0.54$) and of the interaction between treatment and month of lactation ($P = 0.69$) on yield of ECM was observed.

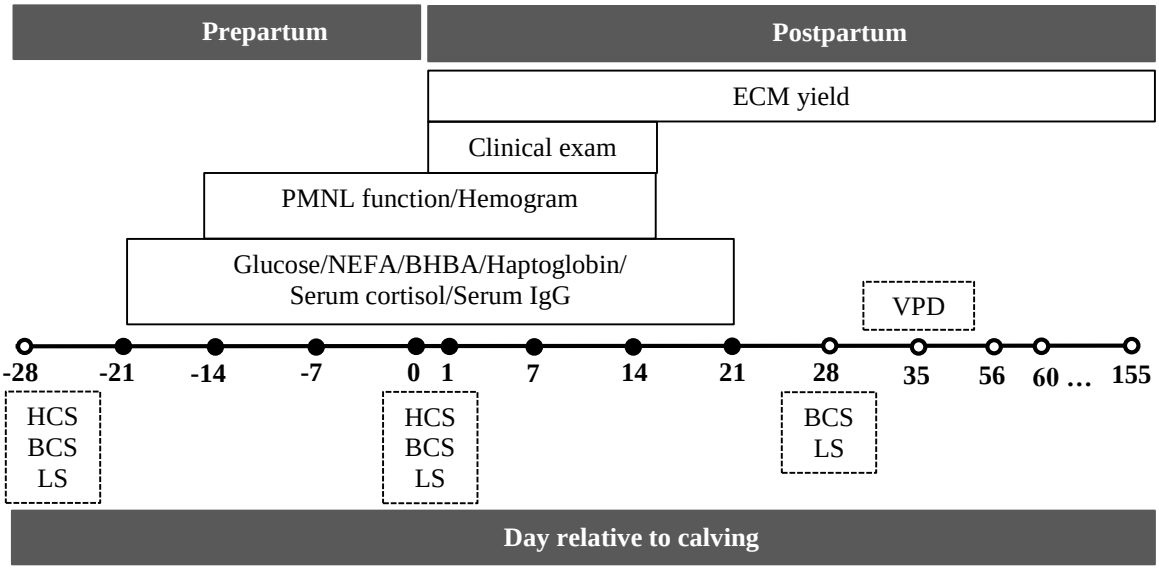


Figure 1. Diagram of activities. Closed circles represent days when blood samples were collected. BCS = body condition score; BHBA= beta-hydroxybutyrate; ECM = energy corrected milk; HCS = hair cortisol sample; LS = locomotion score; NEFA= non-esterified fatty acid; PMNL = polymorphonuclear leukocytes; VPD = vaginal purulent discharge exam.

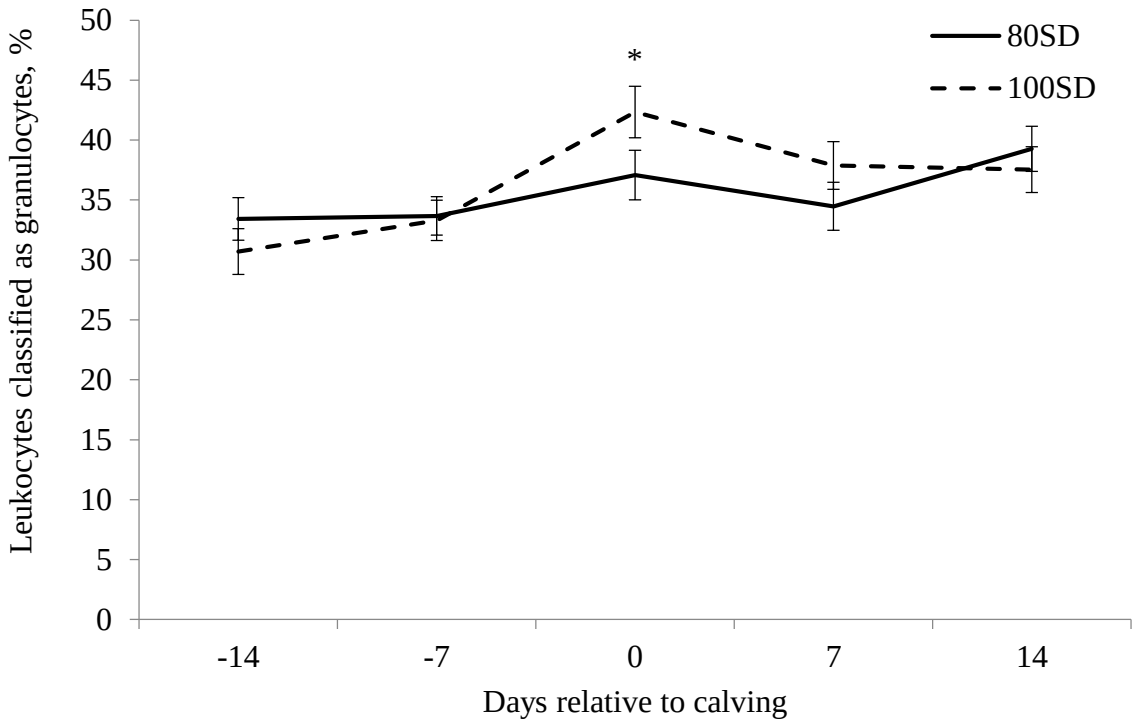


Figure 2. Effect of treatment on percentage of leukocytes classified as granulocytes. Treatment: 80SD – pre-partum pens' target stocking density = 80% of headlocks; and, 100SD – pre-partum pens' target stocking density = 100% of headlocks. Effects: Treatment – $P = 0.69$, day relative to calving – $P < 0.01$, and treatment by day relative to calving – $P = 0.08$. * Indicates a tendency ($P = 0.08$) for treatments to differ.

