

The influence of immune activation on energetic and calcium homeostasis in Holstein dairy cows

by

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DEDICATION

This dissertation is written in honor of my parents, Kevin and Andrea Nolan. I am extremely grateful for your continual support. I would not be where I am today without you. I love you both more than I can say.

-Erin

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ABSTRACT

Nutrient partitioning towards productive processes (i.e., milk synthesis, growth, and reproduction) concomitant with diluting maintenance costs are key to profitable animal agriculture. During immune activation, the hierarchy of coordinated nutrient trafficking is reprioritized towards the immune system at the expense of production. Dairy cows encounter frequent immune challenges, as bacterial insults can originate from a myriad of sources including the uterus, mammary gland, lungs, and gastrointestinal tract. Regardless of origin, immune activation hinders animal welfare and mounting evidence suggests it plays a role in many undesirable phenotypes post-calving (i.e., decreased DMI, increased NEFA, hypocalcemia). Following activation, most leukocytes undergo a metabolic shift from oxidative phosphorylation to aerobic glycolysis (a phenomenon known as the “Warburg effect”) and begin consuming copious amounts of glucose. To ensure adequate glucose delivery to activated leukocytes, several well-characterized metabolic adjustments are employed including: increased insulin and glucagon levels, increased skeletal muscle catabolism, hypertriglyceridemia, and hypoketonemia. The energetic burden of immune activation is intensified by a simultaneous decrease in feed intake and thus reduced intestinally derived nutrients. Identifying dietary strategies with potential to alleviate the negative consequences of immune activation on metabolism and production are of interest. As part of this dissertation, supplementation of dietary zinc hydroxychloride, zinc amino acid complex, and chromium propionate were evaluated in feed-restricted or LPS-infused cows. In addition to energetic metabolism, immune activation induces a marked and sustained decrease in circulating calcium (Ca). In this dissertation we have demonstrated that the total Ca deficit was ~20 g during an acute (12 hour) and intense model of immune activation. Infection-induced hypocalcemia is a species conserved response, yet, it remains largely unknown what role

Ca plays during infection and why it abruptly decreases during immune activation. Evidence suggests it may be a protective strategy to prevent a hyperinflammatory systemic response. Based upon the literature and our supporting work we suggest that post-calving hypocalcemia can be explained, at least partially, by inflammation. In addition to hypocalcemia, research in rodents suggests that inflammation may also be involved in fatty liver development. A final objective of this dissertation was to evaluate if inflammation affected liver fat accumulation in artificially-induced hyperlipidemic cows. In summary, immune activation negatively influences metabolic, hormonal, and Ca homeostasis and these alterations closely mimic changes observed in poorly transitioning dairy cows. Having a better understanding of the impact of immune activation on nutrient trafficking and Ca homeostasis will provide foundational information for developing strategies aimed at minimizing production losses during infection.

CHAPTER 1. LITERATURE REVIEW

The Periparturient Period

Nutrient Partitioning

Early lactation cows are often unable to consume enough energy to meet the requirements of both maintenance and milk synthesis. Consequently, cows typically enter into a state of negative energy balance (**NEBAL**) and marked alterations in whole-body carbohydrate and lipid metabolism are employed to support lactation. Detailed descriptions of these mechanisms have been reported previously (Vernon, 1989; Bell, 1995; Drackley, 1999), but will be summarized briefly herein. During NEBAL, somatotropin promotes non-esterified fatty acid (**NEFA**) export from adipose tissue by increasing the lipolytic response to β -adrenergic signals and reducing lipogenic and antilipolytic responses to insulin (Bauman and Vernon, 1993).

Adipose tissue lipolysis and NEFA mobilization are accentuated by a concomitant decrease in pancreatic insulin secretion (Bauman and Currie, 1980; Rhoads et al., 2004). Reduced insulin action decreases glucose disposal by insulin-sensitive tissues (i.e., adipose tissue and muscle) and reduces inhibition of hepatic gluconeogenesis. The mammary gland's glucose uptake is not insulin dependent (Zhao and Keating, 2007), so the aforementioned metabolic adjustments are key homeorhetic strategies to ensure that glucose uptake is not the bottleneck of milk synthesis.

In the presence of elevated NEFA, skeletal muscle fatty acid oxidation increases and this process blunts glucose uptake and oxidation, this key "glucose-sparing" mechanism is referred to as the "Randle effect" (Randle, 1998). Non-esterified fatty acids not oxidized by systemic tissues are either utilized as precursors for milk fat synthesis or taken up by the liver where they can be completely oxidized to provide energy, partially oxidized for ketone body production, or resynthesized into triglycerides (**TG**) which are secreted (via very low density lipoproteins;

VLDL) or stored (Drackley, 1999). Some tissues (i.e. the central nervous system) and cell types (i.e. red blood cells) are unable to oxidize NEFA and thus require the energy within fatty acids to be interconverted into an alternative form. Thus, liver-derived ketones serve as a substitutable energy source for various tissues. Altogether, these homeorhetic strategies are coordinated to allow for maximal milk synthesis (Bauman and Currie, 1980).

Metabolic and Infectious Disease Traditional Dogmas

Maintaining cow health and productivity during the transition period represents a significant hurdle to the dairy industry. Coinciding with the aforementioned changes in energetic metabolism is an increased risk of metabolic disorders and infectious diseases such as ketosis, fatty liver, milk fever, displaced abomasum (**DA**), retained placenta (**RP**), mastitis, and metritis (Goff and Host, 1997; LeBlanc, 2010). Approximately 75% of disease typically occurs during the first month postpartum (LeBlanc et al., 2006) and studies suggest the disorders are interrelated (Curtis et al., 1984). Long-standing tenets describe a causal role of hypocalcemia, increased NEFA, and hyperketonemia in the incidence of transition diseases and disorders (Figure 1.1).

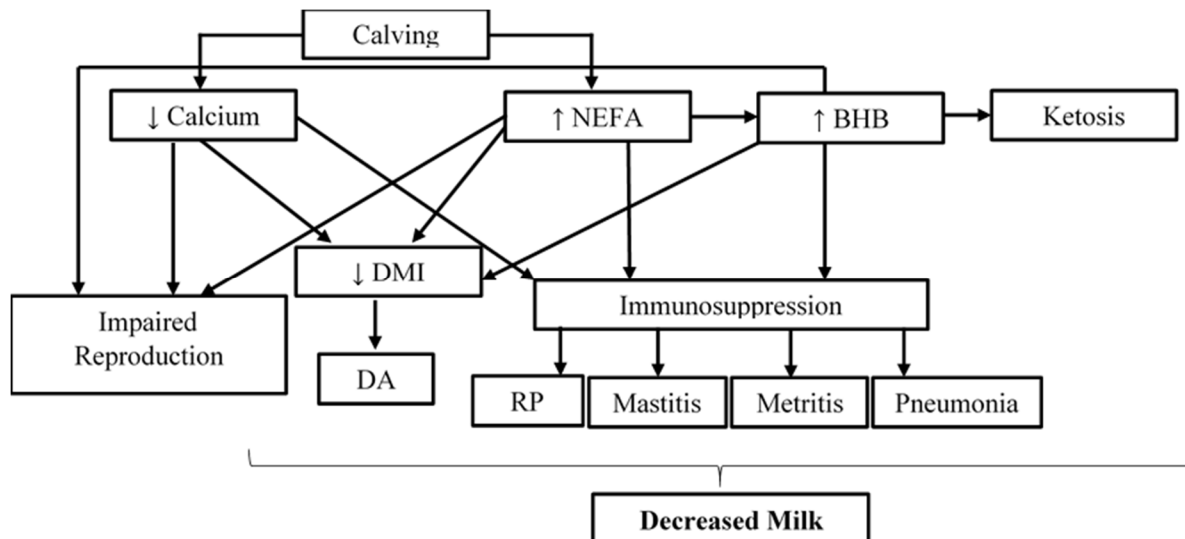


Figure 1.1. Traditional mechanisms by which hypocalcemia and increased NEFA and ketones are thought to cause poor transition cow health and performance.

Research characterizing metabolic disorders by alterations in a single circulating metabolite began as early as the 1920s. In these reports, milk fever was characterized by decreased circulating calcium (Ca; Hayden and Scholl, 1923; Sjollem and Van Der Zande, 1923; Dryer and Greig, 1925) and ketosis was described by increased circulating acetone (Stinson, 1928; Sampson et al., 1933). In the late 1950s and 1960s, ketosis was further characterized by changes in NEFA (Radloff et al., 1966; Radloff and Schultz, 1967) and NEBAL was proposed as the primary cause of ketosis (Shaw, 1956). In the mid-1980s, it became clear that many of the major metabolic and infectious disorders were interrelated (Curtis et al., 1984; Markusfeld, 1986; Gröhn et al., 1989). Associations of increased NEFA, hyperketonemia, and hypocalcemia and the incidence of disease became a topic of intensive investigation in the 1980s (Curtis et al., 1983; Doohoo and Martin, 1984; Markusfeld, 1987; Kaneene et al., 1997; Geishauser et al., 1997; Cameron et al., 1998). Since that point, expansive literature has been published associating alterations in NEFA, ketones, and Ca with cow health and performance. Increased NEFA and hyperketonemia are presumptively causative to illnesses such as DA, RP, metritis, reduced lactation performance, poor reproduction, and an overall increased culling risk (Cameron et al., 1998; LeBlanc et al., 2005; Duffield et al., 2005; Quiroz-Rocha et al., 2009; Ospina et al., 2010a; Chapinal et al., 2011; Huzzey et al., 2011). Similarly, hypocalcemia has traditionally been considered a gateway disorder leading to ketosis, mastitis, metritis, DA, impaired reproduction, and decreased milk yield (Curtis et al., 1983; DeGaris and Lean, 2008; Goff, 2008; Martinez et al., 2012; Chapinal et al., 2012; Riberio et al., 2013; Neves et al., 2018a, b). In addition to specific metabolites, the magnitude and duration of NEBAL is associated with an increased risk of metabolic disorders, health problems (Goff and Horst, 1997; Drackley, 1999; Heuer et al., 1999) and poor reproduction (Lucy et al., 1992; Beam and Butler, 1999). In

particular, the severity and timing of the NEBAL nadir (~5-10 DIM) is related to reproductive success (Beam and Butler, 1999; Butler, 2000). Consequently, transition period energetics and metabolism have been intensely studied, written about, and excessively pontificated on for the last 50 years.

Despite the wide-held global belief, there remains little mechanistic evidence for how changes in these simple and ancient energetic metabolites and Ca can directly have such a large influence on a variety of seemingly unconnected systems and diseases. The best line of evidence is extrapolated from the purported role of elevated NEFA, hyperketonemia, and hypocalcemia on immunosuppression (Ducusin et al., 2003; Lacetera et al., 2004; Scalia et al., 2006; Hammon et al., 2006; Martinez et al., 2012, 2014; LeBlanc, 2020). For example, in vitro incubation of isolated neutrophils with increasing NEFA and BHB concentrations negatively impacts leukocyte function, such as neutrophil oxidative burst (Hoeben et al., 1997; Scalia et al., 2006; Grinberg et al., 2008; Ster et al., 2012) and lymphocyte antibody secretion (Lacetera et al., 2004). Additionally, chemotaxis and myeloperoxidase activity were impaired in neutrophils isolated from cows with elevated NEFA and ketones around parturition (Suriyasathaporn et al., 1999; Hammon et al., 2006). Intracellular calcium signaling is a key initial feature in leukocyte activation (Lewis, 2001) and reports have demonstrated decreased leukocyte intracellular Ca stores in hypocalcemic cows (Ducusin et al., 2003; Kimura et al., 2006). Furthermore, Ca chelators have been shown to reduce neutrophil phagocytosis in vitro (Ducusin et al., 2001) and in vivo (Martinez et al., 2014). However, there are inconsistencies (in vivo and in vitro) in how NEFA, hyperketonemia and hypocalcemia affect leukocyte function (reviewed by LeBlanc, 2020). Extending in vitro results to the whole-animal has obvious limitations and this is especially true when considering the immune system. For example, most leukocyte's function is

integrally dependent upon an intracellular metabolic shift from oxidative phosphorylation to aerobic glycolysis (discussed below; Pálsson McDermott and O'Neill, 2013) and it is highly unlikely that in vitro conditions can mimic the extracellular milieu accompanying normal immune activation. Additionally, we now realize that almost all periparturient dairy cows (even the seemingly healthy ones) experience some extent of immune activation and thus inflammation (discussed more below: Humblet et al., 2006; Bertoni et al., 2008). Consequently, it is not clear whether leukocyte function during the transition period reflects “immunosuppression” or simply the pathology associated with normal immune activation. Regardless, other than the questionable immune suppression theory, there remains scant evidence (or even hypotheses) of how changes in NEFA, ketones and Ca concentrations could have such large impacts on unconnected phenotypes.

We believe there are multiple flaws in the theory connecting NEFA, ketones, and Ca with negative outcomes in the post-fresh dairy cow. In addition to not having causal substantiation nor even biological plausibility, many of the theory’s tenets appear to counter evolutionary adaptations associated with milk synthesis, reproduction, and survival of a species. Below, we outline the inadequacies of the rationale for causation and provide evidence (facts and concepts) demonstrating that changes in circulating NEFA, ketones, and Ca are not causing negative outcomes, but are simply reflective of either normal metabolic changes healthy cows enlist to achieve high production or the metabolic consequences of immune activation-induced hypophagia.

Table 1.1. Limitations to the Dogma that NEFA, Ketones and Ca are causing negative outcomes

-
1. Inconsistent evidence
 2. Association and correlations are not cause and effect
 3. Infusing ketones or NEFA does not cause negative outcomes
 4. Ketones do not decrease feed intake
 5. Preventing adipose tissue mobilization reduces milk yield
 6. Ketotic cows are sometimes not hypoinsulinemic
 7. Some females do not consume food after parturition
-

Inconsistency and Association vs. Causation: Though there are hundreds of peer-reviewed manuscripts demonstrating an association between these metabolites and transition cow problems, importantly some do not. For example, a variety of papers demonstrated no relationship between NEFA, ketones, and Ca and negative outcomes (Burke et al., 2010, Bicalho et al., 2014, 2017; Abdelli et al., 2017). The consistency of an affect is incredibly important when making causal inference from observational and retrospective research. Secondly, these tenets are largely based on associations and not cause and effect relationships garnered from controlled and intervening experimentation. Assessing the strength or robustness of the associations within the literature is difficult due to variability in analysis and statistical methods. In particular, different metabolite thresholds are utilized between studies and for different outcomes and timepoints (pre vs. postpartum, week 1 vs. 2) within an observational study. In addition, uncommon association metrics (e.g., odds ratio, relative risk, hazard ratio) are utilized to assess the relationship. A partial summary of the association studies was compiled by McArt et al. (2013) and Overton et al. (2017). Although these reports illustrate the large number of observational studies demonstrating a relationship of the metabolites (NEFA, BHB, and Ca) with health and performance, they also demonstrate the large variability in metabolite thresholds and association strength. For example, the association (as measured by OR) between postpartum BHB and DA incidence ranges from 1.1 to 27.6 across studies (McArt et al., 2013). Often times these metabolites are actually *positively* associated with milk yield (Duffield et al., 2009; Furken et al., 2015; Belay et al., 2017; Bach et al., 2019). Interestingly, several reports demonstrate both a negative association of elevated NEFA and ketones with health and a positive association with production (Duffield et al., 2009; Ospina et al., 2010b; Belay et al., 2017; Bach et al., 2017). The

conflicting relationships described above illustrate the limitations of the dogma and highlight the restrictions of retrospective classification (healthy vs a negative outcome).

Feed intake: The detrimental effects of high ketone and NEFA levels has been partially attributed to their alleged suppressive effect on feed intake (Allen et al., 2009; Laeger et al., 2012). This is especially a prevalent mindset in veterinary medicine as practicing clinicians often anectodly report that ketones depress feed intake. However, the results of NEFA and ketone infusion studies on feed intake are extremely variable and are further complicated by utilizing animals in positive energy balance receiving an exogenous energy source. In other words, because animals are thought to primarily eat to meet their energetic requirement (Church and Pond, 1988), infusing any energy (glucose, NEFA, BHBA etc.) would conceptually decrease feed intake when animals were in positive energy balance, but would not decrease appetite if animals were on a lowered plane of nutrition. In an elegant series of studies conducted by Zarrin et al. (2013; 2014a, b) they found that BHBA infusion did not affect feed intake or milk yield. Infusion of propionate, but not lipid, decreased dry matter intake in mid-lactation cows (Stocks and Allen, 2014). When examining different fuel sources infused into the brain, Davis et al. (1981) found that glucose and glycerol reduced feed intake while BHBA did not. Furthermore, infusing ketone bodies i.v. actually increased feed intake (Carneiro et al., 2016a, b). With the wide variation in responses to metabolite infusion, it is hard to draw conclusions from these models outside of normal physiological repsonses. Regardless, from an evolutionary perspective it is bioenergeticly diffuclt to hypothesize why NEFA and BHBA would decrease appetite. Adipose tissue mobilization and partial conversion of NEFA into ketones is a key metabolic strategy animals utilize to conserve skeletal muscle and ultimately survive negative energy balance (Sherwin et al., 1975). The importance of ketogenesis to surviving malnutrition is

highlighted by the fact that mutations in the gene regulating ketone synthesis (mitochondrial HMG-CoA synthetase) results in hypoglycemic-induced coma within 24 hours (Thompson et al., 1997). Reliance on stored lipid during energy insufficiency is so conserved that even microorganisms have the capacity to store and oxidize NEFA (Nunn, 1986) and interconvert fatty acid energy into ketones (Wang et al., 2014). Thus, even the simplest of life forms have been utilizing these basic and uncomplicated ancient fuels (NEFA and ketones) since the beginning of time. If NEFA and BHBA actually blunted the urge to eat, a starving animal would choose to be anorexic; a scenario that would hasten their demise, and one, which is obviously not biologically accurate. For eons, animals have ebbed and flowed into and out of negative energy balance (because of food insecurity, hibernation, migration, and lactation) and oxidizing NEFA and ketones are key to this survival strategy, but during the last 50 years dairy scientists have increasingly viewed increased circulating NEFA and ketones as pathologic.

Anorexia during lactation: Increased mobilization of adipose tissue to support lactation is a conserved response in lactating females (Ofstedal, 1992, 2000; McNamara, 1997; Vernon and Pond, 1997; Ramos-Roman et al., 2020). Interestingly, in certain mammals such as bears, seals, dolphins, and baleen whales (i.e. the Bluewhale), lactation occurs concurrently with a prolonged fast and consequently these mammals rely almost entirely on adipose tissue reserves to meet energy demands (Ofstedal, 1992, 1997, 2000; Crocker et al., 2001; Fowler et al., 2016, 2018). In fact, baleen whales will sustain a 6 to 7 month lactation without eating (Ofstedal, 1992) and will mobilize ~33% of their fat stores which is equivalent to 16 ton of their body weight (Ofstedal, 2000). In seals, greater than 90% of the energy requirements of lactation are powered by lipid stores (Crocker et al., 2001; Fowler et al., 2018) and these mammals may lose more than 50% of their body fat reserves (Crocker et al., 2001). This is even more impressive considering most sea

mammals are thought to be unable to perform ketogenesis (Jeff and Hiler, 2018). Regardless, the species conserved reliance on NEFA to support lactation further exemplifies the importance of this strategy. In fact, the extent to which cows incorporate adipose tissue mobilization pales in comparison to many other species (Collier et al., 2005).

Insulin and preventing adipose mobilization: A key strategy (maybe the most integral part) to successfully initiating milk synthesis is the development of insulin resistance in both skeletal muscle and adipose tissue and the decrease in pancreatic insulin secretion (Bauman and Currie, 1980; Baumgard et al., 2017). As already mentioned, this allows adipose tissue mobilization and the exiting NEFA can be used by most cell types and tissues in the body. For the tissues and cells that either do not have the machinery necessary to oxidize NEFA or do not have access to it (i.e. the central nervous system because of the blood brain barrier), the liver interconverts NEFA energy into ketones and this allows almost the entire organism to utilize adipose-derived molecules for energy. This altered use of fuel “saves glucose” for the synthesis of milk and indeed >90% of glucose made by the liver is utilized by the mammary gland in early lactation (Bell, 1995). The bedrock of this homeorhetic principle is a reduction in circulating insulin coupled with insulin resistance in extra-mammary tissue. Not surprisingly then, higher producing cows are more hypoinsulinemic than their lower producing herd mates throughout lactation (Hart et al., 1975, 1978, 1979), early lactating insulin concentrations are inversely related to whole lactation performance (Zinicola and Bicalho, 2019), and insulin clearance is influenced by genetic selection for milk yield (Barnes et al., 1985). Further, administering insulin or insulin sensitizing agents decrease milk yield (Kronfeld et al., 1963; Schmidt, 1966; Yousefi et al., 2016), albeit this was not corroborated in mid-lactation cows receiving a low insulin dose (Winkelman and Overton, 2013).

Given insulin's incredibly potent regulation of intermediary metabolism, high milk production associated excessive adipose tissue-induced ketosis should be accompanied by severe hypoinsulinemia (Hove, 1978). Hypoinsulinemia allows for maximal rates of hepatic glucose export (glycogenolysis and gluconeogenesis; Berg, 2002). The intrahepatocyte biochemistry favoring ketone production centers on oxaloacetate (**OAA**) and acetyl-CoA derived from fatty acid oxidation. If oxaloacetate is plentiful, acetyl-CoA combines with it to make citrate and the TCA cycle progresses (Krebs, 1966). However, when glucose requirements are high and during robust rates of gluconeogenesis (i.e., early lactation) OAA leaves the TCA cycle to form phosphoenolpyruvate. Simultaneously, there is a large amount of acetyl CoA originating from β -oxidation of adipose derived NEFA (Krebs, 1966). The unavailability of OAA is now the metabolic crossroad between carbohydrate and lipid metabolism and accumulated acetyl CoA enters into ketogenesis (Krebs, 1966), an enzymatic pathway inhibited by insulin (Sato et al., 1995). Accordingly, most cows with hyperketonemia are simultaneously hypoinsulinemic (Hove, 1978; Brockman, 1979). However, frequently there are no differences in circulating insulin between ketotic cows and healthy controls (Oikawa et al., 2019; Baumgard unpublished data), and in fact ketosis has been reported to be accompanied with hyperinsulinemia (Kronfeld 1971; Holtenius and Holtenius, 1996; Herdt, 2000). Further, hyperinsulinemia is thought to occur prior to clinical signs of ketosis (Rukkwamsuk et al., 1998, 1999). This is a peculiar pathological endocrine profile as insulin would normally prevent ketosis on multiple levels: 1) blunting adipose tissue mobilization, 2) reduced hepatic gluconeogenesis and thus minimized drain of OAA leaving the TCA cycle, 3) reduced fatty acid transport into the mitochondria via carnitine palmitoyltransferase 1 (**CPT1**) downregulation, and 4) negatively governing the rate limiting enzyme of ketone synthesis (HMG-CoA synthase). Resultantly, there are clearly numerous

metabolic and endocrine footprints associated with ketosis, a controversial concept originally proposed by Holtenius and Holtenius (1996) and supported by Herdt (2000). Regardless, there are obviously key knowledge gaps remaining in the pathophysiology of ketosis.

In summary, it is becoming evident that periparturient diseases and disorders cannot be fully explained by this traditional line of thinking. Instead, recent reports suggest immune activation and inflammation may be involved and this will be discussed throughout this review.

Lipopolysaccharide

Structure

Lipopolysaccharide (**LPS**), also called endotoxin, is a glycolipid present in the outer membrane of gram-negative bacteria, which has a tripartite structure consisting of a lipid A moiety, a core polysaccharide, and an O-polysaccharide. The lipid A domain anchors LPS to the outer membrane and is the endotoxically active part of the molecule (Erridge et al., 2002; King et al., 2009). Structural changes in the lipid A domain, such as phosphorylation state of the disaccharide backbone and nature, number, and length of the acyl chains, influence LPS toxicity (Erridge et al., 2002; Li et al., 2013). The diphosphorylated hexaacyl lipid A structure (commonly found in *Escherichia coli*) elicits a potent inflammatory response due to optimal recognition by the immune system (Erridge et al., 2002). Bacteria modify the lipid A structure in response to environmental stimuli to improve resistance to immune system-derived antimicrobial peptides (King et al., 2009). Although structural variations exist in the lipid A region, it is far more conserved when compared to the polysaccharide domains. The O-polysaccharide, which consists of 0 to 50 repeating oligosaccharides, contains the greatest degree of variability among the domains and is the outermost part of the LPS molecule. Although not pathogenic, the O-polysaccharide (also called the O-antigen) is the immunogenic component of LPS and is associated with virulence (Lerouge and Vanderleyden, 2001). Complement recognition (via

mannose-binding lectin) of the O-antigen triggers activation of the membrane attack complex (**MAC**) resulting in bacterial cell lysis. The presence of an elongated O-antigen chain protects endotoxins from MAC-mediated cell lysis (Zhao et al., 2002; Hölzer et al., 2009) and subsequently reduces the susceptibility to phagocytosis (Eder et al., 2009; Hölzer et al., 2009). Despite its importance to bacterial cell survival, the O-polysaccharide is not ubiquitously expressed in all endotoxins; strains which lack an O-antigen are classified as “rough” whereas those that express it are considered “smooth” (Erridge et al., 2002) and the latter produces more intense inflammatory responses (Védrine et al., 2018). Therefore, the structure of LPS markedly influences its toxicity.

Lipopolysaccharide can circulate as a free molecule when released from the outer membrane following bacterial growth or death. The inner and outer membrane are attached by lipoproteins which separate during normal bacterial division, abnormal growth of the outer membrane, death, or as a response to antibiotic treatment (Crutchley et al., 1967; Tsui and Harrison, 1978; van Langevelde et al., 1998). Lipopolysaccharide translocation into portal and systemic circulation can occur at various epithelial interfaces and those particularly relevant in the transition period will be discussed below (see Sources of LPS in the Transition Cow).

LPS Recognition

Pathogen associated molecular patterns (**PAMP**) present on invading pathogens are sensed by the innate immune system via pathogen recognition receptors (**PRR**) including Toll-like receptors (**TLR**), NOD-like receptors (**NLR**), and RIG-1-like receptors (**RLR**), among others (Kumar et al., 2011). Toll-like receptor 4 (**TLR4**), the PRR which recognizes LPS, is present on leukocytes as well as adipocytes (Mukesh et al., 2010; Vailati Riboni et al., 2015), hepatocytes (Vaure and Liu, 2014; Xu et al., 2017), endometrial cells (Davies et al., 2008; Sheldon and Roberts, 2010), mammary epithelial cells (Ibeagha-Awemu et al., 2008), and

intestinal epithelial cells (Malmuthuge et al., 2012). Lipopolysaccharide interaction with TLR4 is assisted by the accessory proteins LPS-binding protein (**LBP**) and cluster of differentiation 14 (**CD14**). Lipopolysaccharide-binding protein separates LPS monomers from endotoxin aggregates (which form naturally in circulation due to the amphiphilic structure) and facilitates the interaction with membrane bound or soluble CD14. Next, CD14 transfers LPS to myeloid differential protein 2 (**MD2**), a protein complexed with TLR4 on the cell surface, which dimerizes and triggers a signaling cascade through the Toll-interleukin-1 receptor (**TIR**) initiating either the myeloid differentiation factor 88 (**MyD88**)-dependent or independent pathway. The MyD88 dependent pathway leads to translocation of nuclear factor kappa β (**NF κ β**) to the nucleus and initiation of inflammatory cytokine transcription (Lu et al., 2008a). Cytokines play a crucial role in regulating a balanced inflammatory response. Proinflammatory cytokines (e.g., interleukin [**IL**] IL-1, IL-12, tumor necrosis factor alpha [**TNF- α**], interferon gamma [**IFN- γ**]) initiate a local and systemic response resulting in hypothalamic-pituitary-adrenal (**HPA**) axis activation, acute phase protein production, hypophagia, pyrexia, and altered metabolism (Bertoni et al., 2009). Anti-inflammatory cytokines (i.e., IL-4, IL-10, and transforming growth factor beta [**TGF- β**]) depress the inflammatory response to prevent potential deleterious effects of a hyperinflammatory response (i.e., tissue damage; Grimbale, 2001).

Sources of LPS in the Transition Cow

Gastrointestinal Tract

The gastrointestinal tract harbors trillions of microorganisms (Hooper and Macpherson, 2010) and it has been estimated that >1 g of LPS is found continuously within the human intestinal tract (Erridge et al., 2007). Luminal bacteria (and other toxic substances) are separated from circulation by either stratified squamous or columnar epithelia, depending on the intestinal

segment. The stratified squamous epithelium, found in the reticulo-rumen and omasum, is composed of 4 distinct strata including the basale and spinosum, which have metabolic properties, and the granulosum and corneum, which provide a protective barrier (as reviewed by Steele et al., 2016). In contrast to the reticulo-rumen and omasum, the lower gut is composed of a simple columnar epithelium which consists of various specialized cells including absorptive epithelial cells, goblet cells, Paneth cells, Peyer's patches, dendritic cells (**DC**), lymphocytes, and enteroendocrine cells (Peterson and Artis, 2014). Extensive luminal defense mechanisms function to protect the epithelial barrier within the lower gut. An overlying mucus layer, produced by goblet cells, provides a physical barrier between the intestinal microbiota and the epithelium and lubricates the intestine (Capaldo et al., 2017; Martens et al., 2018). A single layer of loosely adherent mucus lines the small intestine whereas two distinct layers are present in the large intestine; an inner layer impenetrable to most bacteria and an outer permeable layer (Pelaseyed et al., 2014). Mucins are the glycoprotein component of mucus, which interact with the previously secreted inner mucus layer to form a net-like gel (Pelaseyed et al., 2014). More than 20 different mucins are produced by the gastrointestinal tract, but the primary intestinal mucin is Muc2 (Pelaseyed et al., 2014; Guerville and Boudry, 2016). Additional luminal defense mechanisms include Paneth cell antimicrobial peptides and enzymes (i.e., α - and β - defensins, lysozyme C, and group IIA phospholipase A2) and enterocyte alkaline phosphatase (**AP**) which are secreted into the mucus layer and induce bacterial cell death (Guerville and Boudry, 2016; Gassler, 2017). Despite extensive luminal defense mechanisms, bacteria are capable of reaching the apical side of the epithelium and can subsequently cross via paracellular (between cells) or transcellular (across cells) routes (Guerville and Boudry, 2016).

Tight junctions (**TJ**) are multiple protein complexes which provide a selectively permeable barrier between adjacent epithelial cells of the stratum granulosum (Graham and Simmons, 2005) and columnar epithelium (Turner et al., 2009). Occludin, claudin, junctional adhesion molecule, and tricellulin are the integral transmembrane proteins (Furuse et al., 1993, 1998; Martin-Padura et al., 1998; Ikenouchi et al., 2005) which are anchored to the cytosolic actin-myosin ring via scaffold proteins, such as zona occludens (Turner, 2006). Phosphorylation of the myosin light chain (**MLC**) via MLC kinase triggers contraction of the actin-myosin ring permitting paracellular permeability (Turner et al., 1997); hyperphosphorylation of MLC occurs in response to various pro-inflammatory cytokines (Bruewer et al., 2005; Ma et al., 2005; Suzuki et al., 2011). Paracellular permeability is also increased in response to proteases (Cenac et al., 2002), histamine (Gardner et al., 1996), and LPS-induced oxidative stress (Han et al., 2004). Even when TJ complexes are maintained, LPS can cross the epithelial barrier transcellularly via TLR4 receptor-mediated endocytosis (Neal et al., 2006). Epithelial cells of the forestomach and lower gut are normally hyporesponsive to bacteria due to inherently low TLR4 and MD-2 expression on the apical side (Abreu et al., 2001; Uehara et al., 2007; Lenoir et al., 2008; Malmuthuge et al., 2012). However, inflammation increases TLR4 expression facilitating increased enterocyte LPS internalization (Abreu et al., 2002). A portion of the internalized LPS is transported to the Golgi apparatus where it becomes bound to newly formed chylomicrons with the help of intestinally-derived LBP, and can enter systemic circulation via the lymph (Ghoshal et al., 2009). Sequestration of LPS with lipoproteins typically reduces LPS bioactivity and promotes hepatic clearance (see Detoxification section), however, loosely attached LPS may be recognized in mesenteric lymph nodes triggering systemic inflammation (Ghoshal et al., 2009; Grunfeld and Feingold, 2009; Moreira et al., 2012). Once in circulation, LPS may be

transferred to other lipoproteins such as high density lipoprotein (**HDL**) and can be endocytosed by adipocytes triggering a localized inflammatory response (Hersoug et al., 2016). The role of lipoproteins in LPS translocation and detoxification remains poorly understood in ruminants and requires further investigation (Eckel and Ametaj, 2016; Khiaosa-Ard and Zebeli, 2018).

A diverse immunological barrier exists below the epithelium (the lamina propria) which recognizes infiltrating pathogens and LPS (via PRR) leading to a localized inflammatory response. Macrophages, DC, and adaptive lymphocytes comprise the immune effector sites whereas Peyer's patches and isolated lymphoid follicles encompass the immune inductive sites (i.e., gut-associated lymphoid tissue [**GALT**]) within the lamina propria (Ohno, 2015). Peyer's patches are aggregated lymphoid follicles contained in the subepithelial dome (Ahluwalia et al., 2017) which are separated from the intestinal lumen by specialized M-cells. M-cells lack microvilli and have a much thinner layer of mucus relative to enterocytes and these structural differences facilitate sampling and transport of luminal antigens to DC (which are also located in the subepithelial dome; Ahluwalia et al., 2017). Dendritic cells can also directly sample antigens from the intestinal lumen by extending their dendrites between TJ of adjacent epithelial cells (Coombes and Maloy, 2007). Dendritic cells phagocytose, process, and present antigens to naïve T-cells triggering their activation and differentiation (Ahluwalia et al., 2017). Plasma cells (i.e., antibody secreting B cells) of the mucosal immune system will secrete secretory immunoglobulin A (**sIgA**) into the gut lumen which binds and neutralizes antigens inhibiting interaction with the epithelium (Corthésy, 2008). In addition, sIgA facilitates antigen uptake into lymphoid compartments (Corthésy, 2008). When the intestinal tract's capacity to detoxify LPS is exceeded, endotoxin can enter into portal and systemic circulation (Guerville and Boudry, 2016).

Consequently, although extensive mechanisms are in place to prevent pathogen translocation, there are a numerous events which reduce barrier effectiveness.

Dairy cows are exposed to a myriad of situations which can negatively impact intestinal barrier integrity including: heat stress (Baumgard and Rhoads, 2013; Koch et al., 2019), subacute rumen acidosis (**SARA**; Emmanuel et al., 2007; Khafipour et al., 2009), and feed restriction (Zhang et al., 2013; Kvidera et al., 2017c). Potential mechanisms by which heat stress and rumen acidosis may impact barrier integrity have been described in detail elsewhere (Baumgard and Rhoads, 2013; Steele et al., 2016). Interestingly, “stress” alone is associated with inflammation (Proudfoot et al., 2018) and gastrointestinal hyperpermeability in production animals (Pohl et al., 2017). In response to stress, the HPA axis is activated, which in turn stimulates nervous system and peripheral tissue production of corticotropin-releasing factor (**CRF**) and subsequent release of adrenocorticotropin hormone from the anterior pituitary gland (Charmandari et al., 2005). Receptors for CRF are widely expressed in both the central and peripheral nervous system where they interact with enteric neurons and epithelial and immune cells (Larauche et al., 2009; Li et al., 2017). Administering CRF induces intestinal barrier dysfunction (Santos et al., 1999; Teitelbaum et al., 2008) and initiates systemic inflammation (Cooke and Bohnert, 2011; Cooke et al., 2012). Consequences of CRF are mediated by stimulation of intestinal mast cell degranulation and release of pre-formed and de-novo mediators such as histamine, proteases, and cytokines which negatively impact intestinal barrier function (Overman et al., 2012). Mechanistically, the effects of CRF on barrier integrity are not fully elucidated, but likely are a consequence of disrupted TJ complexes (Jacob et al., 2005; Groschwitz et al., 2013; Wilcz-Villega et al., 2013). In addition to hypothalamic release, CRF is also produced and released by intestinal cells (including immune and enterochromaffin cells) and the localized production can

also affect intestinal epithelial function (Saunders et al., 2002; Vanuytsel et al., 2014; Albert-Bayo et al., 2019). Stress-mediated effects on the gut barrier may explain why so many seemingly unrelated situations (i.e., heat stress, cold stress, weaning, acidosis, feed restriction, etc.) share a common consequence of leaky gut and systemic inflammation.

Mammary Gland

The mammary gland is highly susceptible to bacterial infections making it a prominent source of LPS in the transition period. Intramammary infections are most prevalent during early involution (i.e., dry off) and colostrogenesis (Ballou, 2012). Abrupt milking cessation at dry off engorges the udder with milk, increasing intramammary pressure and disrupting physical defense mechanisms within the streak canal (i.e., the keratin plug; Tucker et al., 2009); allowing microorganisms to colonize the mammary gland (Bradley and Green, 2004). Bacterial infections often remain quiescent throughout the dry period and clinical disease is not observed until the periparturient period (Bradley and Green, 2004). Interestingly, a previous report estimated that approximately 65% of early lactation clinical coliform mastitis cases originated during the dry period (Smith and Schoenberger, 1985).

Bovine mammary epithelial cells (**BMEC**) serve a dual purpose of producing milk while simultaneously creating a semi-impermeable (although permeability is increased during the dry period) barrier between blood and milk components (the blood-milk barrier). Akin to the intestinal epithelium, integrity of the blood-milk barrier is reliant on TJ proteins which connect adjacent epithelial cells (Burton and Erskine, 2003). Lipopolysaccharide, released during gram-negative bacterial proliferation within the teat and udder cistern, is recognized by resident leukocytes and mammary epithelial cells via TLR4 (Seyfert et al., 2004; Ibeagha-Awemu et al., 2008). Proinflammatory cytokines, produced in response to TLR4 activation, signal recruitment of effector leukocytes into the mammary gland and disrupt TJ integrity (Pappe et al., 2002;

Burton and Erskine, 2003; Xu et al., 2018). Furthermore, leukocyte pathogen elimination triggers epithelial cell damage (Wellnitz et al., 2016). Altogether, these changes disrupt the blood-milk barrier resulting in systemic endotoxemia and inflammation, and this occurs in an alarming number of gram-negative bacterial infections (Wenz et al., 2001). Similarly, incubation of BMEC with LPS increases blood-milk barrier permeability and promotes apoptosis (Sun et al., 2015). In vivo, intramammary LPS infusion induces acute systemic inflammation (Waldron et al., 2003a; Bannerman et al., 2003) and the response is far more potent when compared to lipoteichoic acid (LTA; the PAMP from gram-positive bacteria; Keane, 2019). Disrupting the blood-milk barrier can also be evaluated by decreased milk potassium and increased milk sodium, L-lactate, lactate dehydrogenase, albumin, and IgG and increased circulating α -lactoalbumin and lactose (Stelwagen et al., 1993; Stelwagen et al., 1997; Bruckmaier and Wellnitz, 2017).

Uterus

Bacteria present within the uterine lumen were originally thought to originate exclusively from contamination with environmental pathogens during and after parturition (Sheldon et al., 2006), however, it is now established that a uterine microbiome exists (Karstrup et al., 2017; Moore et al., 2017). Both bacteria adapted to the uterus (part of the existing microbiome before parturition) and originating from the environment contribute to metritis (Sheldon et al., 2019). The most common uterine pathogens are *Escherichia coli* and *Trueperella pyogenes* (Hussain et al., 1990; Brodzki et al., 2014). Infiltration of environmental microorganisms is restricted by anatomical barriers including the vulva, vagina, and cervix; however, dilation of these structures during and after parturition reduces their ability to prevent pathogen entry. The mucosal epithelium lining the genital tract offers a physical defense mechanism which differs in structure depending on the location. The vagina, which frequently encounters microorganisms, contains a

multilayered squamous epithelium (Blazquez et al., 1987) whereas the cervix, uterus, and oviduct contain a single cell layer of columnar epithelium (Kojima and Selander, 1970a,b; Mokhtar, 2015). A mucus layer overlies the apical surface of the epithelium (Dadarwal et al., 2017; Sheldon et al., 2019) and endometrial epithelial cells secrete antimicrobial peptides into the lumen (Ibrahim et al., 2016); these mechanisms work together to prevent bacteria from reaching the epithelium. Tight junction proteins connect adjacent uterine epithelial cells separating the apical and basolateral components of the endometrium and preventing bacteria from penetrating the underlying stroma (Sheldon et al., 2019). Epithelial cells recognize pathogens via PRR, which triggers inflammatory cytokine and antimicrobial peptide production (Davies et al., 2008). Interestingly, both apical and basolateral PRR activation triggers cytokine secretion apically and this aids in immune cell recruitment to the infection site (Healy et al., 2015; Sheldon et al., 2019).

During parturition, the protective uterine epithelium is often damaged allowing bacteria access to the underlying stroma. Stromal cells are less robust than epithelial cells and are particularly sensitive to *Trueperella pyogenes*-secreted pyolysin. Pyolysins bind cholesterol rich domains in the stromal cell plasma membrane forming pores leading to osmotic death (Amos et al., 2014). Bacterial infiltration of the stroma induces cell damage and cytolysis stimulating the release of damage associated molecular pattern (**DAMP**) molecules (Sheldon et al., 2019) which are recognized by epithelial and stromal cells via PRR; a scenario which intensifies the inflammatory response (Blander and Sander, 2012). Lymphoid aggregates consisting of B- and T-lymphocytes are also found throughout the genital tract (as reviewed by Dadarwal et al., 2017) and antibody release into the uterine lumen has been confirmed (Dhaliwal et al., 2001). However, the success of adaptive immunity in countering uterine infections remains unclear

especially since uterine infections are often observed in successive calvings (Sheldon et al., 2019). Despite extensive defense mechanisms, increased circulating inflammatory cytokines are frequently observed in naturally metritic cows (Barragan et al., 2018; Cui et al., 2019) and in severe metritis cases increased circulating LPS has been observed (Mateus et al., 2003). As eluded to above, the act of parturition independently triggers inflammation and the severity of calving difficulty likely predisposes cows to a higher risk of pathogen entry into circulation. In summary, both the act of parturition and bacterial contamination can contribute to systemic inflammation in dairy cows.

Gut-Mammary-Uterus Communication

Communication between distant mucosal sites (particularly the gut and lung) was described more than 40 years ago (Kraft et al., 1976; Bienenstock et al., 1978) and continues to be a topic of intensive investigation (Tulic et al., 2016). Mechanisms mediating cross talk between the gut and lung mucosa remain incompletely understood, however, evidence suggests leukocyte trafficking between epithelial sites and inflammatory mediators play a role (Tulic et al., 2016). Recent research in ruminants suggests that communication also occurs between gut, mammary, and uterine epithelial interfaces. Permeability of the blood-milk barrier and/or increased somatic cell count have been observed in models of impaired intestinal integrity such as heat stress (Weng et al., 2018; Safa et al., 2018; Al-Qaisi et al., 2020a) and feed restriction (Stumpf et al., 2013; Kvidera et al., 2017c). Furthermore, cows with grade 3 metritis often develop mastitis (Sheldon et al., 2019) and experimentally-induced mastitis (via intramammary LPS infusion) negatively influences reproductive performance (Lavon et al., 2008; Campos et al., 2018). Communication across these spatially distinct epithelial interfaces may explain why so many negative health events in the transition period are related.

Inflammatory Responses to LPS

Neutrophil Recruitment

Neutrophils play a crucial role in the defense against infection particularly in the uterus and mammary gland. In cattle, the turnover and production of neutrophils exceeds 10^{11} cells per day and during acute inflammation it increases 10-fold (Van Schyndel et al., 2018; LeBlanc, 2020). Cytokine production by tissue-resident macrophages and epithelial cells during infection triggers alterations on the surface of endothelial cells, which sets neutrophil recruitment in motion (Ley et al., 2007). Endothelial cells upregulate P-, E-, and L-selectins which bind to circulating neutrophils and facilitate rolling adhesion. Chemokines (particularly IL-8) decorating the luminal surface of the endothelium activate neutrophils and induce conformational changes in integrins which facilitate neutrophil adhesion, crawling, and transmigration into the infection site (as reviewed by Liew and Kubes, 2019). Following extravasation, neutrophils eliminate pathogens by phagocytosis and intracellular digestion by oxidation (i.e., oxidative burst), extracellular release of oxidants from granules (i.e. myeloperoxidase), or by casting extracellular traps (NETs) of DNA (LeBlanc, 2020). After completing their cellular function, apoptotic neutrophils are cleared by local macrophages and this process can stimulate the macrophage to move towards an anti-inflammatory phenotype (Greenlee-Wacker, 2016). In addition to IL-8, bovine neutrophils may be activated by platelet activating factor, TNF- α , complement component C5a, and PAMP (i.e., LPS; Bassel and Caswell, 2018). Interestingly, direct neutrophil activation by LPS downregulates the chemokine receptor and inhibits migration (Alves-Filho et al., 2010). Consequently, bacterial clearance at infectious sites is reduced and the risk of further LPS infiltration into circulation is increased. The relevance of this mechanism in cows experiencing endotoxemia requires investigation.