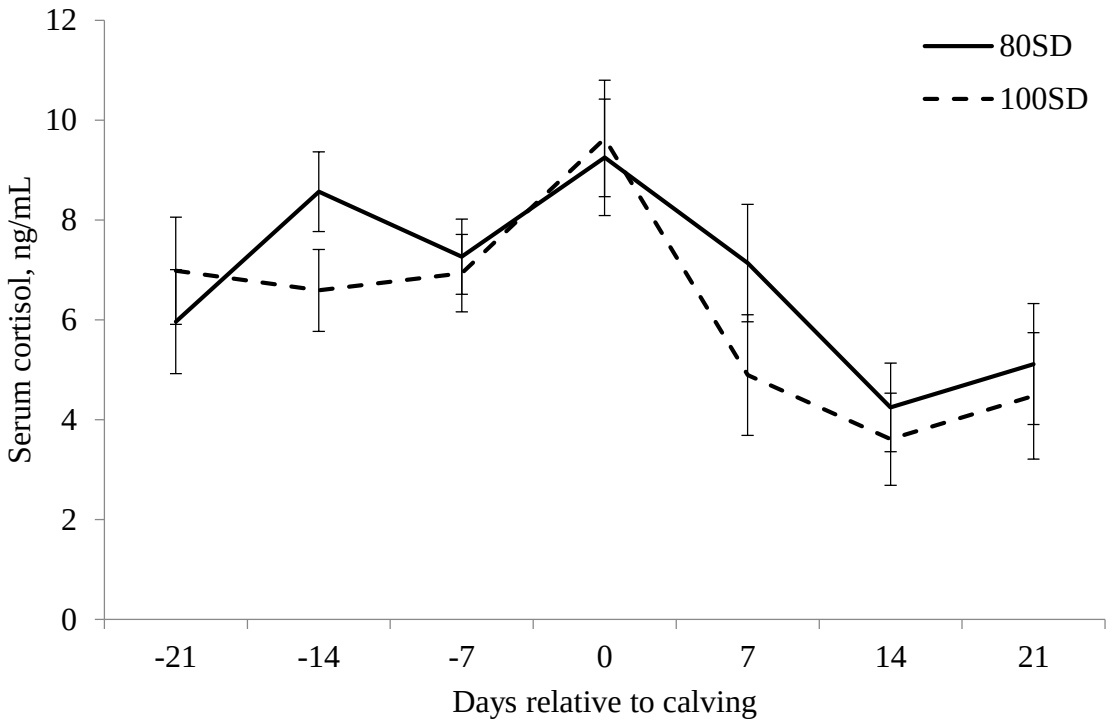


Figure 3A. Effect of treatment on serum cortisol concentration. Treatment: 80SD – pre-partum pens' target stocking density = 80% of headlocks; and, 100SD – pre-partum pens' target stocking density = 100% of headlocks. Effects: Treatment – $P = 0.64$, day relative to calving – $P < 0.01$, and treatment by day relative to calving – $P = 0.81$.

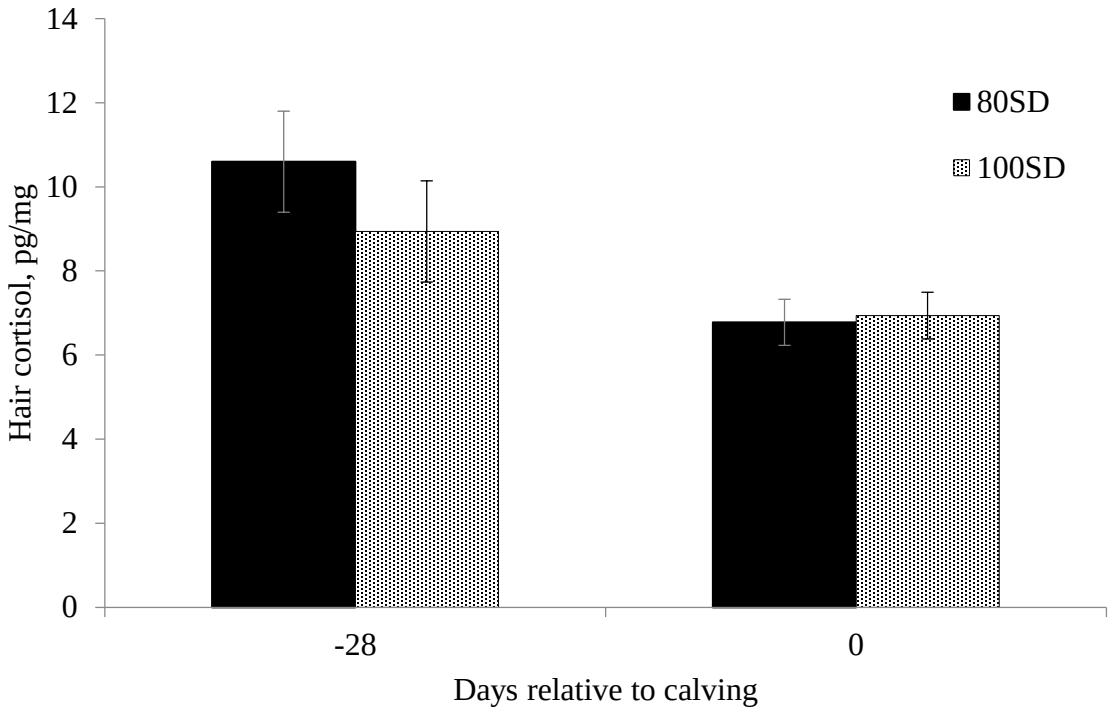
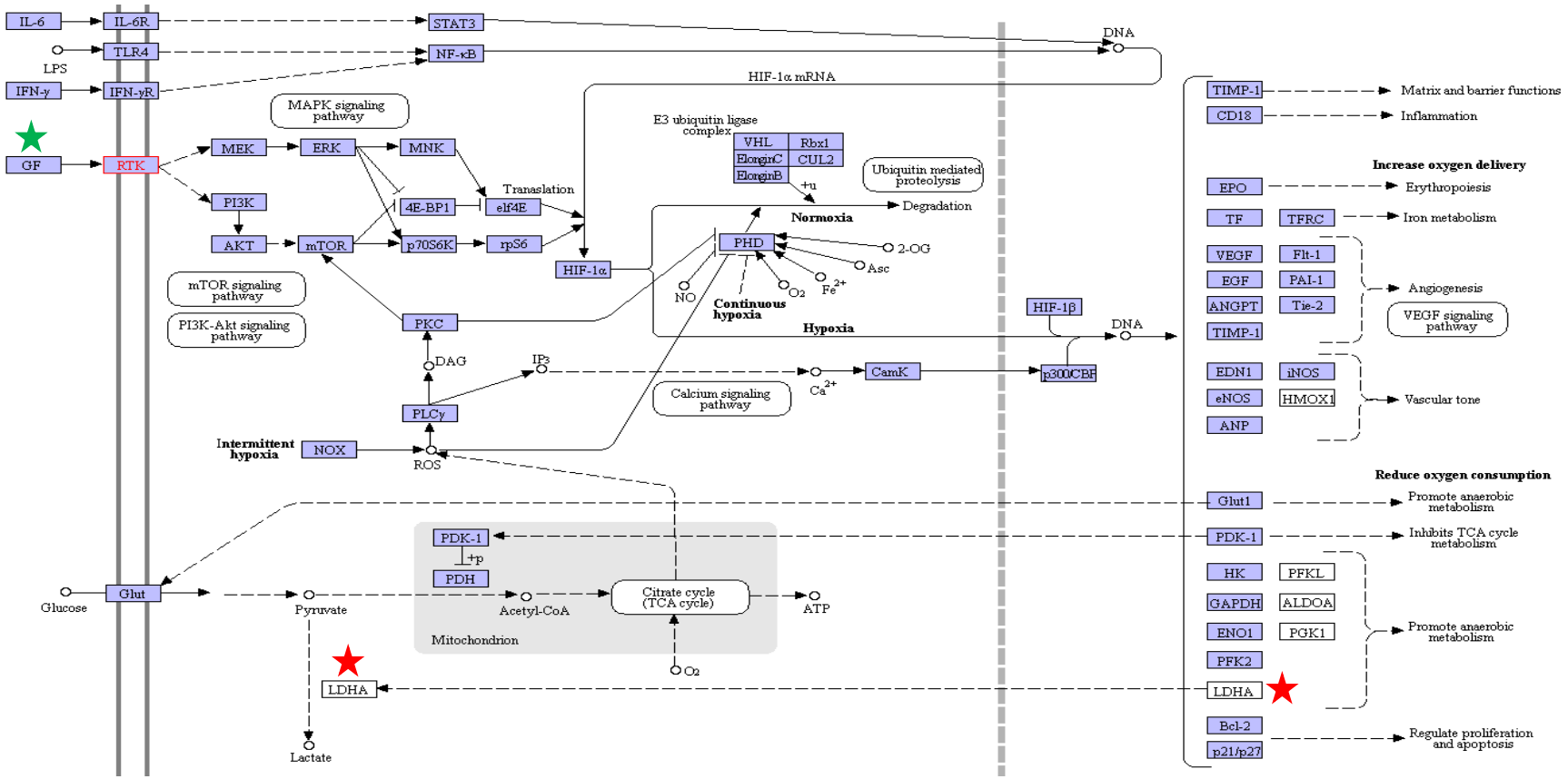


Figure 3B. Effects of treatment on hair cortisol concentrations at enrollment and at calving. Treatment: 80SD – pre-partum pens' target stocking density = 80% of headlocks; and, 100SD – pre-partum pens' target stocking density = 100% of headlocks. Enrollment – P = 0.36; calving – P = 0.84.

APPENDIX

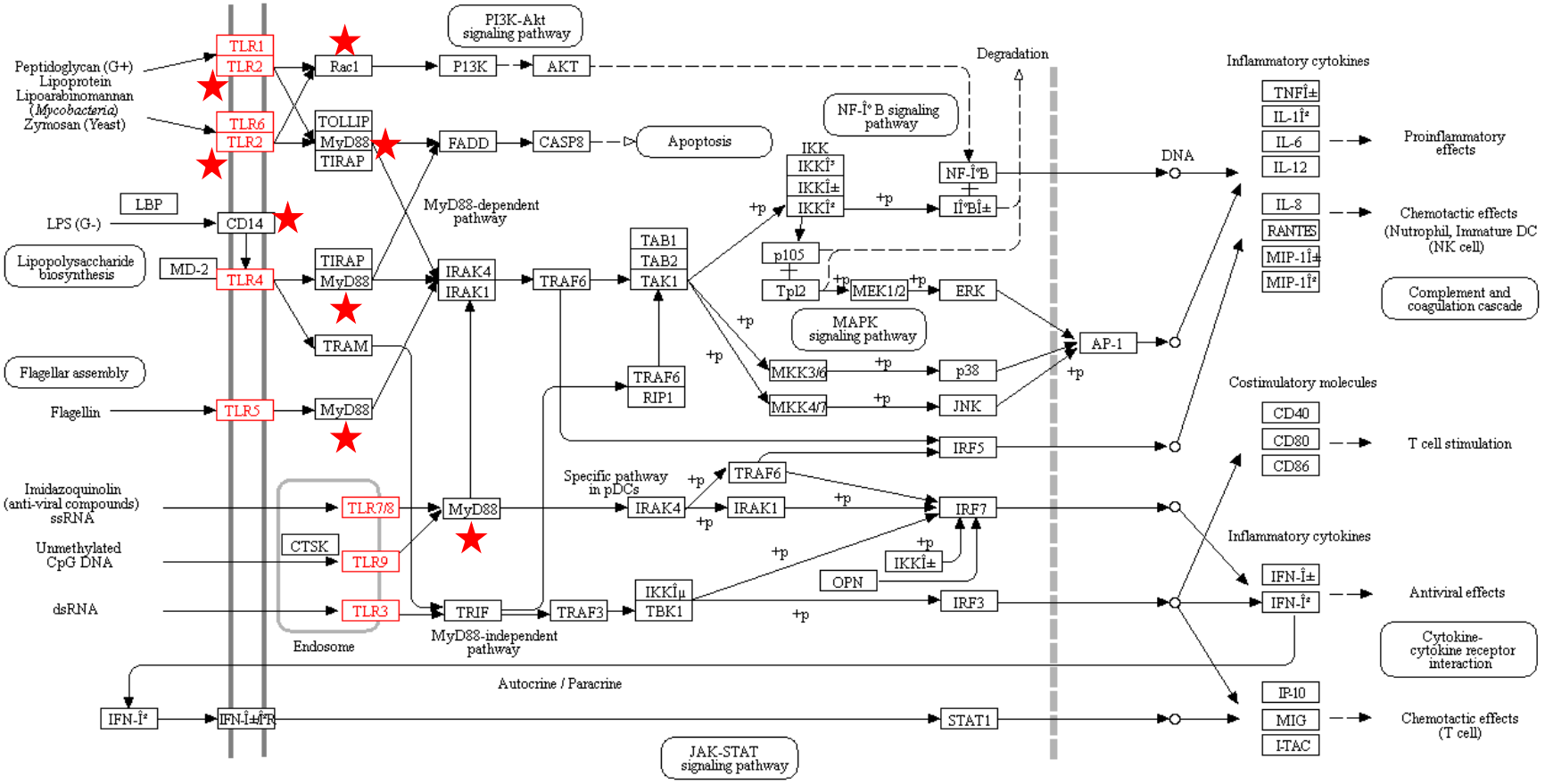
Possible leukocyte pathways and genes modulated by rbST treatments: GF(GH/IGF1)-HIF1A-LDHA



Adapted from KEGG pathways (Kaneisha et al., 2015).