The Hepatic Response

The liver is continuously exposed to a variety of immune stimulants (i.e., endotoxins, danger signals, bacteria, etc.) via portal and systemic circulation and is an essential regulator of immune and inflammatory responses (Strnad et al., 2017). More than 60% of intravenously infused bacteria is sequestered in the liver within 10 minutes of infusion (Yan et al., 2014). Inflammatory cytokines (particularly IL-1, IL-6, and TNF- α) originating from Kupffer cells (in response to hepatic pathogen uptake) or peripheral infection sites, signal a switch in hepatic function from a metabolic to an inflammatory phenotype; a reaction termed the acute phase protein (**APP**) response (Strnad et al., 2017). Acute phase proteins are classified as either negative or positive based on their change in concentration (Kushner and Mackiewicz, 1987; Petersen et al., 2004); circulating positive APP increase transiently in response to proinflammatory cytokines whereas negative APP concomitantly decrease. The contemporaneous change in the plasma protein profile purportedly occurs because of competition for amino acid precursors (Bertoni and Trevisi, 2013). Alterations in the negative APP pattern generally occur quicker and last longer in response to an immune insult when compared to positive APP (Fleck, 1989). Negative acute phase proteins such as albumin, cholesterol (an index of lipoproteins), retinol-binding protein, transferrin, and paraoxonase (associates with HDL to protect from oxidation) play a critical role in regulating normal liver metabolism and function. Although not a protein, bilirubin is also considered a negative APP as its clearance is dependent on liver enzymes (Tennant, 1997). Additional indicators of liver function include AP, aspartate aminotransferase, and glutathione peroxidase.

Positive APP aid in pathogen elimination, removal of toxic substances, and maintenance of a balanced inflammatory response (Ceciliani et al., 2012) and can be further classified as either minor, major, or moderate depending on the magnitude of increase observed following

immune activation. Common positive APP evaluated in ruminants include serum amyloid A (SAA), haptoglobin (**Hp**), and LBP (Ceciliani et al., 2012). Serum amyloid A and Hp are major APP produced primarily by hepatocytes, but also by various extrahepatic tissues including the mammary gland, pancreas, gastrointestinal tract, and ovary, among others (Lecchi et al., 2012). Acute phase SAA is an apolipoprotein which exists in 1 of 3 isoforms: SAA1 and SAA2 produced by hepatocytes and SAA3 produced by extrahepatic tissues (Ceciliani et al., 2012). During inflammation, SAA displaces ApoA1 from HDL and subsequently scavenges cholesterol from dying cells (Coetzee et al., 1986; Liang and Sipe, 1995; Sato et al., 2016). Furthermore, SAA can facilitate bacterial opsonization and leukocyte chemotaxis (Shah et al., 2006; De Buck et al., 2016) and has antimicrobial activity in the mammary gland (Molenaar et al., 2009; Parés et al., 2020). Haptoglobin's most well-known function is binding hemoglobin released during hemolysis, thereby protecting hemoglobin from oxidative damage (Buehler et al., 2009) and reducing iron availability to bacteria (Eaton et al., 1982; Ascenzi et al., 2005). Interestingly, Hp has potent anti-inflammatory actions that are crucial for immune tolerance and maintaining a balanced inflammatory response (Raju et al., 2019). In particular, Hp inhibits leukocyte activities such as respiratory burst by binding to receptor ligand sites (Oh et al., 1990; Arredouani et al., 2005).

Lipopolysaccharide-binding protein is a moderate APP produced primarily by hepatocytes, but also by adipose tissue (Rahman et al., 2015), the gastrointestinal tract, and mammary gland (Rahman et al., 2010). As aforementioned (see LPS Recognition section), LBP facilitates LPS presentation to CD14 for activation of the TLR4 pathway. Although the LPS-CD14-TLR4 interaction can occur independently, LBP markedly enhances responsiveness (i.e., cytokine production) of macrophages to LPS (Martin et al., 1992). Although classically known

for its role in LPS recognition, LBP can also recognize other PAMP such as LTA (Schroder et al., 2003). Interestingly, while constitutive levels of LBP promote immune activation, acute phase levels are anti-inflammatory and inhibit cytokine production in rodents and humans (Lamping et al., 1998; Zweigner et al., 2001). Recombinant LBP decreased LPS-induced cytokine production in a BMEC line (Sun et al., 2015). Lipopolysaccharide-binding protein exerts its anti-inflammatory action by facilitating the transfer of LPS to lipoproteins (see Detoxification section), which are directed to the liver for biliary excretion (Wurfel et al., 1994; Lamping et al., 1998). As a result, LPS binding to leukocytes is markedly reduced which in turn attenuates the inflammatory response (Lamping et al., 1998; Hamann et al., 2005). The temporal pattern of the APP differs such that LBP and SAA typically increase more rapidly whereas the Hp response is delayed. Understanding the temporal profile is an important consideration when trying to identify potential inflammation sources (Humer et al., 2018). Altogether these APP play crucial roles in regulating both local and systemic inflammatory responses.

Detoxification

As previously mentioned (see Gastrointestinal tract and Uterus section), LPS detoxification strategies such as antimicrobial peptides, AP, and acyloxyacyl hydrolase (**AOAH**) aid in immune defense at epithelial interfaces and these will now be discussed in detail. Antimicrobial peptides have a high affinity for LPS and formation of LPS-peptide complexes prevents LPS interaction with TLR4 (Rosenfeld et al., 2008). At the gut level, AP is secreted bidirectionally into the lumen and portal circulation by enterocytes and colonocytes, where it cleaves phosphate groups in positions 1 and 4 of lipid A leading to a nontoxic LPS molecule (Eliakim et al., 1991; Lallés, 2014). However, the dephosphorylated LPS can still bind TLR4, but does not trigger the signaling cascade and this competitive binding prevents interaction of toxic LPS forms with the TLR4 receptor (Lallés, 2014). Interestingly, providing oral AP in

rodent intestinal hyperpermeability models reduces LPS translocation and systemic inflammation (Kaliannan et al., 2013; Hamarneh et al., 2017). Acyloxyacyl hydrolase is a lipase produced by macrophages, neutrophils, and DC, which markedly reduces LPS's immunogenic activity by cleaving the secondary fatty acyl chains from the lipid A moiety (Munford and Hall, 1986; Shao et al., 2006; Lu and Munford, 2011). The immunogenic activity of both free LPS and LPS-bound to lipoproteins can be reduced by AOA (Shao et al., 2012) and this prevents prolonged LPS-induced inflammation of the liver (Shao et al., 2007). Cumulatively, these detoxification strategies reduce LPS toxicity and attenuate the inflammatory response.

The liver is the primary site of LPS detoxification (Strnad et al., 2017) and this is partly mediated by cytokine-producing Kupffer cells that phagocytose and deacetylate LPS (Mathison and Ulevitch, 1979; Shao et al., 2012). Alternatively, LPS removal from circulation can occur via a non-inflammatory pathway involving lipoproteins (as reviewed by Eckel and Ametaj, 2016). Formation of LPS-lipoprotein complexes is facilitated by LBP and soluble CD14 (**sCD14**; Vreugdenhil et al., 2003; Eckel and Ametaj, 2016) and this interaction neutralizes the stimulatory activity of LPS and prevents its interaction with TLR4 (Feingold et al., 1995; We et al., 2004; Feingold and Grunfeld, 2011). In fact, LPS incubation with lipoproteins prior to administration or lipoprotein infusion following LPS reduces mortality and inflammation (Morel et al., 1986; Harris et al., 1993; Hubsch et al., 1995; Read et al., 1995; Guo et al., 2013). In addition to binding free LPS, lipoproteins (particularly HDL) can trigger release of LPS already bound to leukocytes, thereby attenuating the host inflammatory response (Kitchens et al., 1999). Lipopolysaccharide can also be transferred between different lipoprotein classes; LPS associated with chylomicrons can transfer to low density lipoprotein (**LDL**) which is cleared by hepatocytes via LDL-associated receptors (Harris et al., 2002). When bound to lipoproteins, LPS is

preferentially shunted away from macrophages and towards hepatocytes for biliary excretion (Harris et al., 1993; Read et al., 1993).

High density lipoprotein has the greatest LPS-binding ability of the lipoprotein classes (Levels et al., 2001) and is the most abundant lipoprotein in bovine plasma (Bauchart, 1993). During infection, HDL-cholesterol markedly decreases in humans and rodents (Khovidhunkit et al., 2004) and this is consistent with decreased cholesterol reported in ruminants (Bertoni and Trevisi, 2013). The composition of HDL is also markedly altered during the acute phase response such that the major apolipoprotein ApoA1 is displaced by SAA leading to the generation of acute phase (ap)-HDL, which is preferentially removed from circulation by the liver (Hoffman and Benditt, 1983). Both normal and ap-HDL have been shown to reduce the inflammatory response (Thaveeratitham et al., 2007). Collectively, the aforementioned detoxification mechanisms aid in LPS removal while minimizing the negative consequences of inflammation.

Immunometabolism

The Warburg Effect

In most nonproliferating, differentiated mammalian cells, energy is produced via the combined processes of glycolysis, the tricarboxylic acid (**TCA**) cycle, and oxidative phosphorylation which generates approximately 36 to 38 adenosine triphosphate (**ATP**) per molecule of glucose. One glucose molecule is oxidized to two pyruvate via glycolysis, which subsequently enter the mitochondria and are decarboxylated to acetyl-CoA by pyruvate dehydrogenase (**PDH**). Typically, the fate of pyruvate was thought to be dependent on oxygen availability, such that in the presence of oxygen acetyl-CoA enters the TCA cycle by combining with oxaloacetate to form citrate, which will eventually produce ATP via oxidative and substrate-level phosphorylation. However during hypoxia, anaerobic glycolysis occurs in which

pyruvate is shunted away from mitochondrial oxidative phosphorylation towards lactate production via cytosolic lactate dehydrogenase (Berg et al., 2002). Anaerobic glycolysis nets two ATP molecules and regenerates NAD^+ which allows glycolysis to proceed (since NAD^+ is needed to convert glyceraldehyde 3-phosphate to 1,3 bisphosphoglycerate). Interestingly, in 1923, Professor Otto Warburg demonstrated that highly proliferative cancer cells switch to glycolytic metabolism even in the presence of oxygen, a metabolic process termed the Warburg effect (Warburg, 1927; Palsson-McDermott and O'Neill, 2013). It was later noted that this same phenomenon occurred in essentially all rapidly proliferating cells, including immune cells (Warburg, 1958; Sbarra and Karnovsky, 1959; Vander Heiden et al., 2009).

The Warburg effect is utilized by rapidly proliferating cells to support growth. Although ATP production from aerobic glycolysis is inefficient, it supplies energy at a much faster rate when compared to oxidative phosphorylation (Pfeiffer et al., 2001) and this was traditionally thought to be the primary advantage of the Warburg effect. Yet, it is unlikely that the ATP requirement is the primary reason cells undergo the metabolic switch, as ATP availability is apparently not limiting growth in these rapidly proliferating cells (Vander Heiden et al., 2009; Lunt and Vander Heiden, 2011). Rather, glucose oxidation by aerobic glycolysis generates intermediates needed to support biosynthesis and provides a way to maintain cellular redox balance (NAD^+/NADH). For example, nucleotide, amino acid, and NADPH production occurs through the pentose phosphate pathway (**PPP**) via the glycolytic intermediate glucose-6-phosphate, while fatty acids needed for membrane lipid synthesis are synthesized from citrate in the cytosol through ATP-citrate lyase to form acetyl CoA (see Alternative role of the TCA cycle section). The fatty acids can combine with glycerol produced from the glycolytic intermediate dihydroxyacetone phosphate (**DHAP**) to form TG. Furthermore, the intermediate 3-

phosphoglycerate is utilized for the synthesis of the nonessential amino acids cysteine, glycine, and serine while pyruvate is utilized for alanine synthesis (Lunt and Vander Heiden, 2011; Palsson-McDermott and O'Neill, 2013). Although synthesis of these biosynthetic precursors is crucial for supporting cell proliferation, a high glycolytic flux towards lactate must also be maintained to allow NAD^+ regeneration, which is necessary for glycolysis to continue (Lunt and Vander Heiden, 2011). An additional advantage to implementing the Warburg effect is the capacity to continually proliferate in hypoxic environments. Sites of infection and inflammation may become nutrient or oxygen deficient due to increased neutrophil oxygen consumption, increased metabolic activity of cells at the site, or necrosis of infected cells (Pearce et al., 2013). The transcription factor, hypoxia-inducible factor 1 (**HIF-1**), is the master regulator to hypoxia adaptation and is a key mediator of the Warburg effect (Palsson-McDermott and O'Neill, 2013). Increased HIF-1 α induces the expression of glycolytic enzymes such as lactate dehydrogenase and PDH kinase (which inhibits PDH) as well as glucose transporter (**GLUT**) 1 (Semenza et al., 1996; Chen et al., 2001; Kim et al., 2006). Even during normoxia, HIF-1 is increased by succinate-mediated stabilization (Selak et al., 2005). Altogether, these advantages make glycolytic metabolism an ideal method of energy generation for rapidly proliferating cells.

Alternative Role of the TCA Cycle

The TCA cycle is thought to be used mainly by quiescent or non-proliferative leukocytes. However, mitochondrial metabolism does not cease during the switch to glycolytic metabolism, instead the TCA cycle is disrupted in two places (after both citrate and succinate) and becomes anabolic as opposed to catabolic (Palsson-McDermott and O'Neill, 2013). The TCA cycle intermediate citrate, increases in LPS-activated macrophages. Mitochondrial citrate is exchanged with cytosolic malate via the mitochondrial citrate carrier (**CIC**) and is subsequently cleaved by citrate lyase into acetyl-CoA and OAA. Acetyl-CoA supports lipid synthesis whereas OAA

produces the cofactor NADPH used for nitric oxide (**NO**) and reactive oxygen species (**ROS**; via NADPH oxidase) generation and can also be used to produce aspartate for generating nucleotides. Inhibiting CIC markedly reduces LPS-induced NO, prostaglandin, and ROS production, corroborating its crucial role to macrophage inflammatory activity (Infantino et al., 2011). Citrate accumulation also results in synthesis of itaconic acid, a non-amino organic acid which has antibacterial properties (Michelucci et al., 2013). Furthermore, itaconic acid inhibits succinate dehydrogenase resulting in accumulation of succinate (Cordes et al., 2016), the importance of which is described below.

Succinate generation can also occur by either glutamine-dependent anaplerosis (i.e., glutamine produces glutamate and subsequently α -ketoglutarate) or through the γ -aminobutyric acid (**GABA**)-shunt (Palsson-McDermott and O'Neill, 2013). The latter involves transamination of α -ketoglutarate to L-glutamic acid by GABA α -oxoglutarate transaminase, which is subsequently decarboxylated to form GABA via glutamic acid decarboxylase. Subsequently, GABA is converted to succinate through succinic semialdehyde (Palsson-McDermott and O'Neill, 2013). Increased succinate levels have a negative feedback effect on prolyl hydroxylases (the enzymes responsible for succinate generation from α -ketoglutarate) and this allows for the stabilization of HIF-1 α (Selak et al., 2005). Increased HIF-1 α stimulates IL-1 β production (Tannahill et al., 2013) and downregulates oxidative phosphorylation by inducing PDH kinase 1 and 3, which in turn inactivate PDH (Kim et al., 2006; Papandreou et al., 2006; Lu et al., 2008b). Recent reports suggest that succinate oxidation via succinate dehydrogenase rather than succinate itself is responsible for promoting pro-inflammatory functions (Murphy and O'Neill, 2018; O'Neill and Artyomov, 2019), but further investigation is required. Regardless,

succinate plays an important in promoting glycolytic metabolism and pro-inflammatory functions.

Metabolic Reprogramming and Leukocyte Function

Metabolic reprogramming occurs in activated leukocytes of both innate and adaptive immunity and is intimately related to the nature of the immune response (Borregaard and Herlin, 1982; Buck et al., 2015; O'Neill and Pearce, 2016). Warburg metabolism is important even in leukocytes that are terminally differentiated such as neutrophils, macrophages, and DC.

Neutrophils are highly reliant on glycolytic metabolism as they consume little oxygen and have few mitochondria (van Raam et al., 2006). Following neutrophil activation by TLR agonists or phagocytosis of antigens, glucose and oxygen consumption are increased as a result of Warburg metabolism (Borregaard and Herlin, 1982). Glycolytic flux through the PPP generates NADPH which is utilized for the generation of ROS by NADPH oxidase. The ROS produced facilitate formation of extracellular NETs (Rodríguez-Espinosa et al., 2015). Interestingly, Awasthi and colleagues (2019) recently demonstrated that the glycolytic end-product lactate was also essential to the production of NETs. In macrophages, the metabolic profile and function differs markedly depending on the phenotype expressed. Macrophages can differentiate into one of two phenotypes: M1 (classical) or M2 (alternative). Macrophage polarization to the M1 phenotype occurs in response to IFN- γ and/or LPS (as well as other TLR ligands) stimulation; these cells express a pro-inflammatory phenotype and secrete mediators such as IFN- γ , TNF- α , and NO. In contrast, IL-4 and IL-13 activate M2 macrophages, which modulate inflammation and promote tissue repair (Pearce and Pearce, 2013). Following stimulation, M1 macrophages shift towards Warburg metabolism whereas M2 macrophages remain reliant on oxidative phosphorylation. M1 macrophages express a highly active phosphofructokinase-2 (**u-PFK2**) isoform which promotes glycolysis; whereas M2 macrophages express a much less active form (**PFKFB1**; Rodríguez-

Prados et al., 2010; Kelly and O'Neill, 2015) and are more reliant on fatty acid oxidation for feeding the TCA cycle (Vats et al., 2006). Metabolic reprogramming of M1 macrophages allows for the production of several important molecules (many of which originate from citrate and succinate accumulation) which regulate their functional characteristics (i.e., phagocytosis and ROS and IL-1 β production) and these have been described in detail previously (O'Neill and Pearce, 2016). Macrophage exposure to anti-inflammatory cytokines such as IL-10 opposes the switch to glycolytic metabolism, decreases glucose uptake, and promotes mitophagy (Eddie Ip et al., 2017).

Dendritic cells, important for cross talk between innate and adaptive immunity, also undergo metabolic reprogramming in response to TLR agonists (Krawczyk et al., 2010). In the early stages of activation, DC increase glycolytic flux to facilitate citrate accumulation that is utilized for fatty acid synthesis in support of increased size of the endoplasmic reticulum and Golgi apparatus, organelles needed for protein synthesis (Everts et al., 2014). Control of DC metabolic reprogramming occurs through phosphatidyl inositol-3'-kinase (**PI3K**)/Akt signaling (Krawczyk et al., 2010). Downstream signaling of PI3K/Akt activates mammalian target of rapamycin (**mTOR**, comprised of mTORC1 and mTORC2) which regulates anabolic metabolism (Krawczyk et al., 2010). Inhibiting mTORC1 decreases DC number, differentiation, and survival (as reviewed by Pearce and Everts, 2015). Similarly, AMP kinase (**AMPK**), which promotes catabolic metabolism, antagonizes mTOR and DC activation and renders DC tolerogenic (Krawczyk et al., 2010). Thus, anabolic metabolism promotes a pro-inflammatory response whereas catabolic metabolism promotes an anti-inflammatory response (O'Neill and Pearce, 2016). Mammalian target of rapamycin is intimately involved in metabolic

reprogramming in numerous leukocytes including neutrophils (McInturff et al., 2012), macrophages (Kang and Kumanogoh, 2020), and T lymphocytes (Buck et al., 2015).

Much like the innate immune system, T and B lymphocytes of the adaptive immune system rely on Warburg metabolism to regulate their activity. Mature naïve T cells enter the periphery as quiescent cells that rely on fatty acid oxidation and oxidative phosphorylation for ATP generation (Pearce and Pearce, 2013); however, this is markedly altered following antigen stimulation. T cell activation requires three signals: 1) T cell receptor (**TCR**) ligation, 2) binding of costimulatory molecules, and 3) cytokines. Antigen presentation by antigen presenting cells (**APC**) using MHC class II (found on macrophages, DC, and B cells) triggers CD4⁺ T cell differentiation into one of several lineages of helper T cell (**Th**) such as Th1, Th2, Th17, or regulatory T cells (**Treg**). The expressed lineage is dictated by the cytokine milieu (Delgoffe et al., 2009). Ligation of costimulatory molecules and specific cytokines such as IL-2 stimulate metabolic reprogramming through PI3K/Akt signaling and downstream activation of mTOR (Kolev et al., 2015; Buck et al., 2015). Mammalian target of rapamycin promotion of the glycolytic switch supports effector T cell differentiation, growth, and function (Delgoffe et al., 2011). Furthermore, the transcription factor Myc is important in mediating metabolism to support amino acid, nucleotide and lipid biosynthesis needed for cell proliferation (Wang et al., 2011; Buck et al., 2015). Glycolytic metabolism favors CD4⁺ T cells differentiation into Th1, Th2, or Th17 lineages. However, blocking mTOR activation via rapamycin triggers T cells differentiation into Treg (Delgoffe et al., 2009, 2011). This response is consistent with the fact that Treg have high levels of activated AMPK and require lipid oxidation (Michalek et al., 2011). Regulatory T cells predominately have anti-inflammatory properties and thus differentiation to this phenotype is important in regulating a balanced inflammatory response. Interestingly, T cells

retain the ability to become activated and proliferate when aerobic glycolysis is limited, however, effector function (i.e., IFN- γ production) is compromised due to binding of the glycolytic enzyme GAPDH to the 3' untranslated region of IFN- γ mRNA (Chang et al., 2013). This posttranscriptional effect illustrates the profound link between metabolism and leukocyte activity. B cells are activated upon antigen recognition via the B cell receptor and co-stimulation from Th2 cells. Once activated, B cells produce antibodies unique to the presented antigen which neutralize the pathogen and they too upregulate glycolytic metabolism (Jellusova and Rickert, 2016). Altogether, these responses emphasize the link between metabolism and immune cell phenotype; in general glycolytic metabolism leads to acquisition of a more inflammatory phenotype whereas oxidative phosphorylation is associated with an anti-inflammatory function. Understanding how metabolism influences the fate and function of immune cells provides a unique opportunity to modulate immunity across numerous species and physiological states.

Glucose Consumption

Immune cells utilizing Warburg metabolism, markedly increase glucose consumption to meet energy demands and support biosynthetic pathways (Calder et al., 2007; Palsson-McDermott and O'Neill, 2013). Glucose is the preferred fuel for most pro-inflammatory leukocytes and improving glucose availability increases their longevity and function (e.g., phagocytosis, ROS production, NET formation; Furukawa et al., 2000; Healy et al., 2002; Garcia et al., 2015; Rodríguez-Espinosa et al., 2015). Although secondary to glucose, glutamine is also fundamental to effector function (Newsholme and Newshome, 1989; Newsholme et al., 1999). Cellular glucose uptake is dependent on GLUT expression on the plasma membrane. Several GLUT isoforms with varying degrees of affinity exist and cellular expression is driven by physiological function (Gould and Holman, 1993). Expression of GLUT1 allows for glucose uptake in basal conditions, whereas GLUT3 and GLUT4 are insulin dependent (Estrada et al.,

1994; Maratou et al., 2007). Activated monocytes, neutrophils, and T- and B-lymphocytes express GLUT1, GLUT3, and GLUT4 on the plasma membrane, and insulin augments GLUT3 and GLUT4 expression (Maratou et al., 2007). The insulin receptor (**IR**) is expressed on most activated immune cells (Walrand et al., 2006) and insulin increases glucose uptake and modulate immunity (Estrada et al., 1994; Walrand et al., 2004, 2006; Calder et al., 2007). Bovine monocytes and polymorphonuclear leukocytes (**PMNL**) also express GLUT1, GLUT3, GLUT4, and the IR on the plasma membrane (Nielsen et al., 2003; O'Boyle et al., 2012; Garcia et al., 2015). Endotoxin stimulation increased GLUT3 and GLUT4 expression on bovine monocytes (O'Boyle et al., 2012); however, in PMNL and polarized macrophages GLUT3 was the primary isoform (Garcia et al., 2015; Eger et al., 2016). Increased GLUT3 (and possibly GLUT4) may allow for competitive uptake amongst cells when glucose concentrations in the microenvironment are low (i.e., early lactation), as GLUT3 has a higher affinity for glucose than GLUT1 (Gould and Holman, 1993; Zhao and Keating, 2007; Thorens and Mueckler, 2010).

In agreement with changes in transporter expression, in vitro studies have demonstrated increased glucose uptake by activated neutrophils (Borregaard and Herlin, 1982; Healy et al., 2002), macrophages (Newsholme and Newsholme, 1989; Spolarics et al., 1991), DC (Jantsch et al., 2008; Krawczyk et al., 2010; Everts et al., 2012), and T- and B-lymphocytes (Hume et al., 1978; Doughty et al., 2006; Caro-Maldonado et al., 2014). Assessing glucose consumption in vivo is difficult due to the ubiquitous and fluctuating distribution of immune cells. Early investigators demonstrated increased whole-body glucose utilization in endotoxin-administered animals (Wolfe et al., 1977; Lang et al., 1985). However, interpreting changes in whole-body glucose disposal is complicated by the fact that it represents the net effect of tissues which increase glucose uptake and those that decrease glucose dependence. Mészáros and colleagues

(1987) utilized tracer technology to evaluate tissue specific differences in glucose uptake following endotoxin administration, and results demonstrated it was greatest in immune rich tissues (i.e., liver, spleen, skin). In addition, glucose uptake differed within each cell fraction within the liver such that it did not change in parenchymal cells, but markedly increased in Kupffer cells and neutrophils (Mészáros et al., 1991). In contrast to the immune tissues, peripheral tissues (particularly muscle) decreased glucose uptake in response to immune activation (Mészáros et al., 1987; Lang et al., 1993); resulting from insulin resistance (Lang et al., 1989, 1991). The aforementioned studies clearly demonstrated endotoxin-mediated changes in whole-body glucose disappearance reflect increased leukocyte utilization. Understanding the impact of immune activation on whole-body glucose consumption in livestock species is of interest, as glucose is an important fuel for productive purposes (i.e. milk synthesis, fetal growth). However, the techniques used in rodents are less feasible in livestock species and do not assess the quantity of glucose used. Therefore, we recently developed a technique called an LPS-euglycemic clamp, to quantify glucose requirements during immune activation and results in cows demonstrated a requirement of ~1 kg of glucose in 12 hours (Kvidera et al., 2017a), an estimate which is remarkably similar to that hypothesized by Waldron and others (2006). This technique was repeated using other animal models and when evaluated on a metabolic BW basis, the glucose requirement of the immune system was consistent across species with values of 0.66, 1.0, and 1.1 g/kg of BW^{0.75}/h in cows, steers, and pigs, respectively (Kvidera et al., 2016, 2017a,b). The consistency in the glucose requirement across different ages, physiological states, and species suggests that the extent of fuel utilization by activated leukocytes is a conserved process.

Whole Body Response to Immune Activation

Energetic homeostasis is markedly altered in response to immune activation in order to ensure adequate glucose is supplied to immune cells. Understanding the intricate nutrient partitioning strategies employed during infection has captivated researchers for centuries. The response to infection involves dynamic changes in hormonal, metabolic, and mineral homeostasis some of which have been highlighted in Table 1.2. It is important to note that responses to immune activation are not static and thus understanding the temporal etiology requires repeated and frequent measurements. The carbohydrate, protein, lipid, and calcium response to immunoactivation will be discussed in detail below.

Table 1.2. The effects of endotoxin on plasma metabolites and regulatory hormones in various species^a

Metabolite	Species	Response	Reference
Insulin	Bovine	↑ / ↓	3,4,11,19,22,23,27,43,44,46,47 / 28
	Dogs	↑	20,48
	Horses	↑	9
	Pigs	↑ / ↓	36, 45 / 8,36
	Rodent	= / ↑↓	38,40 / 39
	Sheep	↑	10,16,18
Glucagon	Bovine	↑	3,4,47
	Dogs	↑	20, 42
	Rodents	↑	38,39,40
	Sheep	↑	16
Epinephrine	Bovine	↑	31
	Human	↑	25
	Pigs	↑	26
	Rodent	↑	39
Cortisol	Bovine	↑	1,3,4,11,22,23,24,27,31,33,47
	Human	↑	25
	Pig	↑	8,26
	Sheep	↑	10
	Dogs	↑	42
Glucose	Bovine	↑↓ / ↑ / ↓	1,2,3,19,23,24,27,34,43,44 / 4,6,22 / 21,28,29
	Dogs	↓	17,20
	Pigs	↑↓ / ↓	36 / 7,8
	Rodents	↑↓	38,39
	Sheep/Goats	↑↓ / ↓ / ↑	15,16,32 / 10,18,30 / 30
NEFA	Bovine	↑ / = / ↓	2,3,5,6,23,28, 41,44 / 4,19 / 11,22
	Pigs	↑	8,36
	Rodents	↓	37
	Sheep	↓ / =	15 / 30
BHBA	Bovine	↓	3,4,5,6,22,23,24,43,44
	Rodent	↓	37
	Sheep	↓	15
L-lactate	Bovine	= / ↑	3/ 1,21,23,24,34,43
	Pigs	↑	7
	Rodent	↑	40
	Sheep	↑ / =	16 / 15
	Dogs	↑	42

Table 1.2 Continued.

Metabolite	Species	Response	Reference
Calcium	Bovine	↓	2,12,14,34,43,44
	Dogs	↓	17
	Horses	↓	9
	Pigs	↓	13
	Sheep	↓	15
BUN	Bovine	↑ / = / ↓	2,19,27,28,33,43/ 44/ 4
	Goat	=	30,35
	Pigs	↑	7,36

^aAdapted from (Kvidera et al., 2017)

1 Giri et al., 1990	13 Carlstedt et al., 2000	25 Richardson et al., 1989	37 Kaminski et al., 1979
2 Elsasser et al., 1996	14 Griel et al., 1975	26 Williams et al., 2009	38 Yamashita et al., 2015
3 Waldron et al., 2003a	15 Naylor and Kronfeld, 1986	27 Waggoner et al., 2009b	39 Maitra et al., 2000
4 Zarrin et al. 2014	16 Naylor and Kronfeld, 1985	28 Ballou et al., 2008	40 Lang et al., 1991
5 Graugnard et al., 2013	17 Holowaychuk et al., 2012	29 Bieniek et al., 1998	41 Lehtolainen et al., 2003
6 Moyes et al., 2014	18 Southorn and Thompson, 1986	30 Wang et al., 2016	42 McGuinness, 1994
7 Bruins et al., 2003	19 Burdick Sanchez et al., 2014	31 Burdick et al., 2012	43 Kvidera et al., 2016
8 Leininger et al., 2000	20 Blackard et al., 1976	32 Wang et al., 2015b	44 Kvidera et al., 2017a
9 Toribio et al., 2005	21 Gerros et al., 1995	33 Waggoner et al., 2009a	45 Kvidera et al., 2017b
10 Yates et al., 2011	22 Waldron et al., 2006	34 Tennant et al., 1973	46 Gross et al., 2020
11 Vernay et al., 2012	23 Steiger et al., 1999	35 van Miert et al., 1983	47 Gross et al., 2018
12 Waldron et al., 2003b	24 Werling et al. 1996	36 Myers et al., 1997	48 Spitzer et al., 1980

Carbohydrate Metabolism

As previously discussed, immune activation induces marked alterations in whole-body glucose dynamics as a result of increased leukocyte glucose requirements (see Immunometabolism section). Endotoxemia causes whole-body insulin resistance (Lang et al., 1985; McGuinness, 2005; Vernay et al., 2012), which specifically reflects a reduction in insulin-mediated glucose uptake by peripheral tissues such as muscle and adipose (Spitzer et al., 1980; Lang et al., 1990). Some reports indicate increased adipose tissue glucose uptake (Lang et al., 1992; Maitra et al., 2000), however, this is likely explained by the presence of resident macrophages which utilize glucose (Weisberg et al., 2003). The insulin resistant state represents a key glucose-sparing strategy in support of the immune response. Endotoxin administration triggers a biphasic response in circulating glucose, with an initial transient hyperglycemic period followed by chronic hypoglycemia (Blackard et al., 1976; Waldron et al., 2003a; Kvidera et al., 2017a). Hyperglycemia results from increased hepatic glucose output via glycogenolysis and gluconeogenesis, although the latter process is typically delayed (Naylor and Kronfeld, 1985;

Spitzer et al., 1985; McGuinness, 1994; Waldron et al., 2003a, 2006). Increased hepatic glucose output is facilitated by characteristic increases in glucagon and cortisol (see Table 1.2).

Epinephrine may also play a role, however, the liver becomes less sensitive to epinephrine-mediated increases in glucose turnover during immune activation (Hargrove et al., 1989) as a result of downregulation of adrenergic receptors (Gurr and Ruh, 1980; Snavely et al., 1985).

Interestingly, despite being in a catabolic state, LPS-infused animals are hyperinsulinemic, a response which is conserved across most species (see Table 1.2). Glucose infusion has been shown to exacerbate hyperinsulinemia (Blackard et al., 1976), however, we observed no difference in insulin concentrations between cows infused with LPS alone and those infused with LPS in combination with glucose (Kvidera et al., 2017a). In addition, hyperinsulinemia persists even during hypoglycemia (Kvidera et al., 2017a) and when hyperglycemia is prevented by pre-LPS fasting, demonstrating that hyperglycemia is not the primary stimulus for increased pancreatic insulin secretion during an infection (Hand et al., 1983; Kvidera et al., 2017b). The mechanism by which LPS increases insulin remains unclear, but likely involves direct effects of LPS on the pancreas (Vives-Pi et al., 2003; Bhat et al., 2014) or secondary effects by the secretagogue glucagon-like peptide 1 (Nguyen et al., 2014). Together, the peripheral tissue insulin resistance and increased hepatic glucose output provide glucose at a rate exceeding the immune system's requirement, culminating in transient hyperglycemia. However, once the immune system becomes fully engaged leukocyte glucose consumption outpaces these strategies (1-3 hours), often resulting in hypoglycemia. In response to low LPS doses glucose may return to basal levels, however, high doses result in a profound hypoglycemia which in certain cases can be lethal (Lang et al., 1985, 1993).

Lipid Metabolism

Hypertriglyceridemia is a well-characterized response to infection in monogastrics, which develops as a result of reduced TG clearance or increased TG hepatic production, depending on the dose (Blackburn, 1977; Wolfe et al., 1985; Takeyama et al., 1990; Grunfeld and Feingold, 1992; Memon et al., 1992). In response to high LPS doses, hypertriglyceridemia occurs as a result of decreased muscle and adipose tissue clearance mediated by reduced endothelial lipoprotein lipase (Bagby and Spitzer, 1980; Kawakami and Cerami, 1981; Feingold et al., 1992) whereas in response to low LPS doses it is a reflection of increased hepatic production (Feingold et al., 1992). Triglyceride accumulation preferentially occurs in VLDL (Bartolomé et al., 2010). Increased circulating TG likely represent a strategy to promote LPS detoxification as previously discussed (see Detoxifications section). In ruminants LPS-induced changes in TG concentrations are poorly described as both increased (Ballou et al., 2008; Graugnard et al., 2013) and decreased (Wang et al., 2017) concentrations have been reported. Discrepancies in the response may be explained by sampling time, as the increase in TG appears to be short-lived (Ballou et al., 2008; Graugnard et al., 2013). The mode of action for transient hypertriglyceridemia in ruminants remains unclear, but increased hepatic export is unlikely as ruminants have a poor capacity to export VLDL (Kleppe et al., 1988).

The lipolytic response to LPS is variable as both increased and decreased NEFA concentrations have been reported (see Table 1.2). In general, administering LPS increases circulating NEFA, but the response is delayed and dampened when compared to non-inflamed animals on the same plane of nutrition (Kvidera et al., 2017a). In lactating cows, the blunted NEFA response is most likely explained by LPS-induced immediate reduction in milk synthesis, which spares energy, whereas the feed restricted cows maintain a much higher level of production requiring greater mobilization. Other potential factors contributing to the blunted

NEFA response include anti-lipolytic effects of increased insulin (Vernon, 1992) and increased circulating lactate which sensitizes adipocytes to insulin action by signaling through GPR81 (Ahmed et al., 2010). Increased NEFA have also been observed in response to inflammatory cytokine infusion (Kushibiki et al., 2003; Yuan et al., 2013). In immune activated rodents, NEFA delivered to the liver rapidly accumulates into TG resulting in fatty liver (Lanza-Jacoby and Tabares, 1990; Endo et al., 2007; Stienstra et al., 2010); the role of LPS in fatty liver development will be discussed in detail later on (see Fatty Liver section). Even though hepatic NEFA uptake and TG synthesis are increased, the partial oxidation of NEFA via ketogenesis is downregulated in rodents (Takeyama et al., 1990; Maitra et al., 2009). Reduced ketogenesis is hypothesized to occur via a reduction in gene expression of peroxisome proliferator-activated receptor-alpha (PPAR- α ; Beigneux et al., 2000; Maitra et al., 2009) which regulates enzymes involved in fatty acid oxidation including CPT1, acyl-CoA oxidase, and ATP-citrate lyase (Maitra et al., 2009). However, in LPS-administered ruminants, ketogenesis appears to remain functional (Waldron et al., 2003a), yet BHB concentrations markedly decrease (see Table 1.2). In well-fed ruminants, the majority of BHB is produced by the rumen epithelium (Pennington, 1952), thus decreased BHB concentrations are likely at least partially explained by LPS-induced reduced feed intake and this explains why BHB decreases in pair-fed animals as well. Although increased peripheral tissue BHB clearance also plays a prominent role (Zarrin et al., 2014; Horst and Baumgard, unpublished). The LPS-induced changes in lipid metabolism differ markedly between ruminants and monogastrics and further investigation is needed.

Protein Metabolism

Administering LPS in rodents increases muscle protein degradation by more than 60% (Jepson et al., 1986). Immune activation induces muscle proteolysis as a means of providing amino acids (AA) to support gluconeogenesis (Wannemacher et al., 1980) and APP synthesis

(Iseri and Klasing, 2013, 2014). The extent of skeletal muscle protein catabolism in support of APP synthesis far exceeds the true requirement due to a mismatch in the AA composition between muscle and APP (Reeds et al., 1994). Amino acids not incorporated into APP, are deaminated and the carbon skeletons are utilized for glucose synthesis while the amino groups enter ureagenesis. As a result, blood urea nitrogen (**BUN**) concentrations consistently increase in monogastric immunoactivation models (see Table 1.2). In ruminants, changes in circulating BUN are more variable as the increase may be masked by changes in rumen ammonia flux which is altered due to decreased substrate availability and variations in ruminal microbiota composition and function (Gaylean et al., 1981; Jing et al., 2014). In agreement with this, we observed no change in circulating BUN following LPS administration in lactating cows (Kvidera et al., 2017a). A more reliable marker of muscle protein mobilization is 3-methylhistidine (**3-MH**; Blum et al., 1985) and it has been demonstrated to be increased in cows exhibiting a more pronounced inflammatory response postpartum (Zhou et al., 2017). We observed no change in 3-MH with intravenous LPS administration in lactating cows (Appuhamy et al., 2018), however, this inconsistency may be explained by insufficient sampling frequency. Circulating AA such as glutamine, arginine, asparagine, and total branched chain amino acids decreased in lactating cows administered LPS (Appuhamy et al., 2018; Humer et al., 2018). Besides supporting glucose and APP synthesis, AA released from muscle (particularly glutamine and arginine) may also be utilized as a fuel source for activated leukocytes (Newsholme and Newshome, 1989; Newsholme et al., 1999; Pekarova and Lojek, 2015). In summary, muscle protein degradation is crucial to mounting the inflammatory response.

Calcium Homeostasis

In addition to altering energetics, infection markedly reduces circulating Ca (see Table 1.2). Infection-induced hypocalcemia is a species conserved response occurring in humans

(Cardenas-Rivero et al., 1989; Dias et al., 2013), calves (Tennant et al., 1973; Elsasser et al., 1996), dogs (Holowaychuk et al., 2012), horses (Toribio et al., 2005), pigs (Carlstedt et al., 2000), and sheep (Naylor and Kronfeld, 1986). Additionally, hypocalcemia occurs in cows administered LPS (Griel et al., 1975; Waldron et al., 2003b; Kvidera et al., 2017a; Al-Qaisi et al., 2020b) or challenged with experimentally-induced SARA (Minuti et al., 2014; Stefanska et al., 2018). Although LPS-induced hypocalcemia is a commonly observed phenomenon, it remains poorly understood what role Ca plays in inflammation and why it acutely decreases.

Interestingly, septic humans are typically hypocalcemic (Zaloga, 1992; Kelly and Levine, 2013) and early reports indicate that Ca administration to septic patients increases the incidence of organ failure and mortality (Malcolm et al., 1989). It is now hypothesized that sepsis-induced hypocalcemia serves as a protective strategy and should not be considered pathologic (Collage et al., 2013; Eckel and Ametaj, 2016). Early investigators described a critical role of decreased blood Ca for optimal LPS detoxification via non-inflammatory routes (Skarnes and Chedid, 1964). When circulating Ca concentrations are decreased, LPS aggregation is inhibited; a situation that allows LBP to transfer LPS monomers to sCD14 and eventually to lipoproteins for biliary excretion (see Detoxification section). In contrast, during eucalcemia, the disaggregation of LPS monomers is inhibited (Skarnes and Chedid, 1964), and consequently, LPS is recognized by pro-inflammatory mechanisms (i.e. cells containing TLR4 receptors); a scenario contributing to a hyper-inflammatory systemic response. This mechanism may explain why changes in Ca homeostatic regulators (i.e., parathyroid hormone, calcitonin, and Vitamin D) favor a hypocalcemic state during infection. For example, hypoparathyroidism (Nielsen et al., 1997; Holowaychuk et al., 2012), increased circulating calcitonin and procalcitonin (Müller et al., 2000; Bonelli et al., 2018), and decreased 1,25-(OH)₂D concentrations (Waldron et al., 2003b;

Holowaychuk et al., 2012) are observed in immunoactivated states. Although not fully elucidated, Ca removed from circulation may be sequestered in the ascites (Carlstedt et al., 2000). The relationship between immune activation and hypocalcemia has practical relevance to the transition period and will be further discussed below (see Hypocalcemia section).

LPS/Inflammation and Transition Cow Performance

Regardless of health status (Humblet et al., 2006), increased inflammatory biomarkers are observed in nearly all cows during the transition period (Ametaj et al., 2005; Humblet et al., 2006; Bionaz et al., 2007; Bertoni et al., 2008; Mullins et al., 2012). Cows are exposed to a myriad of physiological, environmental, and/or psychological stressors between dry off and the early postpartum period that disrupt barrier integrity at epithelial interfaces, permitting pathogen infiltration and initiation of an inflammatory response (see Sources of LPS in the Transition cow). Exposure to more frequent and severe immune insults perpetuates and intensifies the inflammatory response (Bertoni et al., 2008; Bradford et al., 2015; Trevisi and Minuti, 2018). Bertoni and colleagues (2008) demonstrated that cows with the strongest inflammatory profile (as measured by the liver activity index) were at a substantially higher risk of developing transition disorders. In addition, essentially all of the major transition cow diseases/disorders (i.e., metritis, mastitis, laminitis, ketosis, milk fever, and retained placenta) are preceded by a heightened inflammatory response (Huzzey et al., 2009; Dervishi et al., 2015, 2016a,b; Zhang et al., 2015, 2016; Abuajamieh et al., 2016a). Potential downstream consequences of LPS and the corresponding inflammatory response are highlighted in Figure 1.2 and will be discussed in greater detail below.