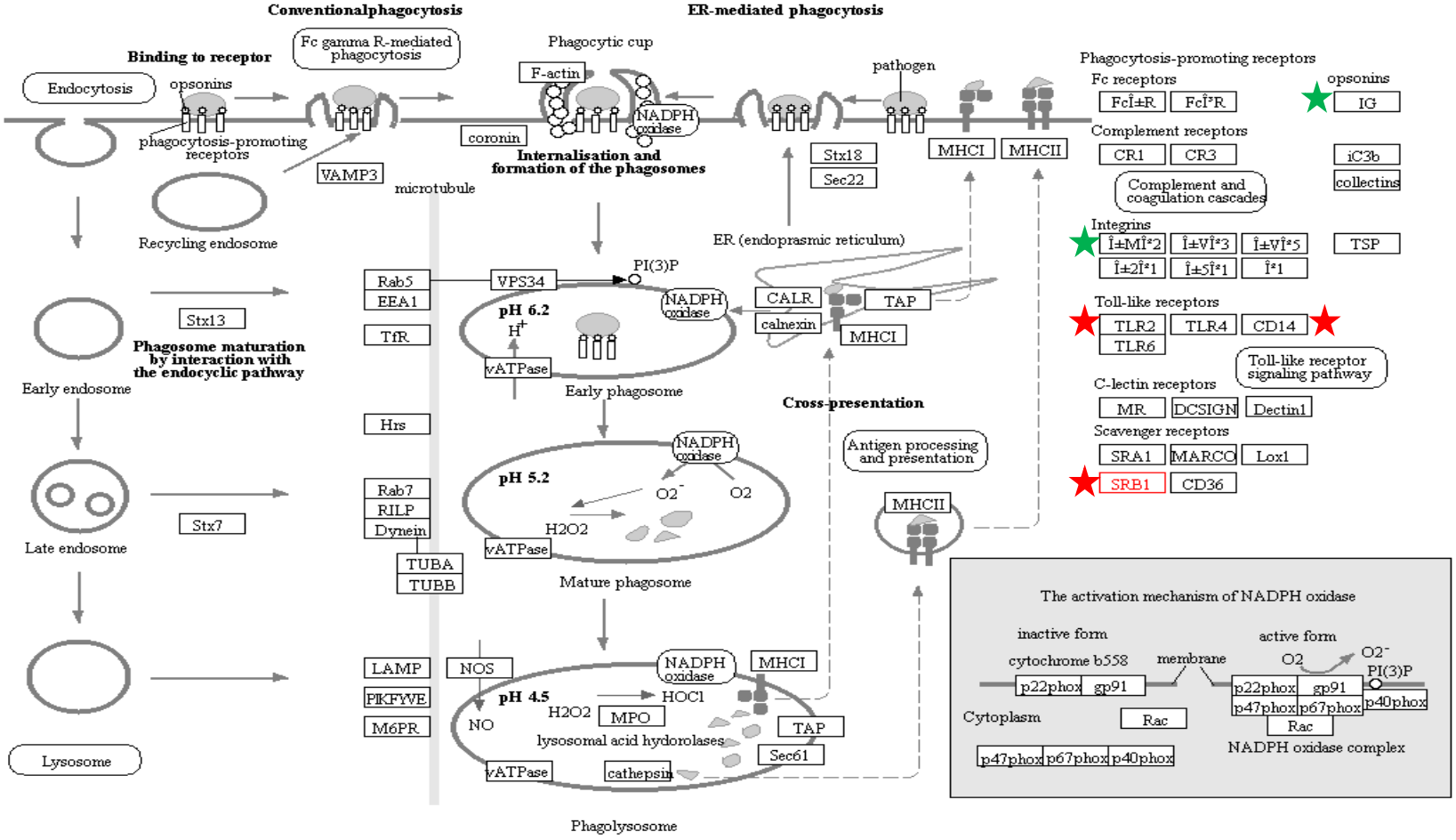
APPENDIX

Possible leukocyte pathways and genes modulated by rbST treatments: TLR2-CD14-MYD88



Adapted from KEGG pathways (Kaneisha et al., 2015).

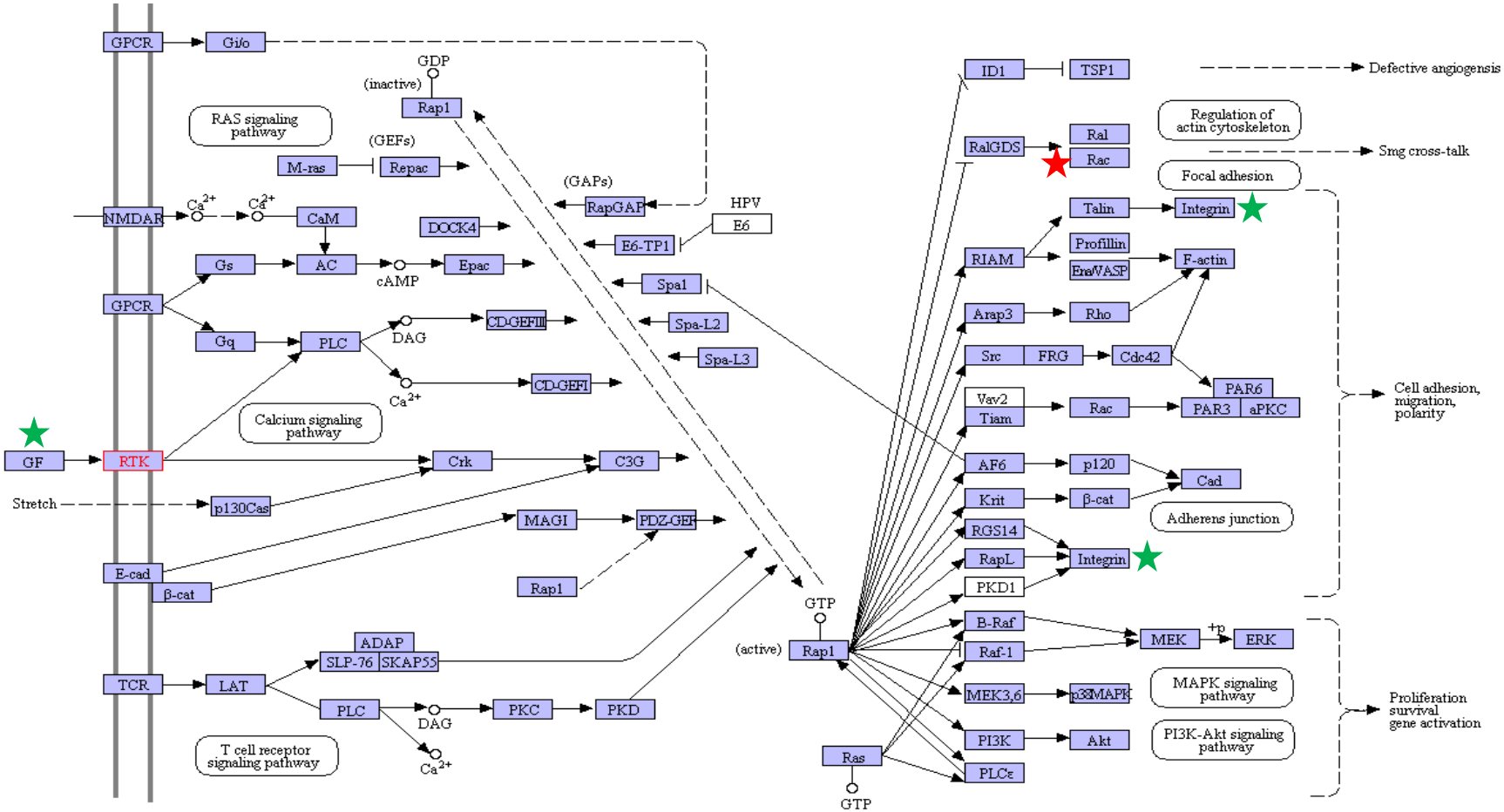
Possible leukocyte pathways and genes modulated by rbST treatments: Phagosome – TLR2-CD14-SCARB1-IgG