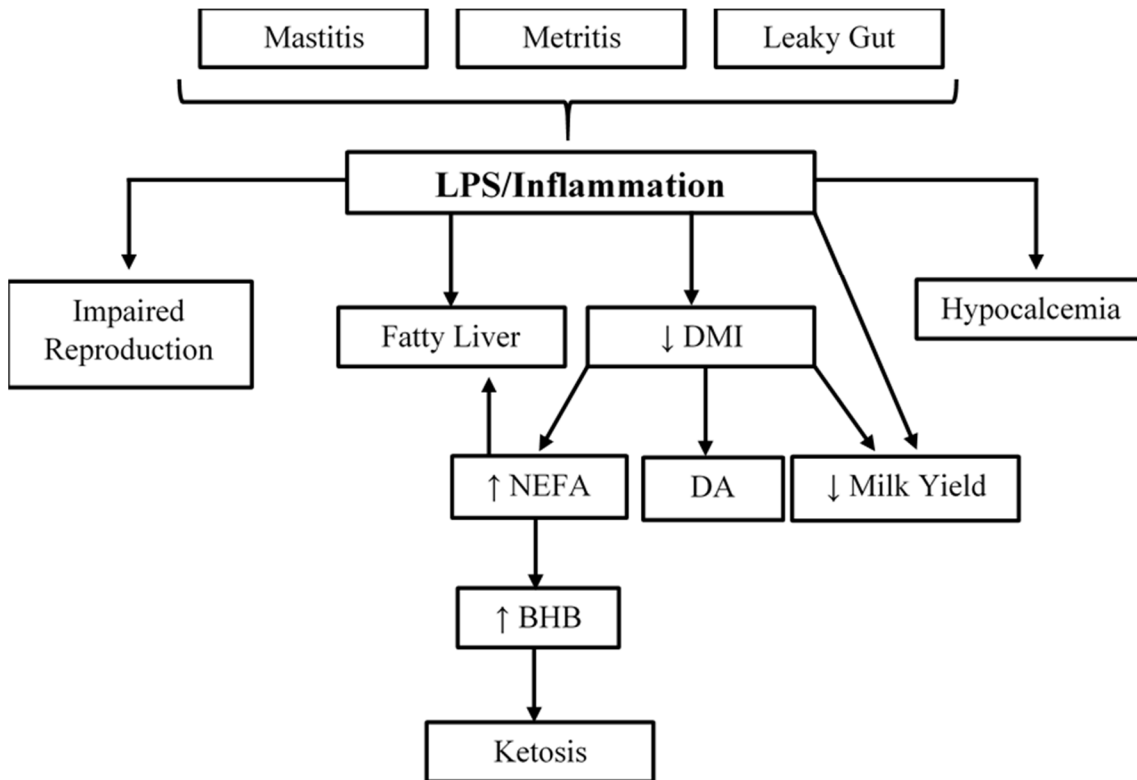


Figure 1.2. Potential downstream consequences of immune activation. In this model, decreased feed intake, hypocalcemia, excessive NEFA, hyperketonemia, and hepatic lipidosis are not causative to poor transition cow performance and health, but rather a reflection of prior immune stimulation.

NEBAL, DA, and Ketosis

As discussed previously (see Nutrient Partitioning and Metabolic and Infectious Disease sections), cows typically enter into NEBAL in the early postpartum period as result of increased energetic requirements and insufficient dietary intake. Depressed feed intake prior to calving is a well-characterized response and is an important determining factor in the severity of NEBAL that ensues (Hayirli et al., 2002a). Inflammatory mediators released during an immune response have potent anorexic effects (Kushibiki et al., 2003) and thus, may contribute to depressed feed intake surrounding calving. In support of this tenant, cows exhibiting an earlier and larger reduction in feed intake prior to parturition had a concomitantly more robust increase in Hp

concentrations (Trevisi et al., 2002). Furthermore, cows with poor liver functionality/activity (a proxy of inflammation) have lower feed intake, decreased rumination time, an exacerbated NEBAL, and increased NEFA and BHB (Trevisi et al., 2010, 2012; Zhou et al., 2016). Immune activation and inflammation-induced reductions in feed intake and rumination time may increase the opportunity for abomasal migration and subsequent DA. Intermittent endotoxin administration during the periparturient period increases the incidence of DA (Zebeli et al., 2011), corroborating inflammation's role in the disorder. Besides DA, inflammation has also been associated with ketosis development. We and others have previously demonstrated that cows which develop ketosis postpartum (and no other overt health event) had higher concentrations of LPS, cytokines, APP, and lactate prior to disease diagnosis (Abuajamieh et al., 2016a; Zhang et al., 2016), and these changes could be observed as early as 8 weeks prior to calving (Zhang et al., 2016). Additional indicators of inflammation such as, increased markers of liver impairment (e.g., glutamic-oxaloacetic transaminase and bilirubin), decreased negative APP (e.g., retinol), increased neutrophil and monocyte activation markers (myeloperoxidase and advanced oxidation protein products), and decreased circulating Zn have also been observed in ketotic cows relative to their "healthy" herdmates (Rodriguez-Jimenez et al., 2018; Mezzetti et al., 2019). Shen and colleagues (2019) observed increased hepatic expression of inflammatory genes (e.g., NF- κ B, pro-inflammatory cytokines, inducible NO synthase) and circulating cytokines in ketotic cows, however the authors concluded that ketosis caused inflammation rather than the vice-versa. We believe inflammation before calving accentuates the reduction in feed intake, stimulates increased adipose tissue mobilization, and thus subsequently promotes ketone synthesis. Evidence suggests inflammatory cytokines may also act directly on the adipocyte to stimulate lipolysis (see Lipid section), further increasing the opportunity for ketone

body production. Therefore, decreased feed intake, increased NEFA, and hyperketonemia are likely a consequence of immune activation and are not themselves causative of transition disorders. It is important to note that increased NEFA and ketones in the absence of inflammation and poor lactational performance should not be considered problematic, as this is a necessary mechanism to spare glucose for copious milk synthesis.

Fatty Liver

Fatty liver is traditionally thought to occur when excessive adipose tissue mobilization (as a result of severe NEBAL) and corresponding hepatic NEFA uptake, exceeds the liver's capacity to fully oxidize fatty acids resulting in conversion to TG (as well as ketone synthesis; Herdt, 1988). Triglycerides are believed to rapidly accumulate because of the ruminant liver's poor capacity to export VLDL (Kleppe et al., 1988). In non-ruminants, hepatic steatosis is commonly observed during intestinal hypermeability pathologies (Ilan, 2012; Hamarneh et al., 2017) and can be induced by inflammatory cytokine infusion (see Lipid section). Inflammatory cytokines produced in response to LPS recognition, can negatively impact hepatic lipid trafficking (Lanza-Jacoby and Tabares, 1990; Endo et al., 2007; Stienstra et al., 2010). Inflammation's role on hepatic lipid metabolism is confirmed by LPS and cytokine recognition interference experiments that ameliorate liver fat accumulation (Endo et al., 2007; Spruss et al., 2009; Jin et al., 2017; Jia et al., 2018). It is generally believed that increased hepatic NEFA delivery and inflammation must coincide for fatty liver disease progression; a concept known as the two-hit hypothesis (Day and James, 1998; Csak et al., 2011). Therefore, strong evidence demonstrates a role of immune activation and inflammation in fatty liver development.

In transition cows, heightened circulating inflammatory markers precede fatty liver development (Ohtsuka et al., 2001; Ametaj et al., 2005, 2010), suggesting the same relationship between hepatic inflammation and lipid accumulation likely exists in ruminants. Additionally,

once-daily subcutaneous TNF- α infusion in late lactation cows altered hepatic lipid handling and increased TG storage (Bradford et al., 2009). However, no change in liver TG content was observed with continuous TNF- α infusion in late lactation (Martel et al., 2014) or repeated infusions in early lactation cows (Yuan et al., 2013). Graugnard and others (2013) demonstrated an exacerbated increase in liver TG content in cows overfed prepartum and administered intramammary LPS postpartum. Effects of immune activation on adipose tissue mobilization (both by direct action on adipocytes and as a result of decreased feed intake) and hepatic NEFA delivery coupled with inflammation-induced alterations in hepatic lipid handling may culminate in fatty liver in transition cows. An additional mechanism by which inflammation may increase fatty liver is through the lipoprotein pathway (see Detoxification section). Preferential shunting of lipoprotein-LPS complexes to hepatocytes at a rate exceeding biliary excretion may result in TG accumulation.

Milk Fever

At lactation onset, dairy cows experience a marked increase (>65%; DeGaris and Lean, 2008) in Ca requirements to support colostrum and milk synthesis (Horst et al., 2005). The dairy industry has long hypothesized that the mammary gland's Ca demand is so extensive and acute that it often exceeds the homeostatic mechanisms (i.e., PTH and Vitamin D) employed to replenish it and as a result clinical or subclinical hypocalcemia (**SCH**) occurs (Horst et al., 2005; Goff, 2008). Clinical hypocalcemia incidence has been markedly reduced with the introduction of therapeutic and prophylactic strategies (Charbonneau et al., 2006; Reinhardt et al., 2011), however, SCH remains prevalent. It has recently been recognized that the temporal pattern of circulating Ca differs markedly across SCH cases, such that in some cases it is transient whereas in others it is persistent or delayed (Caixeta et al., 2017; McArt and Neves, 2020). For example, McArt and Neves (2020) classified cows into 1 or 4 groups based on post-calving Ca

concentrations: normocalcemia (>2.15 mmol/L at 1 and 2 DIM), transient SCH (≤ 2.15 mmol/L at 1 DIM), persistent SCH (≤ 2.15 mmol/L at 1 and 2 DIM), or delayed SCH (> 2.15 mmol/L at 1 DIM and ≤ 2.15 mmol/L at 2 DIM). Interestingly, cows experiencing transient SCH produced more milk and were no more likely to experience a negative health event when compared to normocalcemic cows, whereas the opposite (i.e., higher health risk and hindered productivity) was observed in cows experiencing either persistent or delayed SCH. The distinguishing feature between these different SCH “types” may be LPS and immune activation.

As discussed previously (see Calcium section), hypocalcemia is a well-characterized response to LPS exposure, which presumably occurs to permit LPS detoxification via lipoproteins. Impressively, immune activation was originally hypothesized by early investigators to be involved with milk fever (Thomas, 1889; Hibbs, 1950), but until recently (Aiumlamai et al., 1992; Eckel and Ametaj, 2016) it has rarely been considered a contributing factor to hypocalcemia. It is of interest to elucidate whether inflammation can explain the manifestation of the different hypocalcemia “types”, especially considering their associations with poor performance. Akin to increased NEFA and hyperketonemia, strong evidence suggests that hypocalcemia is a consequence of immune activation and is not itself causative to transition disorders.

Immunosuppression

More than 30 years ago dairy scientists described impairments in leukocyte cellular functions during the periparturient period (Kehrli et al., 1989), and this “immunosuppressive” state has continued to be a topic of intensive investigation (Goff and Horst, 1997; Kimura et al., 2002; Lacetera et al., 2004, 2005; LeBlanc, 2020). Cellular leukocyte functions such as neutrophil phagocytosis, the ability of lymphocytes to respond to mitogens and produce antibodies, peripheral blood mononuclear cell DNA synthesis, immunoglobulin concentration,

INF γ , complement, and lysozyme are often depressed prepartum (Kehrli et al., 1989; Goff and Horst, 1997; Mallard et al., 1998; Lacetera et al., 2005; Trevisi and Minuti, 2018). Although in large part changes in cellular function are most evident in the immediate postpartum period (Goff and Horst, 1997; Trevisi and Minuti, 2018). In contrast to past literature, recent transcriptome analysis reports have demonstrated that many leukocyte cellular functions are actually upregulated postpartum (Mann et al., 2019; Minuti et al., 2020). Therefore, recent literature suggests that the immune system is not necessarily “suppressed”, but rather dysregulated around parturition (Trevisi and Minuti, 2018; Minuti et al., 2020). Interestingly, Mann and others (2019) demonstrated that leukocyte inflammatory pathways were upregulated to a larger extent in cows with a greater energy deficit (as determined by NEFA, BHB, and glucose concentrations). Although the exact sequence of events cannot be confirmed, it could be suggested that cows with an exacerbated inflammatory response have a subsequent greater magnitude of nutrient deficit and metabolic disease as suggested above (see NEBAL, DA, and Ketosis and Fatty Liver sections). Heightened leukocyte inflammatory pathways may occur as a result of successive insult exposure, as recently hypothesized by Trevisi and Minuti (2018). In this context, each additional immune insult triggers an amplified inflammatory response, which in turn attenuates certain cellular functions (Heyland et al., 2006). Therefore, the heightened inflammatory state resulting from repeated insults could explain changes to cellular immune function and may prevent adequate immune responses to fight secondary infections.

Retained Placenta and Reproductive Performance

Expulsion of fetal membranes necessitates an immune response as leukocytes facilitate the degradation of the cotyledon-caruncle attachment that separates placental membrane from maternal tissue (LeBlanc, 2008). The importance of inflammation to this response is made evident by the fact that blocking inflammation (via administering non-steroidal anti-

inflammatory drugs) increases the incidence of RP (Newby et al., 2017). Interestingly, intermittent endotoxin administration during the periparturient period increases the incidence of RP (Zebeli et al., 2011). Results of this repeated insult model are consistent with changes in cellular function that may occur as a result of an amplified inflammatory response as mentioned above (see Immunosuppression section). Retained placenta increases the risk of uterine infections and this appears to be at least partially mediated by its effects on the elimination of lochia, which contains high concentrations of LPS (Ametaj, 2017b).

Both localized and systemic inflammation can negatively impact reproductive performance (Peter and Bosu, 1988; Williams et al., 2008; Sheldon et al., 2009; Lavon et al., 2011; Asaf et al., 2014). For example, uterine infection prolongs the luteal phase (Peter and Bosu, 1988; Williams et al., 2008; Sheldon et al., 2009), disrupts ovarian steroidogenesis (Sheldon et al., 2009), and results in abnormal or delayed folliculogenesis after parturition (Huszenicza et al., 1999). In addition, remote inflammation (i.e., mastitis) disrupts follicular steroid concentrations and hinders oocyte maturation (Lavon et al., 2011; Asaf et al., 2014). Cows diagnosed with mastitis prior to first service have an increased number of days to first service and days open (Barker et al., 1998). Administering LPS intravenously results in marked disruptions in hypothalamic and pituitary hormone release and ovarian responsiveness (Coleman et al., 1993; Battaglia et al., 2000) and induces abortion (Giri et al., 1990). Presumably, regardless of the origin, infection negatively influences immediate and future reproductive performance. The direct effects of endotoxin and inflammation on reproduction likely explain the associated effects that NEFA, ketones and calcium have with fertility (because immune activation also directly affects these metabolites).

Dietary Mitigation Strategies

Dietary mitigation strategies capable of improving barrier defense and/or modulating immunity are currently of great interest, especially considering the relevance of immune activation to transition cow disease and performance. This review focuses on describing the use of chromium (**Cr**) and zinc (**Zn**) and their role in improving animal health and performance.

Chromium

Chromium is a micronutrient which can exist in multiple valence states. Trivalent Cr, the most stable and biologically active form, is a nutrient which potentiates the action of insulin in insulin-sensitive tissues (Kegley et al., 2000). Chromium's mode of action was first described by Yamamoto and colleagues (1984) after the discovery of a Cr-binding oligopeptide termed "low molecular weight Cr binding substance" or chromodulin, which consists of four amino acid residues (glycine, cysteine, aspartate and glutamate; Yamamoto et al., 1987). Pancreatic insulin released in response to a meal binds to its transmembrane IR on the surface of target cells triggering tyrosine kinase activation and eliciting Cr uptake into insulin-sensitive cells. The cytosolic protein apochromodulin (metal free form of chromodulin) then sequesters four Cr ions and becomes its active form, chromodulin. Once activated, chromodulin binds to the IR, purportedly maintaining the receptor in its active conformation, thus amplifying its kinase activity. Subsequently, GLUT-4 translocation to the plasma membrane is improved and glucose uptake is increased (Chen et al., 2006). Once insulin levels decline, chromodulin is released from the cell and excreted in the urine (Vincent et al., 2015).

It was originally thought that basal diets provided adequate Cr to meet requirements in production animals and while this may be true in healthy animals, during stress (i.e., weaning, transport, feedlot acclimation, transition period, etc.) animals are often Cr deficient (Burton, 1995). In the 1990s, researchers began evaluating effects of supplemental Cr in farm animals. In

particular, several studies investigated effects of supplementing organic Cr sources (such as Cr picolinate, Cr methionine, or Cr propionate) which are 10 to 30 times more bioavailable than inorganic sources (Forbes and Erdman, 1983). In accordance with its proposed mode of action, supplemental Cr improved whole-body insulin sensitivity in monogastrics and ruminants (see Table 1.3). In addition, dietary Cr supplementation increases milk production in lactating cows (Hayirli et al., 2001; Smith et al., 2005; Sadri et al., 2009; Soltan, 2010) and positively influences production traits in pigs (Page et al., 1993; Liu et al., 2017; Mayorga et al., 2019). Several studies have also shown that Cr may have immunomodulatory effects (see Table 1.3). Although not always consistent (Chang et al., 1996; Kegley et al., 1997), Cr supplementation increases mitogen-stimulated lymphocyte blastogenesis and antibody production (Burton et al., 1993; Moonsie-Shageer and Mowat, 1993; Chang et al., 1994), improves macrophage phagocytosis (Lee et al., 2000), and alters cytokine production in vivo and in vitro (Burton et al., 1996; Burdick et al., 2012; Yuan et al., 2014). Additionally, Cr increases neutrophil number in circulation (Kafilzadeh et al., 2012; Mayorga et al., 2019) and within tissue (Yasui et al., 2014). Effects of Cr on immune function have been attributed to its counteractive effect on immunosuppressive glucocorticoids such as cortisol (Kafilzadeh et al., 2012). Although, another possible mechanism may be a Cr-mediated increase in leukocyte insulin sensitivity, which improves leukocyte glucose uptake and function (see Immunometabolism section). It is of interest to further evaluate effects of Cr supplementation on leukocyte metabolism. In 2009, the U.S. Food and Drug Administration Center for Veterinary Medicine permitted the use of Cr propionate as a supplemental Cr source in cattle diets. Chromium propionate remains the only source permitted for use in ruminant diets. However, research continue to be generated evaluating various Cr sources.

Table 1.3. Effects of chromium supplementation on insulin sensitivity, stress, and immunity

Variable	Form of Cr	Species	Response	Reference
Whole-body Insulin Sensitivity	CrMet	Bovine	↑	1, 2,3
		Pigs	↑	15
	CrPic	Bovine	↑/=	4, 5, 17/6, 12
		Pigs	↑	13, 14
		Bovine	↑	16, 27
Cortisol	CrMet	Bovine	=/↓	25,35/27
	CrYeast	Bovine	↓	11,33
		Pigs	↓	30
	Chelated Cr	Bovine	↓	28
	CrPic	Pigs	=	29
	CrProp	Bovine	=	24
	Cr Nicotinic Acid	Bovine	=	32
Cytokine Production	CrPic	Pigs	↑/↓	8/9
		Bovine	↑	10
	CrYeast	Pigs	↓	30
	CrProp	Bovine	↑	12, 24
	Chelated Cr	Bovine	↓	19
Phagocytosis	Chelated Cr	Bovine	=	22
	CrPic	Pigs	↑	31
Neutrophil Number	CrProp	Pigs	↑	23
		Bovine	↑	26
	CrMet	Bovine	↑	25
	Chealted Cr	Bovine	=	34
Antibody Response	Chelated Cr	Bovine	↑	18,34
	CrYeast	Bovine	↑	11,33
	Cr Nicotonic Acid	Bovine	↑/=	34/32
Blastogenesis	Chelated Cr	Bovine	↑	18, 20, 21, 27
¹ Hayirli et al., 2001	¹⁰ Zhang et al., 2014	¹⁹ Burton et al., 1996	²⁸ Mowat et al., 1993	
² Kegley et al., 2000	¹¹ Moonsie-Shageer and Mowat, 1993	²⁰ Chang et al., 1994	²⁹ Kim et al., 2010	
³ Habibi et al., 2019	¹² Yuan et al., 2014	²¹ Burton et al., 1995	³⁰ Song et al., 2014	
⁴ Spears et al., 2012	¹³ Amoikon et al., 1995	²² Chang et al., 1996	³¹ Lee et al., 2000	
⁵ Leiva et al., 2018	¹⁴ Liu et al., 2017	²³ Mayorga et al., 2019	³² Kegley et al., 1997	
⁶ Kneeskern et al., 2016	¹⁵ Mathews et al., 2001	²⁴ Burdick et al., 2012	³³ Chang and Mowat, 1992	
⁷ Leiva et al., 2017	¹⁶ Bunting et al., 1994	²⁵ Kafilzadeh et al., 2012	³⁴ Faldyna et al., 2003	
⁸ Myers et al., 1995	¹⁷ Sumner et al., 2007	²⁶ Yasui et al., 2014	³⁵ Nikkhah et al., 2011	
⁹ Mvers et al., 1997	¹⁸ Burton et al., 1993	²⁷ Soltan, 2010		

Zinc

Zinc is a trace element which is involved in a number of important biological processes and is indispensable to life. It serves as a catalytic cofactor, has structural functions in a number of proteins, and modulates signaling events (Stefanidou et al., 2006; Kambe et al., 2015).

Although Zn is involved in numerous physiological processes, it has received considerable attention for its regulatory role in immune function and epithelial barrier integrity (Wessels and Cousins, 2015; Ohashi and Fukada, 2019). For example, leukocyte formation, maturation and

differentiation, activation, cellular function, and apoptotic rate (among others; Haase and Rink, 2014; Wessels and Cousins, 2015) are Zn dependent and are impaired during Zn deficiency. Furthermore, Zn regulates epithelial barrier integrity by increasing cell proliferation (Shao et al., 2017), turnover and repair (Cario et al., 2000), and abundance of tight junction protein complexes (Finamore et al., 2008; Miyoshi et al., 2016; Sarkar et al., 2019). Zinc deficiency impairs barrier function (Finamore et al., 2008; Zhong et al., 2010; Miyoshi et al., 2016), whereas supplementing Zn improves intestinal integrity across species and experimental models (Rodriguez et al., 1996; Sturniolo et al., 2001; Zhang and Guo, 2009; Bückner et al., 2020). Due to its importance to physiological functions, Zn homeostasis is tightly regulated by the gastrointestinal system via absorption of dietary Zn and secretion/excretion of endogenous Zn into the gut lumen (Krebs, 2000).

In animal diets, Zn can be supplemented in various forms including; Zn sulfate, Zn methionine, Zn amino acid complex, or Zn hydroxychloride, and the latter three are considered more bioavailable. Our group has previously demonstrated improved intestinal integrity with partial replacement of Zn sulfate with Zn amino acid complex in heat-stressed pigs (Sanz-Fernandez et al., 2014; Pearce et al., 2015) and steers (Abuajamieh et al., 2016b). For example, Pearce et al. (2015) observed increased epithelial resistance, decreased circulating LPS, and increased LBP in heat-stressed pigs fed Zn amino acid complex. Similarly, Abuajamieh et al. (2016b) reported Zn supplementation improved villi architecture and reduced the febrile response in chronically heat-stressed steers. Although barrier integrity was not measured, Mayorga et al. (2018) observed altered inflammatory cytokine production with Zn amino acid complex supplementation in heat-stressed pigs. Effects of Zn source on epithelial barrier integrity metrics in lactating cows is scarce. Shaffer et al. (2019) reported no differences in

mammary epithelial integrity between cows fed Zn sulfate and cows fed Zn methionine. However, Weng et al. (2018) observed improved mammary integrity metrics when Zn hydroxychloride was partially replaced with Zn methionine. Further investigation into how Zn effects both intestinal and mammary epithelial integrity is warranted. From a ruminant perspective, enhanced bioavailability of improved Zn sources is ostensibly explained by reduced ruminal solubility (when compared to inorganic sulfates) and thus decreased formation of insoluble complexes between Zn and feed constituents, ruminal metabolites, and microorganisms (Shaeffer, 2017; Caldera et al., 2019), thereby increasing Zn delivery throughout the intestinal tract. Altogether, strong evidence suggests supplementation of more bioavailable Zn sources is a promising strategy to improve animal health, especially during periods of stress which may negatively impact barrier function.

Conclusion

Compromised intestinal, uterine, and/or mammary epithelial integrity during the transition period may permit LPS translocation into portal and systemic circulation. Leukocyte recognition of infiltrating PAMPs results in immune activation and systemic inflammation. This has obvious implications to animal agriculture, as supporting the immune response drives nutrients away from economically productive processes. Furthermore, immune activation and inflammation impact hepatic lipid handling and calcium homeostasis. These alterations have practical relevance to the transition cow, as LPS exposure is often inevitable. Therefore, objectives of this dissertation were to gain a better understanding of how immune activation alters nutrient partitioning and Ca homeostasis as well as to identify potential dietary strategies which may help to alleviate the negative consequences of immune activation.

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CHAPTER 2. EVALUATING EFFECTS OF ZINC HYDROXYCHLORIDE ON BIOMARKERS OF INFLAMMATION AND INTESTINAL INTEGRITY FEED RESTRICTION

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Abstract

Objectives were to evaluate effects of supplemental zinc (Zn) hydroxychloride (HYD; Micronutrients, Indianapolis, IN) on intestinal permeability, metabolism, and inflammation during feed restriction (FR). Holstein cows ($n = 24$; 159 ± 8 d in milk; parity 3 ± 0.2) were enrolled in a 2×2 factorial design and randomly assigned to 1 of 4 treatments: 1) ad libitum-fed (AL) and control diet (ALCON; 75 mg/kg Zn from Zn sulfate; $n=6$), 2) ad libitum-fed and HYD diet (ALHYD; 75 mg/kg Zn from HYD; $n=6$), 3) 40% of ad libitum feed intake and control diet (FRCON; $n=6$), or 4) 40% of ad libitum feed intake and HYD diet (FRHYD; $n=6$). Prior to study initiation, cows were fed their respective diets for 21 d. The trial consisted of 2 experimental periods (P) during which cows continued to receive their respective dietary treatments. Period 1 (5 d) served as the baseline for P2 (5 d), during which cows were fed ad libitum or restricted to 40% of P1 feed intake. In vivo intestinal integrity was evaluated on d 4 of P1 and d 2 and 5 of P2 using the paracellular permeability marker chromium (Cr)-EDTA. All cows were euthanized at the end of P2 to assess intestinal architecture. As anticipated, FR cows lost body weight (~ 46 kg), entered into calculated negative energy balance (-13.86 Mcal/d), and had decreased milk

yield. Circulating glucose, insulin, and glucagon decreased and nonesterified fatty acids and β -hydroxybutyrate increased in FR relative to AL cows. Relative to AL cows, FR increased lipopolysaccharide-binding protein, serum amyloid A (SAA), and haptoglobin (Hp) concentrations (2-, 4- and 17-fold, respectively); and peak SAA and Hp concentrations were observed on d 5. Circulating SAA and Hp from FRHYD tended to be decreased (47 and 61%, respectively) on d 5 relative to FRCON. Plasma Cr AUC increased (32%) in FR treatments on d 2 and tended to be increased (17%) on d 5 of P2 relative to AL-fed treatments. No effects of diet were observed on Cr appearance. Relative to AL cows, FR increased jejunum villus width and decreased jejunum crypt depth and ileum villus height and crypt depth. Relative to FRCON, ileum villus height tended to increase in FRHYD cows. Feed restriction tended to decrease jejunum and ileum mucosal surface area, but the decrease in the ileum was rescued by dietary HYD. In summary, FR induced intestinal hyperpermeability and feeding HYD appeared to benefit some key metrics of barrier integrity.

Keywords: inflammation, leaky gut, Cr-EDTA

Introduction

Livestock are often challenged with transient periods of insufficient feed intake, which jeopardize animal performance and profitability. Recent ruminant literature demonstrated deleterious effects of feed restriction (**FR**) on intestinal barrier integrity (Gäbel and Aschenbach, 2002; Zhang et al., 2013; Kvidera et al., 2017b); a phenomenon which had previously been well-characterized in avian (Yamauchi et al., 1996; Gilani et al., 2017) and monogastric species (Rodriguez et al., 1996; Boza et al., 1999; Pearce et al., 2013). Feed restriction negatively impacts intestinal architecture as a consequence of decreased epithelial cell proliferation and migration and increased apoptosis (Ferraris and Carey, 2000). Furthermore, physical and chemical defense mechanisms (i.e., mucus production, tight junction proteins, and Paneth cell

function) are compromised during FR (Thompson and Applegate, 2006; Hodin et al., 2011; Kvidera et al., 2017a). Mechanistically, what triggers the alterations in barrier defense strategies remains poorly understood, however, convincing evidence suggests a neuroendocrine role (Horn et al., 2014; Najafi et al., 2017). Regardless of the mechanism, alterations in physical and chemical defenses contribute to the loss of barrier function, which increases the risk of pathogen entry into portal, lymph, and systemic circulation (Deitch et al., 1990; Kvidera et al., 2017b). Leukocyte recognition of the infiltrating pathogens initiates a cascade of events culminating in immune activation and inflammation; a process which is energetically demanding (Johnson, 2012; Kvidera et al., 2017c; Horst et al., 2018, 2019). Fueling the energetic requirement of the immune system markedly disrupts the hierarchy of nutrient partitioning away from profitable functions. Therefore, dietary strategies which may strengthen the epithelial barrier and reduce the risk of immune activation are currently of great interest.

Zinc (**Zn**) is an essential trace mineral which plays a role in variety of biological processes including immune regulation and epithelial barrier defense (Wessels et al., 2017; Ohashi and Fukada, 2019). Effects of Zn on gut integrity are conserved across species and experimental models of intestinal barrier dysfunction (Rodriguez et al., 1996; Sturniolo et al., 2001; Zhang and Guo, 2009). Zinc increases cell proliferation (Shao et al., 2017), turnover and repair (Cario et al., 2000) and increases tight junction protein complexes (Finamore et al., 2008; Sarkar et al., 2019). Dietary Zn's efficacy is generally more evident when improved sources with better stability and bioavailability relative to zinc sulfate are used (Pearce et al., 2015; Abuajamieh et al., 2016; Horst et al., 2019). Enhanced bioavailability with hydroxychlorides is ostensibly explained by reduced ruminal solubility (when compared to inorganic sulfates) and thus decreased formation of insoluble complexes between Zn and feed constituents, ruminal

metabolites, and microorganisms (Shaeffer, 2017; Caldera et al., 2019); a scenario that increases Zn delivery throughout the intestinal tract. Therefore, we hypothesized that feeding Zn hydroxychloride would strengthen epithelial barrier integrity in response to FR and alter the pattern of inflammatory biomarkers. Thus, experimental objectives were to evaluate the effects of replacing Zn sulfate with Zn-hydroxychloride on intestinal architecture, paracellular permeability, and the acute phase protein response following 5 d of FR in lactating Holstein cows.

Materials and Methods

Animals and Experimental Design

All procedures were approved by the Iowa State University Institutional Animal Care and Use Committee. Twenty-four lactating Holstein cows (685 ± 9 kg; 159 ± 8 DIM; parity 3 ± 0.2) were utilized in an experiment conducted in two replications (12 cows/replicate). Cows were randomly assigned to 1 of 2 dietary treatments: 1) control (75 mg/kg supplemental Zn from Zn sulfate) or 2) Zn hydroxychloride (75 mg/kg supplemental Zn from Zn HYD; Micronutrients USA LLC, Indianapolis, IN). Prior to the start of the study (i.e., before cows were moved into individual pens), cows were fed their respective diets for 21 d. After the initial feeding phase, cows were moved to individual box-stalls (4.57×4.57 m) at the Iowa State University Dairy Farm. Cows were allowed 3 d to acclimate to housing conditions during which they were implanted with jugular catheters. The trial consisted of two experimental periods. Period 1 (P1) lasted 5 d and served as the baseline, which yielded data for covariate analysis. Period 2 (P2) lasted 5 d during which cows were assigned to 1 of 2 treatments: 1) 100% of ad libitum feed intake (**AL**; $n = 12$) or 2) 40% of ad libitum feed intake (**FR**; $n = 12$). Dietary and FR combinations resulted in 4 total treatments: 1) ad libitum-fed and control diet (**ALCON**; $n = 6$), 2) ad libitum-fed and HYD diet (**ALHYD**; $n = 6$), 3) 40% of ad libitum feed intake and control

diet (**FRCON**; n = 6), or 4) 40% of ad libitum feed intake and HYD diet (**FRHYD**; n = 6). One cow from the ALCON treatment was removed from the experiment due to health issues and her data was not included in the final dataset.

Throughout the experiment, all cows were fed a TMR formulated to meet or exceed the predicted requirements for energy, protein, minerals, and vitamins (NRC, 2001). Samples of TMR were obtained daily and composited into weekly samples for nutrient analysis (Dairyland Laboratories Inc., Arcadia, WI; Table 2.1). Trace mineral analysis was conducted on the same weekly composited TMR samples as well as the premix and vitamin trace mineral mix at preparation of each batch (SDK Laboratories, Inc., Hutchinson, KS; Table 2.1 and 2.2). Daily feed intake during P2 was determined as a percentage (40%) of each cow's mean daily ad libitum intake during P1. Feed was provided once daily during P1 for all cows (0800 h), once daily during P2 for AL treatments (0800 h), and divided into three equal portions during P2 for FR treatments (0800, 1300, 1800 h). Drinking water samples were obtained at the beginning of the pre-feeding phase and at the end of P2 for analysis of trace mineral content and hardness (SDK Laboratories, Inc., Hutchinson, KS; Table 2.3). Energy balance (EBAL) was calculated using the following equations: $EBAL = \text{energy intake} - \text{energy output}$, where $\text{energy intake} = 1.6 \text{ Mcal/kg} \times \text{DMI}$ and $\text{energy output} = (NE_M = 0.08 \text{ Mcal/kg} \times BW^{0.75}) + [NE_L = \text{milk yield} \times (0.0929 \times \text{fat \%} + 0.0547 \times \text{protein \%} + 0.0395 \times \text{lactose \%})]$. Body weights and body condition scores (BCS) were obtained on d 1 of acclimation and on d 5 of P2 to calculate BW and BCS change.

Cows were milked twice daily (0600 and 1800 h) and yield was recorded. A sample for composition analysis was obtained at each milking. Samples were stored at 4°C with a preservative (bronopol tablet; D & F Control System, San Ramon, CA) until analysis by Dairy

Lab Services (Dubuque, IA) using AOAC approved infrared analysis equipment and procedures (AOAC International, 1995). Rectal temperature (**Tr**) and respiration rate (**RR**) were recorded after each milking. Respiration rate was measured as flank movements during a 15 s interval and later transformed to breaths/min (**bpm**). Rectal temperature was measured using a digital thermometer (GLA M700 Digital Thermometer, San Luis Obispo, CA).

Blood samples for metabolite and inflammatory biomarker analysis were obtained on d 3 and 5 of P1 and d 1, 3, and 5 of P2 following the AM milking. Samples were collected from the catheter and divided equally between a tube containing K₂EDTA (BD, Franklin Lakes, MJ; for plasma collection) and an empty glass tube (for serum collection). Samples for glucagon analysis (3 mL) were collected from the catheter into K₂EDTA vacutainers containing 150 µL of Aprotinin (BP2503-10; Fisher BioReagents, Fair Lawn, NJ). Serum samples were allowed to clot at room temperature for 1 h prior to centrifugation. Plasma and serum were harvested following centrifugation at $1,500 \times g$ for 15 min at 4°C and were subsequently frozen at -20°C until analysis. Samples for complete blood count analysis were collected on d 3 and 5 of P1 and twice daily during P2 following milking. A 3 mL blood sample was collected from the catheter (K₂EDTA; BD Franklin Lakes, NJ) and stored at 4°C for ~12 h before submitting to the Iowa State University's Department of Veterinary Pathology for analysis.

Laboratory Analysis

Serum cortisol and plasma insulin, non-esterified fatty acids (**NEFA**), BHB, blood urea nitrogen (**BUN**), glucose, glucagon, lipopolysaccharide (**LPS**)-binding protein (**LBP**), serum amyloid A (**SAA**), haptoglobin (**Hp**), and L-lactate concentrations were determined using commercially available kits according to manufacturers' instructions (cortisol, Enzo Life Sciences, Farmingdale, NY; insulin, Mercodia AB, Uppsala, Sweden; NEFA, Wako Chemicals USA, Richmond, VA; BHB, Pointe Scientific Inc., Canton, MI; BUN, Teco Diagnostics

Anaheim, CA; glucose, Wako Chemicals USA Inc., Richmond, VA; glucagon, RD Systems, Inc., Minneapolis, MN; LBP, Hycult Biotech, Uden, the Netherlands; SAA, Tridelata Development Ltd., Kildare, Ireland; Hp, Life Diagnostics, Inc., West Chester, PA; and L-lactate, Biomedical Research Service Center, Buffalo, NY). The inter- and intra-assay coefficients of variation for insulin, NEFA, BHB, BUN, glucose, glucagon, cortisol, LBP, SAA, Hp, and L-lactate, were 2.2 and 6.5%, 5.6 and 3.5%, 4.9 and 12.6%, 6.7 and 5.1%, 4.3 and 4.7%, 8.4 and 6.7%, 14.1 and 6.2%, 6.7 and 6.5%, 10.6 and 4.9%, 8.7 and 6.6%, and 9.3 and 11.6%, respectively.

Neutrophil Isolation and Functional Analysis

Blood samples (32 mL) for neutrophil isolation were collected on d 3 of P1 and on d 3 and 5 of P2 into 50 mL conical tubes containing acid citrate dextrose (8 mL). Samples were immediately transported to the laboratory for neutrophil isolation and functional analysis as previously described (Kimura et al., 2014; Horst et al., 2019). Neutrophil function was assessed by oxidative burst (cytochrome C reduction) and extracellular release of myeloperoxidase (MPO). Three cell preparations were used to assess MPO activity: 1) cells were lysed by treatment with cetyltrimethylammonium bromide solution as a measure of total MPO, 2) PMN were stimulated with equal parts Ca ionophore A23187 and cytochalasin B in HBSS to assess release of MPO with stimulation, and 3) PMN treated with HBSS alone as a measure of unstimulated MPO release.

$$\text{Exocytosis (\%)} = [(\text{OD of stimulated PMN})/(\text{OD of lysed PMN})] \times 100$$

In vivo Intestinal Tract Barrier Function

Intestinal barrier function was evaluated in vivo using the paracellular permeability marker chromium (Cr)-EDTA as previously described (Wood et al., 2015). Beginning at 0700 h (after milking, before feeding) on d 4 of P1 and d 2 and 5 of P2, a 180 mM solution of Cr-EDTA

(1.5 L) was pulse dosed into the rumen. Blood samples were collected at 0, 1, 2, 4, 8, 12, 18, and 24 h relative to Cr-EDTA administration into a tube containing K₂EDTA (BD, Franklin Lakes, Reference#368381, NJ; plasma for trace element analysis). Plasma was harvested following centrifugation at $1,500 \times g$ for 15 min at 4°C and subsequently frozen at -20°C until analysis. Samples were submitted to Brooks Applied Labs (Bothell, WA) for analysis of total Cr using inductively coupled plasma triple quadrupole mass spectrometry (ICP-QQQ-MS; Agilent 8900).

Tissue Collection

At the end of P2, all cows were transported to the Iowa State Livestock Infectious Disease Isolation Facility and euthanized with a CASH Special captive bolt gun (Accles & Shelvoke Ltd., Sutton Coldfield, West Midlands) using a large animal charge followed by exsanguination. Liver and intestinal tissues (duodenum, jejunum, ileum, and colon) were harvested immediately following euthanasia. Liver was obtained from the right lobe and was snap-frozen in liquid nitrogen and stored at -80°C until later analysis. Intestinal samples were obtained as follows: duodenum was collected 20 cm distal to the pyloric sphincter, jejunum was collected 1 m proximal to the ileocecal junction, ileum was collected 18 cm proximal to the ileocecal junction, and colon was collected 50 cm proximal to the rectum. All intestinal samples were flushed with saline in an attempt to remove any intestinal content and a 4 cm section from the middle of the segment was collected and fixed in 10% neutral buffered formalin for later histological analysis as previously described (Kvidera et al., 2017a,b).

Liver Analysis

Hepatic triglyceride (TG) content was measured as previously described (Morey et al., 2011). A ~20 mg liver sample was weighed and homogenized with 500 µL chilled PBS. The homogenate was then centrifuged at $8,000 \times g$ for 2 min at 4°C. Free glycerol was immediately determined using 10 µL of supernatant via enzymatic glycerol phosphate oxidase method

(Sigma-Aldrich, St. Louis, MO). An additional 300 μ L of supernatant was removed and incubated with 75 μ L lipase (MP Biomedicals, Solon, OH) at 37°C for 16 h before determining total glycerol using the same method. Free glycerol (before lipase digestion) was subtracted from total glycerol (after lipase digestion) in order to determine TG content, and this was expressed as a percentage of wet weight of the original sample. The intra-assay coefficients of variation for free glycerol and total glycerol were 9.4 and 2.0%, respectively.

Histological Analysis

For histological analysis, 10% neutral buffered formalin-fixed duodenum, jejunum, ileum, and colon were submitted to the Iowa State University Veterinary Comparative Pathology Core for sectioning and staining. Hematoxylin and eosin (**H&E**) staining was used for morphology, periodic acid-Schiff (**PAS**) for goblet cell area (**GCA**) quantification, and May-Grunwald Giemsa for mast cell quantification. One slide per cow per tissue was generated. Using a microscope (Leica DMI3000 B Inverted Microscope, Bannockburn, IL) with an attached camera (QImaging 12-bit QICAM Fast 1394, Surrey, BC), 5 images per intestinal section were obtained at 5 \times magnification for the H&E and PAS stains and at 40 \times magnification for the Giemsa stain. All image processing and quantification was done using ImageJ 1.48v (National Institutes of Health, USA). Goblet cell area, villus morphology, and mucosal surface area were quantified using previously described methods (Kvidera et al., 2017a,b). Mast cells were counted in a grid fashion using ImageJ software. Cells falling within a single grid (20,000 micrometer²) on each image were counted and later summed. The cell number was then divided by the area to obtain cells per micrometer squared and multiplied by 1×10^6 to express values as cells per millimeter squared of tissue area.

Statistical Analyses

The sample size calculation was determined using PROC Power and was based on the effects of FR on inflammation using 80% power and a 95% confidence level. The sample size of 12 cows per group was based on a detection of difference of 363 ± 299 $\mu\text{g/mL}$ (mean \pm SD) in plasma SAA (Kvidera et al., 2017a). Data were analyzed using SAS version 9.4. For production and postmortem parameters (DMI, energy balance, BW, BCS, milk yield, energy corrected milk, feed efficiency, milk composition, rectal temperature, respiration rate, intestinal histology) effects of group (AL or FR), diet (CON or HYD), and their interaction were assessed using PROC MIXED and cow was included as a random effect. For blood parameters, a repeated measures analysis with an autoregressive covariance structure and time as the repeated effect was used to determine effects of time (d or h), group, diet, and their interactions. Each specific variable's P1 value was used as a covariate. Data are reported as least squares means and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.15$.

Results

Production

By design, DMI was decreased (60%) in FR relative to AL-fed cows during P2 ($P < 0.01$; Table 2.4) and no effect of diet was observed ($P > 0.85$). As anticipated, FR cows lost BW (~46 kg) and entered into calculated negative energy balance (-13.86 Mcal/d) during P2 ($P < 0.01$; Table 2.4). Dietary HYD improved EBAL similarly in both groups (AL and FR) when compared to CON cows ($P = 0.05$; Table 2.4). No effects of group or diet were observed for BCS (Table 2.4). Regardless of Zn source, FR decreased milk yield (33%) during P2 relative to AL-fed cows ($P < 0.01$; Table 2.4). No main effects of diet were observed on milk yield ($P > 0.37$). Similarly, FR decreased ECM (31%) during P2 when compared to AL-fed cows ($P < 0.01$). Regardless of group, HYD supplementation tended to decrease ECM (6%) relative to CON ($P = 0.11$; Table

2.4). Feed efficiency increased (76%) in FR relative to AL-fed cows ($P < 0.01$), although no dietary effect was detected ($P > 0.37$; Table 2.4).

Overall, milk lactose content decreased (2%) in FR relative to AL-fed cows ($P < 0.01$; Table 2.4). Milk lactose tended to decrease (1%) in ALHYD relative to ALCON, whereas it was similar between FRCON and FRHYD cows ($P = 0.12$; Table 2.4). Milk fat percentage increased (12%) and yield decreased (26%) in FR relative to AL-fed cows ($P < 0.01$). Regardless of feed intake, HYD supplementation decreased milk fat percentage (5%; $P = 0.05$) and tended to decrease milk fat yield (6%; $P = 0.12$; Table 2.4). Relative to AL cows, FR decreased milk protein content and protein yield during P2 (5 and 37%, respectively; $P < 0.01$; Table 2.4), but no main effects of diet were observed ($P \geq 0.23$). Overall, MUN increased (5%) in FR relative to AL-fed cows ($P < 0.01$), but no dietary effect was observed ($P > 0.90$; Table 2.4). Feed restriction tended to increase somatic cell score relative to AL cows during P2 (38%; $P = 0.08$; Table 2.4). No main effects of diet were observed for these parameters ($P > 0.42$). Rectal temperature was similar across diets in AL-fed groups, however, during FR it decreased (0.1°C) in HYD-supplemented cows relative to CON ($P = 0.02$; Table 2.4). Respiration rate decreased (8 bpm) in FR cows relative to AL-fed cows ($P < 0.01$; Table 2.4). Regardless of feed intake (AL or FR), HYD-supplementation tended to decrease respiration rate (3 bpm; $P = 0.11$).

Metabolism

Glucose and insulin concentrations from FR cows were consistently decreased (8 and 57%, respectively) throughout P2 relative to AL-fed cows ($P \leq 0.05$; Figure 2.1A and B). Feed restriction decreased circulating glucagon (31%; $P < 0.01$; Figure 2.1C) and markedly increased NEFA relative to AL-fed cows ($P = 0.03$; Figure 2.1D). Circulating BHB from FR cows increased (37%) on d 3 and 5 of P2, whereas in AL-fed cows it did not change from P1 ($P = 0.09$; Figure 2.1E). Blood urea nitrogen concentrations initially increased (19% on d 1) and then

steadily declined throughout P2 in FR relative to AL-fed cows ($P < 0.01$; Figure 2.1F). No main effects of diet or interactions between group and diet were observed in any of the aforementioned blood metabolites and hormones ($P \geq 0.24$).

Inflammation

Although circulating insulin was decreased with FR, no differences were observed in the insulin-to-DMI ratio ($P > 0.85$; Table 2.5). Overall, circulating LBP was increased in FR relative to AL-fed cows (63%; $P = 0.02$) and tended to be increased in HYD relative to CON cows (38%; $P = 0.10$) throughout P2. Circulating LBP increased (94%) in FRHYD cows on d 1 and 3 of P2 whereas concentrations from all other treatments remained similar ($P = 0.14$; Figure 2.2A). Serum amyloid A concentrations from FR cows increased progressively throughout P2 ($P = 0.07$) and were increased 4-fold overall in FR vs. AL cows ($P = 0.04$). There was a tendency for a diet by day interaction for SAA such that it increased in CON cows from d 3 to 5 of P2 whereas it decreased in HYD cows ($P = 0.14$) and this effect was more pronounced in the FR cows (Figure 2.2B). Overall, Hp concentrations tended to increase 17-fold in FR relative to AL-fed cows ($P = 0.08$) and the increase was largely driven by differences on d 5 of P2 (Figure 2.2C). Dietary HYD tended to decrease Hp concentrations in FR cows on d 5 of P2 relative to CON, however, no dietary effects were observed in AL-fed cows at the end of P2 ($P = 0.10$; Figure 2.2C). Regardless of group, circulating L-lactate tended to decrease (18%) in HYD-supplemented cows ($P = 0.12$; Figure 2.2D). No effects of group or diet were observed for circulating cortisol during P2 ($P > 0.43$; Figure 2.2E). Feed restriction increased liver TG content 2-fold at the end of P2 relative to AL-fed cows ($P = 0.04$; Table 2.4), however, no main effects of diet ($P > 0.56$) nor an interaction were observed.

White blood cell counts tended to increase throughout P2 in HYD supplemented cows (regardless of group) compared to CON (6%; $P = 0.10$; Table 2.5). Circulating neutrophil counts

from FRCON cows decreased (25%) relative to ALCON, but counts did not differ between AL and FR groups fed dietary HYD ($P = 0.05$; Table 2.5). No effects of group, diet, or their interaction were observed on neutrophil oxidative burst ($P \geq 0.27$; Figure 2.3A) or neutrophil total MPO activity ($P \geq 0.23$; Figure 2.3B). However, oxidative burst tended to be decreased in FRCON vs. ALCON cows on d 5 (16%; $P = 0.15$) and was numerically increased (19%) in FRHYD relative to FRCON cows on d 5 ($P > 0.17$). Myeloperoxidase activity following in vitro neutrophil stimulation did not differ across dietary treatments during AL-fed conditions, however, during FR, HYD supplementation increased stimulated MPO release (24%; $P = 0.01$; Figure 2.3C). On d 5, stimulated MPO release tended to decrease in FRCON relative to ALCON cows (27%; $P = 0.07$). Myeloperoxidase exocytosis tended to decrease in HYD supplemented cows regardless of group ($P = 0.15$; Figure 2.3D).

Relative to AL cows, circulating platelets decreased (14%) in both dietary treatments during feed restriction ($P = 0.03$; Table 2.5). No effects of group or diet were detected for circulating monocytes ($P \geq 0.39$; Table 2.5). Lymphocytes were similar in ALCON and FRCON cows, but increased (7%) in FRHYD relative to ALHYD cows ($P = 0.02$, Table 2.5). There was a tendency for eosinophil counts to decrease (21%) in FRHYD relative to FRCON cows, but there were no dietary treatment differences in AL-fed cows ($P = 0.11$; Table 2.5). Overall, HYD supplementation tended to increase basophil counts (17%) during P2 ($P = 0.10$), but no differences were detected across groups ($P > 0.67$; Table 2.5). Feed restriction and HYD supplementation increased hemoglobin and hematocrit throughout P2 ($P \leq 0.01$; Table 2.5), and the greatest differences were detected from d 1.5 to 5 ($P \leq 0.01$).

Intestinal Integrity

Plasma Cr AUC increased (32%) in FR treatments on d 2 ($P = 0.01$; Figure 2.4A) and tended to be increased (17%) on d 5 of P2 relative to AL-fed treatments ($P = 0.09$; Figure 2.4B).

No effects of diet were observed on Cr AUC. No effects of group or diet were observed on duodenum villus height, width, crypt depth, or villus height-to-crypt depth ratio at the end of P2 ($P > 0.32$; Figure 2.5A). Feed restriction increased jejunum villus width and decreased crypt depth ($P = 0.02$; Figure 2.5B), but no differences were detected for jejunum villus height or the villus height-to-crypt depth ratio ($P \geq 0.32$). Feed restriction tended to decrease ileum villus height and crypt depth relative to AL-fed cows ($P \leq 0.14$; Figure 2.5C). Ileum villus height was similar across diets during AL conditions, but tended to be increased in FRHYD cows relative to FRCON ($P = 0.09$). No treatment differences were observed for colon crypt depth ($P > 0.34$; data not shown). No effects of group or diet were observed on duodenum mucosal surface area ($P > 0.50$; Figure 2.5D). Overall, the jejunum mucosal surface area tended to be decreased in FR relative to AL cows ($P = 0.15$; Figure 2.5E). Ileum mucosal surface area tended to decrease in FRCON relative to ALCON cows, but was similar in ALHYD and FRHYD treatments ($P = 0.12$; Figure 2.5F). Duodenum GCA was similar in ALCON and FRCON cows, however, in HYD supplemented animals FR increased GCA ($P = 0.03$; Table 2.6). No effects of group or diet were observed for jejunum or ileum GCA ($P \geq 0.17$; Table 2.6). Feed restriction decreased colonic GCA (40%) at the end of P2 ($P = 0.02$; Table 2.6). Feed restriction increased mast cell number similarly across diets within the duodenum ($P = 0.02$). No effects of group or diet were observed for the quantity of mast cells in the jejunum, ileum, or colon (Table 2.6).

Discussion

Weaning (Moeser et al., 2007), heat stress (Baumgard and Rhoads, 2013; Pearce et al., 2015), rumen acidosis (Emmanuel et al., 2007; Khafipour et al., 2009), and the periparturient period (Bertoni et al., 2008; Trevisi et al., 2012; Abuajamieh et al., 2016) are just a few of the stressors production animals frequently encounter which are characterized by gut barrier dysfunction, inflammation, hypophagia and compromised productivity. The short-term FR which

accompanies these situations independently affects intestinal permeability and pathogen translocation (Zhang et al., 2013; Pearce et al., 2015; Kvidera et al., 2017a,b). Feed restriction decreases intestinal cell number, proliferation, and migration rates while simultaneously increasing cell loss and apoptosis (Bayer et al., 1981; Goodlad et al., 1988; Ferraris and Carey, 2000). Consequently, villus height and mucosal surface area are reduced and ultimately intestinal barrier function is compromised (Mayhew, 1990; Ferraris and Carey, 2000). Loss of barrier integrity permits LPS infiltration (as well as other noxious substances) into circulation and consequently initiates a systemic immune response (Pearce et al., 2013; Kvidera et al., 2017b). When activated, most leukocytes switch their metabolism from oxidative phosphorylation to aerobic glycolysis and begin utilizing copious amounts of glucose (Palsson-McDermott and O'Neill, 2013). As a means of supporting the increased glucose demand, several marked alterations in nutrient partitioning are employed, which ultimately deemphasizes productive processes (i.e., milk and meat synthesis; Waldron et al., 2003; Kvidera et al., 2017c; Horst et al., 2018, 2019). Dietary mitigation strategies aimed at enhancing barrier integrity have the potential to improve animal performance.

Zinc is an essential micronutrient crucial for maintaining mammary (Weng et al., 2018), lung (Bao and Knoell, 2006), and intestinal epithelial integrity (Sanz-Fernandez et al., 2014; Abuajamieh et al., 2016; Miyoshi et al., 2016). Zinc ameliorates gut permeability in a number of leaky-gut models (Rodriguez et al., 1996; Lambert et al., 2003; Weng et al., 2018) and plays an essential role in cellular processes such as metabolism, inflammation, and oxidative stress (as reviewed by Olechnowicz et al., 2018). The efficacy of Zn is improved when more bioavailable sources are provided such as amino acid complexes and hydroxychlorides (Sanz-Fernandez et al., 2014; Pearce et al., 2015; Abuajamieh et al., 2016; Horst et al., 2019). Therefore, we

hypothesized that replacing Zn sulfate with HYD would alleviate the impact of FR on intestinal permeability and modify the inflammatory response in lactating Holstein cows.

In the current experiment, FR cows lost significant BW (~46 kg), entered into calculated negative EBAL (-13.86 Mcal/d), and had reduced milk and component yields, indicating successful implementation of our experimental protocol. The decrease in production metrics was consistent with our previous report executing the same magnitude and duration of FR (Kvidera et al., 2017b) as well as others utilizing a comparable approach (Velez and Donkin, 2005; Carlson et al., 2006; Ferrareto et al., 2014). Dietary Zn source had no effect on DMI or milk yield in our study which agrees with past studies evaluating improved Zn sources (Weng et al., 2018; Horst et al., 2019), but contradicts others (Kellogg et al., 2004; Cope et al., 2009; Nayeri et al., 2014; Osorio et al., 2016). However, our experimental design (FR) prevented HYD from being able to influence DMI and thus other DMI-dependent production metrics in half of the groups (the FR treatments). Regardless production metrics were not the primary objective of our experiment.

Feed restriction induced well-characterized changes in metabolism including decreased circulating glucose and insulin, increased NEFA and BHB. These changes are consistent with previous FR studies in ruminants (Carlson et al., 2006; Ferraretto et al., 2014; Lérias et al., 2015; Kvidera et al., 2017b) and demonstrate the coordinated changes in nutrient partitioning animals implement to survive insufficient feed intake (McCue, 2010). Interestingly, despite the catabolic state, we observed decreased circulating glucagon (a potent stimulator of hepatic glucose output; Faulkner and Pollock, 1990) in FR relative to AL-fed groups. Hypoglucagonemia in response to FR has been reported in sheep (Carruthers et al., 1974), whereas others have observed no FR-induced change in glucagon concentrations (de Boer et al., 1985; Drackley et al., 1989, 1991). Unlike monogastrics, glucagon concentrations increase in response to a meal in ruminants as a

means of stimulating hepatic glucose production from ruminally-derived substrates (Mineo et al., 1994). Thus, the decrease in glucagon concentrations observed herein is likely a reflection of a fasted versus a fed state and species differences in how gluconeogenesis is controlled.

Although the negative consequences of FR on production and metabolism have been well-described in ruminant literature, its impact on intestinal tract barrier function has only recently received attention (Zhang et al., 2013; Kvidera et al., 2017a,b). Besides regulating digestion and nutrient uptake, the intestinal epithelium serves as a crucial barrier against bacteria (commensal and pathogenic) and toxins residing within the gut lumen. Two physical strategies imperative to gut barrier integrity are the mucus layer (produced by Paneth and goblet cells) and tight junction complexes (as reviewed by Steele et al., 2016). The mucus layer serves as a physical barrier to prevent pathogen penetration (Barreau and Hugot, 2014), whereas tight junction complexes allow for selective permeability (Mani et al., 2012). Interestingly, intestinal mucus production is decreased during insufficient feed intake (Núñez et al., 1996; Thompson and Applegate, 2006; Kvidera et al., 2017a), thereby permitting pathogen colonization at the epithelium. In accordance, we detected decreased colonic GCA in response to FR, but no changes were detected in the duodenum, jejunum or ileum. Reasons for not observing differences in small intestinal GCA are not clear and contradict our previous report implementing the same magnitude and duration of FR (Kvidera et al., 2017b). Even though we did not detect an overall effect of FR on duodenum GCA, it was increased in HYD cows during FR. Additionally, we observed a numerical increase in ileum GCA from HYD supplemented cows independent of feed intake. Increased GCA agrees with a previous report feeding high levels of Zn oxide in weaned pigs (Hedemann et al., 2006). The mode of action by which Zn

(especially Zn source) may alter GCA and mucus production remains incompletely understood and warrants further investigation as it likely has multiple implications to animal agriculture.

In agreement with changes in GCA, FR negatively influenced villus morphology. These architectural changes were characterized particularly by decreased jejunum and ileum crypt depth and decreased ileum villus height; changes which corroborate our previous study in cows (Kvidera et al., 2017b) as well as reports in other species (Holt et al., 1986; Thompson and Applegate, 2006; Pearce et al., 2013). The decrease in villus height and crypt depth likely reflects a reduction in cellular proliferation as previously described (Kvidera et al., 2017b). Alterations in villus morphology do not directly indicate changes in permeability, therefore, we utilized Cr-EDTA as an indicator of paracellular leakiness; a technique adapted from Zhang et al. (2013). Feed restriction increased intestinal permeability acutely and chronically as indicated by increased plasma Cr AUC (discussed below).

In agreement with past reports (Pearce et al., 2015; Abuajamieh et al., 2016; Li et al., 2019), improved Zn source ameliorated the negative effects of FR on ileum villus architecture and mucosal surface area. Besides improved bioavailability, source-mediated differences in gut parameters may be explained by altered release of free metal along the tract. Sulfate sources of trace minerals dissociate quickly in an aqueous environment, which can lead to a high metal load in the proximal intestine and less extractable mineral in the distal intestine (Klasing and Naziripour, 2010). In vitro, high levels of zinc salts (including zinc sulfate) caused rapid accumulation of Zn^{2+} in porcine jejunum epithelial cells, leading to cell damage and oxidative stress compared to zinc glycinate, a more stable and non-reactive form (Chen et al., 2019). However, high Zn concentrations were used in these studies, making interpretation in a practical dairy setting difficult. Improved morphology was not reflected by a corresponding change in

total tract permeability (plasma Cr concentrations). Reasons for the discrepancy in our intestinal integrity metrics are not fully understood, but may be explained by evaluation at different timepoints or differences in sensitivity/variation of the different techniques. In addition, the Cr-EDTA technique measures total tract permeability and therefore it is difficult to isolate where the greatest degree of “leakiness” occurred. Although measuring circulating Cr levels in response to ruminal Cr-EDTA administration appears to be a promising technique to evaluate intestinal permeability, there are some limitations needing consideration. First, it is not entirely clear how altered passage rate (which presumably occurred during FR) influences Cr-EDTA appearance along the intestinal tract. However, because the temporal pattern of circulating Cr following Cr-EDTA administration was similar and Cr concentrations were comparable at 24 h across challenges, it is likely that passage rate only had a marginal affect. Second, it is not clear whether FR influenced renal and milk Cr clearance rate. However, Zhang et al. (2013) previously reported increased urinary Cr recovery in FR relative to AL-fed heifers suggesting that the increased plasma appearance is not simply a reflection of decreased renal removal from circulation. Additionally, it is reasonable to assume that effects of FR on clearance rate were similar on d 2 and 5, yet we observed larger Cr AUC on d 2 suggesting increased Cr reflected increased gut leakiness. Regardless, further validation of this technique is warranted.

Fasting-induced intestinal damage occurs rapidly with several reports in pigs and chickens observing detrimental consequences within 24 h of FR (Thompson and Applegate, 2006; Horn et al., 2014; Gilani et al., 2017). Interestingly, herein we observed increased permeability (as indicated by increased Cr AUC) on d 2 compared to d 5 of FR. Rapid effects of FR on gut permeability suggest a mechanism other than decreased luminal nutrient contents is responsible, and some reports point to a neuroendocrine role (Horn et al., 2014; Najafi et al.,

2017). In response to stress, the hypothalamic pituitary axis (HPA) is activated, which stimulates nervous system and peripheral tissue production of corticotropin-releasing factor (CRF). Administering CRF induces intestinal barrier dysfunction in rodents (Santos et al., 1999; Teitelbaum et al., 2008) and initiates systemic inflammation in ruminants (Cooke and Bohnert, 2011; Cooke et al., 2012). Consequences of CRF are mediated by stimulation of intestinal mast cell degranulation and release of pre-formed and de-novo mediators such as histamine, proteases, and cytokines which negatively affect intestinal barrier function (Abraham and St. John, 2010; Overman et al., 2012). Stress-mediated effects on the gut barrier may explain why so many seemingly unrelated situations (i.e., heat stress, weaning, acidosis, etc.) share a common consequence of leaky gut and systemic inflammation (Mayorga et al., 2020).

In the current study, we observed no effect of FR on circulating cortisol concentrations suggesting the HPA axis may not have been activated. Reasons for not detecting increased cortisol are likely explained by insufficient sample collection during the acute phase of FR as several reports demonstrate resolution of the cortisol response within 24 h of an insult (Markos Chouzouris et al., 2018; Marques et al., 2019). In agreement, we detected no treatment differences in mast cell abundance in the jejunum, ileum, or colon, although FR increased duodenum mast cell number (5 d following FR initiation). To the authors' knowledge, no reports in ruminants exist demonstrating effects of stress on mast cell activation. Additionally, it is of interest to evaluate Zn's influence on the stress response as previous reports have demonstrated attenuated hypercortisolemia when improved Zn sources were provided (Lippolis et al., 2017; Xu et al., 2017; Horst et al., 2019). Furthermore, Zn may act as a mast cell stabilizer within the intestinal tract (Penissi et al., 2003). Consequently, there remain multiple mechanistic questions about how Zn sources attenuates intestinal hyperpermeability during stress.

Our results indicate that 5 d of FR increased intestinal epithelial permeability and this ostensibly permitted pathogen infiltration into systemic circulation. In corroboration, we observed increased acute phase proteins (LBP, SAA, and Hp) and altered neutrophil function during FR. Despite detecting increased permeability on d 2 of FR, meaningful increases in SAA and Hp were not observed until d 5 of FR, which agrees with our previous report (Kvidera et al., 2017b). The delayed increase in SAA and Hp concentrations was expected based on patterns previously observed in response to intravenous LPS administration (Horst et al., 2018, 2019) and CRF infusion (Cooke et al., 2012). Herein we observed a tendency for decreased SAA and Hp concentrations on d 5 of FR in HYD supplemented cows. Both increased (Pearce et al., 2015; Jarosz et al., 2019; Horst et al., 2019) and decreased (Mayorga et al., 2018) inflammatory mediators have been reported in previous studies feeding improved Zn sources. Based on the HYD-mediated changes in intestinal morphology, decreased acute phase proteins presumably reflect improved barrier integrity and reduced passage of luminal antigens across the intestinal barrier. However, Zn's inhibitory effect on the nuclear factor kappa-light-chain-enhancer of activated B-cells pathway (Prasad, 2008; Foster and Samman, 2012) which is crucial in regulating the immune response may be another explanation. In addition to stimulating an acute phase protein response, FR negatively influenced neutrophil oxidative burst and stimulated MPO activity. Haptoglobin directly inhibits leukocyte activity by binding to receptor-ligand sites (Oh et al., 1990; Arredouani et al., 2005). Haptoglobin's potent anti-inflammatory actions are crucial for the development of immune tolerance and preventing an over-exaggerated inflammatory response (Raju et al., 2018). Interestingly, no differences were observed in neutrophil function in FRHYD relative to AL-fed groups, which is likely explained by the decreased Hp concentrations also observed in this treatment. The immunosuppressive actions of Hp warrant further

investigation, especially considering its relevance to the dysregulated immune response present around parturition.

Conclusion

In corroboration with our previous report, 5 d of FR negatively impacts intestinal morphology and initiates a systemic inflammatory response. Furthermore, FR impacts permeability both acutely and chronically, and the effects appear to be more pronounced after 2 d of FR. The temporal intestinal barrier response to inadequate nutrient intake has practical implications to multiple scenarios in a dairy cow's life-cycle. Replacing Zn sulfate with Zn HYD improves barrier morphology and modulates the acute phase protein response and neutrophil function.

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Tables and Figures

Table 2.1. Ingredients and nutrient composition of the total mixed ration¹

Ingredient, % of DM	Control	HYD
Ingredient		
Corn silage	30.34	30.34
Alfalfa hay	23.50	23.50
Ground corn	18.80	18.80
Dry corn gluten pellets	10.26	10.26
Premix ²	6.84	6.84
Soy plus	4.27	4.27
Whole cottonseed	3.42	3.42
Soybean meal	1.71	1.71
Molasses	0.85	0.85
Chemical Analysis, % of DM ³		
Starch, %	24.6	24.9
Crude protein, %	16.2	16.6
NDFom, %	32.5	32.0
ADF, %	23.4	22.6
NE _L , Mcal/kg	1.61	1.60
Mineral, % of DM ⁴		
Calcium, %	1.00 ± 0.44	0.90 ± 0.21
Phosphorus, %	0.39 ± 0.04	0.38 ± 0.04
Potassium, %	1.31 ± 0.10	1.34 ± 0.08
Magnesium, %	0.34 ± 0.02	0.34 ± 0.02
Sodium, %	0.51 ± 0.05	0.56 ± 0.08
Sulfur, %	0.22 ± 0.02	0.21 ± 0.03
Aluminum, ppm	293 ± 123	281 ± 82
Cobalt, ppm	0.57 ± 0.21	0.52 ± 0.15
Copper, ppm	15.64 ± 2.83	16.80 ± 2.56
Iron, ppm	252 ± 124	258 ± 52
Manganese, ppm	60.90 ± 9.80	63.35 ± 11.68
Molybdenum, ppm	1.00 ± 0.32	1.01 ± 0.21
Selenium, ppm	0.61 ± 0.05	0.60 ± 0.06
Zinc, ppm	92.39 ± 24.98	92.54 ± 16.45

¹Diet dry matter = 49.45% for Control and 51.59% for HYD

²See Table 2 for premix ingredients and composition

³A composite of all weekly samples was sent off for analysis

⁴Mineral composition was analyzed weekly throughout the trial. Analysis presented as the mean ± standard error.

Table 2.2. Ingredients and analyzed mineral composition of premix

	Control	HYD
Soybean meal	27.33	27.33
Bloodmeal	16.00	16.00
Pork meat and bone meal	11.39	11.39
Sodium bicarbonate	10.29	10.29
MagnaPalm	9.14	9.14
Calcium carbonate	9.11	9.11
Salt	5.49	5.49
VTM ¹	2.85	2.85
Urea	2.29	2.29
Magnesium oxide	2.06	2.06
FloMatrix	1.37	1.37
Alimet	0.69	0.69
Diamond V XPC	0.68	0.68
Choice white grease	0.58	0.58
Smartamine M	0.58	0.58
Rumensin 90	0.10	0.10
Biotin 2%	0.05	0.05
Mineral Analysis, % of DM ²		
Calcium, %	5.10 ± 0.06	5.61 ± 0.43
Phosphorus, %	0.72 ± 0.12	0.72 ± 0.09
Potassium, %	0.95 ± 0.05	0.86 ± 0.06
Magnesium, %	1.22 ± 0.03	1.32 ± 0.04
Sodium, %	5.22 ± 0.31	6.21 ± 0.30
Sulfur, %	0.85 ± 0.24	0.63 ± 0.15
Aluminum, ppm	476 ± 113	633 ± 142
Cobalt, ppm	3.21 ± 0.01	2.31 ± 0.78
Copper, ppm	142 ± 46	143 ± 10
Iron, ppm	380 ± 153	613 ± 194
Manganese, ppm	474 ± 11	552 ± 71
Molybdenum, ppm	0.78 ± 0.04	0.92 ± 0.47
Selenium, ppm	4.48 ± 1.11	4.18 ± 0.06
Zinc, ppm	1368 ± 855	1162 ± 499

¹Analyzed mineral composition of vitamin trace mineral mix was as follows.

Control diet: Ca 15.12%, P 0.09%, K 0.21%, Mg 0.12%, Na 0.13%, S 3.07%, Al 1002 mg/kg, Co 119 mg/kg, Cu 4336 mg/kg, Fe 763 mg/kg, Mn 18,375 mg/kg, Mo 1.76 mg/kg, Se 103 mg/kg, and Zn 32,549 mg/kg. **HYD diet:** Ca 15.12%, P 0.04%, K 0.21%, Mg 0.13%, Na 0.06%, S 1.42%, Al 771 mg/kg, Co 106 mg/kg, Cu 4495 mg/kg, Fe 454 mg/kg, Mn 18527 mg/kg, Mo 0.77 mg/kg, Se 121 mg/kg, Zn 31673 mg/kg

²Samples collected each time a new batch of premix was made (Twice for the control premix and 4 times for the HYD premix). Analysis presented as the mean ± standard error.

Table 2.3. Water sample analysis \pm S.D.

	Lactating Barn – Control Pen	Lactating Barn – HYD Pen	Maternity Barn – Control & HYD
Number of samples ¹	2	2	2
pH	7.90 \pm 1.33	7.75 \pm 1.17	8.84 \pm 0.20
Chloride, mg/L	21.25 \pm 5.30	23.75 \pm 1.77	22.50 \pm 3.54
Total hardness, mg/L	126.0 \pm 4.24	121.5 \pm 6.36	121.5 \pm 6.36
Nitrate-Nitrogen, mg/L	2.16 \pm 0.57	2.23 \pm 0.78	2.17 \pm 0.52
Calcium, mg/L	33.25 \pm 0.78	31.8 \pm 2.40	31.9 \pm 2.55
Magnesium, mg/L	10.40 \pm 0.57	10.25 \pm 0.21	10.20 \pm 0.00
Sodium, mg/L	11.75 \pm 0.92	12.25 \pm 2.33	13.8 \pm 2.69
Sulfate, mg/L	57.25 \pm 2.47	56.00 \pm 7.07	53.00 \pm 8.49
Iron, mg/L	<0.01	<0.01	<0.01
Manganese, mg/L	<0.01	<0.01	<0.01
EC ² , umhos/cm	347.5 \pm 17.68	331.0 \pm 26.87	333.5 \pm 20.51
TDS ³ , mg/L	246.5 \pm 12.02	234.5 \pm 19.09	236.5 \pm 14.85

¹Water samples were collected at the beginning and end of the experiment²Electrical conductivity³Total dissolved solids - calculated

Table 2.4. Effects of group (ad libitum intake or feed restriction) and diet (Zn sulfate or Zn hydroxychloride) on production parameters during P2¹

Parameters	AL		FR		SEM	P		
	CON	HYD	CON	HYD		G ²	Diet	G × Diet
Number of animals (n=23)								
DMI, kg/d	25.9	27.1	11.1	10.2	0.9	<0.01	0.85	0.24
Milk yield, kg/d	38.8	39.1	27.6	24.3	1.3	<0.01	0.37	0.22
ECM ³ , kg/d	44.3	43.0	31.8	28.6	1.1	<0.01	0.11	0.49
Milk composition								
Fat, %	4.36	4.15	4.86	4.64	0.09	<0.01	0.05	0.97
Fat, kg	1.70	1.62	1.27	1.18	0.04	<0.01	0.12	0.92
Lactose, %	4.61 ^z	4.57 ^y	4.50 ^x	4.53 ^x	0.02	<0.01	0.59	0.12
Protein, %	3.23	3.29	3.08	3.13	0.04	<0.01	0.23	0.94
Protein, kg	1.26	1.27	0.86	0.75	0.04	<0.01	0.32	0.19
MUN, mg/dL	14.45	14.52	15.29	15.16	0.20	<0.01	0.90	0.63
SCS ⁴	1.84	2.25	2.85	2.79	0.41	0.08	0.70	0.57
FE ⁵ , ECM:DMI	1.72	1.59	2.94	2.89	0.08	<0.01	0.37	0.65
BW loss ⁶ , kg	-2.05	-10.61	39.39	53.41	9.52	<0.01	0.78	0.25
BCSΔ ⁷	-0.10	-0.04	-0.25	-0.25	0.12	0.16	0.80	0.80
EBAL, Mcal/d	1.40	3.94	-14.30	-13.42	0.82	<0.01	0.05	0.31
Rectal temperature, °C	37.9 ^{ab}	38.0 ^b	38.0 ^b	37.8 ^a	0.1	0.22	0.73	0.02
Respiration rate, bpm	45	42	37	35	1	<0.01	0.11	0.73
Liver TG, %	1.08	0.90	2.02	1.94	0.40	0.04	0.56	0.69

¹During period 2, cows were either fed ad libitum (AL) or feed restricted (FR) and fed a diet with supplemental Zn from Zn sulfate (CON) or Zn hydroxychloride (HYD)

²Group

³Energy corrected milk

⁴Somatic cell score

⁵Feed efficiency

⁶Initial body weight – final body weight

⁷Final body condition score – initial body condition score

Table 2.5. Effects of group (ad libitum intake or feed restriction) and diet (Zn sulfate or Zn hydroxychloride) on complete blood count and insulin:DMI during P2¹

Parameters	AL		FR		SEM	P						
	CON	HYD	CON	HYD		G ²	Diet	G × Diet	Day	G × Day	Diet × Day	G × Diet × Day

Number of animals (n=23)												
CBC Parameters												
WBC, ×10 ³ /μL	6.63	6.75	6.35	7.04	0.21	0.99	0.10	0.17	0.19	0.87	0.48	0.98
Neutrophils, ×10 ³ /μL	3.55 ^b	3.24 ^{ab}	2.84 ^a	3.41 ^{ab}	0.22	0.22	0.60	0.05	0.01	0.45	0.26	0.77
Platelets, ×10 ³ /μL	346	367	306	320	19	0.03	0.34	0.84	<0.01	0.59	0.25	0.78
Monocytes, ×10 ³ /μL	0.45	0.38	0.38	0.40	0.03	0.39	0.39	0.16	0.05	0.83	0.56	0.85
Lymphocytes, ×10 ³ /μL	2.75 ^{ab}	2.60 ^a	2.64 ^{ab}	2.78 ^b	0.06	0.52	0.90	0.02	0.23	0.11	0.18	0.96
Eosinophils, ×10 ³ /μL	0.32 ^x	0.34 ^x	0.42 ^y	0.33 ^x	0.03	0.20	0.38	0.11	0.18	0.01	0.26	0.72
Basophils, ×10 ³ /μL	0.062	0.070	0.065	0.066	0.002	0.67	0.10	0.20	0.40	0.77	0.48	0.66
Hemoglobin, gm/dL	10.01	10.15	10.33	10.68	0.09	<0.01	0.01	0.29	0.04	<0.01	0.56	0.55
Hematocrit, %	27.74 ^x	28.25 ^{xy}	28.32 ^y	29.75 ^z	0.24	<0.01	<0.01	0.10	0.12	0.01	0.20	0.52
Insulin:DMI	0.03	0.02	0.03	0.02	0.00	0.85	0.37	0.54	0.38	0.28	0.91	0.51

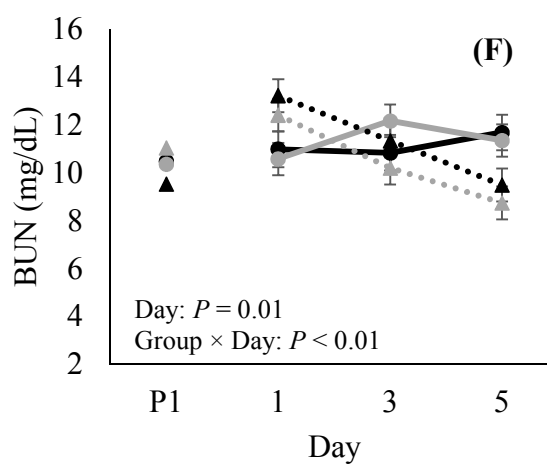
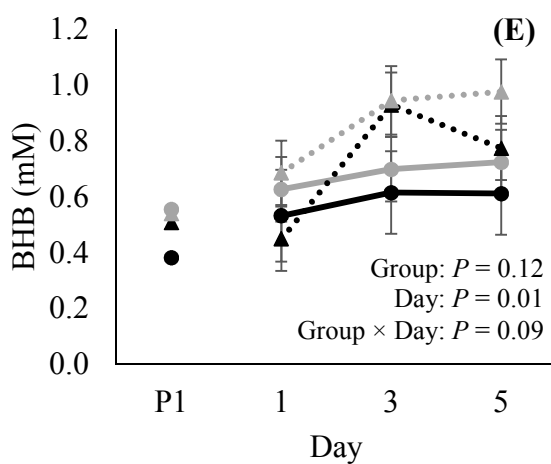
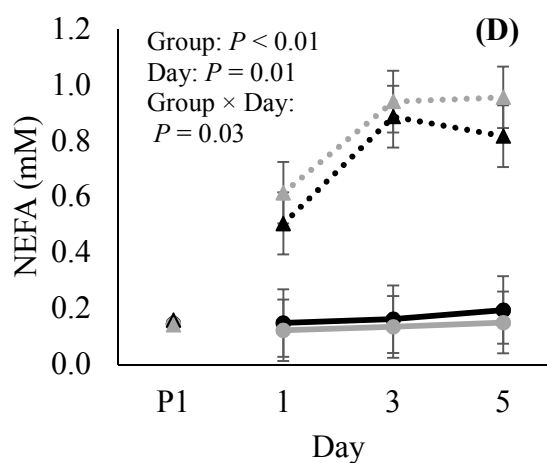
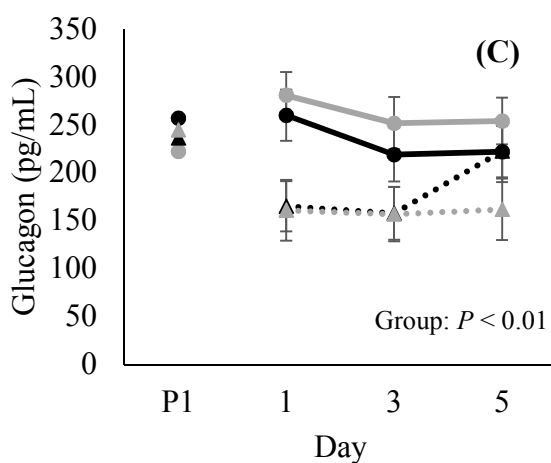
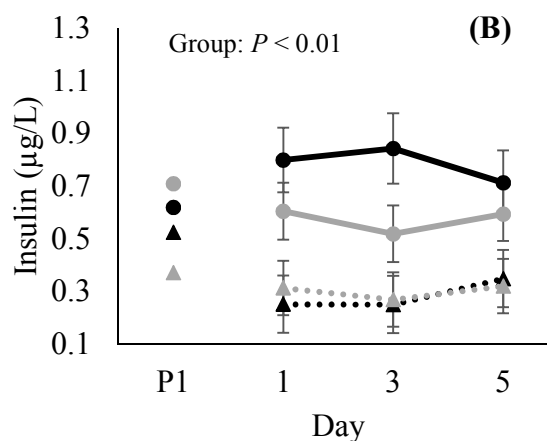
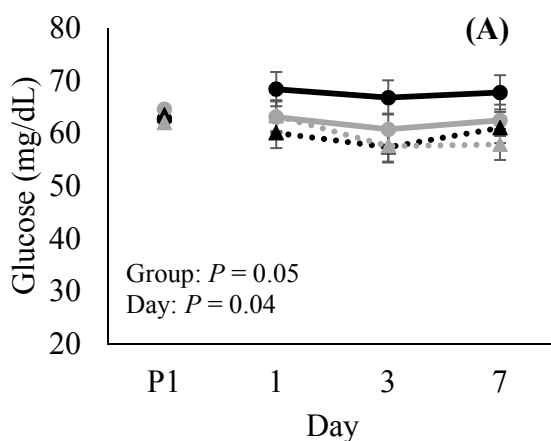
¹During period 2, cows were either fed ad libitum (AL) or feed restricted (FR) and fed a diet with supplemental Zn from Zn sulfate (CON) or Zn hydroxychloride (HYD)²Group^{a,b}Values within a row with differing superscripts denote difference ($P < 0.05$)^{x,y,z}Values within a row with differing superscripts denote tendency ($0.05 > P \leq 0.15$)

Table 2.6. Effects of group (ad libitum intake or feed restriction) and diet (Zn sulfate or Zn hydroxychloride) on intestinal goblet cell area (GCA), mast cell number, and myeloperoxidase at the end of P2 P2¹

	AL		FR			P		
Parameters	CON	HYD	CON	HYD	SEM	G ²	Diet	G × Diet
Number of animals (n=23)								
Duodenum								
Goblet cell area, %	6.75 ^{ab}	4.89 ^a	5.55 ^{ab}	7.72 ^b	0.85	0.35	0.86	0.03
Mast cell #, cells/mm ²	288.1	253.3	383.3	396.7	46.9	0.02	0.82	0.62
Jejunum								
Goblet cell area, %	3.58	3.56	3.41	3.14	0.51	0.57	0.78	0.81
Mast cell #, cells/mm ²	370.5	383.3	370.0	450.0	50.4	0.52	0.37	0.52
Ileum								
Goblet cell area, %	3.96	5.01	3.56	5.30	0.98	0.96	0.17	0.73
Mast cell #, cells/mm ²	353.0	381.2	375.0	388.3	39.1	0.72	0.60	0.85
Colon								
Goblet cell area, %	15.27	17.00	10.27	9.06	2.45	0.02	0.92	0.56
Mast cell #, cells/mm ²	161.7	168.3	218.3	198.3	41.3	0.31	0.87	0.75

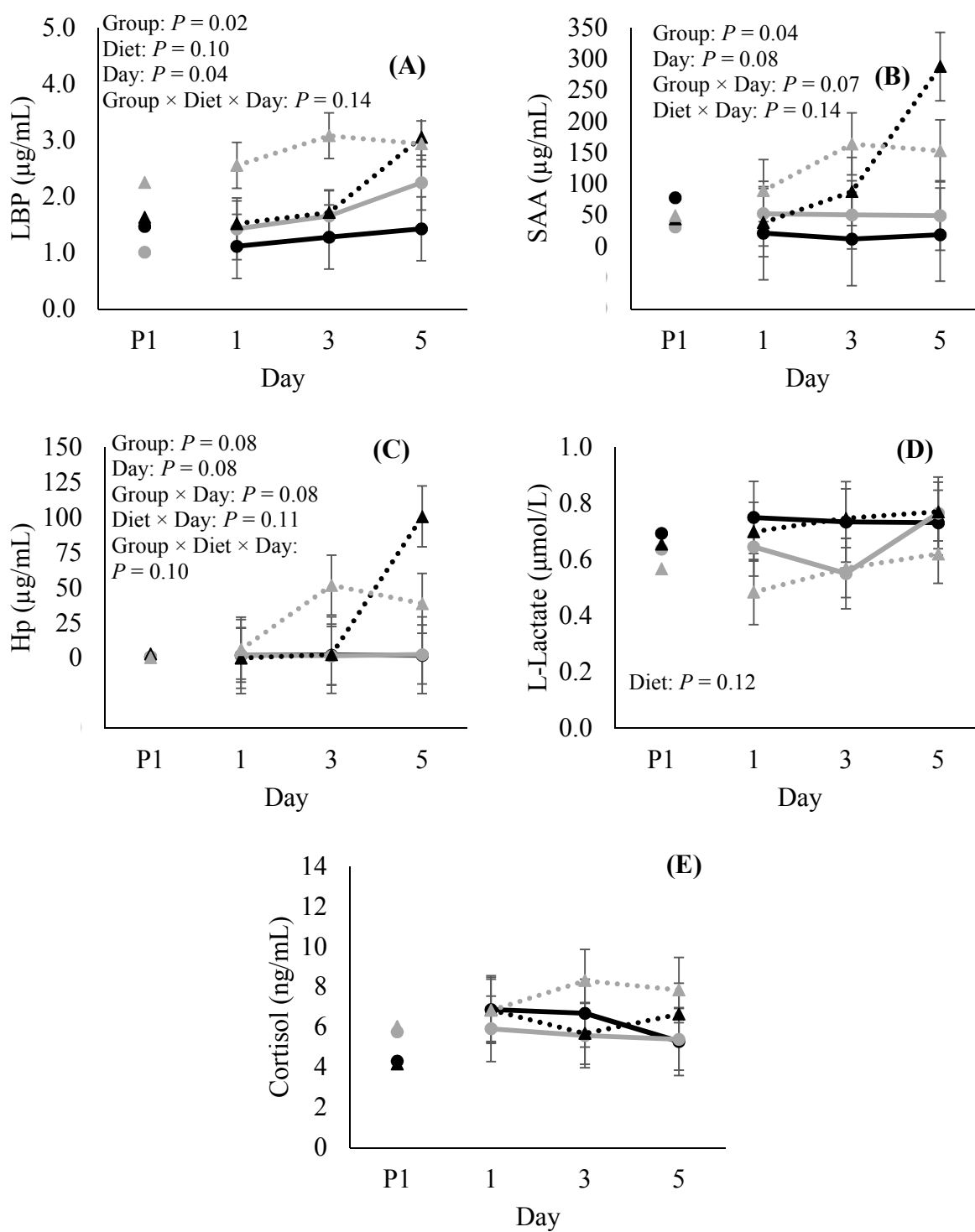
¹During period 2, cows were either fed ad libitum (AL) or feed restricted (FR) and fed a diet with supplemental Zn from Zn sulfate (CON) or Zn hydroxychloride (HYD)

²Group



—●— ALCON —●— ALHYD ···★··· FRCON ···★··· FRHYD

Figure 2.1. Effects of group (ad libitum intake or feed restricted) and diet (Zn sulfate or Zn hydroxychloride) on circulating (A) glucose, (B) insulin, (C) glucagon, (D) non-esterified fatty acids (**NEFA**), (E) β -hydroxybutyrate (**BHB**), and (F) blood urea nitrogen (**BUN**) during P2. Data were analyzed using PROC MIXED and included fixed effects of group, diet, day, and their interactions. P1 represents an average of measurements obtained during the 5 d of P1 and was utilized as a covariate. Data are represented as least squares means \pm standard error of the mean and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.15$.



—●— ALCON —●— ALHYD ..★.. FRCON ..★.. FRHYD

Figure 2.2. Effects of group (ad libitum intake or feed restricted) and diet (Zn sulfate or Zn hydroxychloride) on circulating (A) lipopolysaccharide-binding protein (LBP), (B) serum amyloid A (SAA), (C) haptoglobin (Hp), (D) L-lactate, and (E) cortisol during P2. Data were analyzed using PROC MIXED and included fixed effects of group, diet, day, and their interactions. P1 represents an average of measurements obtained during the 5 d of P1 and was utilized as a covariate. Data are represented as least squares means \pm standard error of the mean and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.15$.