Adapted from KEGG pathways (Kaneisha et al., 2015).

APPENDIX

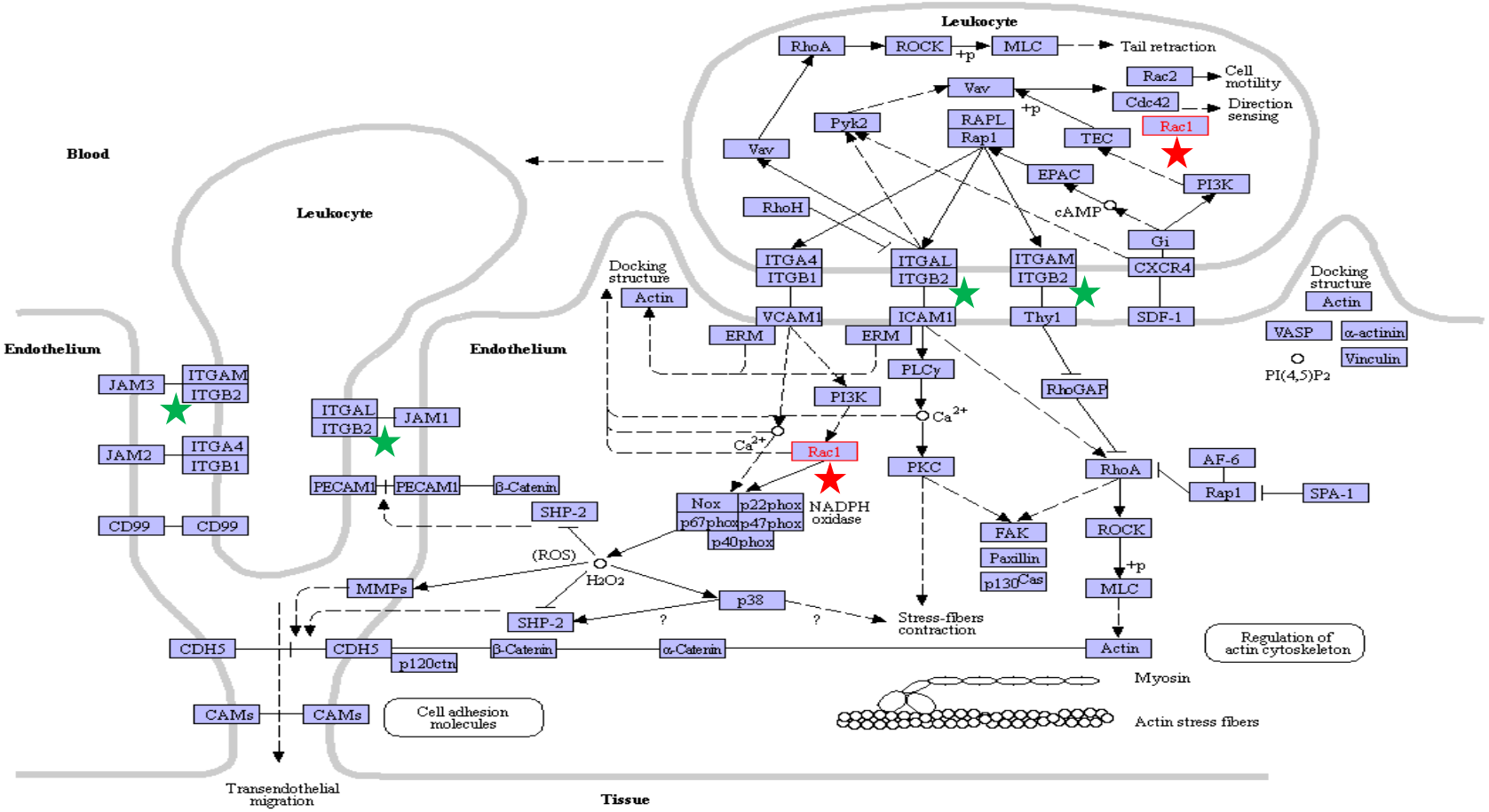
Possible leukocyte pathways and genes modulated by rbST treatments: GF(GH/IGF1)-Rap1-Rac-Integrins (CD18)



Adapted from KEGG pathways (Kaneisha et al., 2015).