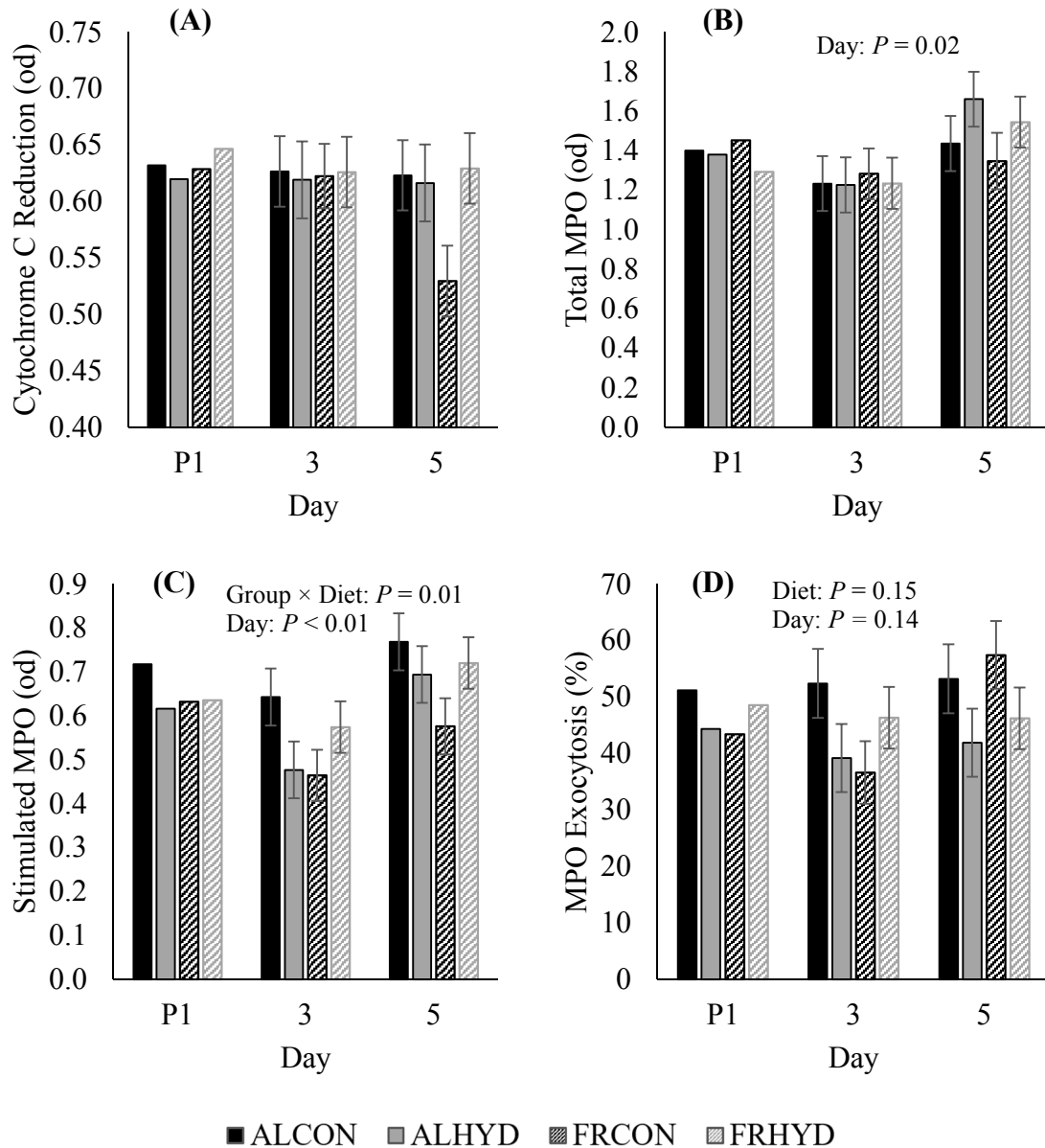


Figure 2.3. Effects of group (ad libitum intake or feed restricted) and diet (Zn sulfate or Zn hydroxychloride) on neutrophil (A) oxidative burst, (B) total myeloperoxidase (MPO) release, (C) stimulated MPO release, and (D) MPO exocytosis. Data were analyzed using PROC MIXED and included fixed effects of group, diet, day, and their interactions. P1 represents an average of measurements obtained d 3 of P1 and was utilized as a covariate. Data are represented as least squares means \pm standard error of the mean and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.15$.

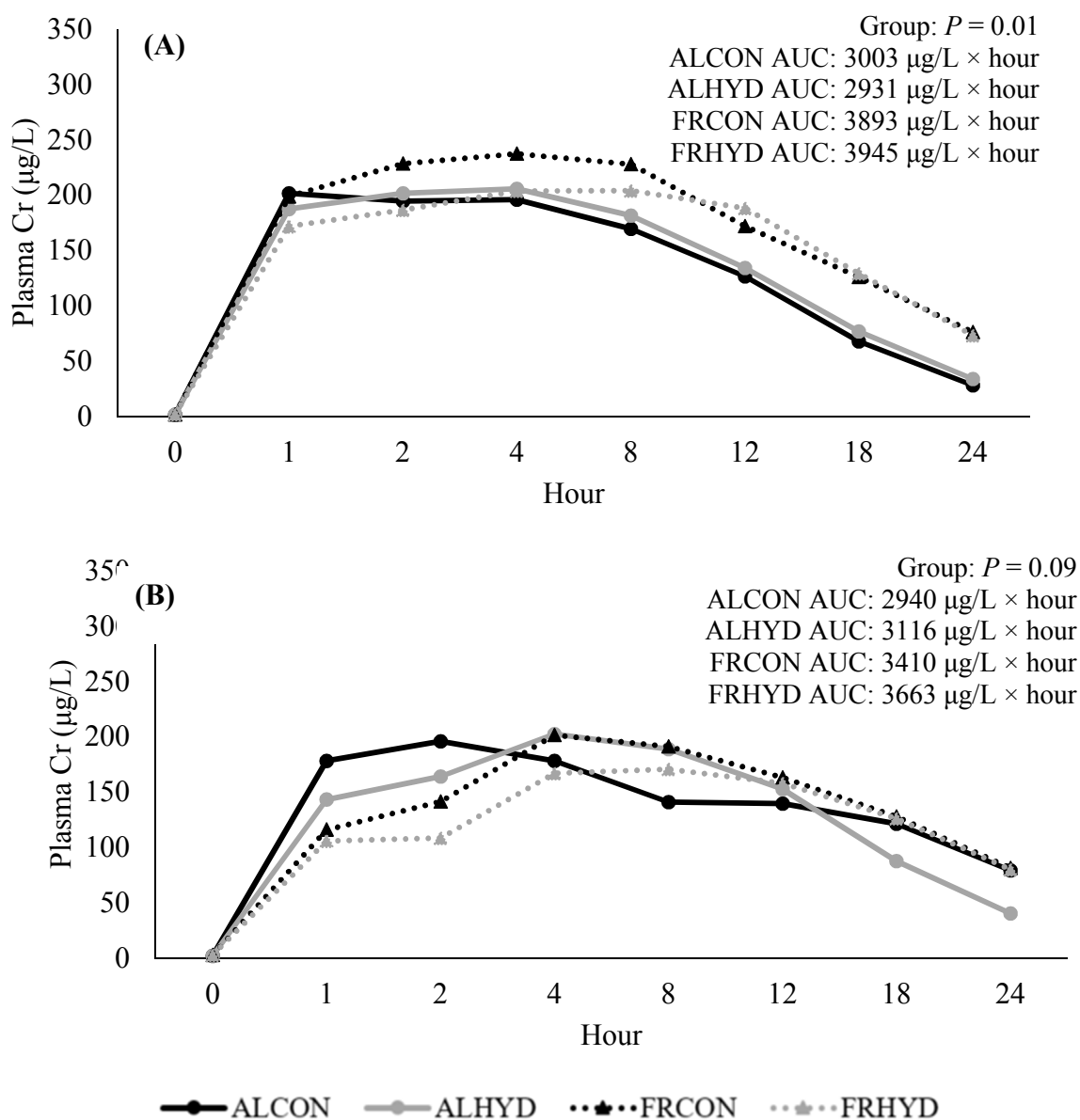


Figure 2.4. Effects of group (ad libitum intake or feed restricted) and diet (Zn sulfate or Zn hydroxychloride) on circulating Cr concentrations on (A) d 2 and (B) d 5 of P2. Data were analyzed using PROC MIXED and included fixed effects of group, diet, and their interaction. P1 represents an average of measurements obtained during the 5 d of P1 and was utilized as a covariate. Data are represented as least squares means \pm standard error of the mean and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.15$.

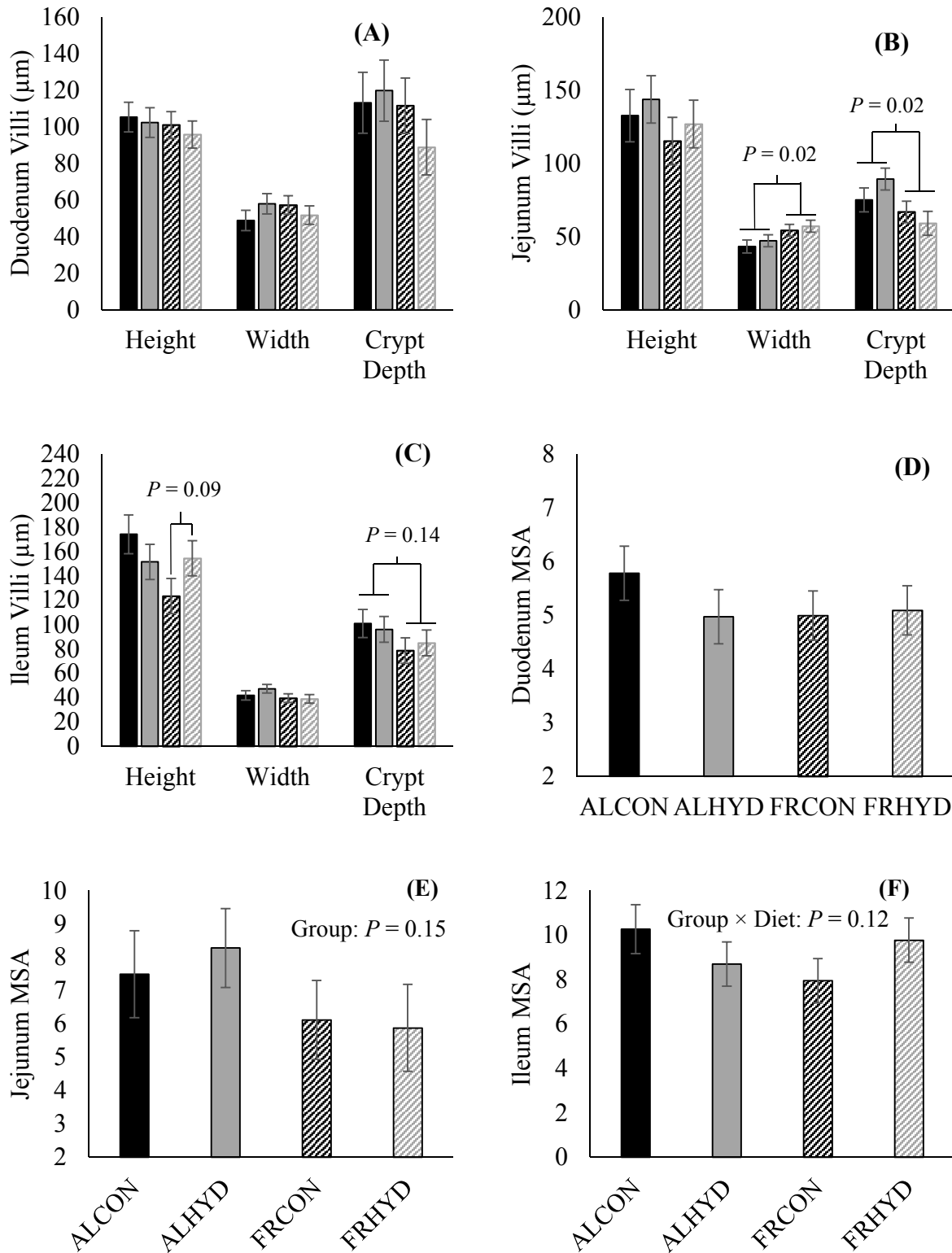


Figure 2.5. Effects of group (ad libitum intake or feed restricted) and diet (Zn sulfate or Zn hydroxychloride) on (A) duodenum villus morphology, (B) jejunum villus morphology, (C) ileum villus morphology, (D) duodenum mucosal surface area, (E) jejunum mucosal surface area, and

(F) ileum mucosal surface area. Data were analyzed using PROC MIXED and included fixed effects of group, diet, and their interactions. Data are represented as least squares means \pm standard error of the mean and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.15$.

CHAPTER 3. CHROMIUM'S IMPACT ON BIOENERGETICS AND LEUKOCYTE DYNAMICS FOLLOWING IMMUNOACTIVATION IN LACTATING HOLSTEIN COWS

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Abstract

Activated immune cells are insulin sensitive and utilize copious amounts of glucose. Since chromium (Cr) increases insulin sensitivity and may be immunomodulatory, our objectives were to evaluate supplemental Cr (KemTRACE® chromium propionate, 20 g/d; Kemin Industries Inc., Des Moines, IA) on immune system glucose utilization and immune system dynamics following an i.v. endotoxin challenge in lactating Holstein cows. Twenty cows (320 ± 18 DIM) were randomly assigned to 1 of 4 treatments: 1) pair-fed (PF) control (PF-CON; 5 mL saline; n=5), 2) PF and Cr supplemented (PF-Cr; 5 mL saline; n=5), 3) LPS-euglycemic clamp and control supplemented (LPS-CON; 0.375 µg/kg BW LPS; n=5), and 4) LPS-euglycemic clamp and Cr supplemented (LPS-Cr; 0.375 µg/kg BW LPS; n=5). The experiment was conducted serially in 3 periods (P). During P1 (3 d) cows received their respective dietary treatments and baseline values were obtained. At the initiation of P2 (2 d), either a 12 h lipopolysaccharide (LPS)-euglycemic clamp was conducted or cows were PF to their respective dietary counterparts. During P3 (3 d), cows consumed feed ad libitum and continued to receive their respective dietary treatment. During P2, LPS administration decreased DMI (40%) similarly amongst diets, and by experimental design the pattern and magnitude of reduced DMI

was similar in the PF cohorts. During P3, LPS-Cr fed cows tended to have decreased DMI (6%) relative to LPS-CON. Relative to controls, milk yield from LPS-challenged cows decreased (58%) during P2 and LPS-Cr cows produced less (16%) milk than LPS-Con. During P3 milk yield progressively increased similarly in LPS administered cows but overall milk yield remained decreased (24%) compared to PF controls. There were no dietary treatment differences in milk yield during P3. Circulating insulin increased 9 and 15-fold in LPS administered cows at 6 and 12 h postbolus, respectively, compared to PF controls. Compared to LPS-CON cows, circulating insulin in LPS-Cr cows was decreased (48%) at 6 h postbolus. Relative to PF cows, circulating LPS-binding protein and serum amyloid A from LPS administered cows increased 2 and 5-fold, respectively. Compared to PF cows, blood neutrophil counts in LPS infused cows initially decreased, then gradually increased 163%. Between 18 and 48 h postbolus, the number of neutrophils were increased (12%) in LPS-Cr vs. LPS-CON cows. The 12-h total glucose deficit was 220 and 1,777 g for the PF and LPS treatments, respectively, but glucose utilization following immune activation was not influenced by Cr. In summary, supplemental Cr reduced the insulin response and increased circulating neutrophils following an LPS challenge, but did not appear to alter the immune systems glucose requirement following acute and intense activation.

Keywords: chromium, lipopolysaccharide, insulin, neutrophil

Introduction

Dairy cows employ homeorhetic mechanisms to support dominant physiological states (i.e., growth, reproduction, lactation; Bauman and Currie, 1980) and during lactation this is in large part characterized by decreased circulating insulin coupled with reduced insulin sensitivity in adipose tissue and skeletal muscle (De Koster, and Opsomer, 2013; Baumgard et al., 2017). However, immunoactivation markedly reprioritizes the hierarchy of coordinated nutrient

trafficking (Bradford et al., 2015) at the expense of milk synthesis. Inflammation is caused by multiple pathologies including, mastitis, metritis, heat stress, intestinal barrier dysfunction, and feed restriction (Lambert et al., 2002; Sheldon et al., 2008; Ballou, 2012; Zhang et al., 2013; Kvidera et al., 2017b, 2017d). While these insults have differing etiologies, inflammation and suboptimal production are common outcomes; therefore, identifying strategies amenable to dietary manipulation that could facilitate the immune response and/or ameliorate production losses during immunoactivation would largely impact animal health and farm profitability.

Upon activation, immune cells become obligate glucose utilizers and shift ATP production from oxidative phosphorylation to aerobic glycolysis, a process known as the “Warburg effect” (Palsson-McDermott and O’Neill, 2013). During an immune challenge circulating insulin increases, likely because insulin facilitates immune cell glucose uptake and improves function (Walrand, 2004). In an attempt to spare glucose for the immune system, muscle and adipose tissue become insulin resistant (Lang et al., 1990) and milk synthesis is reduced (Kvidera et al., 2017b). However, the ubiquitous nature of leukocytes makes quantifying the immune system’s *in vivo* glucose consumption difficult. By employing an LPS-euglycemic clamp technique we have recently demonstrated that an activated immune system utilizes > 1 kg of glucose within 12 h in the lactating cow model (Kvidera et al., 2017a). Thus, mounting an immune response is energetically expensive and strategies that divert glucose towards activated leukocytes may allow for a more efficient immune response.

Chromium (Cr) potentiates insulin action (Chen et al., 2006), and while the exact mechanism is not fully understood, it appears to work intimately with chromodulin (or low-molecular-weight Cr-binding substance; Vincent, 2015). In response to insulin, Cr enters insulin sensitive cells and, in combination with chromodulin, binds to the insulin receptor, which

amplifies downstream signaling cascades (Vincent, 2015). Subsequently, GLUT-4 translocation to the plasma membrane is improved and glucose uptake is increased (Chen et al., 2006), a potential benefit to insulin sensitive immune cells upon activation. Therefore, study objectives were to evaluate the effects of supplemental Cr on glucose consumption and circulating leukocyte dynamics in an acute and intensely activated immune system via the LPS-euglycemic clamp technique.

Materials and Methods

Animals and Experimental Design

All procedures were approved by the Iowa State University Institutional Animal Care and Use Committee. Twenty non-pregnant lactating Holstein cows (781 ± 21 kg BW; 320 ± 18 DIM; parity 3 ± 0.3) were utilized in an experiment conducted in two replications (10 cows/replicate). Cows were housed in individual box-stalls (4.57×4.57 m) at the Iowa State University Dairy Farm. Cows were allowed 4 d to acclimate to housing and feeding conditions; during this time they were implanted with bilateral jugular catheters. Beginning on d 1 of acclimation through study completion, cows received 1 of 2 dietary treatments: 1) a control supplement (20 g/d calcium carbonate) or 2) a Cr supplement (KemTRACE® chromium propionate, 20 g/d to deliver 8 mg of Cr/d; Kemin Industries Inc., Des Moines, IA). Supplements were provided as a “top-dress” premixed with ground corn at a rate of 200 g/d. Period 1 (P1) lasted 3 d and served as the baseline (data generated for covariate analysis) for periods 2 (P2) and 3 (P3). At the initiation of P2, which lasted 48 h, animals experienced either a 12 h LPS-euglycemic clamp as previously described (Kvidera et al., 2017a) or were pair-fed (**PF**) to their respective dietary counterparts for the entire 48 h in order to eliminate the confounding effects of dissimilar nutrient intake; dietary and challenged combinations resulted in four total treatments: 1) PF and control supplemented (**PF-CON**; 5 mL sterile saline with control supplement; $n = 5$), 2) PF and

Cr supplemented (**PF-Cr**; 5 mL sterile saline with Cr propionate supplement; n = 5), 3) LPS-euglycemic clamp and control supplemented (**LPS-CON**; 0.375 $\mu\text{g/kg}$ BW LPS with control supplement; n = 5), and 4) LPS-euglycemic clamp and Cr supplemented (**LPS-Cr**; 0.375 $\mu\text{g/kg}$ BW LPS with Cr propionate supplement; n = 5). Period 3 lasted 3 d during which all animals continued to receive their dietary treatment, but were allowed to consume feed ad libitum.

All cows were fed a diet formulated to meet or exceed the predicted requirements (NRC, 2001; Table 3.1) of energy, protein, minerals, and vitamins. Reduced feed intake in LPS-treated cows during P2 was determined as a percentage of their mean daily ad libitum intake during P1. Throughout the experiment, PF cows lagged 1 d behind LPS infused cows to allow for pair-feeding calculations as we have previously described (Baumgard et al., 2011).

Cows were milked four times daily (0000, 0600, 1200, and 1800 h) during P1 and P2, and twice daily (0600 and 1800 h) during P3. Milk yield was recorded and a sample for composition analysis was obtained at each milking. Samples were stored at 4°C with a preservative (bronopol tablet; D & F Control System, San Ramon, CA) until analysis by Dairy Lab Services (Dubuque, IA) using AOAC approved infrared analysis equipment and procedures (AOAC International, 1995). Rectal temperature (**Tr**), respiration rate (**RR**), and heart rate (**HR**), were recorded after each milking. Heart rate and respiration rate were measured as beats or flank movements during a 15 s interval and later transformed to beats/min and breaths/min (bpm), respectively. Rectal temperature was measured using a digital thermometer (GLA M700 Digital Thermometer, San Luis Obispo, CA).

To estimate the glucose requirements of an activated immune system, we employed the LPS-euglycemic clamp technique as we have recently described (Kvidera et al., 2016, 2017a). Selecting the LPS dose was influenced by the magnitude of hypoglycemia observed in earlier

reports and expected to cause a 40-50% decrease in milk production (Giri et al., 1990; Waldron et al., 2003a; Waggoner et al., 2009; Kvidera et al., 2017a). Lipopolysaccharide (*Escherichia coli* O55:B5; Sigma Aldrich, St. Louis, MO) was dissolved in sterile saline at a concentration of 500 µg/mL and passed through a 0.2 µm sterile syringe filter (Thermo Scientific; Waltham, MA). The total volume of LPS solution administered was approximately 4 mL. Maintaining the euglycemic clamp involved i.v. infusing 50% glucose (as dextrose; VetOne, Boise, ID) at a known and adjustable rate utilizing a modular pump (Deltec 3000, Deltec Inc., St. Paul, MN) in order to maintain the pre-LPS administration blood glucose concentrations.

Catheters were inserted into both jugular veins in all cows prior to P1 as previously described (Baumgard et al., 2011). Blood samples were obtained at -30, -20, and 0 min relative to LPS or saline bolus administration to establish baseline glucose levels. Each respective treatment bolus was administered immediately following the 0 min blood sample collection. For cows receiving LPS, postbolus blood samples (1 mL) were collected every 10 min and immediately analyzed for glucose concentration (TRUEbalance glucometer; McKesson, San Francisco, CA). Glucose infusion began when blood glucose content declined below baseline levels, and its rate of infusion was adjusted as necessary to maintain euglycemia ($\pm 5\%$). The rate of 50% glucose infusion (mL/h) was transformed to rate of glucose infusion (**ROGI**; g/h). The total glucose infused for each cow was calculated using the ROGI for each 10 min interval (72 intervals in total) according to the following equation:

$$\sum_{i=0}^{72} ROGI \left(\frac{g}{h} \right)_i * \frac{1 h}{60 min} * 10 min$$

Blood glucose was measured every 3 h in PF cows. Vital measurements (Tr, RR, and HR) were obtained at -0.5 and 0 h relative to LPS administration, every 3 h for the first 12 h postbolus, and every 6 h thereafter.

Blood samples were collected daily at 1800 h during P1 from the catheter and divided equally between a tube containing K₂EDTA (BD®, Franklin Lakes, NJ; for plasma collection) and an empty glass tube (for serum collection). Additional plasma and serum samples (~10 mL each) were collected from all treatments at -0.5, -0.33, 0, 3, 6, 9, 12, 18, 24, 30, 36, 42, and 48 h relative to bolus administration during P2. Serum samples were allowed to clot at room temperature for 1 h prior to centrifugation. Plasma and serum were harvested following centrifugation at $1500 \times g$ for 15 min at 4°C and were subsequently frozen at -20°C until analysis.

Plasma insulin, non-esterified fatty acids (**NEFA**), BHB, LPS binding protein (**LBP**), serum amyloid A (**SAA**), haptoglobin (**Hp**), BUN, and glucose concentrations were determined using commercially available kits according to manufacturers' instructions (insulin, Mercodia AB, Uppsala, Sweden; NEFA, Wako Chemicals USA, Richmond, VA; BHB, Pointe Scientific Inc., Canton, MI; LBP, Biomedical Research Service Center, Buffalo, NY; SAA, Tridelata Development Ltd., Kildare, Ireland; Hp, Immunology Consultants Laboratory Inc., Portland, OR; BUN, Teco Diagnostics Anaheim, CA; glucose, Wako Chemicals USA Inc., Richmond, VA). The inter- and intra-assay coefficients of variation for glucose, insulin, NEFA, BHB, SAA, LBP, and BUN assays were 7.2 and 4.1%, 7.1 and 5.6%, 4.0 and 3.2%, 6.9 and 6.2%, 20.5 and 11.8%, 12.3 and 3.9%, and 6.2 and 4.6%, respectively, and the intra-assay coefficient of variation for Hp was 7.8%.

Samples for complete blood count analysis were collected at -30 min, -20 min and 0, 3, 6, 9, 12, 18, 24, 30, 36, 42, and 48 h relative to bolus administration. A 3 mL blood sample was collected from the catheter (K₂EDTA; BD Franklin Lakes, NJ) and stored at 4°C for ~12 h before submitting to the Iowa State University's Department of Veterinary Pathology for

analysis. Samples for iSTAT analysis were measured using an iSTAT handheld machine and cartridge (CG8⁺; Abbott Point of Care, Princeton, NJ) and were obtained at 0, 12, 24, and 48 h relative to bolus administration.

Calculations and Statistical Analyses

Administering LPS reduces milk yield and therefore decreases glucose utilized for milk synthesis. The decrease in milk yield allows us to estimate the amount of glucose conserved (milk glucose deficit) due to reduced feed intake alone (PF-CON and PF-Cr cows) and decreased feed intake coupled with ostensible glucose utilization by the immune system (LPS-CON and LPS-Cr cows). The amount of glucose utilized for milk synthesis was calculated for each milking based on Kronfeld's (1982) estimation of 72 g glucose required to synthesize 1 kg of milk. Milk glucose output prior to the challenge was averaged in order to establish a baseline. Milk glucose utilization at both 6 and 12 h was subtracted from the baseline in order to calculate the milk glucose deficit. For PF-CON and PF-Cr cows, milk glucose deficit was solely used to calculate total glucose deficit. For LPS-CON and LPS-Cr cows, milk glucose deficit plus the amount of glucose infused to maintain euglycemia were combined to obtain the total glucose deficit. After cessation of the euglycemic clamp (12 h post-bolus), milk glucose deficit continued to be calculated every 6 h until 48 h postbolus.

Each animal's respective response variable was analyzed using repeated measures with an autoregressive covariance structure for feed intake and iSTAT parameters and spatial power law structure for vitals, milk yield, milk composition, complete blood cell count, and blood metabolites. The repeated effect was time relative to bolus administration. There were no treatment differences in any measured variable during P1; thus each specific variable's pre-bolus values (i.e. P1 average, when available) served as a covariate for analysis of P2 and P3 (each period was statistically analyzed separately). Effects of treatment, time (h relative to bolus),

treatment by time interaction, and replicate were assessed as a completely randomized design using PROC MIXED (SAS Inst. Inc., Cary, NC). Pre-formed contrasts were used to estimate differences between control and Cr supplemented cows, PF and LPS-administered cows, as well as LPS-CON and LPS-Cr cows. Data are reported as least squares means and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$.

Results

During P2 and irrespective of Cr, LPS-administered cows had mild hyperthermia ($+0.82^{\circ}\text{C}$) between 3 and 6 h postbolus, relative to PF cows, whose Tr remained unchanged ($P < 0.01$; Figure 3.1A). Respiration rate was decreased 5 bpm while HR was increased 8 bpm in LPS relative to PF cows ($P < 0.01$; data not shown). Heart rate of LPS-Cr cows tended to be increased (4 bpm) in comparison to LPS-CON cows ($P = 0.07$; data not shown). Period 3 HR of LPS treatments was increased 4 bpm relative to their PF counterparts ($P = 0.04$; data not shown) but Tr and RR did not differ between treatments.

Lipopolysaccharide administration decreased DMI (40%) relative to baseline during P2, and the pattern and extent of reduced DMI was similar for cows in the PF treatments by experimental design ($P = 0.36$; Figure 3.1B). Feed intake increased similarly (115%) from P2 d1 to P2 d2 postbolus for all treatments ($P < 0.01$). Relative to PF cows, DMI of LPS-administered cows remained decreased during P3 (13%; $P < 0.01$; Figure 3.1B). Cr-supplementation tended to decrease DMI (6%) relative to control cows during P3 ($P = 0.10$; Figure 3.1B). Relative to PF controls, milk yield from LPS-administered cows was decreased (58%) during P2 ($P < 0.01$; Figure 3.1C), and P2D1 and P2D2 milk yields were decreased 66 and 49%, respectively ($P \leq 0.01$; Figure 3.1C). Chromium-supplemented cows produced less milk (16%) than control cows throughout the 8 milkings during P2 ($P = 0.05$; Figure 3.1C). The milk yield nadir of LPS treatments occurred 6 h postbolus and was decreased 86% relative to baseline value ($P < 0.01$).

By the end of P2, milk yield from LPS-administered cows increased relative to P2 d1, but remained 61% decreased compared to baseline ($P < 0.01$). After 12 h of pair-feeding, milk yield from PF-Cr cows decreased 40% relative to baseline values ($P = 0.03$), milk yield of PF-CON did not differ from baseline ($P = 0.11$), but was numerically decreased 26%. During the LPS-euglycemic clamp, maintaining euglycemia via exogenous glucose infusion did not rescue milk yield as LPS-euglycemic cows had a 74% decrease in milk yield relative to PF cows. During P3, there was a tendency for a treatment by time interaction where control cows' milk yield remained unchanged while LPS cows gradually approached the yield of the PF cows ($P = 0.06$; Figure 3.1C). Overall during P3, daily milk yield of LPS administered cows remained decreased (24%) relative to PF cows ($P < 0.01$; Figure 3.1C). No effect of diet on milk yield was observed during P3.

Relative to PF cows, milk lactose content from LPS-infused cows decreased (15%; $P < 0.01$; Table 3.2) while milk fat content increased (35%; $P < 0.01$; Table 3.2). Milk protein content of LPS-treated cows increased relative to PF treatments (21%; $P < 0.01$; Table 3.2). There was a treatment by time interaction ($P = 0.04$; Table 3.2) for MUN such that LPS-Cr cows experienced an increase between 18 and 42 h postbolus, while MUN from PF-Cr cows decreased. All treatments returned to baseline values by 48 h postbolus. Milk SCC from all treatments increased with time ($P = 0.05$; Table 3.2). No treatment differences were observed in any milk component content during P3.

A period of hyperglycemia (~180 min) was observed post-LPS administration, after which continuous glucose infusion was necessary to maintain euglycemia ($P < 0.01$; Figure 3.2A). The ROGI increased with time ($P < 0.01$), but was not affected by diet (Figure 3.2A). Total mean glucose deficit accumulated over the 12 h was 120, 319, 1,772, and 1,781 g for PF-

CON, PF-Cr, LPS-CON, and LPS-Cr treatments, respectively (Figure 3.2B). Chromium supplementation had no effect on the total glucose deficit. The 48-h milk glucose deficit was increased 4-fold in LPS treatments, relative to PF controls ($P < 0.01$, data not shown). Interestingly, the milk glucose deficit of Cr supplemented cows was numerically increased, relative to CON supplemented cows ($P = 0.15$).

Insulin concentrations were increased 9- and 15-fold in LPS administered cows at 6 and 12 h, respectively, relative to PF cows ($P < 0.01$; Figure 3.3A). Circulating insulin from LPS cows remained increased (81%) between 18 and 48 h postbolus, relative to PF cows ($P = 0.04$). At 6 h postbolus, insulin concentrations from the LPS-Cr cows were decreased (48%), relative to LPS-CON cows ($P = 0.02$). Circulating NEFA in PF cows increased between 6 and 18 h postbolus, while NEFA from LPS treatments did not increase until 18 h postbolus. Circulating NEFA from cows in all treatments gradually declined between 18 and 48 h ($P < 0.01$; Figure 3.3B). Circulating BHB tended to be decreased in LPS versus PF treatments (16%; $P = 0.07$; Figure 3.3C) and increased in all treatments with time ($P < 0.01$). Blood urea nitrogen concentrations increased in LPS administered cows compared to PF cows (31%; $P = 0.02$; Figure 3.3D). There was a tendency for a treatment by time interaction, such that BUN from LPS-Cr cows continued to increase between 24 and 36 h postbolus, while BUN in cows from all other treatments began to decrease ($P = 0.08$; Figure 3.3D). Except insulin, Cr supplementation had no effect on the aforementioned circulating metabolites.

In LPS-administered cows, circulating LBP was increased relative to PF cows (78%; $P < 0.01$; Figure 3.4A), peaking at a 121% increase 24 h postbolus. Relative to PF cows, SAA concentrations were increased 5-fold ($P < 0.01$; Figure 3.4B) and continually increased over all time points measured ($P < 0.01$). There was a treatment by time interaction for haptoglobin

concentrations, such that LPS treatments increased between 24 and 48 h postbolus, while PF treatments remained at baseline ($P < 0.01$; Figure 3.4C). Interestingly, there was a decrease (51%) at 48 h postbolus in LPS-Cr, relative LPS-CON cows ($P = 0.02$). Blood ionized calcium of LPS administered cows was decreased 39% at 12 h postbolus and temporally increased to within 12% of PF cows by 48 h postbolus ($P < 0.01$; Figure 3.4D).

Circulating white blood cells (**WBC**) were initially decreased between 3 and 12 h postbolus (about 60%) in LPS cows, relative to PF cows ($P < 0.01$; Figure 3.5A), after which LPS cows developed leukocytosis (59% increase, relative to PF; $P < 0.01$). Circulating neutrophils reflected a similar pattern to that of circulating WBCs ($P < 0.01$; Figure 3.5B). While Cr supplementation had no effect on overall neutrophil number during P2, post-hoc analysis revealed LPS-Cr cows had a 16% increase in their peak neutrophil number at 30 h postbolus ($P = 0.05$) and a 14% increase at 36 h postbolus, relative to LPS-CON ($P = 0.09$; Figure 3.5B). Further, between 18 and 48 h postbolus LPS-Cr cows had increased neutrophil number (12%; $P = 0.04$), relative to LPS-CON cows.

Patterns of circulating lymphocytes and monocytes also mirrored total WBC patterns with an initial marked decrease followed by a steady increase ($P = 0.01$; Figure 3.5C; Table 3.3). Circulating lymphocytes and monocytes of LPS treatments exceeded PF treatments at 48 h postbolus and were increased 3 and 28%, respectively ($P < 0.01$). Overall, Cr-supplemented cows had a 22% increase in monocyte cell number, relative to control ($P = 0.03$; Table 3.3). Chromium supplementation decreased (24%) eosinophil number in PF-Cr cows relative to PF-CON cows ($P = 0.01$; Table 3.3). Platelets were decreased in LPS-administered cows relative to PF cows (52%; $P < 0.01$; Table 3.3). Platelet counts from LPS-Cr cows were decreased 54% compared to LPS-CON cows ($P < 0.01$).

Discussion

Immunostimulation homeorhetically alters systemic metabolism in a coordinated effort to meet the energetic demands of leukocytes. In response to immunogenic stimuli, immune cells become obligate glucose utilizers, and they switch their energetic metabolism from oxidative phosphorylation to aerobic glycolysis. This is known as the Warburg effect and allows for more rapid ATP production (Palsson-McDermott and O'Neill, 2013). Additionally, glucose derived carbon is directed through the pentose phosphate pathway to produce important intermediates to support proliferation and production of reactive oxygen species (Calder et al., 2007; Palsson-McDermott and O'Neill, 2013).

Delivering energetic substrates to activated leukocytes is a critical component in successfully mounting an effective immune response (MacIver et al., 2008). Bovine monocytes, neutrophils, and lymphocytes have GLUT3 and GLUT4 transporters (Maratou et al., 2007; O'Boyle et al., 2012) and O'Boyle and others (2012) have demonstrated increased mRNA expression of GLUT3 and GLUT4 upon endotoxin stimulation. Improved glucose availability to activated leukocytes increases their longevity and function (e.g., phagocytosis, reactive oxygen species production; Sagone et al., 1974; Furukawa et al., 2000; Healy et al., 2002; Garcia et al., 2015). Lee et al. (2000) reported increased glucose uptake and macrophage function with Cr supplementation in vitro, likely due to Cr's role in improving insulin sensitivity. Ensuring an adequate glucose supply for immune cells is not limited to leukocyte glucose uptake mechanisms, but also relies on altered systemic glucose partitioning and availability. During immunoactivation, skeletal muscle and adipose tissue become insulin resistant and the mammary gland reduces glucose uptake; these energetic shifts reflect synchronized efforts to spare glucose for the new dominant physiological state (Maitra et al., 2000). Therefore, we hypothesized Cr

would increase leukocyte glucose consumption and thus increase the total glucose deficit during an LPS-euglycemic clamp.

Herein, we demonstrated successful immunoactivation as reflected by increased circulating acute phase proteins (APP; including SAA, Hp, and LBP), mild febrile response, initial leukopenia and subsequent leukocytosis, hypogalactia, and hypophagia. The transient febrile response observed is similar to previous studies (Giri et al., 1990; Waldron et al., 2003a; Vernay et al., 2012; Moyes et al., 2014) and agrees with Lang et al. (1987) that increased glucose utilization continues independently of increased rectal temperature during immunoactivation.

Leukopenia occurred immediately following LPS administration and this agrees with previous ruminant reports (Griel et al., 1975; Gerros et al., 1995; Bieniek et al., 1998). Leukopenia likely represents immune cell infiltration into tissues or may be explained by shifts from circulating systemic leukocytes into the margined pool (Hoedemaker et al., 1992; Lang et al., 1992). Within 12-18 h postbolus, lymphocyte and monocyte counts had returned to pre-infusion levels; however, neutrophil counts exceeded baseline values by 12 h postbolus. Subsequent neutrophilia may indicate increased neutrophil production, demargination from pulmonary circulation, or decreased endothelial adherence (Jagels and Hugli, 1994; Opdenakker et al., 1998). Bannerman and others (2008), observed a similar dynamic change in circulating WBC and neutrophils when evaluated over time. The fluctuating temporal pattern of leukocytes demonstrates the importance of frequent and repeated sampling in accurately assessing changes in number and function of WBC following immunoactivation.

The role of Cr in improving leukocyte insulin action has led several to investigate whether it can improve immunity. Although not always consistent (Chang et al., 1996; Kegley et al., 1997b), previous studies have reported improved immune function (e.g., phagocytosis,

blastogenic response, antibody production) of activated leukocytes when animals were supplemented with Cr (Moonsie-Shageer and Mowat, 1993; Chang et al., 1996; Lee et al., 2000). Increased cytokine production with Cr supplementation has also been observed in mice, steers, and lactating cows (Burdick et al., 2011; Yuan et al., 2014; Jin et al., 2016). Herein we observed increased circulating neutrophil counts in LPS-administered cows supplemented with Cr, and this agrees with others (Kafilzadeh et al., 2012; Yasui et al., 2014; Mayorga et al., 2016). While mechanisms for increased circulating neutrophils with Cr supplementation are not fully understood, it may relate to increased insulin sensitivity within bone marrow, the site of immune cell proliferation (Faulhaber et al., 2009). Increased neutrophils in the blood of Cr-supplemented cows may also be explained by reduced rates of apoptosis mediated by Cr-induced increased glucose uptake (Healy et al., 2002). Immunomodulatory effects of Cr have also been attributed to its counteractive effects on immunosuppressors such as glucocorticoids, namely by decreasing circulating cortisol (Kafilzadeh et al., 2012). Regardless of the mechanism, it is of interest to determine whether Cr-induced increases in neutrophils translate into improved whole-animal “well-being” and productivity.

In addition to markedly altering immune cell dynamics, LPS initiates a well-characterized biphasic response in circulating glucose with an initial period of hyperglycemia followed by severe hypoglycemia (Lohuis et al., 1988; Waldron et al., 2003a; Zarrin et al., 2014). In agreement with Kvidera and colleagues (2017a), LPS administration induced marked hyperglycemia for the first ~180 min postbolus after which continuous glucose infusion was necessary to maintain euglycemia. Bernhard and others (2012) reported increased peak circulating glucose in Cr-supplemented steers relative to controls immediately following LPS exposure. The increased hyperglycemic response may reflect an increased capacity to store liver

glycogen and thus Cr-fed animals would have more extensive capacity for glycogenolysis and hepatic glucose output. Improved liver glycogen storage with Cr supplementation has been observed previously (Rosebrough and Steele, 1981; Brooks et al., 2016). However, no differences in peak glucose response following LPS administration were observed in the present study and this agrees with others (Lee et al., 2000; Lein et al., 2005). Reasons for inconsistencies in the LPS-induced hyperglycemic response with Cr supplementation are not clear, but may be explained by small sample size, differences in species and/or physiological states, Cr dose, or LPS dose and administration routes.

We predicted the LPS dose selected for the current study would decrease milk yield by 40-50%; however, despite the LPS dose being only 25% of that of our previous report (Kvidera et al., 2017a), the milk production response and the total glucose deficit accumulated over 12 h were similar amongst studies. This likely indicates the dose in both experiments (0.375 and 1.5 $\mu\text{g/kg BW}$) was large enough to induce the maximal response in leukocyte glucose consumption. When evaluated on a metabolic BW basis, the glucose requirement of the immune system in the current study is approximately 1.0 g/kg of BW^{0.75}/h, comparable to 0.66, 1.0, and 1.1 g/kg of BW^{0.75}/h reported in cows, steers, and pigs, respectively (Kvidera et al., 2016, 2017a,c). In contrast to our hypothesis, no differences in the total glucose deficit were detected between LPS-CON and LPS-Cr cows. However, the current model does not account for liver's contribution (via gluconeogenesis or glycogenolysis) to the circulating glucose pool, a clear limitation to the experimental model. In particular, not accounting for glycogenolysis during the hyperglycemic phase causes the total glucose deficit to be underestimated (i.e. we are unable to estimate immune system's glucose use during the first ~180 min). Additionally, increased gluconeogenic rates occur following LPS exposure in small animal models (Spitzer et al., 1985; McGuinness,

1994) and ruminants (Waldron et al., 2003b), thus hepatic glucose output during immunoactivation increases because of both glycogenolysis and gluconeogenesis. Further, previous studies have reported increased circulating glucagon in Cr supplemented cows (Smith et al., 2008; Sadri et al., 2012) and, although speculation, increased gluconeogenesis in response to increased glucagon may allow for increased glucose availability to immune cells, which could not be detected with the current model. Interestingly, we observed an improved immune response, as indicated by increased neutrophil number, in LPS-Cr cows despite no differences in glucose deficit. Therefore, further investigating the contribution of glycogenolysis and gluconeogenesis to leukocyte glucose utilization and the potential beneficial effect Cr has on this response is of both academic and practical interest.

Determining differences in production outcomes was not the primary objective, as the experiment was knowingly underpowered to detect treatment differences in such variable measurements. Nonetheless, analyzing and presenting production variables allows for the primary aims (glucose dynamics and immune responses) to be put into context. Despite maintaining euglycemia, milk yield was not rescued with i.v. glucose infusion (74% decrease at 12 h postbolus), and this agrees with our previous study (Kvidera et al., 2017a) and confirms hypoglycemia is not responsible for reduced milk synthesis during an infection. Decreased milk yield is typical during infection and it represents an important glucose-sparing strategy during immunoactivation. The effects of LPS on milk synthesis may be either direct via binding to mammary epithelial Toll-like receptor 4 or indirect by increased proinflammatory cytokines (Verheijden et al., 1983; Ibeagha-Awemu et al., 2008). Interestingly, milk yield from Cr supplemented cows was decreased 16%, relative to controls throughout the LPS challenge, potentially resulting from Cr-induced increased leukocyte glucose consumption. This tenet is

partially supported by the milk glucose deficit ($P = 0.15$) of Cr supplemented cows being numerically increased, relative to controls. However, the biological importance of this trend and how it relates to our hypothesis requires further investigation.

In order to eliminate the confounding effects of dissimilar nutrient intake, a pair-feeding model was utilized throughout the LPS challenge. Total glucose deficit of PF treatments was accounted for exclusively by the decrease in milk yield, whereas the deficit of LPS treatments included the decrease in milk yield in addition to infused glucose. Although cows remained on the same plane of nutrition, the milk glucose deficit of LPS treatments was 4-fold greater than the PF controls. Despite a variety of studies demonstrating beneficial effects of Cr supplementation on DMI and milk yield (Hayirli et al., 2001; Smith et al., 2005; McNamara and Valdez, 2005) we observed little or no differences in traditional productive traits between control and Cr treatments during P2. At the initiation of P3 all treatments were allowed to consume feed ad libitum; however, DMI and milk yield from previously LPS infused cows remained below that of PF treatments. Further, DMI of Cr supplemented treatments tended to be decreased relative to controls. The delayed return to baseline feed intake and milk yield agrees with others (Waldron et al., 2003a; Bannerman et al., 2008; Aditya et al., 2017) and demonstrates the long term consequences of inflammation. It is possible the improved DMI previously reported by others supplementing Cr was not observed in the current study due to small sample size and severity of the immune insult.

Despite being in a catabolic state, ruminant and monogastric animals become hyperinsulinemic following LPS exposure (Waldron et al., 2003a; Zarrin et al., 2014; Kvidera et al., 2017a). The mechanism of LPS-induced hyperinsulinemia has not been fully elucidated but it could be indirect via glucagon-like peptide 1 (Nguyen et al., 2014) or the direct result of LPS on

glucose stimulated insulin secretion as we have recently reviewed (Baumgard et al., 2016); Regardless, insulin serves as an important mediator of glucose uptake by activated immune cells (Calder et al., 2007) and LPS-triggered hyperinsulinemia maybe a conserve evolutionary strategy. Continuous glucose infusion may partially explain the hyperinsulinemic state, as an interaction between LPS and glucose infusion has been suggested (Blackard et al., 1976). However, our previous study observed no differences in insulin between cows maintained at euglycemia and cows allowed to develop hypoglycemia, confirming glucose infusion is not the prime mediator of hyperinsulinemia and the continued increase in circulating insulin occurs for hours despite eventual LPS-induced hypoglycemia (Kvidera et al., 2017a). Consequently, acute hyperglycemia is not responsible for the chronic hyperinsulinemia. Further, fasted pigs administered LPS become hyperinsulinemic, despite having a blunted hyperglycemic response (Kvidera et al., 2017c). Interestingly, we observed an attenuated insulin response during the hyperglycemic phase in cows supplemented with Cr. There is conflicting evidence on Cr's role in the insulin response to a glucose tolerance test as both increased (Striffler et al., 1995; Kegley et al., 2000) and decreased (Subiyatno et al., 1996; Kegley et al., 1997a; Hayirli et al., 2001; Stahlhut et al., 2006; Leiva et al., 2015) concentrations have been observed. With no differences observed in total glucose deficit, the decreased insulin response in Cr supplemented cows suggests increased insulin sensitivity, presumably, of activated leukocytes.

As would be expected with insulin's antilipolytic characteristics, there was a blunted circulating NEFA response during the first 12 h of the LPS challenge, after which NEFA began to increase. This disagrees with many studies which observe a steady increase in NEFA post LPS bolus (Waldron et al., 2003a; Graugnard et al., 2013; Moyes et al., 2014). Insulin-mediated lipolysis inhibition may also be influenced by hyperlactatemia as previously described by Ahmed

and colleagues (2010). Despite previous transition cow literature (Hayirli et al., 2001; Kafilzadeh et al., 2012) no effects of Cr were observed on NEFA mobilization in the current study. The temporal pattern of ketones was very different than NEFA, as circulating BHB initially decreased in response to LPS before gradually returning to baseline values, and this agrees with other ruminant models (Waldron et al., 2003a; Graugnard et al., 2013; Zarrin et al., 2014). The mechanism behind decreased BHB is not fully understood, but it may be due to increased ketone utilization by peripheral tissue in an effort to spare glucose for the immune system (Zarrin et al., 2014). Understanding the LPS-mediated changes in NEFA and BHB metabolism would presumably provide key insight to how fuel preferences and nutrient partitioning are reprioritized during immunoactivation.

Sepsis-induced muscle proteolysis is another strategy employed to support the demand for gluconeogenic precursors and amino acids for APP synthesis. Due to differences in amino acid composition between APP and skeletal muscle, a considerable amount of muscle proteolysis must occur to support APP synthesis (Reeds et al., 1994). In ruminant models, BUN can be hard to interpret as muscle proteolysis is not the sole source of urea. Similar to the other metabolites, little change in BUN was observed during the first 12 h postbolus; however, beginning after 12 h, there was a progressive increase over time. This may be due to cessation of the clamp and thus greater need for gluconeogenic precursors to supply glucose. Interestingly, BUN from LPS-Cr cows was increased relative to all other treatments. In a non-inflammatory state, Cr supplementation would normally increase muscle insulin sensitivity; a scenario that would presumably blunt proteolysis, but this is clearly not the case in an immunoactivated state. Evaluating direct markers of protein degradation, such as 3-methyl histidine, may allow for a more accurate assessment of Cr's role in LPS induced proteolysis.

Severe hypocalcemia is a well described response to endotoxin exposure (Waldron et al., 2003b; Holowaychuk et al., 2009) and it was sustained throughout the sampling period in the current study. Calcium prevents LPS disaggregation and interferes with LPS binding to lipoproteins (Munford et al., 1981). Thus, LPS-induced hypocalcemia may be a survival mechanism to promote LPS detoxification via lipoproteins in order to prevent an over-inflammatory response (Skarnes, 1968). Gaining a better understanding of the direct and indirect role Ca plays in immune function has practical implications to the dairy industry.

In agreement with our previous clamp studies, it is clear that immunoactivation causes drastic changes in glucose metabolism. Taken together, our results suggest Cr has little effect on total glucose deficit in response to severe LPS challenge. However, future work should focus on evaluating whether Cr increases hepatic gluconeogenesis rates during immunoactivation, as this may explain why no overall differences were observed in glucose deficit in the current study. Further, focusing on the molecular changes in insulin signaling and GLUT expression of immune cells isolated from Cr-supplemented cows administered LPS may allow for a better understanding of the mechanism by which Cr is functioning. Interestingly, in the current study the increased neutrophil number occurred despite no differences in total glucose deficit, suggesting more efficient neutrophil glucose utilization with Cr supplementation.

Conclusion

Our results corroborate data collected from our previous clamp studies indicating that a substantial amount of glucose is utilized in response to an immunogenic stimuli. Further, we have demonstrated potential immunomodulatory effects (e.g., increased circulating neutrophils) of Cr supplementation following immunoactivation. The increase in circulating immune cells was observed despite no detected effect of Cr on total glucose deficit following LPS administration. In addition, Cr supplementation decreased circulating insulin in response to LPS,

highlighting its potential role in improving immune cell insulin sensitivity. Future work should focus on the direct effects of Cr on immune cell signaling and glucose utilization, and hepatic glucose metabolism in response to inflammation.

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Tables and Figures

Table 3.1. Ingredients and composition of diet

Ingredient	% of DM ²
Corn Silage	33.6
Corn Gluten Feed	19.1
Alfalfa Hay	15.8
Lactation Grain	11.7
Ground Corn	8.2
Whole Cottonseed	4.8
Molasses	2.8
Expeller Soybean Meal	2.4
Soybean Meal	1.7
Chemical analysis, % of DM	
Starch	20.7
CP	19.2
NDF	34.8
ADF	21.5
Fat	4.98
NE _L Mcal/kg DM	1.57

¹Values represent an average of ration nutrient summary reports collected throughout the trial. Diet moisture averaged 52.49%.

²Average nutrient levels: 0.99% Ca, 0.53% P, 0.39% Mg, 0.25% S, 1.42% K, 0.51% Na, 0.55% Cl, 68.25 ppm of Zn, 45.66 ppm of Mn, 3.62 ppm of Fe, 13.92 ppm of Cu, 0.78

Table 3.2. Milk composition of cows fed a control (CON) diet or a diet supplemented with chromium propionate (Cr) followed by administration of a saline or LPS bolus

of a sum of LPS doses											
Parameters	Trt ¹				SEM	<i>P</i>			Contrasts ²		
	PF-CON	PF-Cr	LPS-CON	LPS-Cr		Trt	Time	Trt × Time	CON v Cr	PF v LPS	LPS-CON v LPS-Cr
Period 2											
Milk Fat, %	4.19 ^a	4.33 ^a	5.32 ^{ab}	6.16 ^b	0.42	0.01	<0.01	0.25	0.25	<0.01	0.16
Lactose, %	4.62 ^b	4.53 ^b	3.89 ^a	3.85 ^a	0.11	<0.01	0.17	0.26	0.61	<0.01	0.83
Protein, %	3.38 ^a	3.24 ^a	3.70 ^a	4.31 ^b	0.18	<0.01	<0.01	0.01	0.21	<0.01	0.03
SCC	331	525	554	616	135	0.44	0.05	0.35	0.38	0.26	0.78
MUN, mg/dL	15.57	14.40	16.52	18.13	1.21	0.20	0.05	0.04	0.86	0.06	0.36
Period 3											
Milk Fat, %	3.95	4.03	4.58	5.15	0.51	0.36	0.24	0.50	0.54	0.12	0.45
Lactose, %	4.55	4.56	4.35	4.46	0.11	0.55	0.02	0.46	0.64	0.22	0.54
Protein, %	3.46	3.29	3.68	3.76	0.29	0.68	0.04	0.75	0.87	0.27	0.86
SCC	305	343	943	436	435	0.79	0.05	0.83	0.62	0.43	0.49
MUN, mg/dL	14.78	13.88	12.32	14.70	0.91	0.25	0.05	0.65	0.43	0.37	0.09

¹PF-CON=Pair-fed, saline bolus, control; PF-Cr=Pair-fed, saline bolus, chromium; LPS-CON=ad libitum, LPS bolus, control; LPS-Cr=ad libitum, LPS bolus, chromium

²CON = PF-CON and LPS-CON treatments; Cr = PF-Cr and LPS-Cr treatments; LPS = LPS-CON and LPS-Cr treatments

^{a,b}Values within a row with differing superscripts denote differences ($P < 0.05$) between treatments.

Table 3.3. Complete blood count parameters in cows fed the control (CON) diet or chromium propionate (Cr) followed by administration of a saline or LPS bolus

Parameters	Trt ¹				SEM	<i>P</i>			Contrasts ²		
	PF-CON	PF-Cr	LPS-CON	LPS-Cr		Trt	Time	Trt × Time	CON v Cr	PF v LPS	LPS-CON v LPS-Cr
Platelets, ×10 ³ /μL	310.7 ^c	344.1 ^c	214.4 ^b	98.8 ^a	28.3	<0.01	0.39	0.16	0.18	<0.01	0.01
Monocytes, ×10 ³ /μL	0.33 ^b	0.43 ^c	0.22 ^a	0.24 ^a	0.03	<0.01	<0.01	<0.01	0.03	<0.01	0.60
Eosinophils, ×10 ³ /μL	0.45 ^c	0.34 ^b	0.21 ^a	0.23 ^a	0.03	<0.01	<0.01	<0.01	0.10	<0.01	0.67

¹PF-CON=Pair-fed, saline bolus, control; PF-Cr=Pair-fed, saline bolus, chromium; LPS-CON=ad libitum, LPS bolus, control; LPS-Cr=ad libitum, LPS bolus, chromium

²CON = PF-CON and LPS-CON treatments; Cr = PF-Cr and LPS-Cr treatments; LPS = LPS-CON and LPS-Cr treatments, PF = PF-CON and PF-Cr treatments

^{a,b,c}Values within a row with differing superscripts denote differences ($P < 0.05$) between treatments.

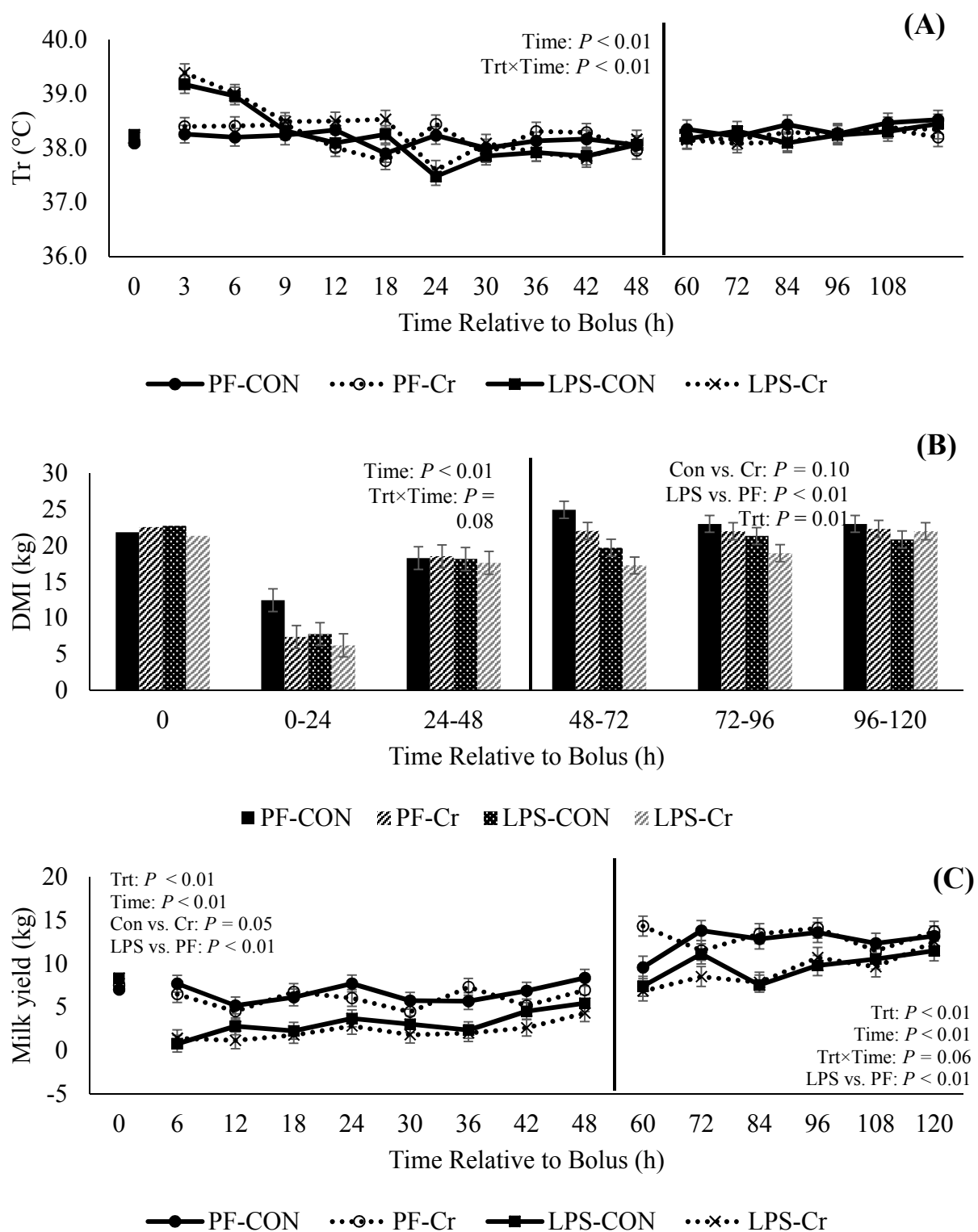


Figure 3.1. Effects of a LPS bolus or pair-feeding in cows fed a control diet (CON) or chromium propionate (Cr) supplemented diet on (A) rectal temperature, (B) DMI, and (C) milk yield. The solid line separates P2 from P3 and represents two separate statistical models.

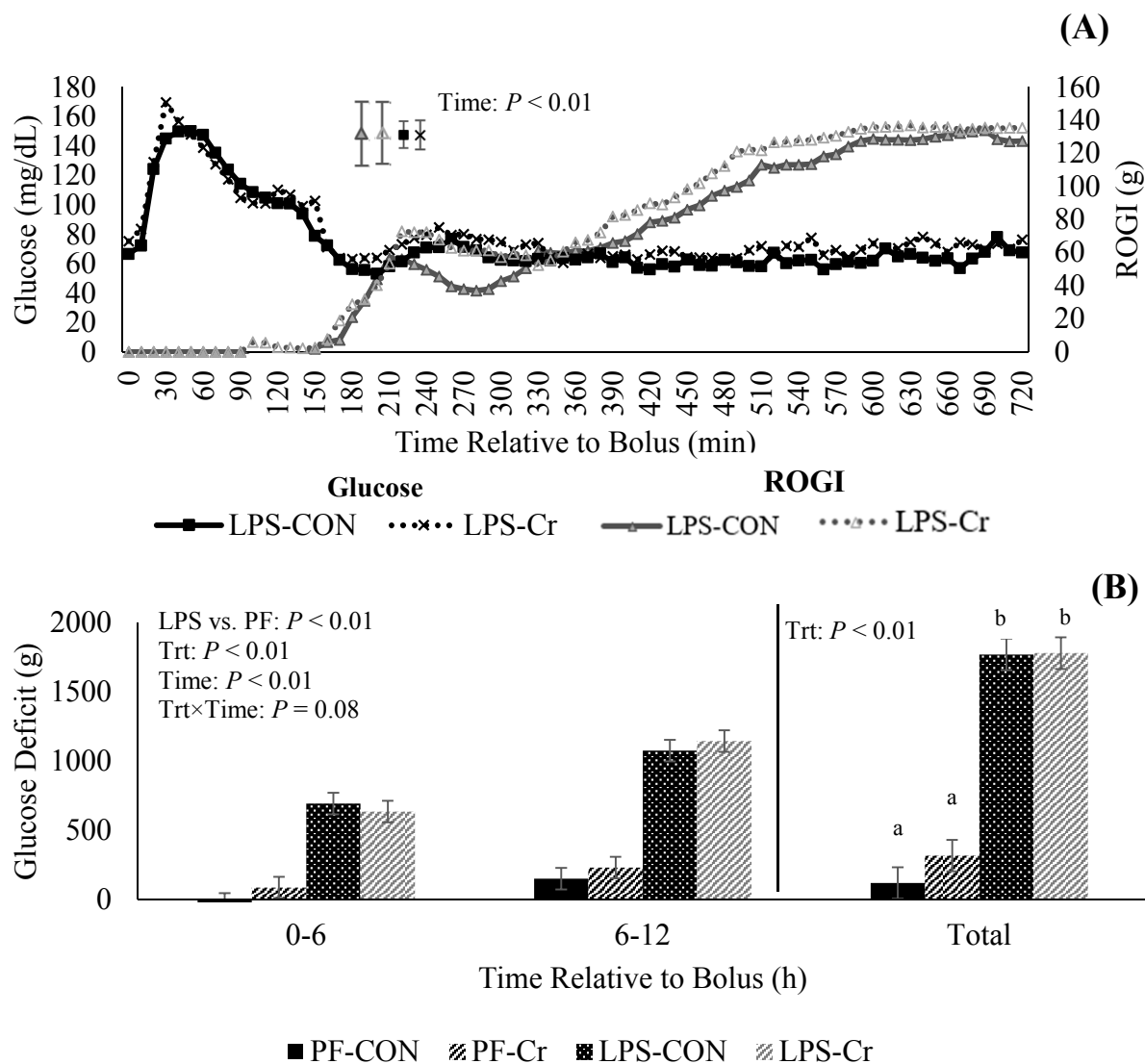


Figure 3.2. Effects of a LPS bolus in cows fed a control diet (CON) or chromium (Cr) propionate supplemented diet on (A) circulating glucose and rate of glucose infusion (ROGI). Effects of a LPS bolus or pair-feeding in cows fed a control diet (CON) or chromium (Cr) propionate supplemented diet on (B) milk and/or total glucose deficit from 0-6, 6-12, and accumulated over 12 h. ^{a,b,c}Values with differing superscripts denote differences ($P < 0.05$) between treatments. Standard error of the mean is represented by the vertical bars.

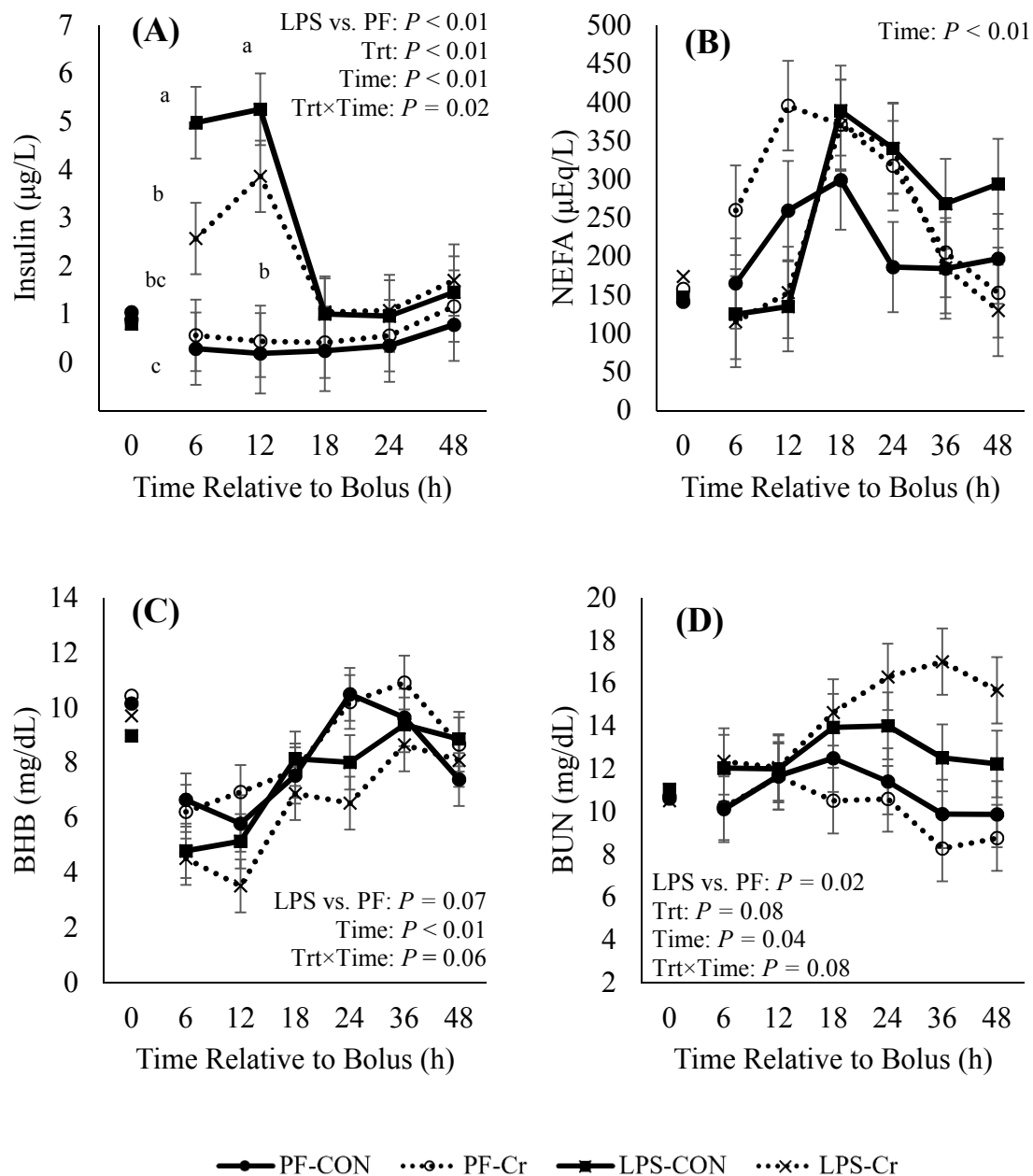


Figure 3.3. Effects of a LPS bolus or pair-feeding in cows fed a control diet (CON) or chromium (Cr) propionate supplemented diet on circulating (A) insulin, (B) nonesterified fatty acids (NEFA), (C) BHB, and (D) BUN. ^{a,b,c}Values with differing superscripts denote differences ($P < 0.05$) between treatments.

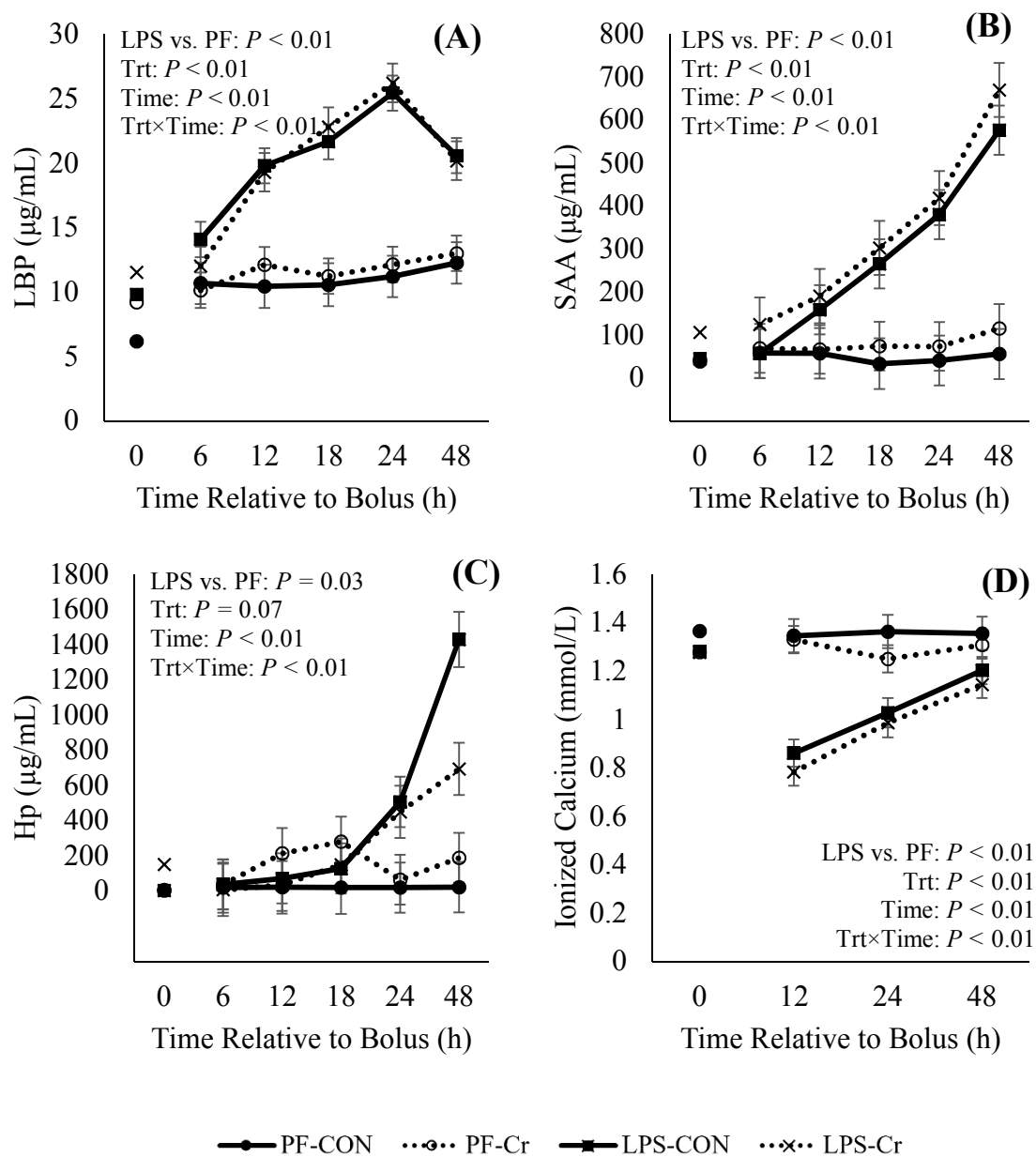


Figure 3.4. Effects of a LPS bolus or pair-feeding in cows fed a control diet (CON) or chromium (Cr) propionate supplemented diet on circulating (A) LPS-binding protein (LBP), (B) serum amyloid A (SAA), (C) haptoglobin (Hp), and (D) ionized calcium.

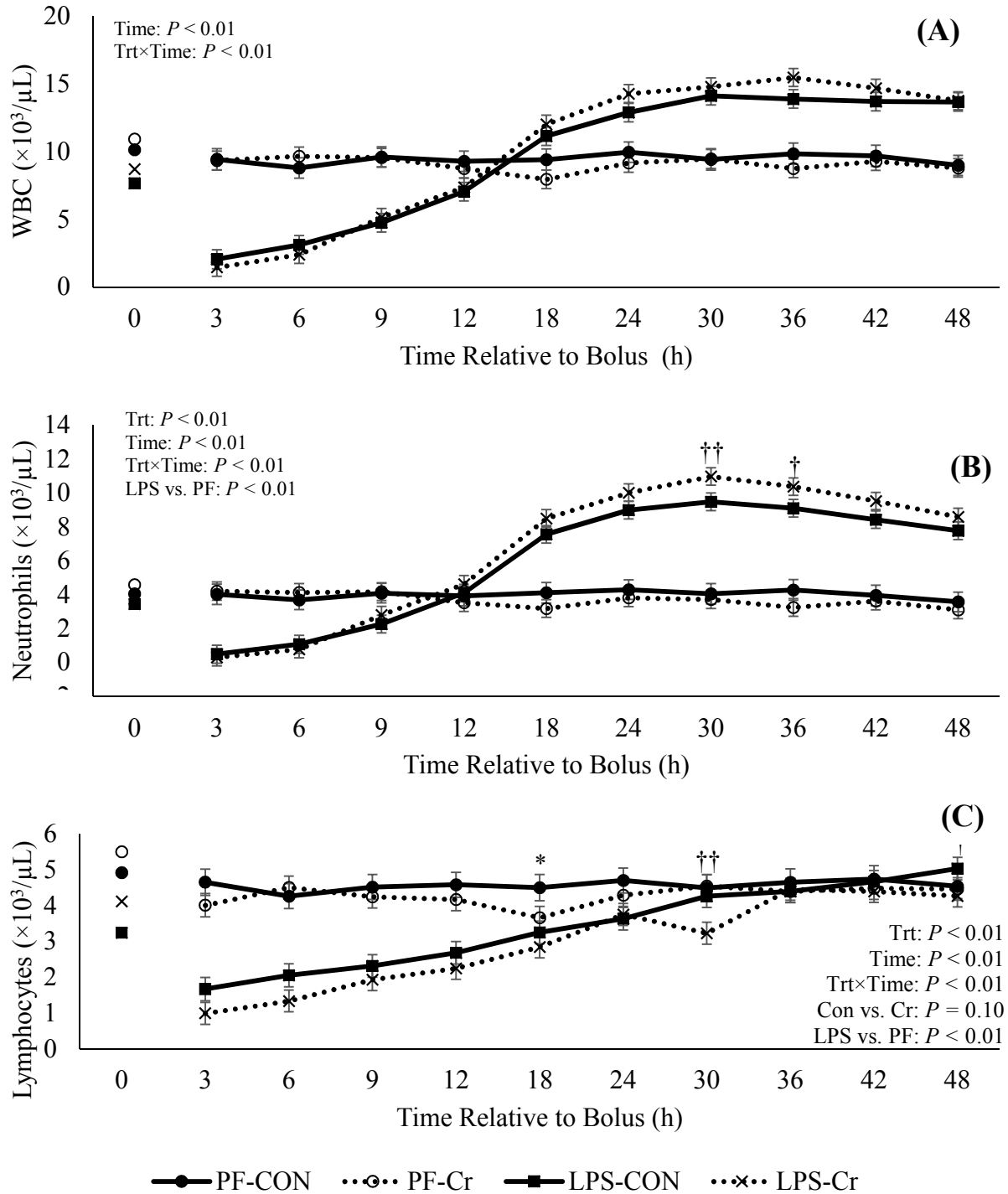


Figure 3.5. Effects of a LPS bolus or pair-feeding in cows fed a control diet (CON) or chromium (Cr) propionate supplemented diet on circulating (A) white blood cell (WBC), (B) neutrophil, and (C) lymphocyte counts. † represents a tendency for a difference between LPS-CON and LPS-Cr treatments ($P < 0.10$), †† represents a significant difference between LPS-CON and LPS-Cr treatments ($P < 0.05$), * represents a tendency for a difference between PF-CON and PF-Cr treatments ($P < 0.10$), and ** represents a significant difference between PF-CON and PF-Cr treatments ($P < 0.05$).

CHAPTER 4. EFFECTS OF DIETARY ZINC SOURCE ON THE METABOLIC AND IMMUNOLOGICAL RESPONSE TO LIPOPOLYSACCHARIDE IN LACTATING HOLSTEIN DAIRY COWS

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Abstract

Objectives were to evaluate the effects of replacing 40 ppm of Zn from Zn sulfate (CON) with Zn amino acid complex (AvZn) on metabolism and immunological responses following an i.v. lipopolysaccharide (LPS) challenge in lactating cows. Cows were randomly assigned to 1 of 4 treatments: 1) pair-fed (PF) control (PF-CON; 5 mL saline; n=5), 2) PF AvZn (PF-AvZn; 5 mL saline; n=5), 3) LPS-euglycemic clamp control (LPS-CON; 0.375 µg LPS/kg BW; n=5), 4) LPS-euglycemic clamp AvZn (LPS-AvZn; 0.375 µg LPS/kg BW; n=5). Cows were enrolled in 3 experimental periods (P); during P1 (3 d), cows received their respective dietary treatments and baseline data was obtained. During P2 (2 d), a 12 h LPS-euglycemic clamp was conducted or cows were PF to their respective dietary counterparts. During P3 (3 d), cows received their dietary treatment and consumed feed ad libitum. Mild hyperthermia (1°C) was observed in LPS cows at 3 h postbolus. Throughout P2, rectal temperature of LPS-AvZn cows was decreased (0.3°C) relative to LPS-CON cows. Administering LPS decreased dry matter intake (DMI; 47%) during P2, and by experimental design the pattern was similar in PF cohorts. During P3, DMI from LPS cows remained decreased (15%) relative to PF cows. Milk yield from LPS cows decreased (54%) during P2 relative to PF cows, but it was similar during P3. During P2, somatic cell count (SCC) increased (3-fold) in LPS cows, relative to PF controls. Dietary AvZn tended to

decrease SCC (70%) during P3 relative to LPS-CON cows. Insulin increased 7-fold in LPS cows at 12 h postbolus and remained increased (4-fold) for the duration of P2. Circulating glucagon from LPS cows increased (65%) during P2, and supplementing AvZn blunted the increase (30% relative to LPS-CON). During P2, circulating cortisol increased (7-fold) post-LPS infusion relative to PF cows and supplementing AvZn decreased cortisol (58%) from 6-48 h postbolus relative to LPS-CON cows. Administering LPS increased circulating LPS-binding protein and serum amyloid A (SAA; 3- and 9-fold, respectively) relative to PF cows. Compared to LPS-CON, LPS-AvZn cows had increased circulating SAA (38%) 24 h postbolus. The 12 h total glucose deficit was 36 and 1,606 g for the PF and LPS treatments, respectively, but was not influenced by Zn source. In summary, replacing a portion of the Zn sulfate with Zn amino acid complex appeared to reduce the inflammatory response, but had no effect on the glucose deficit.

Keywords: leukocyte, cortisol, somatic cell count

Introduction

Uterine, mammary, and intestinal epithelial can become compromised due to a variety of stressors (Khafipour et al., 2009; Kvidera et al., 2017c; Koch et al., 2019) and hyperpermeability of these barriers permits pathogen infiltration and ultimately immune stimulation (van Miert and Frens, 1968). Regardless of the pathogen's origin, a robust and transient immune response is imperative to successful pathogen clearance, resolution of inflammation, and return to optimal production ([i.e., milk production, growth, reproduction]; Bertoni et al., 2008; Bradford et al., 2015; Trevisi and Minuti, 2018). Mounting a successful immune response is dependent on effective leukocyte communication (cytokine and chemokine production), extravasation, cellular function (i.e., intracellular killing, reactive oxygen species production, cytokine production), and the elimination of cell debris and harmful byproducts. The energetic costs of initiating an immune response are substantial (Johnson, 2012) and this is particularly true for glucose

(Kvidera et al., 2016, 2017a,b; Horst et al., 2018). Therefore, dietary strategies capable of improving barrier integrity and immune responses are of practical and economic interest, particularly in lactating dairy cows because glucose is critical for copious milk synthesis (Baumgard et al., 2017).

Zinc plays a pivotal role as a structural, catalytic, and signaling component. While it has multiple responsibilities, its immunomodulatory effects have received considerable attention (Osorio et al., 2016; Wessels et al., 2017). Zinc is required for proper epithelial barrier function and regulates damaged epithelium regeneration (Alam et al., 1994). Furthermore, leukocyte formation, maturation and differentiation, activation, cellular function, and apoptotic rate (among others) are Zn dependent and are largely influenced by changes in Zn homeostasis (Haase and Rink, 2014; Wessels and Cousins, 2015). Thus, providing dietary sources of Zn (with optimal bioavailability), may benefit leukocyte function/communication and consequently could influence the overall energetic cost of immunoactivation. Research suggests organic trace minerals improve bioavailability and mineral retention in comparison to inorganic forms (Spears, 1989). Further, altered cytokine and acute phase protein (**APP**) production have been observed with supplemental complexed Zn in ruminants and monogastrics when compared to inorganic forms (Abuajamieh, 2016b; Mayorga et al., 2018).

We hypothesized that feeding complexed Zn would improve the immune response to an LPS challenge and alter leukocyte glucose consumption. Therefore, study objectives were to evaluate the effects of supplemental Zn amino acid complex on productivity, metabolism, glucose consumption and circulating leukocyte dynamics and function in an acute and intensely activated immune system via the lipopolysaccharide (LPS)-euglycemic clamp technique.

Materials and Methods

Animals and Experimental Design

All procedures were approved by the Iowa State University Institutional Animal Care and Use Committee. Twenty non-pregnant lactating Holstein cows (715 ± 19 kg BW; 284 ± 29 DIM; parity 2 ± 0.2) were utilized in an experiment conducted in two replications (10 cows/replicate). Cows were randomly assigned to 1 of 2 dietary treatments: 1) control (**CON**; 64 ppm supplemental Zn from ZnSO₄) or 2) replacement of 40 ppm ZnSO₄ with Zn amino acid complex (**AvZn**; AvailaZn, Zinpro Corporation, Eden Prairie, MN). Actual dietary Zn concentrations (Dairyland Laboratories Inc. Arcadia WI), were 111 and 112 ppm for the CON and AvZn diets, respectively. Prior to the start of the study (i.e., before cows were moved into individual pens), cows were fed their respective diets for 42 d at the Iowa State University Dairy Farm (Ames). After the initial feeding phase, cows were moved to individual box-stalls (4.57×4.57 m) at the Iowa State University Dairy. Cows were allowed 4 d to acclimate to housing conditions; during this time they were implanted with bilateral jugular catheters as previously described (Baumgard et al., 2011). The trial consisted of 3 experimental periods (P). Period 1 (P1) lasted 3 d and served as the baseline (data generated for covariate analysis) for periods 2 (P2) and 3 (P3). At the initiation of P2, which lasted 48 h, animals experienced either a 12 h lipopolysaccharide (LPS)-euglycemic clamp as previously described (Kvidera et al., 2016, 2017a; Horst et al., 2018) or were pair-fed (PF) to their respective dietary counterparts for the entire 48 h to eliminate the confounding effects of dissimilar nutrient intake. Dietary and challenged combinations resulted in 4 total treatments: 1) PF control diet (**PF-CON**; 5 mL sterile saline with control diet; $n = 5$), 2) PF AvZn diet (**PF-AvZn**; 5 mL sterile saline with Availa-Zn diet; $n = 5$), 3) LPS-euglycemic clamp control diet (**LPS-CON**; $0.375 \mu\text{g}$ LPS/kg BW with control diet; $n = 5$), and 4) LPS-euglycemic clamp AvZn diet (**LPS-Zn**; $0.375 \mu\text{g}$ LPS/kg BW with Availa-Zn diet; $n = 5$).

During P2, cows receiving LPS were allowed to eat ad libitum, but the saline-infused cows were pair-fed to their dietary counterparts to eliminate the confounding effects of dissimilar feed intake. For pair-feeding calculations, feed intake was averaged for each cow and used as a baseline. During P2, the decrease in feed intake in LPS-CON and LPS-AvZn cows was calculated every day as a percentage of feed intake reduction during P1. The percentage of feed intake reduction was averaged for all cows in the LPS-CON and LPS-AvZn treatments on the two days of P2 and applied individually to the baseline of each cow in the PF-CON and PF-AvZn treatments. During P3 (3 d), all animals continued to receive their dietary treatment, but were allowed to consume feed ad libitum.

All cows were fed a diet formulated to meet or exceed the predicted requirements of energy, protein, minerals and vitamins (NRC, 2001; Tables 4.1 and 4.2). Reduced feed intake in LPS-treated cows during P2 was determined as a percentage of their mean daily ad libitum intake during P1. Throughout the experiment, PF cows lagged 13 d behind LPS infused cows to allow for pair-feeding calculations as we have previously described (Baumgard et al., 2011). In order to more accurately mirror the altered feed intake pattern observed in LPS-administered cows, the amount of feed offered to the PF cows was separated into 3 portions: 10% at 1200, 30% at 1800, and 60% at 0000 h on d 1 of P2 and then 50% at 0600 and 1800 h on d 2 of P2, respectively. Cows were milked 4 times daily (0000, 0600, 1200, and 1800 h) and yield was recorded. A sample for composition analysis was obtained at each milking. Samples were stored at 4°C with a preservative (bronopol tablet; D & F Control System, San Ramon, CA) until analysis by Dairy Lab Services (Dubuque, IA) using AOAC approved infrared analysis equipment and procedures (AOAC International, 1995). Rectal temperature (**Tr**) and respiration rate (**RR**) were recorded after each milking. Rectal temperature was measured using a digital

thermometer (GLA M700 Digital Thermometer, San Luis Obispo, CA). Respiration rate was measured as flank movements during a 15 s interval and later transformed to breaths/min (**bpm**).

To estimate the glucose requirements of an intensely activated immune system, we employed the LPS-euglycemic clamp technique as we have recently described (Kvidera et al., 2016, 2017a; Horst et al., 2018). Selecting the LPS dose was influenced by the magnitude of hypoglycemia observed in earlier reports (Giri et al., 1990; Waldron et al., 2003; Waggoner et al., 2009; Kvidera et al., 2017a). Lipopolysaccharide (*Escherichia coli* O55:B5; Sigma Aldrich, St. Louis, MO) was dissolved in sterile saline at a concentration of 66.6 µg/mL and passed through a 0.2 µm sterile syringe filter (Thermo Scientific; Waltham, MA). The total volume of LPS solution administered was approximately 4 mL and was infused within ~30 sec. Maintaining the euglycemic clamp involved infusing 50% glucose (as dextrose; VetOne, Boise, ID) intravenously at a known and adjustable rate utilizing a modular pump (Deltec 3000, Deltec Inc., St. Paul, MN) in order to maintain the pre-LPS administration blood glucose concentrations.

Blood samples were obtained at -30, -20, and 0 min relative to LPS or saline bolus administration to establish baseline glucose levels. Each respective treatment bolus was administered immediately following the 0 min blood sample collection. For cows receiving LPS, postbolus blood samples (1 mL) were collected every 10 min and immediately analyzed for glucose concentration (TRUEbalance glucometer; McKesson, San Francisco, CA). Glucose infusion began when blood glucose content declined below baseline levels, and infusion rates were adjusted as necessary to maintain euglycemia ($\pm 5\%$ of baseline). The rate of 50% glucose infusion (mL/h) was transformed to rate of glucose infusion (**ROGI**; g/h). The total glucose infused for each cow was calculated using the ROGI for each 10 min interval (72 intervals in total) according to the following equation:

$$\sum_{i=0}^{72} ROGI \left(\frac{g}{h} \right)_i * \frac{1 h}{60 min} * 10 min$$

Blood glucose was measured every h in PF cows. Vital measurements (Tr and RR) were obtained at -0.5 and 0 h relative to LPS administration, every 3 h for the first 12 h postbolus, and every 6 h thereafter. Additional blood samples were collected daily at 1800 h during P1 from the catheter and divided equally between a tube containing K₂EDTA (BD, Franklin Lakes, NJ; for plasma collection) and an empty glass tube (for serum collection). Additional plasma and serum samples (~10 mL each) were collected from all treatments at -0.5, -0.33, 0, 3, 6, 9, 12, 18, 24, 30, 36, 42, and 48 h relative to bolus administration. Serum samples were allowed to clot at room temperature for 1 h prior to centrifugation. Plasma and serum were harvested following centrifugation at 1500 × g for 15 min at 4°C and were subsequently frozen at -20°C until analysis.

Serum cortisol and plasma insulin, glucagon, non-esterified fatty acids (**NEFA**), BHB, LPS binding protein (**LBP**), serum amyloid A (**SAA**), BUN, glucose, and lactose concentrations were determined using commercially available kits according to manufacturers' instructions (cortisol, Enzo Life Sciences Inc., Farmingdale, NY; insulin, Mercodia AB, Uppsala, Sweden; glucagon, RD Systems, Inc., Minneapolis, MN; NEFA, Wako Chemicals USA, Richmond, VA; BHB, Pointe Scientific Inc., Canton, MI; LBP, Hycult Biotech, Uden, the Netherlands; SAA, Tridelata Development Ltd., Kildare, Ireland; BUN, Teco Diagnostics Anaheim, CA; glucose, Wako Chemicals USA Inc., Richmond, VA; Lactose, BioVision Inc., Milpitas, CA). The inter- and intra-assay coefficients of variation for cortisol, insulin, glucagon, NEFA, BHB, BUN, glucose, LBP, SAA, and lactose were 7.6 and 9.2%, 6.8 and 6.0%, 7.7 and 9.8%, 6.4 and 2.7%,

7.0 and 6.2%, 4.2 and 4.5%, 3.3 and 2.0%, 16.1 and 6.4%, 12.9 and 6.8%, and 7.9 and 4.4%, respectively.

Samples for complete blood count (**CBC**) analysis were collected at -0.5, -0.33, 0, 3, 6, 9, 12, 18, 24, 30, 36, 42, and 48 h relative to bolus administration. A 3 mL blood sample was obtained from the catheter and stored at 4°C for ~12 h before submitting to the Iowa State University's Department of Veterinary Pathology for analysis. Samples for evaluating blood physiology were measured using an iSTAT handheld machine and cartridge (CG8⁺; Abbott Point of Care, Princeton, NJ) and were obtained at 0, 12, 24, and 48 h relative to bolus administration.