APPENDIX

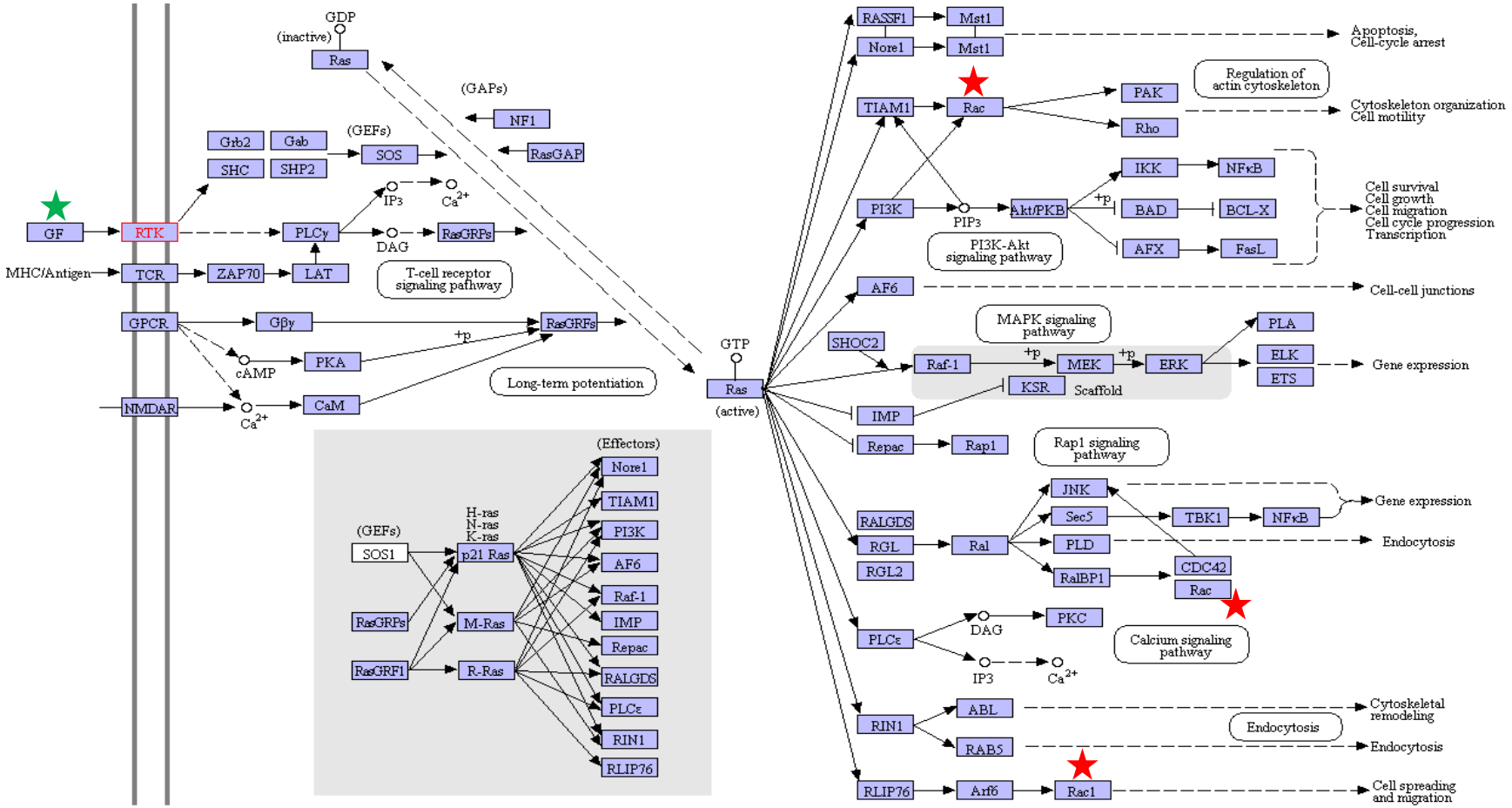
Possible leukocyte pathways and genes modulated by rbST treatments: Transendothelial migration – Rac1-ITGB2 (CD18)



Adapted from KEGG pathways (Kaneisha et al., 2015).

APPENDIX

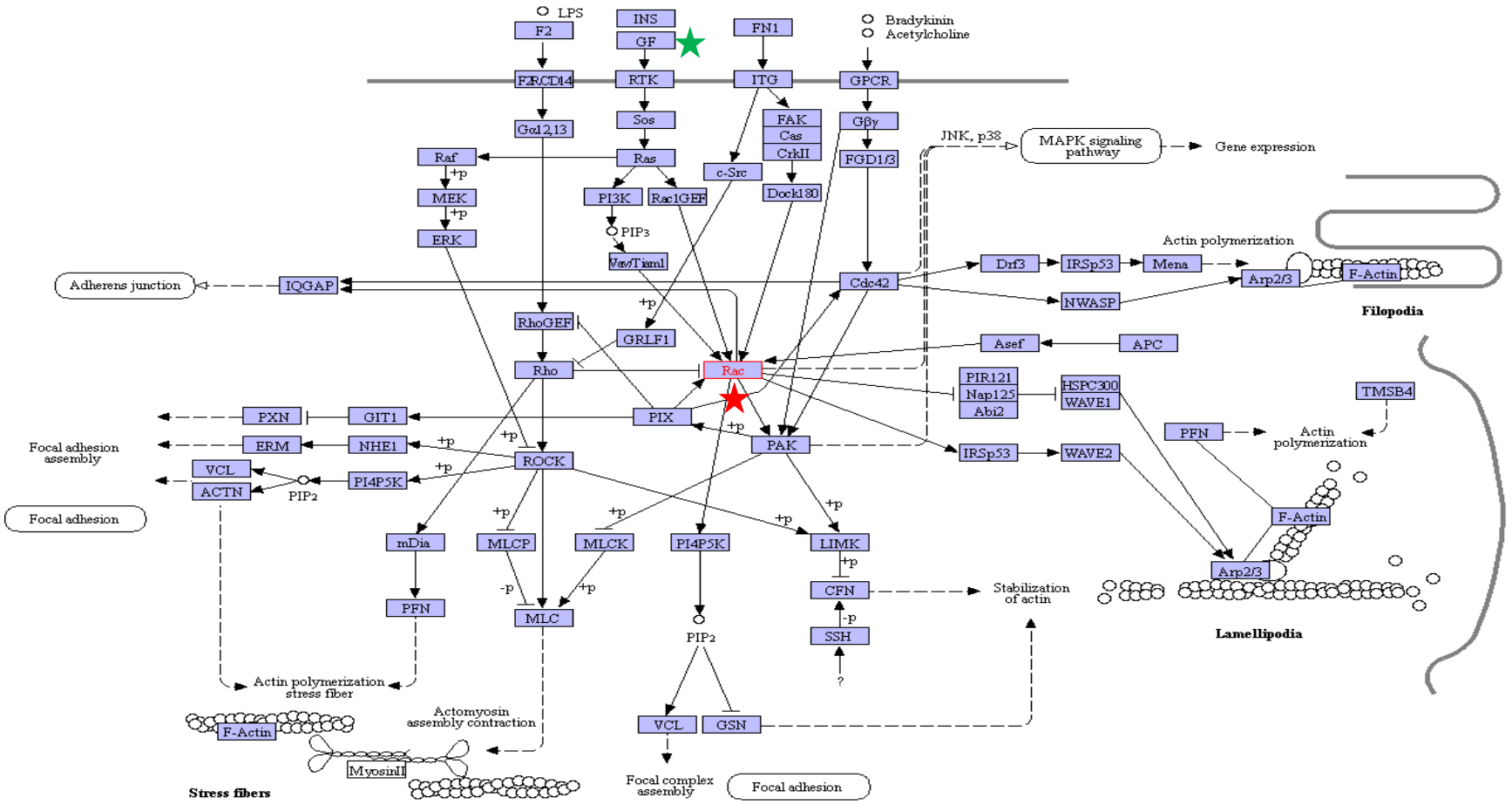
Possible leukocyte pathways and genes modulated by rbST treatments: GF(GH/IGF1)-Ras-Rac