Neutrophil Isolation and Functional Assays

Neutrophil isolation and functional assay techniques were performed on P1D5 and at 12, 24, and 48 h relative to LPS administration using previously described methods (Kimura et al., 2014). In brief, blood samples (32 mL) for neutrophil isolation were collected into 50 mL conical tubes containing acid citrate dextrose (8 mL) and immediately transported to the laboratory for neutrophil isolation. Samples were centrifuged at $1000 \times g$ for 20 min at room temperature and the plasma, buffy coat and upper portion of packed red blood cells were removed. The remaining red blood cells were lysed twice and neutrophils were pelleted following centrifugation at $300 \times g$ for 5 min. Neutrophils were resuspended in phosphate buffered saline (**PBS**) and standardized to 5.0×10^7 cells/mL. Neutrophil function was assessed by extracellular release of myeloperoxidase (MPO) and oxidative burst (cytochrome C reduction).

To assess oxidative burst, 2.5×10^6 polymorphonuclear leukocytes (**PMN**) were treated with either hank's balanced salt solution (**HBSS**) or HBSS plus phorbol myristate acetate (**PMA**) followed by treatment with cytochrome C. Cells were incubated at 39°C for 15 min and immediately read at 2 wavelengths (550 nm and 650 nm) using a spectrophotometer.

Three cell preparations were used to assess MPO activity: 1) cells were lysed by treatment with cetyltrimethylammonium bromide solution as a measure of total MPO, 2) PMN were stimulated with equal parts calcium ionophore A23187 and cytochalasin B in HBSS to assess release of MPO with stimulation, and 3) PMN treated with HBSS alone as a measure of unstimulated MPO release. Each preparation was loaded with 1.25×10^6 cells in a microtiter plate and incubated at 39°C for 20 min. Following incubation, 50 μ L/well of 3,3', 5,5'-tetramethylbenzidine dihydrochloride was added (3.25 mM) followed promptly with 50 μ L/well of H₂O₂. The reaction was stopped after a 2 min incubation with the addition of 50 μ L/well 2N H₂SO₄. Plates were centrifuged for 1 min at 600 \times g and the supernatant was transferred to a second plate. Optical density was determined at 405 nm using a spectrophotometer. Percentage of MPO released from PMN was determined using the following equation:

$$\text{Exocytosis (\%)} = [(\text{OD of stimulated PMN})/(\text{OD of lysed PMN})] \times 100$$

Calculations and Statistical Analysis

Administering LPS reduces milk yield and therefore decreases glucose utilized for milk synthesis. The decrease in milk yield allows us to estimate the amount of glucose conserved (milk glucose deficit) due to reduced feed intake alone (PF-CON and PF-AvZn cows) and decreased feed intake coupled with ostensible glucose utilization by the immune system (LPS-CON and LPS-AvZn cows). The amount of glucose utilized for milk synthesis was calculated for each milking based on Kronfeld's (1982) estimation of 72 g glucose required to synthesize 1 kg of milk. Milk glucose output prior to the challenge was averaged in order to establish a baseline. Milk glucose utilization at both 6 and 12 h was subtracted from the baseline in order to calculate the milk glucose deficit. For PF-CON and PF-AvZn cows, milk glucose deficit was solely used to calculate total glucose deficit. For LPS-CON and LPS-AvZn cows, milk glucose deficit plus the amount of glucose infused to maintain euglycemia were combined to obtain the total glucose

deficit. After cessation of the euglycemic clamp (12 h postbolus), milk glucose deficit continued to be calculated every 6 h until 48 h postbolus.

Sample size determination (5 animals/treatment) was based on previous reports and logistical constraints (Kvidera et al., 2017; Horst et al., 2018). Each animals' respective parameter was analyzed using repeated measures with an autoregressive covariance structure for milk yield and composition and DMI, and a spatial power law for rectal temperature and respiration rate, iSTAT, CBC, neutrophil assays, and blood parameters. The repeated effect was time relative to LPS administration. There were no treatment differences due to diet in any parameter during P1; thus, each specific variable's pre-bolus values (i.e., P1 average, when available) served as a covariate for analysis of P2 and P3 (each period was statistically analyzed separately). Effects of treatment, time (h or d relative to bolus), and treatment \times time interactions were assessed as a completely randomized design using PROC MIXED (SAS Inst. Inc., Cary, NC). A separate analysis was utilized to make statistical comparisons to baseline in which the average P1 value for each parameter was included as an additional time point. Pre-planned contrasts were used to estimate differences between CON and AvZn-supplemented cows, PF and LPS-administered cows, as well as LPS-CON and LPS-AvZn cows and these multiple comparisons were not mathematically adjusted for (Rothman, 1990). Data are represented as least squares means \pm standard error of the mean and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$.

Results

Mild hyperthermia (1°C , relative to PF cows; $P = 0.02$) was observed in LPS administered cows at 3 h postbolus. Throughout P2, Tr of LPS-AvZn cows was decreased (0.3°C), relative to LPS-CON cows ($P = 0.01$; Figure 4.1A). No treatment differences were observed in Tr during P3. No treatment differences were observed in RR during P2 (data not

shown). However, during P3, RR of AvZn-supplemented cows (LPS and PF) tended to be increased (5 bpm; $P = 0.06$) from 60 to 72 h postbolus, while CON-supplemented cows (LPS and PF) remained the same (data not shown).

Lipopolysaccharide administration decreased DMI (47%; $P \leq 0.05$) relative to baseline, and the pattern and extent of reduced DMI was similar for cows in the PF treatments by experimental design ($P > 0.82$; Figure 4.1B). Dry matter intake increased similarly from P2 d 1 to P2 d 2 for all treatments ($P < 0.01$). During P3, DMI of LPS-administered cows remained decreased (12%; $P < 0.01$) relative to PF controls. Zinc source did not have a detectable effect on DMI.

Despite similar DMI, milk yield from LPS-administered cows decreased (54%; $P < 0.01$; Figure 4.1C) throughout P2, and was decreased 69 and 38% on d 1 and 2 of P2 relative to PF controls, respectively ($P < 0.01$). No dietary effect was observed on daily milk yield. When evaluated on an hourly basis during P3, milk yield from LPS-administered cows remained decreased (8%; $P = 0.01$; data not shown) relative to PF cows. When P3 milk yield was condensed to daily averages no treatment differences were observed ($P > 0.48$), although, milk yield increased in all treatments with time ($P < 0.01$). During P2 and P3, the rate of milk yield recovery (as measured by slope), was increased in LPS-administered cows relative to PF controls ($P < 0.01$; Table 4.3). The slope of milk yield recovery following LPS administration (for 72 h) increased (49%) in LPS-AvZn cows, relative to LPS-CON cows ($P = 0.05$; Table 4.3).

Milk urea nitrogen in LPS infused cows gradually increased between 12 and 30 h postbolus, while it decreased in PF controls ($P = 0.05$, Table 4.3). Milk urea nitrogen of LPS-AvZn cows tended to be increased (11%) relative to LPS-CON cows ($P = 0.09$; Table 4.3). During P3 there was a treatment by time interaction for LPS-CON cows, such that MUN content

remained constant from 54 to 78 h postbolus, while in all other treatments it gradually decreased ($P < 0.01$; Table 4.3). Throughout P3, MUN from LPS-AvZn cows tended to be decreased (10%) relative to LPS-CON cows ($P = 0.06$; Table 4.3). During P2, LPS administration decreased milk lactose content (17%; $P < 0.01$; Table 4.3) and increased milk fat and protein concentrations (32 and 19%, respectively; $P < 0.01$; Table 4.3) relative to PF cows. Milk lactose content of all treatments increased throughout P3 ($P = 0.01$; Table 4.3). Overall during P3, milk fat content from LPS-administered cows remained increased (11%) relative to PF controls ($P = 0.04$; Table 4.3). Milk fat and protein content of all treatments gradually returned to baseline with time ($P < 0.01$). Other than slightly increasing MUN in LPS-AvZn cows, AvZn supplementation had little to no effect on milk composition during P2 (Table 4.3).

Milk SCC from LPS-administered cows tended to increase with time, while in PF cows it remained unchanged ($P = 0.10$; Figure 4.1D). During P3, no treatment differences were observed in SCC, however, post-hoc analysis revealed SCC of LPS-AvZn cows tended to be decreased (70%) from 54 to 78 h postbolus relative to LPS-CON cows ($P = 0.10$; Figure 4.1D).

Hyperglycemia occurred in LPS infused cows for ~200 min postbolus, after which continuous glucose infusion was necessary to maintain euglycemia (Figure 4.2A). Rate of glucose infusion did not differ due to dietary Zn source, but did increase with time ($P < 0.01$; Figure 4.2A). For the first 70 min of the hyperglycemic phase, LPS-AvZn cows tended to have decreased (19%) circulating glucose relative to LPS-CON cows ($P = 0.09$; Figure 4.2A). Relative to PF cows, the 48-h milk glucose deficit was increased (75%) in LPS administered cows throughout P2 ($P < 0.01$; data not shown). Similarly, the total glucose deficit was 44-fold more in LPS relative to PF cows. Total glucose deficit accumulated over the 12 h was 5, 67,

1,500, and 1,712 g for PF-CON, PF-AvZn, LPS-CON, and LPS-AvZn cows, respectively (Figure 4.2B). Dietary Zn source did not affect the glucose deficit metrics.

For the duration of glucose infusion, circulating insulin in LPS administered cows was 7-fold more than in PF controls ($P = 0.05$), and it remained increased 5-fold after the 12 h-infusion period ($P < 0.01$; Figure 4.3A). Although not significant ($P > 0.41$), insulin concentrations were numerically decreased (51%) in LPS-AvZn from 3 to 12 h relative to LPS-CON (Figure 4.3A). Circulating glucagon in LPS-administered cows increased (65%) throughout P2 relative to PF controls ($P < 0.01$; Figure 4.3B). Supplementing AvZn decreased glucagon concentrations (30%) following LPS administration relative to LPS-CON cows ($P = 0.02$; Figure 4.3B). From 3 to 12 h postbolus, circulating NEFA in LPS-AvZn cows remained similar to baseline levels, while in all other treatments it gradually increased ($P = 0.02$; Figure 4.3C). Throughout P2, circulating NEFA from LPS-infused cows decreased (39%) relative to PF controls ($P = 0.05$; Figure 4.3C). Circulating BHB in LPS administered cows decreased relative to the PF controls during P2 ($P < 0.01$; Figure 4.3D). Blood urea nitrogen concentrations in LPS-infused cows increased (29%) during P2 relative to PF controls ($P < 0.01$). From 12 to 24 h postbolus, circulating BUN from LPS treatments continued to increase while in PF cows it gradually decreased ($P < 0.01$; Table 4.4). During P2, plasma lactose concentrations from LPS cows increased (26%) relative to PF cows ($P = 0.05$; Table 4.4). Following LPS administration, ionized Ca (iCa) markedly decreased (33% at 12 h postbolus) and it gradually returned to baseline while in PF controls iCa levels remained similar to baseline concentrations ($P < 0.01$; Figure 4.3E). Interestingly, iCa from LPS-AvZn cows tended to be increased (19%) relative to LPS-CON cows at 12 h postbolus ($P = 0.10$).

Circulating LBP from LPS-administered cows increased (3-fold; $P < 0.01$; Figure 4.4A) throughout P2 relative to PF controls. Similarly, circulating SAA increased in LPS challenged cows with time, while in PF controls it remained similar to baseline (9-fold increase relative to PF; $P < 0.01$; Figure 4.4B). At 24 h postbolus, circulating SAA from LPS-AvZn cows was increased (38%) relative to LPS-CON cows ($P = 0.03$). Post-hoc analysis revealed increased SAA from 12-24 h postbolus (43%; $P = 0.05$) in LPS-AvZn cows relative to LPS-CON control (Figure 4.4B). Administering LPS increased circulating cortisol (7-fold) throughout P2 relative to PF cows ($P < 0.01$; Figure 4.4C) and supplementing AvZn decreased cortisol (37%) from 6-48 h postbolus relative to LPS-CON cows ($P = 0.04$; Figure 4.4C).

Circulating white blood cells (WBC) from LPS infused cows were initially decreased between 3 and 12 h postbolus (51%) relative to PF controls ($P < 0.01$; Table 4.5), after which cows from the LPS treatments developed leukocytosis (36% increase, relative to PF; $P < 0.01$). Relative to PF-CON cows, circulating WBC counts of PF-AvZn cows tended to be increased (31%) at 30 h postbolus ($P = 0.09$; Table 4.5). Circulating neutrophils reflected a similar biphasic response ($P < 0.01$; Figure 4.5A). Patterns of circulating monocytes and lymphocytes in LPS-infused cows were comparable to WBC counts, although, the leukopenia phase was prolonged (61 and 45% decrease from 3 to 36 h postbolus, relative to PF cows; $P < 0.01$; Table 4.5). Circulating basophils in LPS cows tended to be decreased from 3 to 6 h and increased from 30 to 48 h postbolus, while in PF controls it did not change ($P = 0.06$; Table 4.5). During P2, LPS-administration decreased circulating platelets (48%) relative to PF cows ($P < 0.01$; Table 4.5).

During P2, no treatment differences were observed in neutrophil oxidative burst (data not shown). However, relative to baseline, oxidative burst from LPS-AvZn cows increased (44%) throughout P2 ($P \leq 0.05$; data not shown). Oxidative burst of LPS-CON cows tended to be

increased (31%) 48 h post-LPS administration relative to baseline ($P = 0.07$; data not shown). Stimulated MPO activity increased similarly in all treatments during P2 (Figure 4.5B). In LPS infused cows, the MPO released increased for the first 12 h postbolus (38%, relative to baseline; $P \leq 0.05$) and then returned to baseline ($P < 0.01$; Figure 4.5B), while in PF cows it remained increased throughout all of P2 ($P \leq 0.05$). Dietary Zn source had no effect on stimulated MPO release. Total MPO from LPS infused cows (determined by cell lysis), decreased (23%, relative to PF cows) from 24 to 48 h postbolus, while in PF controls it increased ($P < 0.01$; Figure 4.5C). Relative to baseline, total MPO of LPS-AvZn cows was decreased (10%, $P = 0.02$) at 24 h postbolus, while LPS-CON cows it remained decreased (11%) from 24 to 48 h postbolus ($P \leq 0.02$). Total MPO in PF treatments increased (11%) relative to baseline from 24 to 48 h postbolus ($P \leq 0.03$). Throughout P2, LPS-administered cows had increased (18%) MPO exocytosis relative to PF controls ($P < 0.01$; Figure 4.5D). Relative to baseline, the percentage of MPO released after in vitro stimulation increased (41%) in all treatments at 12 h postbolus ($P < 0.01$; Figure 4.5D). Myeloperoxidase exocytosis was or tended to be increased throughout P2 in LPS-CON and LPS-AvZn treatments relative to baseline (37 and 22%, respectively; $P < 0.01$ and $P \leq 0.09$; Figure 4.5D).

Discussion

Maintenance of epithelial barrier function is crucial for preventing noxious substances and pathogen infiltration. When the intestinal barrier is compromised, antigens can infiltrate into portal and systemic circulation and initiate a cascade of events culminating in immunoactivation and inflammation. In dairy cows, there are a myriad of possible routes for pathogen entry including the mammary gland, uterus, lungs, and gastrointestinal tract (Bradford et al., 2015). While insult etiology may differ, animal welfare and economic profitability are almost always compromised by epithelial hyperpermeability, due in large part to high energetic demands of

activated leukocytes (Kvidera et al., 2017ab). Successful immunoactivation relies on effective leukocyte communication, extravasation, cellular function, elimination of cell debris and harmful byproducts, and return to homeostasis (Kennedy, 2010). Identifying mitigation strategies aimed at improving barrier integrity (thus reducing pathogen entry) and immunocompetence have the potential to improve productivity over a range of physiological stressors. Zinc is involved in more than 300 enzymes and plays a pivotal role in multiple systems (Prasad, 2012; Kambe et al., 2015). Furthermore, Zn improves epithelial integrity characteristics (mammary and intestinal; Sanz-Fernandez et al., 2014; Weng et al., 2017) and immune function (immune function; cell adherence, clearance of *E. coli*, cytokine production; Finamore et al., 2008; Kidd et al., 1994a). Therefore, we hypothesized supplemental complexed Zn would modify the inflammatory response to an i.v. LPS challenge and in turn alter the leukocyte energetic requirement.

Successful immunoactivation was induced herein as indicated by mild hyperthermia, increased circulating inflammatory biomarkers, altered leukocyte dynamics, and reduced productivity. Cows administered LPS experienced a mild and transient hyperthermia and the extent and pattern agrees with previous reports (Waldron et al., 2003; Moyes et al., 2014a; Horst et al., 2018). Interestingly, relative to LPS-CON cows, feeding complexed Zn reduced the febrile response to LPS. Both an increased (Roberts et al., 2002; Sanz-Fernandez et al., 2014) and decreased (Chirase et al., 1991; Pearce et al., 2015; Abuajamieh et al., 2016) Tr response has been observed with complexed Zn following immunoactivation and the variability is presumably cytokine-driven. Circulating cytokines (such as tumor necrosis factor- α [TNF- α] or interleukin- 1β) have pyrogenic properties and are altered by Zn in pigs (Klosterhalfen et al., 1996; Paulk et al., 2014; Mayorga et al., 2018), humans (Bao et al., 2010), chickens (Jarosz et al., 2017a), and cattle (Batistel et al., 2016). Although not always consistent (Driessen et al., 1994; Klosterhalfen

et al., 1996; Batistel et al., 2016), Zn supplementation decreases circulating cytokines during inflammation via Zn-finger protein inhibition of the nuclear factor kappa-light-chain-enhancer of activated B cells pathway (Prasad, 2008; Foster and Samman, 2012; Jarosz et al., 2017b).

Therefore, inconsistencies in the febrile response may be explained by the transient and variable pattern of circulating cytokines, the type of inflammatory insult, or stage of infection.

In response to immunogenic stimuli and cytokines, circulating APP (i.e., LBP and SAA) concentrations are markedly increased (Waldron et al., 2003; Kvidera et al., 2017a; Horst et al., 2018), which corroborates our results. During infection, circulating Zn is rapidly redistributed into cells and tissues (particularly the liver), where it alters APP production (Liuzzi et al., 2005; Liu et al., 2014). In the present study, feeding complexed Zn increased circulating SAA post-LPS infusion. Similarly, feeding complexed Zn increased APP production in heat-stressed calves and pigs (Pearce et al., 2015; Abuajamieh et al., 2016), chickens (Jarosz et al., 2019), and transitioning dairy cows (Batistel et al., 2016). Interestingly, Liu and co-workers (2014) demonstrated Zn deficiency enhances APP production via activating the JAK-STAT3 pathway in a murine model. Regardless of the aforementioned inconsistencies, it is clear that Zn can influence the APP response.

Cytokines and APP produced in response to LPS direct cell traffic, activate circulating immune cells and facilitate “killing” capacity, which markedly alters temporal leukocyte patterns. In the current study, leukopenia occurred immediately following LPS infusion, likely due to immune cell infiltration (into presumed infection sites) or increased leukocyte appearance in the marginated pool (Hoedemaker et al., 1992; Lang et al., 1992). After approximately 12 h, leukocytosis developed and was most pronounced in the neutrophil population. Neutrophilia may be due to increased neutrophil production, demargination from pulmonary circulation, or

decreased endothelial adherence (Jagels and Hugli, 1994; Opdenakker et al., 1998). These fluctuating temporal patterns are consistent with LPS infusion in lactating cows (Bannerman et al., 2008; Horst et al., 2018).

Depending on the severity of infection, populations of immature or banded neutrophils are increased during neutrophilia, and the immature population's function is not fully operational (Van Eeden et al., 1995; Pappe et al., 2003). Reduced ROS production, increased rates of apoptosis, and reduced adhesion molecule expression (Mehrzhad et al., 2000; Yagi et al., 2002) have been observed in circulating neutrophils isolated from LPS-infused cows. However, improved function of bovine PMN has also been reported following LPS infusion or incubation (Sohn et al., 2007; Moyes et al., 2014a,b; Moraes et al., 2017). We detected no differences in respiratory burst or release of MPO in LPS-infused cows relative to their PF counterparts. Lipopolysaccharide administration decreased total MPO content and increased MPO exocytosis. Although LPS depleted MPO stores, it did not appear to affect the neutrophils capacity to release MPO, suggesting an improved efficiency of neutrophils isolated from LPS-administered cows. Improved PMN activity in LPS cows may be explained by the maintenance of euglycemia following LPS administration. Infusing LPS markedly alters metabolic status (i.e., hypoglycemia, hyperinsulinemia, hypertriglyceridemia, hypergluconemia) and this metabolic milieu may contribute to immunosuppression (Moyes et al., 2010), thus maintenance of euglycemia may have masked LPS-induced hypoglycemia effects on neutrophil function. No differences were observed due to Zn source on the temporal pattern of circulating leukocytes which agrees with previous studies (Kidd et al., 1994b). Moreover, Zn source did not alter neutrophil oxidative burst or MPO production. Dietary Zn's effect on cellular and humoral immunity are extremely and unexplainably variable across species (Kidd et al., 1994a,b; Kincaid

et al., 1997; Nemec et al., 2012; Dietz et al., 2017; Kloubert et al., 2018). The aforesaid inconsistencies may be due to cell type measured, methods for assessing neutrophil function, or pre-existing Zn status.

Although the mechanisms underlying immunosuppression in immature leukocyte populations remain unclear, one hypothesis is an increase in immunosuppressive glucocorticoids, such as cortisol (Burton et al., 1995; Burton et al., 2005). Surprisingly, despite hypercortisolemia we observed no effect or even improved PMN function (i.e. increased MPO exocytosis) following LPS infusion. Additionally, LPS-AvZn cows had decreased circulating cortisol relative to LPS-CON cows, which agrees with previous studies (Lippolis et al., 2017; Dang et al., 2013). Explanations for why AvZn-induced decreased cortisol did not impact PMN function are not clear, however, cortisol concentrations had nearly returned to baseline values prior to PMN isolation, and it is not clear how long neutrophils need to be exposed to corticosteroids before becoming less functional. Additionally, leukocytes can develop steroid-intolerance to immunosuppressive glucocorticoids (Briggs et al., 1983; Snavely et al., 1985). Even though the decrease in cortisol did not coincide with changes in neutrophil activity, it may suggest that the LPS insult was less severe with complexed Zn supplementation and this agrees with the reduced febrile response. The biological significance of Zn-mediated decreased cortisol warrants further investigation as it has relevance to multiple on-farm situations (the transition period, heat stress, transport stress etc.).

Lipopolysaccharide i.v. infusion reduces the integrity of mammary (Wellnitz et al., 2016), and intestinal epithelial barriers (Klunker et al., 2013). In the current study we observed increased SCC in LPS-infused cows relative to PF controls during P2 which agrees with previous work (Shuster et al., 1991; Kvidera et al., 2017a). Additionally, plasma lactose (a biomarker of

reduced mammary barrier function; Shennan and Peaker, 2000) concentrations increased post-LPS infusion. Interestingly, during P3 SCC of LPS-AvZn cows was decreased relative to their LPS-CON counterparts. Reduced milk SCC has been observed repeatedly with dietary Zn supplementation (Kellogg et al., 2004; Nayeri et al., 2014; Machado et al., 2013) and recently Weng et al. (2017) observed reduced circulating lactose with Zn supplementation in heat-stressed cows. Zinc is required for normal epithelial barrier function and has been shown to improve integrity characteristics of mammary (Weng et al., 2017), lung (Bao et al., 2006), and intestinal epithelial barriers (Sanz Fernandez et al., 2014; Pearce et al., 2015; Abuajamieh et al., 2016). Therefore, Zn appears to be a promising mitigation strategy for strengthening the integrity of multiple epithelial barriers and it is of interest to evaluate if dietary Zn can improve uterine epithelial integrity.

In addition to markedly initiating an inflammatory response and compromising mammary epithelial integrity, several well-characterized metabolic adjustments including hyperglycemia or hypoglycemia (depending on the stage of infection), hyperglucagonemia and hyperinsulinemia, increased markers of proteolysis, and hypertriglyceridemia occur post-LPS infusion (Lohuis et al., 1988; Lanza-Jacoby et al., 1998; McGuinness, 2005). These changes, along with reduced insulin sensitivity in skeletal muscle and adipose tissue coupled with decreased milk synthesis, reflect coordinated strategies which facilitate glucose “sparing” for immune cells. Most leukocytes become obligate glucose utilizers when activated (Palsson-McDermott and O’Neill, 2013) and require copious amounts of glucose. In the present study the glucose requirement of the immune system was approximately 0.96 g/kg of BW^{0.75}/h, which is remarkably comparable to our previous studies in monogastrics and ruminants (Kvidera et al., 2016, 2017a,b; Horst et al., 2018). However, despite numerous indications of an altered inflammatory response (i.e.,

reduced pyrexia, increased SAA, and reduced cortisol) dietary Zn source did not appear to affect overall glucose use by the immune system. However, a clear limitation to our model is the inability to account for the liver's contribution (i.e., glycogenolysis and gluconeogenesis) to the circulating glucose pool. Both glycogenolytic and gluconeogenic rates increase following LPS administration (Spitzer et al., 1985; McGuinness, 1994; Waldron et al., 2003), and we are unable to quantify the liver's glucose output; a scenario that causes the total glucose deficit to be underestimated. Additionally, we observed increased circulating glucagon in LPS-infused cows, and glucagon is a potent stimulator of both glycogenolysis and gluconeogenesis (Faulkner and Pollock, 1990). Interestingly, feeding complexed Zn decreased the acute hyperglycemic response to LPS and blunted the post-LPS glucagon surge. Both of the aforementioned changes may suggest that there was less of a glucose demand in complexed Zn-fed cows and this is corroborated by altered inflammation parameters. Further investigation into how dietary Zn modifies the immune system and immunometabolism is needed.

Many of the well-characterized glucose-sparing mechanisms mentioned previously occurred post-LPS administration in our study. Marked hyperinsulinemia developed immediately postbolus and was sustained throughout the sampling period. Increased insulin presumably supports insulin-mediated glucose uptake and function of activated leukocytes (Calder et al., 2007). Although not significant, we observed a numerical decrease in circulating insulin in LPS-AvZn cows relative to their LPS-CON counterparts during the first 12 h postbolus. Zinc plays a pivotal role in processing, storage, secretion, and action of insulin (Myers et al., 2012); furthermore, Zn has been shown to attenuate glucose-stimulated insulin secretion (Slepchenko et al., 2013) via inhibiting tyrosine phosphatases (Haase and Maret, 2005). Literature demonstrating a beneficial effect of organic vs. inorganic forms of Zn on circulating insulin is

limited and warrants further investigation. Regardless, the combined effects of complexed Zn on post-LPS circulating insulin and glucagon suggest a coordinated response reflective of a reduced glucose requirement during the acute stage of infection.

Non-esterified fatty acids decreased following LPS administration and then gradually increased with time. Interestingly, circulating NEFA from LPS-AvZn cows decreased relative to LPS-CON cows. Two possible mechanisms for blunted lipolysis may be: 1) increased insulin antilipolytic sensitivity or 2) antilipolytic effects of increased circulating lactate. While Zn has been shown to alter insulin sensitivity (Slepchenko et al., 2013), the results should be interpreted with caution as pre-existing Zn status (i.e., deficiency) may influence this response. Amplified leukocyte proliferation and activity increases lactate efflux and this can cause infection induced hyperlactatemia (Haji-Michael et al., 1999). Although not observed in the neutrophil parameters we measured, if Zn increased cellular activity of another WBC type, hyperlactatemia may have developed and altered lipolysis. Regardless of the mechanism the biological significance of reduced lipolysis remains unclear and warrants further investigation. Despite observing an altered NEFA pattern, circulating BHB concentrations did not differ in LPS-CON and LPS-AvZn cows. Circulating BHB remained decreased during P2 possibly due to increased utilization by peripheral tissues (Zarrin et al., 2014). However, decreased BHB production from the liver (Takeyama et al., 1990; Gitomer et al., 1995) and rumen epithelium may also contribute to the LPS-induced hypoketonemia. Further investigation into the LPS-induced hypoketonemia is warranted especially considering its relevance to the transition cow's bioenergetics. Skeletal muscle mobilization, as measured by circulating BUN, increased throughout P2. Proteolysis provides substrate for gluconeogenesis and amino acids for APP synthesis. However, BUN can be difficult to interpret in ruminant models as muscle proteolysis is not the sole source of urea.

Regardless, these metabolic adjustments are consistent with previous literature (Waldron et al., 2003; Graugnard et al., 2013; Zarrin et al., 2014) and discussed further in our recent publications (Kvidera et al., 2016, 2017a,b; Horst et al., 2018).

Hypocalcemia develops in infection models across a range of species including calves (Elsasser et al., 1996; Tennant et al., 1973), dogs (Holowaychuk et al., 2012), horses (Toribio et al., 2005), pigs (Carlstedt et al., 2000), sheep (Naylor and Kronfeld, 1986), and cows (Griel et al., 1975; Waldron et al., 2003; Kvidera et al., 2017a). The biological significance of infection-induced hypocalcemia is not fully understood, but past literature has demonstrated a relationship between Ca and LPS detoxification (Skarnes and Chedid, 1964) and this has recently been reviewed by Eckel and Ametaj (2016). Interestingly, complexed Zn supplementation attenuated LPS-induced hypocalcemia at 12 h postbolus (Figure 3E). This may suggest the LPS load was dampened in LPS-AvZn cows at 12 h postbolus compared to LPS-CON, perhaps due to improved integrity of mammary and intestinal epithelial barriers or improved LPS detoxification methods. A reduced LPS load is fitting with the other observations described previously (i.e., reduced fever, increased SAA, reduced cortisol, reduced SCC, decreased insulin and glucagon). The biological significance of ameliorated hypocalcemia due to Zn source warrants further investigation as it has practical relevance to periparturient metabolic disorders.

Determining differences in production outcomes was not the primary objective, as the experiment was knowingly underpowered to detect treatment differences in such variable measurements. Nonetheless, analyzing and presenting production variables allows for the primary aims (i.e., immune response, glucose deficit, and metabolism) to be put into context. Administering LPS markedly decreased DMI and by experimental design the PF controls mirrored this pattern and magnitude of reduced DMI during P2. The total glucose deficit of PF

treatments was accounted for exclusively by the decrease in milk yield because they did not become hypoglycemic, whereas the glucose deficit from the LPS treatments included the decrease in milk yield in addition to infused glucose. Although cows remained on the same nutritional plane, the milk glucose deficit from LPS treatments was increased 75% relative to the PF controls. In agreement with our previous studies (Kvidera et al., 2017a; Horst et al., 2018), maintaining euglycemia did not rescue milk yield in LPS administered cows, confirming that hypoglycemia is not responsible for reduced milk synthesis during infection. Reduced milk production represents an important glucose-sparing mechanism during infection and may occur either directly via LPS binding to mammary epithelial Toll-like receptor 4 or indirectly by increased proinflammatory cytokines (Verheijden et al., 1983; Ibeagha-Awemu et al., 2008). During P3, all treatments were allowed to consume feed ad libitum; however, DMI and milk yield remained decreased in cows previously administered LPS relative to PF controls, demonstrating the prolonged consequences of inflammation. Interestingly, although overall DMI and milk yield were not affected by diet during P2 and P3, LPS-AvZn cows had an increased rate (49%) of recovery in milk yield from d 1 to 3 relative to their LPS-CON counterparts. While not observed as frequently for DMI (Osorio et al., 2016), complexed Zn has been shown to improve milk yield in lactating dairy cows (Kellogg et al., 2004; Nayeri et al., 2014; Osorio et al., 2016). Reasons for not detecting more Zn differences in production parameters may be explained by small sample size, severity of the immune insult, and length of study.

Conclusion

In conclusion, our results indicate that immunoactivation requires copious amounts of glucose, but Zn source did not appear to affect the immune system's fuel consumption. However, replacing a portion of Zn sulfate with Zn amino acid complex improved some inflammatory, metabolic, and some production responses to LPS. The reduced hyperglycemic phase coupled

with decreased circulating glucagon and insulin concentrations demonstrate a dampened metabolic requirement following immunoactivation with complexed Zn supplementation. Further, multiple changes in the inflammatory response coincided with altered energetics including reduced fever, circulating cortisol and SCC, increased SAA concentrations, and attenuated hypocalcemia. Our results demonstrate well-coordinated alterations in metabolic and inflammatory parameters with complexed Zn supplementation during immunoactivation.

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Tables and Figures

Table 4.1. Ingredient and analyzed chemical composition of diet¹

Ingredient	% of DM
Corn silage	26.5
Ground corn	25.7
Alfalfa hay	19.8
Corn gluten feed	8.8
Expeller soybean ²	4.5
Soybean meal	4.5
Mineral mix	4.2
Whole cottonseed	3.5
Molasses supplement ³	1.7
Bypass fat ⁴	0.8
Chemical analysis, % of	
Dry matter, %	47.5
Starch, %	25.5
CP, %	16.7
NDF, %	32.3
ADF, %	21.8
Ether extract, %	4.83
Ca, %	0.96
P, %	0.43
S, %	0.23
K, %	1.41
Na, %	0.48
Cl, %	0.66
NE _L Mcal/kg DM	1.62
Vitamin A, IU/kg DM	6733.3
Vitamin D, IU/kg DM	636.3
Vitamin E, IU/kg DM	18.7

¹Values represent an average of ration nutrient summary reports collected throughout the trial. Average values between both diets containing dietary treatments of 64 mg/kg Zn from ZnSO₄ (control diet) or 40 mg/kg Zn from Availa-Zn (Zinpro Corporation, Eden Prairie, MN) plus 24 mg/kg Zn from ZnSO₄ (AvZn diet). All diets contained 40 mg/kg Mn from Mn sulfate, 6.5 mg/kg Mn from Availa-Mn (manganese AA complex; Zinpro Corporation), 10.1 mg/kg Cu from copper sulfate, 4.1 ppm of Cu from Availa-Cu (copper AA complex; Zinpro Corporation), 0.79 mg/kg Co from Copro (cobalt glucoheptonate; Zinpro Corporation), 0.8 mg/kg I from ethylenediamine dihydroiodide, and 0.3 mg/kg Se, with 50% from sodium selenite and 50% from selenium yeast. Analyzed Zn concentrations were 111 and 112 mg/kg for the control and AvZn diets, respectively.

²Mechanically processed soybean meal (Dairy Nutrition Plus, Ralston, IA).

³Quality Liquid Feeds (Dodgeville, WI).

⁴MagnaPalm (Energy Feeds International, Lago Vista, TX).

Table 4.2. Ingredient and composition of vitamin and mineral mix

Ingredient	% of DM ¹	
	Control ²	AvZinc ³
Calcium carbonate	28.78	28.70
Sodium carbonate	20.50	20.50
Bloodmeal	20.00	20.00
Salt	9.59	9.59
Magnesium oxide	4.53	4.53
Urea	4.01	4.01
DDGS	3.05	2.57
Potassium chloride	2.40	2.40
Yeast culture ⁴	1.61	1.61
Liquid MHA	1.19	1.19
Rumen-protected	1.02	1.02
Mineral oil	1.00	1.00
Availa-Zn 120 ⁶	0.00	0.83
Zinc sulfate	0.45	0.17
Manganese sulfate	0.31	0.31
Selenium premix 0.16%	0.26	0.26
Selenium yeast 2000	0.21	0.21
Availa-Mn 80 ⁶	0.20	0.20
Vitamin E 50%	0.19	0.19
Rumensin 90.7 ⁷	0.18	0.18
Availa-Cu 100 ⁶	0.10	0.10
Dairy trace mineral mix	0.10	0.10
Copper sulfate	0.10	0.10
Biotin 2%	0.09	0.09
COPRO 25 ⁶	0.08	0.08
Vitamin A 650 KIU	0.05	0.05
Vitamin D ₃ 500 KIU	0.01	0.01
EDDI-99	0.002	0.002

¹Average nutrient levels were as follows. Control diet: 30.6% CP, 11.33% Ca, 0.06% P, 2.62% Mg, 1.35% K, 0.50% S, 19.6 mg/kg Co from Copro 25 (Zinpro Corporation, Eden Prairie, MN), 19.6 mg/kg Co total, 101 mg/kg Cu from Availa-Cu 100 (Zinpro Corporation), 351 mg/kg Cu total, 19.6 mg/kg I total, 160.7 mg/kg Mn from AvailaMn 80 (Zinpro Corporation), 1,152 mg/kg Mn total, 4.22 mg/kg Se from selenium yeast, 8.43 mg/kg Se total, 0 mg/kg Zn from Availa-Zn (Zinpro Corporation), 1,588.68 mg/kg Zn total, 17.71 mg/kg Biotin (DSM Nutritional Products, Ames, IA), 33,634 kIU/kg vitamin A, 31.79 kIU/kg vitamin D₃, 937 IU/kg vitamin E, and 0.321 g/kg monensin. AvZn diet: 30.7% CP, 11.33% Ca, 0.06% P, 2.62% Mg, 1.35% K, 0.47% S, 19.6 mg/kg Co from Copro 25, 19.6 mg/kg Co total, 101 mg/kg Cu from Availa-Cu 100, 351 mg/kg Cu total, 19.6 mg/kg I total, 160.7 mg/kg Mn from Availa-Mn, 1,152 mg/kg Mn total, 4.22 mg/kg selenium yeast, 8.43 mg/kg Se total, 1,000 mg/kg Zn from Availa-Zn, 1,588.68 mg/kg Zn total, 17.71 mg/kg Biotin, 33,634 kIU/kg vitamin A, 31.79 kIU/kg vitamin D₃, 937 IU/kg vitamin E, and 0.321 g/kg monensin.

²Control = 64 mg/kg Zn from ZnSO₄. AvZn = 40 mg/kg Zn from Availa-Zn plus 24 mg/kg Zn from ZnSO₄.

³NutriTek (Diamond V, Cedar Rapids, IA).

⁴Smartamine-M (Adisseo, Alpharetta, GA).

⁵Availa-Zn, Availa-Mn, and Availa-Cu = Zn, Mn, and Cu AA complexes, respectively; Copro = cobalt glucoheptonate. ⁶Rumensin-90 (Elanco, Greenfield, IN).

Table 4.3. Milk yield recovery and composition of cows fed a control (CON) diet or a diet supplemented with Availa-Zn¹ following administration of saline or LPS

Parameters	Treatment ²				SEM	<i>P</i>			Contrasts ⁴		
	PF- CON	PF- AvZn	LPS- CON	LPS- AvZn		Trt ³	Time	Trt × Time	CON v AvZn	LPS v PF	LPS-CON v LPS-AvZn
MY <i>m</i> ⁵ d1-2, kg/d	-0.77 ^a	-0.97 ^a	5.31 ^b	8.75 ^b	1.93	0.01			0.41	<0.01	0.21
MY <i>m</i> d1-3, kg/d	0.61 ^a	0.92 ^a	6.49 ^b	9.66 ^c	1.06	<0.01			0.12	<0.01	0.05
MY <i>m</i> d1-4, kg/d	1.15 ^a	1.45 ^a	5.94 ^b	7.56 ^b	0.89	<0.01			0.30	<0.01	0.20
MY <i>m</i> d1-5, kg/d	1.03 ^a	1.73 ^a	5.19 ^b	6.04 ^b	0.82	<0.01			0.36	<0.01	0.46
MY <i>m</i> d2-4, kg/d	1.97 ^a	2.43 ^a	5.97 ^b	6.47 ^b	1.26	0.05			0.71	0.01	0.78
MY <i>m</i> d2-5, kg/d	1.39 ^a	2.37 ^a	4.83 ^b	4.73 ^b	1.03	0.08			0.67	0.01	0.95
P2 ⁶ Milk Parameters											
Fat, %	3.95 ^a	4.13 ^a	5.22 ^b	5.40 ^b	0.31	<0.01	<0.01	0.12	0.57	<0.01	0.68
Lactose, %	4.75 ^b	4.76 ^b	3.94 ^a	3.99 ^a	0.11	<0.01	0.01	0.05	0.76	<0.01	0.73
Protein, %	3.21 ^a	3.32 ^a	4.01 ^b	3.75 ^b	0.14	<0.01	<0.01	<0.01	0.61	<0.01	0.19
MUN, mg/dL	12.62	12.95	12.89	14.32	0.69	0.14	<0.01	0.05	0.22	0.41	0.09
P3 ⁷ Milk Parameters											
Fat, %	3.80	3.79	4.32	4.05	0.17	0.14	<0.01	0.98	0.43	0.04	0.28
Lactose, %	4.72	4.77	4.56	4.69	0.07	0.19	0.01	0.43	0.17	0.13	0.16
Protein, %	3.23	3.30	3.39	3.19	0.14	0.76	<0.01	0.22	0.64	0.87	0.32
MUN, mg/dL	10.59	10.13	10.70	9.62	0.49	0.15	<0.01	0.01	0.11	0.75	0.06

¹Zinc amino acid complex; Zinpro Corporation, Eden Prairie, MN

²PF-CON = Pair-fed, saline bolus, control; PF-AvZn = Pair-fed, saline bolus, Availa zinc; LPS-CON = ad libitum, LPS bolus, control; LPS-AvZn = ad libitum, LPS bolus, Availa zinc

³Treatment

⁴CON = PF-CON and LPS-CON treatments; AvZn = PF-AvZn and LPS-AvZn treatments; LPS = LPS-CON and LPS-AvZn treatments

⁵MY slope post LPS or saline administration

⁶During period 2 cows were either pair-fed or experienced a 12 h LPS-euglycemic clamp

⁷During period 3 cows were fed ad libitum

^{a,b,c}Values within a row with differing superscripts denote differences ($P < 0.05$) between treatments.

Table 4.4. Metabolic parameters of cows fed a control (CON) diet or a diet supplemented with Availa-Zn¹ following administration of saline or LPS during P2²

Parameters	Treatment ³				SEM	<i>P</i>			Contrasts ⁵		
	PF-CON	PF-AvZn	LPS-CON	LPS-AvZn		Trt ⁴	Time	Trt × Time	CON v AvZn	LPS v PF	LPS-CON v LPS-AvZn
BUN, mg/dL	10.0 ^a	9.0 ^a	12.2 ^b	12.5 ^b	0.6	0.01	<0.01	<0.01	0.58	<0.01	0.72
Lactose, μ M	26.0	26.2	30.6	32.6	2.6	0.22	0.08	0.33	0.68	0.05	0.59

¹Zinc amino acid complex; Zinpro Corporation, Eden Prairie, MN

²During period 2 cows were either pair-fed or experienced a 12 h LPS-euglycemic clamp

³PF-CON = Pair-fed, saline bolus, control; PF-AvZn = Pair-fed, saline bolus, Availa zinc; LPS-CON = ad libitum, LPS bolus, control; LPS-AvZn = ad libitum, LPS bolus, Availa zinc

⁴Treatment

⁵CON = PF-CON and LPS-CON treatments; AvZn = PF-AvZn and LPS-AvZn treatments; LPS = LPS-CON and LPS-AvZn treatments

^{a,b}Values within a row with differing superscripts denote differences ($P < 0.05$) between treatments.

Table 4.5. Complete blood cell count of cows fed a control (CON) diet or a diet supplemented with Availa-Zn¹ following administration of saline or LPS during P2²

Parameters	Treatment ³				SEM	<i>P</i>			Contrasts ⁵		
	PF- CON	PF- AvZn	LPS- CON	LPS- AvZn		Trt ⁴	Time	Trt × Time	CON v AvZn	LPS v PF	LPS-CON v LPS-AvZn
WBC, ×10 ³ /μL	7.60	8.66	9.53	9.12	0.71	0.31	<0.01	<0.01	0.65	0.11	0.68
Platelets, ×10 ³ /μL	305.8 ^b	340.3 ^b	174.2 ^a	160.4 ^a	16.6	<0.01	<0.01	0.21	0.54	<0.01	0.54
Monocytes, ×10 ³ /μL	0.41 ^b	0.41 ^b	0.22 ^a	0.20 ^a	0.03	<0.01	<0.01	<0.01	0.78	<0.01	0.74
Lymph, ×10 ³ /μL	3.94 ^b	4.13 ^b	2.34 ^a	2.48 ^a	0.20	<0.01	<0.01	<0.01	0.39	<0.01	0.61
Basophils, ×10 ³ /μL	0.05	0.06	0.06	0.06	0.01	0.48	<0.01	0.06	0.40	0.26	0.97

¹Zinc amino acid complex; Zinpro Corporation, Eden Prairie, MN

²During period 2 cows were either pair-fed or experienced a 12 h LPS-euglycemic clamp

³PF-CON = Pair-fed, saline bolus, control; PF-AvZn = Pair-fed, saline bolus, Availa zinc; LPS-CON = ad libitum, LPS bolus, control; LPS-AvZn = ad libitum, LPS bolus, Availa zinc

⁴Treatment

⁵CON = PF-CON and LPS-CON treatments; AvZn = PF-AvZn and LPS-AvZn treatments; LPS = LPS-CON and LPS-AvZn treatments

^{a,b}Values within a row with differing superscripts denote differences ($P < 0.05$) between treatments.