Adapted from KEGG pathways (Kaneisha et al., 2015).

APPENDIX

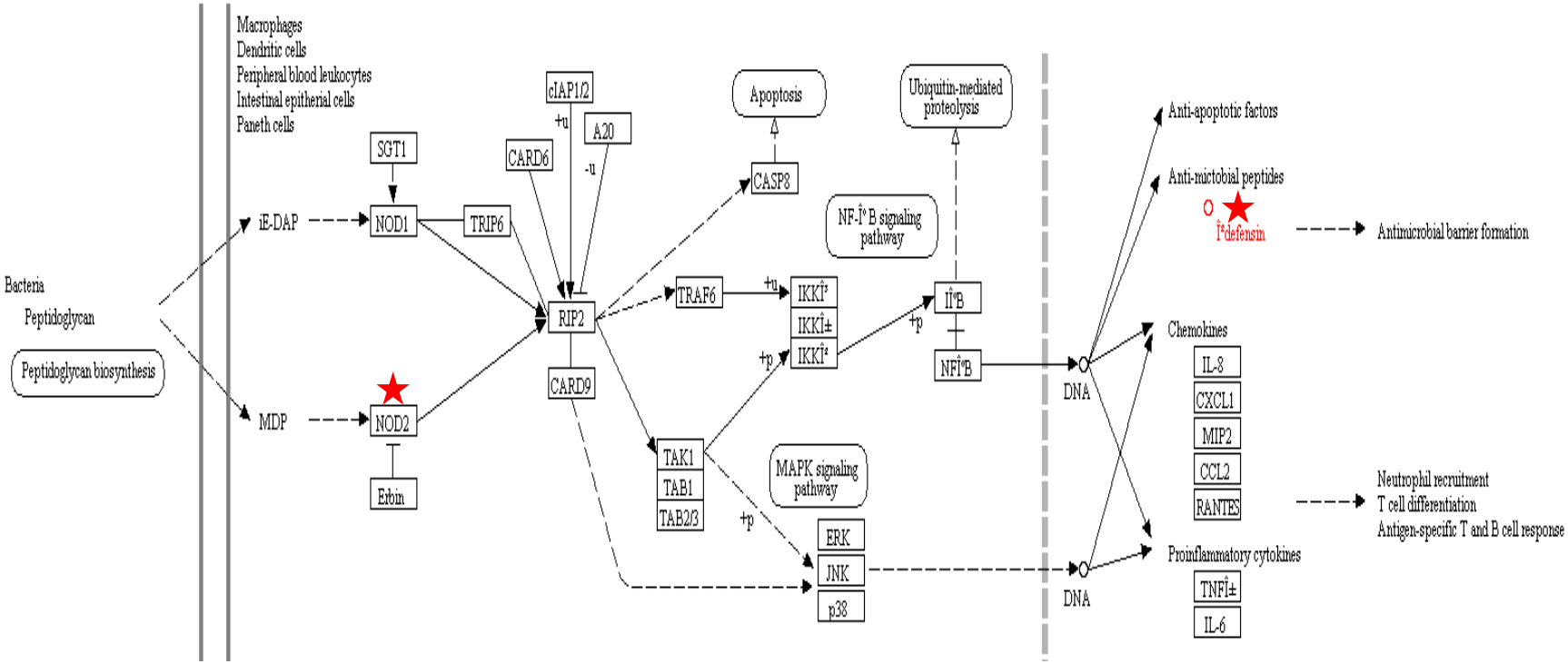
Possible leukocyte pathways and genes modulated by rbST treatments: Actin cytoskeleton regulation – GF(GH/IGF1)-INS-Rac



Adapted from KEGG pathways (Kaneisha et al., 2015).

APPENDIX

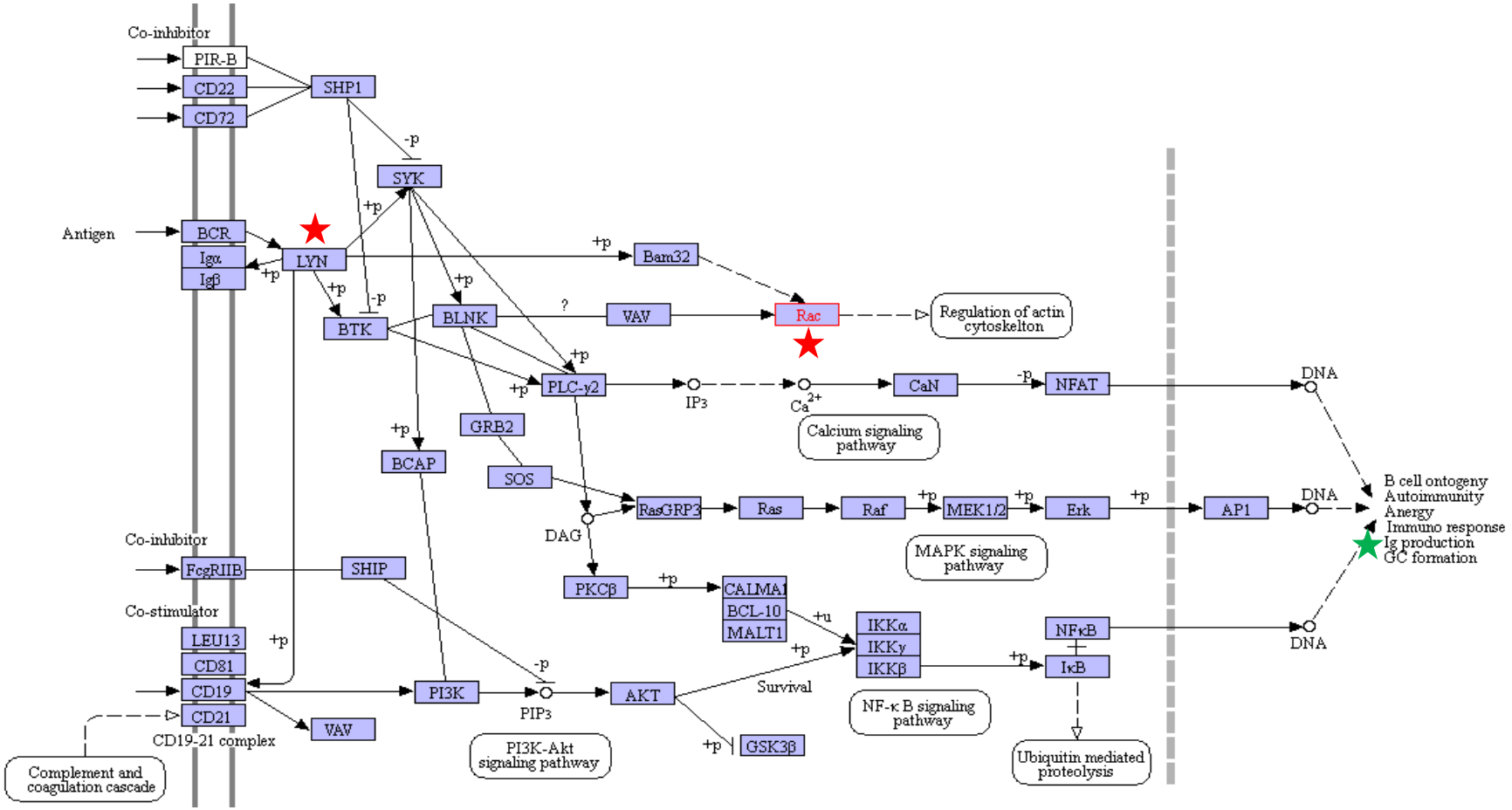
Possible leukocyte pathways and genes modulated by rbST treatments: NOD2-Defensins (DEFB3 and DEFB7)



Adapted from KEGG pathways (Kaneisha et al., 2015).

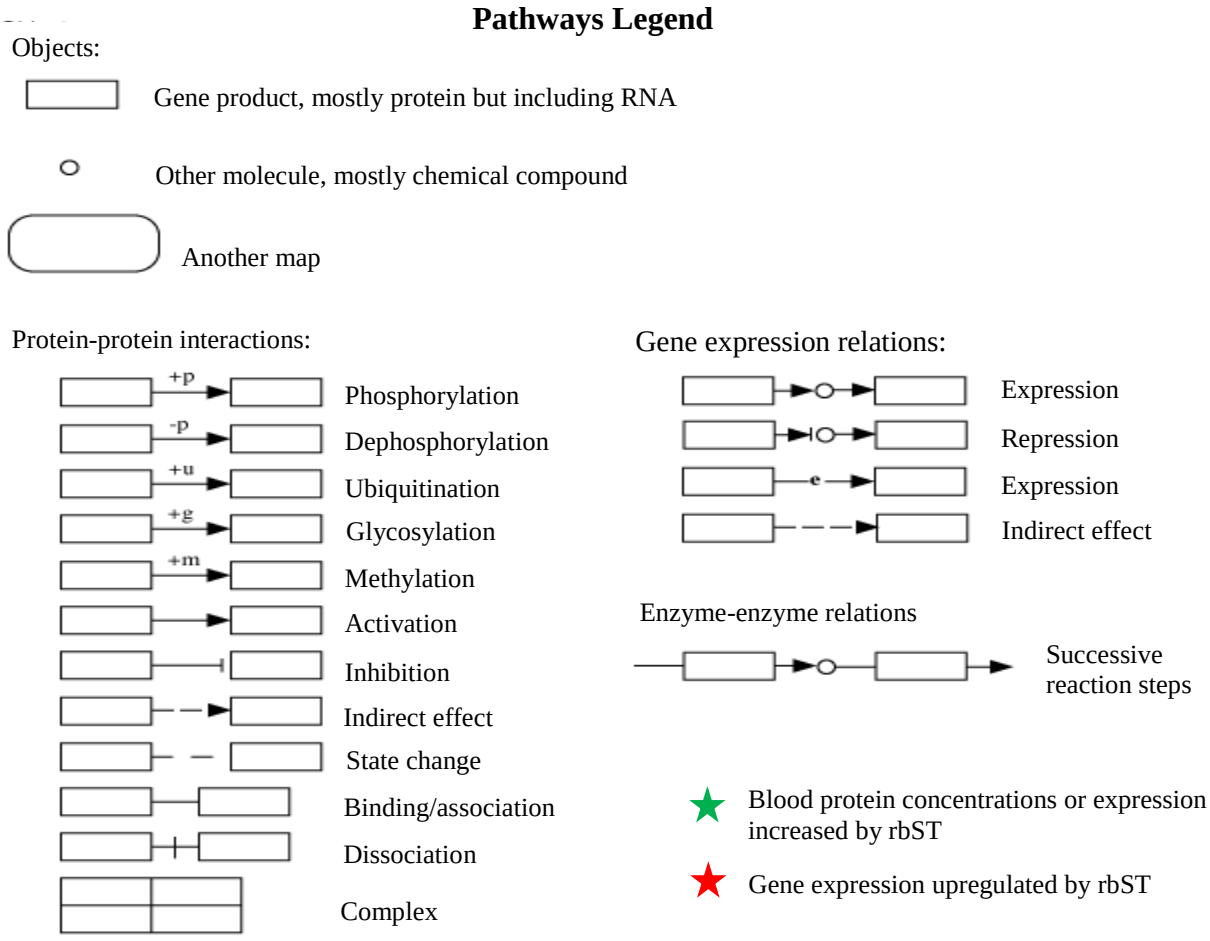
APPENDIX

Possible leukocyte pathways and genes modulated by rbST treatments: B-cell receptor (BCR) signaling – Lyn-Rac



Adapted from KEGG pathways (Kaneisha et al., 2015)

APPENDIX



Adapted from KEGG pathways (Kaneisha et al., 2015).