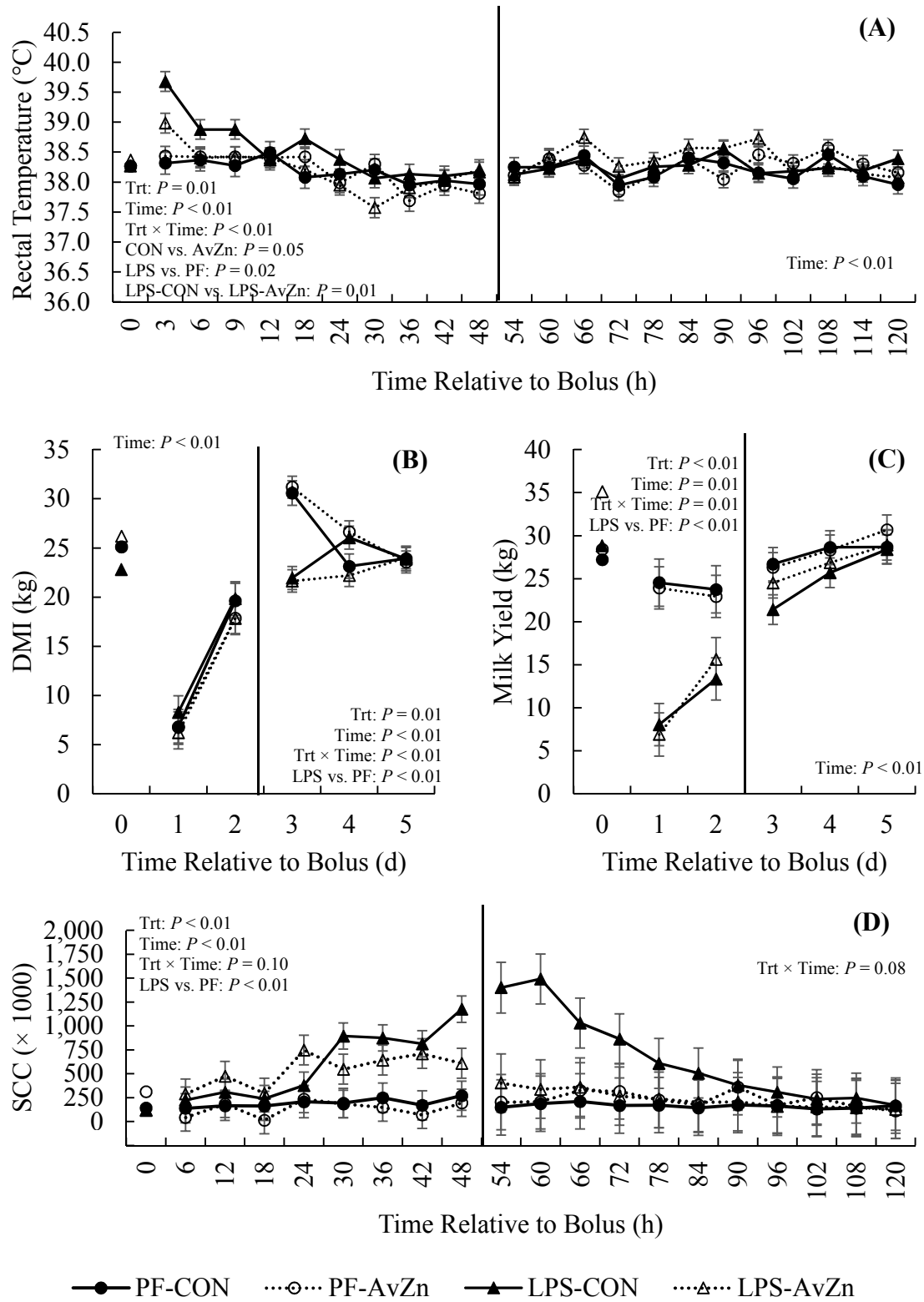


Figure 4.1. Effects of an LPS bolus or pair-feeding in dairy cows fed a control diet (CON) or Availa-Zn (AvZn) supplemented diet on (A) rectal temperature, (B) DMI, (C) milk yield, and (D) milk somatic cell count. Time 0 represents an average of measurements obtained during the baseline and used as a covariate for P2 and P3 analysis. The solid line separates P2 from P3 and represents two separate statistical analyses. Fixed effects of treatment, time, and treatment \times time were assessed as a completely randomized design using PROC MIXED. Data are represented as least squares means \pm standard error of the mean and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$. When < 0.10 the P-value for the fixed effects and the pre-planned contrasts were listed on the figure.

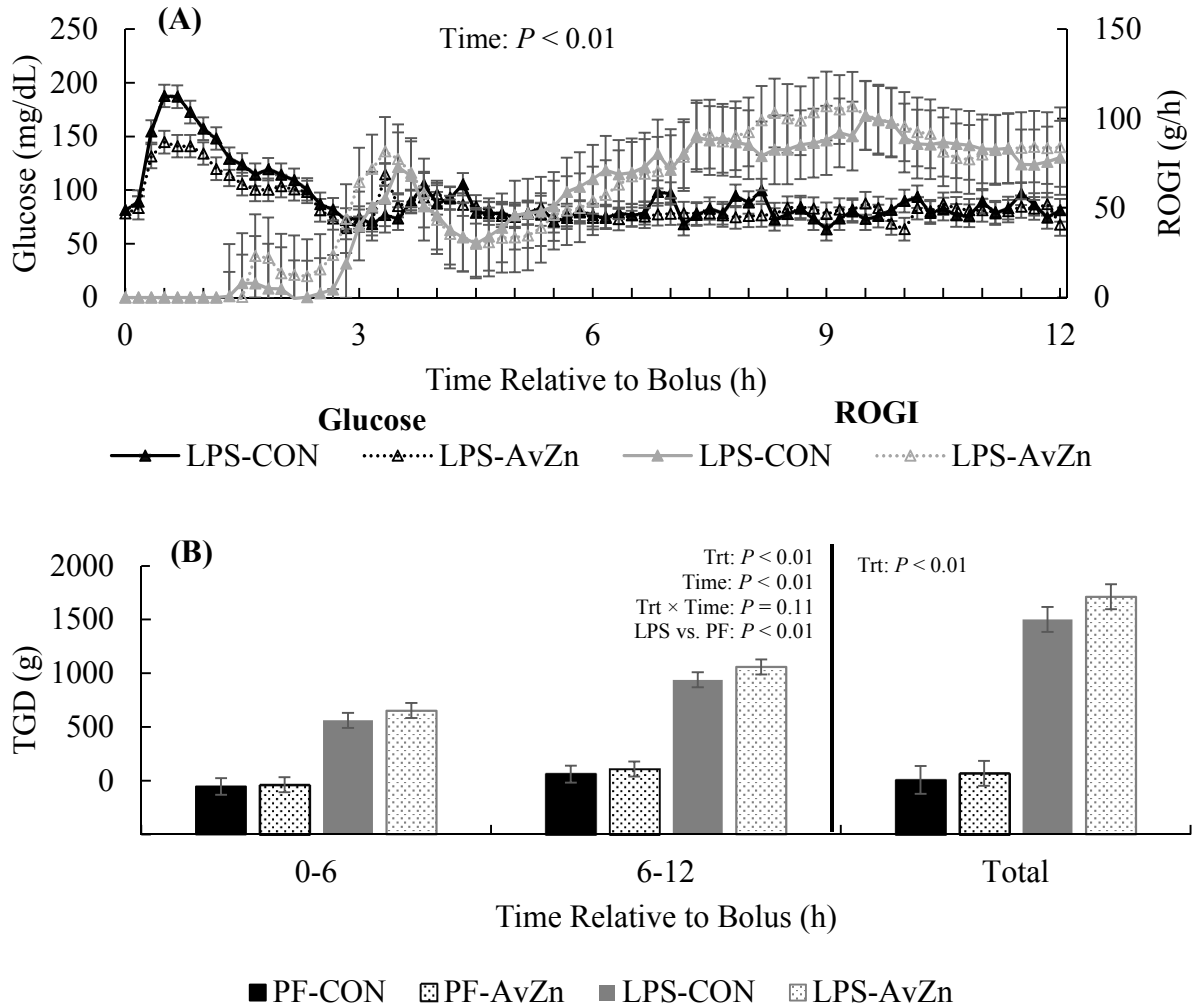


Figure 4.2. Effects of an LPS bolus or pair-feeding in dairy cows fed a control diet (CON) or Availa-Zn (AvZn) supplemented diet on (A) circulating glucose and rate of glucose infusion (ROGI) and (B) milk and/or total glucose deficit (TGD) from 0-6, 6-12 and accumulated over 12 h. Fixed effects of treatment, time, and treatment \times time were assessed as a completely randomized design using PROC MIXED. Data are represented as least squares means \pm standard error of the mean and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$. When < 0.10 the P-value for the fixed effects and the pre-planned contrasts were listed on the figure.

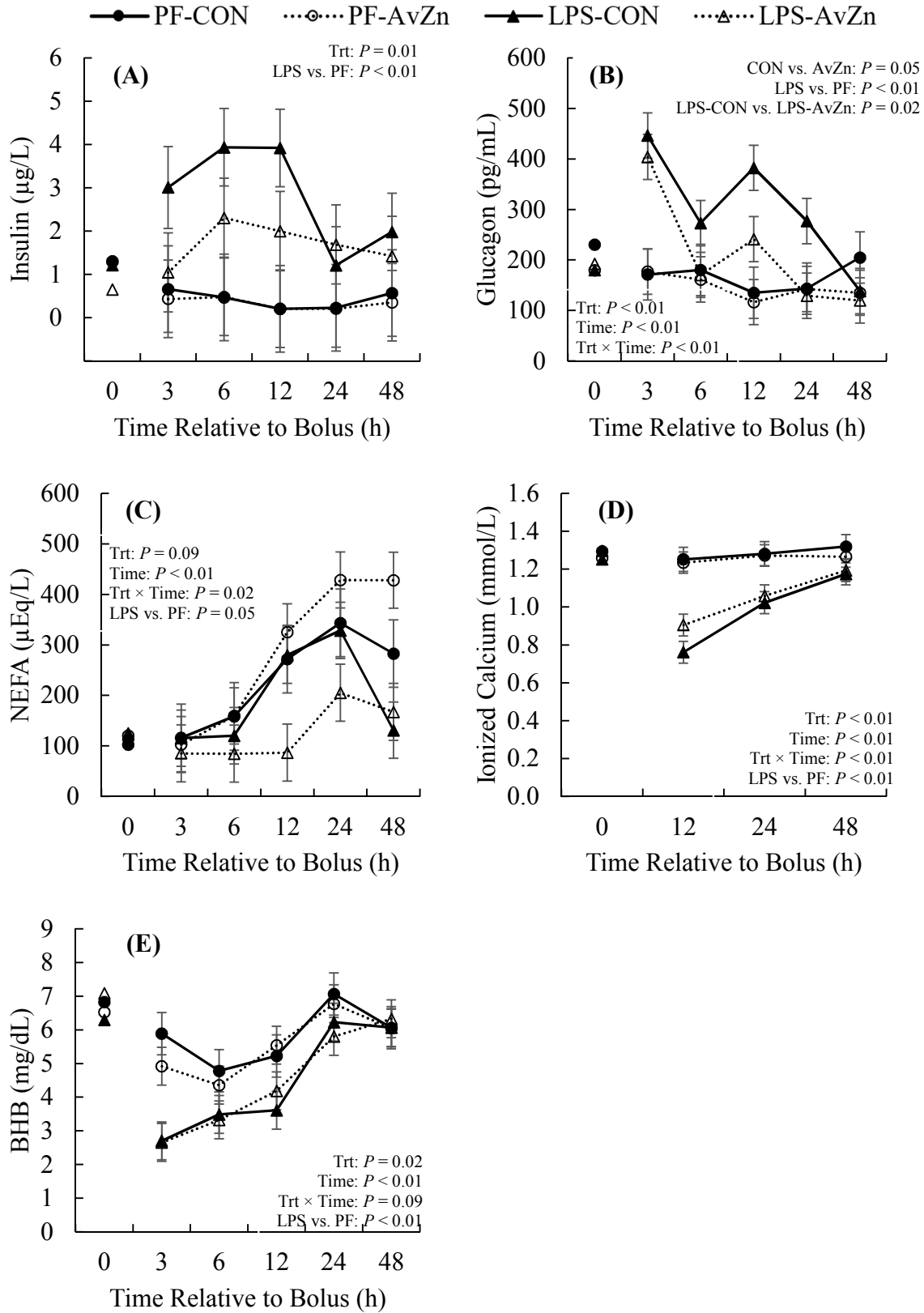


Figure 4.3. Effects of an LPS bolus or pair-feeding in dairy cows fed a control diet (CON) or Availa-Zn (AvZn) supplemented diet on circulating (A) insulin, (B) glucagon, (C) non-esterified fatty acids (NEFA), (D) β -hydroxybutyrate, and (E) ionized calcium. Time 0 represents an average of measurements obtained during the baseline and used as a covariate for P2 and P3 analysis. Fixed effects of treatment, time, and treatment \times time were assessed as a completely randomized design using PROC MIXED. Data are represented as least squares means \pm standard error of the mean and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$. When < 0.10 the P-value for the fixed effects and the pre-planned contrasts were listed on the figure.

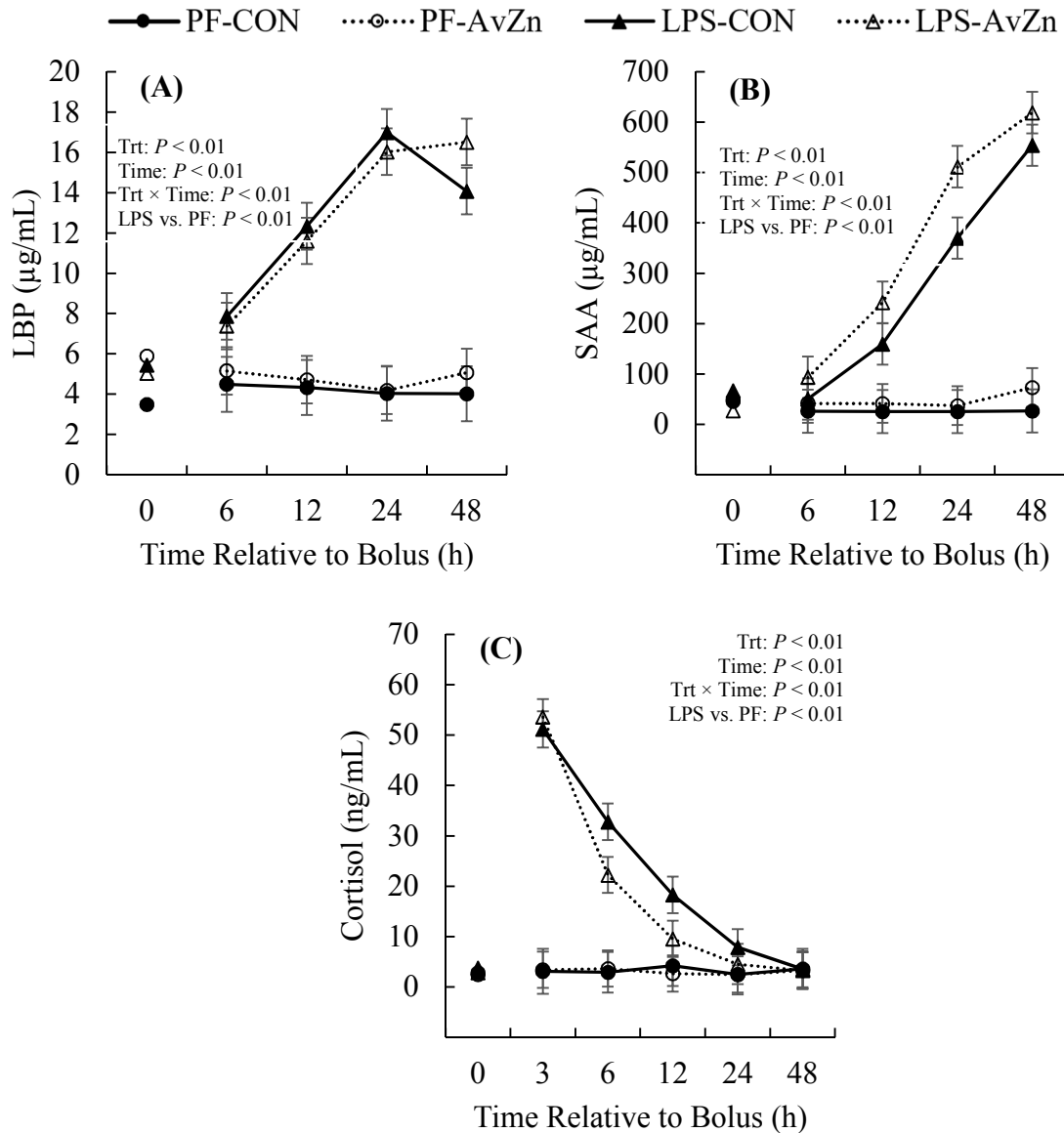


Figure 4.4. Effects of an LPS bolus or pair-feeding in dairy cows fed a control diet (CON) or Availa-Zn (AvZn) supplemented diet on circulating (A) LPS-binding protein (LBP), (B) serum amyloid A (SAA), and (C) cortisol. Time 0 represents an average of measurements obtained during the baseline and used as a covariate for P2 and P3 analysis. Fixed effects of treatment, time, and treatment \times time were assessed as a completely randomized design using PROC MIXED. Data are represented as least squares means \pm standard error of the mean and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$. When < 0.10 the P-value for the fixed effects and the pre-planned contrasts were listed on the figure.

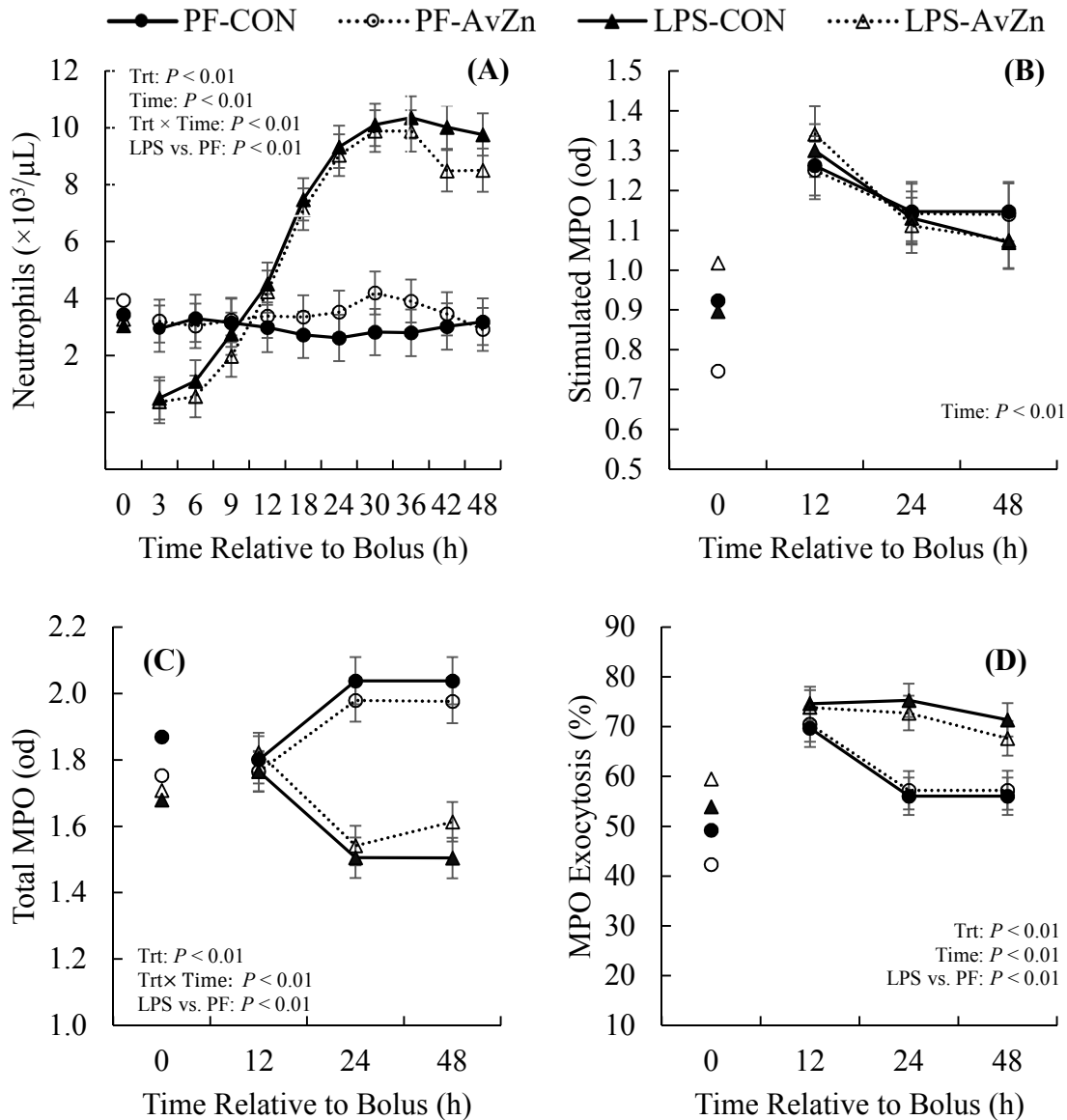


Figure 4.5. Effects of an LPS bolus or pair-feeding in dairy cows fed a control diet (CON) or Availa-Zn (AvZn) supplemented diet on (A) circulating neutrophils, (B) neutrophil stimulated myeloperoxidase (MPO) release, (C) total MPO release, and (D) MPO exocytosis. Time 0 represents an average of measurements obtained during the baseline and used as a covariate for P2 and P3 analysis. Fixed effects of treatment, time, and treatment \times time were assessed as a completely randomized design using PROC MIXED. Data are represented as least squares means \pm standard error of the mean and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$. When < 0.10 the P-value for the fixed effects and the pre-planned contrasts were listed on the figure.

CHAPTER 5. EFFECTS OF MAINTAINING EUCALCEMIA FOLLOWING IMMUNOACTIVATION IN LACTATING HOLSTEIN DAIRY COWS

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Abstract

Periparturient hypocalcemia is a common metabolic disorder and it is ostensibly associated with negative health and production outcomes. Acute infection also markedly decreases circulating calcium (Ca), but the reasons for and consequences of it on physiological and immunological parameters are unknown. Objectives were to evaluate the effects of maintaining eucalcemia on production, metabolic, and immune variables following an intravenous lipopolysaccharide (LPS) challenge. Twelve multiparous lactating Holstein cows (717 ± 20 kg BW; 176 ± 34 DIM; parity 3 ± 0.2) were enrolled in a study containing 2 experimental periods (P); during P1 (3d), cows consumed feed ad libitum and baseline values were obtained. At the initiation of P2 (4d), cows were randomly assigned to 1 of 2 treatments: 1) LPS administered (LPS-Con; $0.5 \mu\text{g/kg}$ of BW LPS; $n=6$) or 2) LPS administered + eucalcemic clamp (LPS-Ca; $0.5 \mu\text{g/kg}$ of BW LPS; Ca infusion; $n=6$). Cows were fasted for the first 12h during P2. After LPS administration, ionized Ca (iCa) was determined every 15 min for 6h and every 30 min for an additional 6h and intravenous Ca infusion was adjusted in LPS-Ca cows to maintain eucalcemia. Blood iCa was decreased 23% for the first 12h postbolus in LPS-Con cows, and by design, Ca infusion prevented hypocalcemia. To maintain eucalcemia for the 12h, 13.7 g of Ca was infused. The total Ca deficit (including Ca not secreted into milk) accumulated over the 12h was 10.4 and 20.2 g for the LPS-Con and LPS-Ca treatments, respectively. Mild

hyperthermia (0.8°C) occurred for ~6h post-LPS administration relative to P1. From 6-7 h postbolus rectal temperature from LPS-Ca cows was increased (0.6°C) relative to LPS-Con cows. On d1 of P2, milk yield decreased (61%) in both treatments relative to P1. Relative to LPS-Con cows, milk yield decreased (15%) in LPS-Ca cows during P2. Overall, circulating LPS-binding protein (LBP) continuously increased postbolus, and at 24h LBP levels in LPS-Ca cows were increased (80%) relative to LPS-Con cows. During P2, serum amyloid A increased (4-fold) in both treatments relative to P1. Administering LPS initially decreased circulating neutrophils then cell counts progressively increased with time. Calcium infusion decreased neutrophil counts (40%) from 9-12h postbolus relative to LPS-Con cows. Neutrophil function, as assessed by oxidative burst and myeloperoxidase production, did not differ due to treatment. In summary, maintaining eucalcemia (via intravenous Ca infusion) during an immune challenge appeared to intensify inflammation and adversely affect lactation performance.

Keywords: calcium, endotoxin, inflammation, infection

Introduction

At the onset of lactation, the mammary gland's use of plasma calcium (**Ca**) is so extensive that it often exceeds the capacity of homeostatic mechanisms (i.e., parathyroid hormone [PTH] and vitamin D) to replenish it and, consequently, cows develop clinical or subclinical hypocalcemia (SCH; Horst et al., 2005; Goff, 2008). Hypocalcemia has been loosely associated with reduced milk production and increased disease incidence (Chapinal et al., 2011; Martinez et al., 2012, 2014; Venjakob et al., 2017) and is believed to be a causative factor in periparturient immunosuppression (Ducusin et al., 2003; Kimura et al., 2006; Martinez et al., 2012, 2014). Despite the purported immune “dysfunction”, nearly all transition cows (even seemingly healthy ones) experience some degree of inflammation postpartum (Humblet et al., 2006). The magnitude and persistency of the inflammatory state appears dependent on the

frequency and type of immune insults (Bertoni et al., 2008; Bradford et al., 2015; Trevisi and Minuti, 2018) and is predictive of transition cow performance (Ohtsuka et al., 2001; Ametaj et al., 2005, 2010; Abuajamieh et al., 2016).

Interestingly, independent of the transition period, immune activation decreases circulating Ca and the response is conserved across species including humans (Cardenas-Rivero et al., 1989; Dias et al., 2013), calves (Tennant et al., 1973; Elsasser et al., 1996), dogs (Holowaychuk et al., 2012), horses (Toribio et al., 2005), pigs (Carlstedt et al., 2000), and sheep (Naylor and Kronfeld, 1986). We and others have repeatedly demonstrated lipopolysaccharide (LPS)-induced hypocalcemia in lactating cows (Griel et al., 1975; Waldron et al., 2003; Kvidera et al., 2017; Horst et al., 2018). Thus, because most periparturient cows are inflamed, immunoactivation may be contributing (at least to some extent) to hypocalcemia during the transition period; a theory originally proposed by early investigators (Thomas, 1889; Hibbs, 1950). Inflammation's effect on Ca homeostasis may explain why SCH remains common, despite successfully implementing pre-partum prophylactic and therapeutic strategies (i.e., acidifying rations, low Ca diets, Ca chelators, etc.) aimed at preventing milk fever (Reinhardt et al., 2011).

Post-calving oral Ca supplementation is an additional approach to mitigate SCH (Oetzel and Miller, 2012; Oetzel, 2013). Aside from improving plasma Ca concentrations and despite some inconsistencies (Domino et al., 2017), oral Ca boluses have been shown to improve milk production and some health metrics (Oetzel et al., 1996; Oetzel and Miller, 2012). We recently demonstrated that oral Ca (in combination with live yeast) prior to and following LPS administration ameliorates hypocalcemia (although it did not prevent it) and improves production (feed intake and milk yield) performance (Al-Qaisi et al., 2020). Unfortunately, we

were unable to distinguish the effects of Ca and live yeast, however, considering Ca's presumed role in immune function and transition cow performance, we conjectured the beneficial effects of the oral bolus were primarily due to alleviating hypocalcemia. Therefore, we hypothesized that maintaining eucalcemia during immunoactivation in lactating dairy cows would improve production and inflammatory outcomes. Study objectives were to evaluate the effects of preventing hypocalcemia on production parameters, inflammatory biomarkers, and neutrophil function in intensely immune activated lactating dairy cows.

Materials and Methods

Animals and Experimental Design

All procedures were approved by the Iowa State University Institutional Animal Care and Use Committee. Twelve non-pregnant lactating Holstein cows (717 ± 20 kg BW; 176 ± 34 DIM; parity 3 ± 0.2) were housed in individual box-stalls (4.57×4.57 m) at the Iowa State University Dairy Farm (Ames, IA). Cows were allowed 3 d to acclimate during which they were implanted with bilateral jugular catheters. Cows were fed ad libitum once daily (0600 h) with a diet formulated to meet or exceed the predicted requirements (NRC, 2001) of energy, protein, minerals, and vitamins (Table 5.1). Cows were milked twice daily (0600 and 1800 h) throughout the experiment and yield was recorded. A sample for composition analysis (milk fat, protein, lactose, MUN, and SCC) was obtained at each milking and stored at 4°C with a preservative (bronopol tablet; D & F Control System, San Ramon, CA) until analysis by Dairy Lab Services (Dubuque, IA) using AOAC approved infrared analysis equipment and procedures (AOAC International, 1995). Additional milk samples were collected and stored at -20°C until analysis by the Iowa State University Veterinary Diagnostic Lab (Ames, IA) for total Ca concentrations using inductively coupled plasma mass spectrometry (ICP-MS; Analytic Jena Inc. Woburn, MA,

USA). Rectal temperature was measured using a digital thermometer (GLA M700 Digital Thermometer, San Luis Obispo, CA) and was recorded after each milking.

The trial consisted of 2 experimental periods (**P**); P1 lasted 3 d and served as the baseline which yielded data for covariate analysis. During P2, which lasted 4 d, animals were randomly assigned to 1 of 2 intravenous bolus treatments: 1) LPS administered in which hypocalcemia was allowed to develop (**LPS-Con**; 0.5 µg/kg of BW of LPS; n = 6) and 2) LPS administered in which eucalcemia was maintained (**LPS-Ca**; 0.5 µg/kg of BW of LPS; Ca infusion; n = 6). Lipopolysaccharide (*Escherichia coli* O55:B5; Sigma Aldrich, St. Louis, MO) was dissolved in sterile saline at a concentration of 89.6 µg/mL and passed through a 0.2 µm sterile syringe filter (Thermo Scientific; Waltham, MA). The total volume of LPS solution administered intravenously was approximately 4 mL. Each respective treatment bolus was administered immediately following the AM milking and 0 min blood sample collection. In the LPS-Ca treatment, we performed a eucalcemic clamp, where a 23% Ca gluconate solution (Agri Laboratories, Ltd. St. Joseph, MO 64503) was intravenously infused at a known and adjustable rate utilizing a modular pump (Deltec 3000, Deltec Inc., St. Paul, MN) in order to maintain the pre-LPS administration blood iCa concentrations (\pm 10% of baseline). To ensure infusion rate accuracy, 250 mL of the Ca gluconate solution was further diluted in 750 mL of sterile saline. Cows were tethered during the 12 h challenge period (but allowed to stand up and lay down) to allow for frequent sampling. Water was provided ad libitum and feed was removed from all cows ~0.5 h prior to treatment administration. Animals remained fasted during the 12 h data collection period to eliminate the confounding effect of dissimilar nutrient intake.

To establish baseline iCa levels, blood samples were obtained at -30 and 0 min relative to LPS administration and immediately analyzed using an iSTAT handheld machine and cartridge

(CG8+; Abbott Point of Care, Princeton, NJ). Blood iCa was measured hourly for LPS-Con cows. For cows in the LPS-Ca treatment, blood samples were obtained every 15 min for the first 6 h and every 30 min for the next 6 h thereafter. Ionized Ca infusion began when blood iCa content declined below baseline concentrations. The rate of Ca infusion (**ROCI**) was transformed from mL/h to g/h. The total Ca infused for each cow was calculated using the ROCI for each interval (36 intervals in total) according to the following equation:

$$\sum_{i=0}^{24} ROCI \left(\frac{g}{h} \right)_i * \frac{1 h}{60 min} * 15 min + \sum_{i=0}^{12} ROCI \left(\frac{g}{h} \right)_i * \frac{1 h}{60 min} * 30 min$$

Milk total Ca content was assessed at the AM and PM milkings on d 1 of P2 and analyzed for total Ca concentrations. Additional plasma samples were collected from all treatments daily at 0600 h during P1 and at -0.5, 0, 6, 12, 24, 48, and 96 h relative to bolus administration during P2. Plasma was harvested following centrifugation at $1500 \times g$ for 15 min at 4°C and was subsequently frozen at -20°C until analysis.

Plasma insulin, glucagon, non-esterified fatty acids (**NEFA**), BHB, BUN, LPS-binding protein (**LBP**), and serum amyloid A (**SAA**) concentrations were determined using commercially available kits according to manufacturers' instructions (insulin, Mercodia AB, Uppsala, Sweden; Glucagon, RD Systems, Inc., Minneapolis, MN; NEFA, Wako Chemicals USA, Richmond, VA; BHB, Pointe Scientific Inc. Canton, MI; BUN Teco Diagnostics Anaheim, CA; LBP, Hycult Biotech, Uden, the Netherlands; SAA, Tridelata Development Ltd., Kildare, Ireland). The inter- and intra- coefficients of variation for insulin, glucagon, NEFA, BHB, BUN, LBP, and SAA assays were 4.3 and 6.0%, 10.5 and 14.1%, 3.0 and 3.6%, 4.2 and 7.1%, 3.4 and 3.6%, 11.3 and 8.6%, and 22.3 and 5.3%, respectively. For complete blood count (**CBC**) analysis a 3 mL blood sample was collected at 0, 1, 2, 3, 4, 5, 6, 9, 12, 24, 36, 48, 72, and 96 h relative to

bolus administration (K₂EDTA; BD Franklin Lakes, NJ) and stored at 4°C for ~12 h before submission to the Iowa State University's Department of Veterinary Pathology.

PMN Isolation and Function

Blood samples (32 mL) for neutrophil isolation were collected on d 4 of P1, and at 6, 12, 24, and 48 h post-LPS administration into 50 mL conical tubes containing acid citrate dextrose (8 mL). Samples were immediately transported to the laboratory for neutrophil isolation as previously described (Kimura et al., 2014). In brief, samples were centrifuged at $1,000 \times g$ for 20 min at room temperature and the plasma, buffy coat, and upper portion the packed red blood cell (RBC) pellet were removed. The remaining RBC were lysed twice using hypotonic phosphate buffered deionized water, and neutrophils were pelleted following centrifugation at $300 \times g$ for 5 min. Neutrophils were resuspended in PBS and standardized to 5.0×10^7 cells/mL. Neutrophil function was assessed by extracellular release of myeloperoxidase (MPO) and oxidative burst (cytochrome C reduction) using methods previously described by Kimura et al. (2014).

To assess oxidative burst, 2.5×10^6 neutrophils were treated with either Hanks' balanced salt solution (HBSS) or HBSS plus phorbol myristate acetate (PMA) followed by treatment with cytochrome C. Cells were incubated at 39°C for 15 min and immediately read at 2 wavelengths (550 nm and 650 nm) using a spectrophotometer.

Three cell preparations were used to assess MPO activity: 1) cells were lysed by treatment with cetyltrimethylammonium bromide solution as a measure of total MPO, 2) PMN were stimulated with equal parts Ca ionophore A23187 and cytochalasin B in HBSS to assess release of MPO with stimulation and, 3) PMN treated with HBSS alone as a measure of unstimulated MPO release. Each preparation was loaded with 1.25×10^6 cells in a microtiter plate and incubated at 39°C for 20 min. Following incubation, 50 μ L/well of 3,3', 5,5'-tetramethylbenzidine dihydrochloride was added (3.25 mM) followed promptly with 50 μ L/well

of H₂O₂. The reaction was stopped after a 2 min incubation with the addition of 50 µL/well 2N H₂SO₄. Plates were centrifuged for 1 min at 600 × g and the supernatant was transferred to a second plate. Optical density was determined at 405 nm using a spectrophotometer. Percentage of MPO released from PMN was determined using the following equation:

$$\text{Exocytosis (\%)} = [(\text{OD of stimulated PMN})/(\text{OD of lysed PMN})] \times 100$$

Calculations and Statistical Analysis

Administering LPS decreases milk yield and therefore decreases Ca uptake by the mammary gland for milk synthesis. The decrease in milk yield permitted us to estimate the amount of Ca conserved (milk Ca deficit) in cows administered LPS and allowed to develop hypocalcemia and those maintained at eucalcemia. Total Ca output in milk before the challenge was calculated to establish a baseline. Milk Ca content at 12 h was subtracted from the baseline to calculate the milk Ca deficit. For LPS-Con cows, milk Ca deficit was used solely to calculate the total Ca deficit. For LPS-Ca cows, milk Ca deficit plus the amount of Ca infused to maintain eucalcemia were combined to obtain the total Ca deficit, and this approach is similar to how we calculated the glucose deficit in our previous LPS-euglycemic clamp papers (Kvidera et al., 2017, Horst et al., 2018, 2019).

Sample size determination (6 animals/treatment) was based on previous reports and logistical constraints (Horst et al., 2019; Al-Qaisi et al., 2020). Post-hoc power analysis (PROC POWER; SAS Inst. Inc., Cary, NC) based on the results of our primary objective (ionized calcium) indicated a statistical power of >90% (alpha = 0.05). Each animal's respective parameter was analyzed using a repeated measures ANOVA with an autoregressive covariance structure for milk yield, DMI, and milk composition and a spatial power law covariance structure for blood metabolites, inflammatory biomarkers, neutrophil function assays, CBC, iSTAT, and rectal temperature data. The repeated effect was time relative to LPS administration. Each

specific variable's pre-bolus values (i.e., P1 average or 0 h when available) served as a covariate for analysis of P2. Effects of treatment, time (h or d relative to bolus administration), and treatment \times time interactions were assessed as a completely randomized design using PROC MIXED (SAS Inst. Inc., Cary, NC). In order to evaluate effects of LPS administration, a separate analysis (using the same model) was used to make statistical comparisons with baseline (pre- vs. post-LPS) in which the average P1 value (DMI, milk yield, and neutrophil function) or 0 h (rectal temperature and blood variables) for each parameter was included as an additional timepoint. Data are reported as least squares means and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$.

Results

Calcium and Blood Gas Analysis

Administering LPS decreased blood iCa (23%) for the first 12 h postbolus in LPS-Con cows and, by design, Ca infusion prevented hypocalcemia in LPS-Ca cows ($P < 0.01$; Figure 5.1). Collectively, 13.7 g of Ca was infused to maintain eucalcemia during the 12 h clamp. Milk Ca concentrations averaged 1.24 ± 0.04 and 0.94 ± 0.04 at the AM milking and 1.55 ± 0.13 and 1.71 ± 0.09 at the PM milking for LPS-Con and LPS-Ca cows, respectively. Milk yield averaged 18.1 and 20.2 kg at the AM milking and 7.3 and 7.9 kg at the PM milking for LPS-Con and LPS-Ca cows, respectively. The total Ca deficit accumulated over the 12 h was 10.4 and 20.2 g for the LPS-Con and LPS-Ca treatments, respectively. During P2, Ca infusion increased HCO_3^- , base excess, and pH relative to LPS-Con cows ($P \leq 0.03$; Table 5.2). Mild hyperthermia (0.8°C) was observed for ~ 5.5 h post-LPS administration relative to P1 ($P \leq 0.05$). From 6-7 h postbolus, rectal temperature in LPS-Ca cows increased (0.6°C) relative to LPS-Con cows ($P < 0.01$; Figure 5.2A).

Production Metrics

Overall, DMI did not differ between treatments ($P > 0.25$); however, hypophagia was observed for 2 d postbolus relative to baseline (34 and 45% for LPS-Con and LPS-Ca, respectively; $P < 0.01$; Figure 5.2B). On d 3 postbolus, DMI in LPS-Ca cows tended to remain below baseline (15%; $P = 0.07$), while it returned to pre-LPS infusion levels in LPS-Con cows (Figure 5.2B). Relative to baseline, milk yield decreased (45%) in both treatments for the first 2 d postbolus (61% on d 1 and 29% on d 2; $P < 0.01$; Figure 5.2C). Overall during P2, milk yield from LPS-Ca cows tended to be decreased (15%) relative to LPS-Con cows ($P = 0.07$; Figure 5.2C) and was decreased on d 1 of P2 (37%; $P = 0.04$; Figure 5.2C). Milk lactose content decreased in both treatments postbolus, but the magnitude of decrease was greater in LPS-Ca cows relative to LPS-Con ($P < 0.01$; Table 5.3). Milk lactose gradually returned to baseline in both treatments with time ($P < 0.01$). Milk fat, protein, and MUN content increased in both treatments at 24 h following LPS administration ($P < 0.01$). From 12-24 h milk fat, protein, and MUN content were increased in LPS-Ca compared to LPS-Con cows (26, 11, and 17%, respectively; $P \leq 0.05$; Table 5.3). Milk SCC did not differ due to treatment ($P > 0.24$; Table 5.3).

Metabolic Variables

Regardless of treatment, administering LPS increased circulating glucose for 2 h postbolus relative to P1 (28%; $P < 0.05$), after which hypoglycemia developed (Figure 5.3A). Glucose concentrations returned to baseline at 24 h postbolus. Relative to P1, insulin concentrations increased post-LPS (60%) similarly amongst treatments ($P \leq 0.04$; Figure 5.3B). Overall, circulating glucagon increased for 12 h postbolus, then gradually decreased below baseline with time ($P = 0.04$; Figure 5.3C) and was unaffected by Ca infusion. Regardless of infusing Ca, NEFA concentrations increased (3-fold, relative to P1; $P < 0.01$) at 12 h postbolus,

then progressively decreased with time ($P < 0.01$; Figure 5.4A). Administering LPS decreased circulating BHB (33%, relative to P1) for 12 h postbolus, after which levels returned to baseline ($P \leq 0.05$). At 48 h postbolus, BHB concentrations from LPS-Ca cows tended to be decreased (20%) relative to controls ($P = 0.07$; Figure 5.4B). Blood urea nitrogen increased (41% for first 24 h; $P \leq 0.01$) similarly in both treatments postbolus relative to P1. Throughout P2, BUN concentrations from LPS-Ca cows increased (8%) relative to LPS-Con cows ($P = 0.05$; Figure 5.4C).

Inflammatory Biomarkers and Immune Metrics

Overall during P2, circulating LBP and SAA increased post-LPS administration (3- and 4-fold, respectively; $P < 0.01$; Figure 5.5A and 5.5B) relative to P1. At 24 h postbolus, circulating LBP from LPS-Ca cows increased (80%) relative to controls ($P = 0.02$; Figure 5.5A). Lipopolysaccharide administration initially decreased (66% for 12 h) circulating WBC then they progressively increased with time (33% from 24-48 h relative to baseline; $P < 0.01$). Circulating monocytes and lymphocytes initially decreased following LPS administration and gradually increased with time ($P < 0.01$). Maintaining eucalcemia had no effect on circulating WBC, monocytes, or lymphocytes (Table 5.4). The pattern of circulating neutrophils reflected the WBC, however, from 9-12 h postbolus neutrophils from LPS-Ca cows were decreased (40%) relative to LPS-Con ($P \leq 0.05$; Figure 5.5C). During P2, there were no overall treatment effects on neutrophil oxidative burst. However, when compared to P1, oxidative burst from LPS-Ca cows tended to increase at 6 h postbolus (31%; $P = 0.07$), whereas in LPS-Con cows it remained similar to baseline (Figure 5.6A). Similarly, MPO exocytosis and total MPO were not influenced by Ca infusion during P2. Neutrophil MPO exocytosis increased (76%) in both treatments for 12 h postbolus relative to P1 ($P \leq 0.10$; Figure 5.6B). Despite increased exocytosis, total MPO did not differ from P1 in LPS-Con cows; however, it was increased (33%) 6 h postbolus in LPS-Ca

cows ($P = 0.03$; Figure 5.6C). In agreement with the other neutrophil function metrics, stimulated MPO release did not differ between treatments during P2. However, relative to P1, MPO release increased in both treatments, although the increase was prolonged in LPS-Con cows (66% over the 48 h; $P \leq 0.05$; Figure 5.6D).

Discussion

At lactation onset, dairy cows experience a marked increase in Ca requirements (>65%; DeGaris and Lean, 2008) to support colostrum and milk synthesis (Horst et al., 2005). Mammary Ca uptake is so acute and extensive it often outweighs the homeostatic mechanisms employed to replenish it and consequently clinical or SCH occurs (Horst et al., 2005; Goff, 2008). Implementing therapeutic and prophylactic strategies has markedly reduced the incidence of clinical hypocalcemia (Charbonneau et al., 2006), but SCH continues to afflict ~25% of primiparous and ~50% of multiparous cows in the United States (Reinhardt et al., 2011). Although asymptomatic, SCH has been loosely associated with reduced production performance (i.e., milk yield and feed intake), increased risk of DA, immunosuppression (and consequently an increased susceptibility to infectious disease), impaired reproduction, and an overall higher culling risk (Seifi et al., 2011; Oetzel and Miller, 2012; Martinez et al., 2014). Interestingly, the severity of the maladies associated with hypocalcemia appear to be dependent on the magnitude, persistency, and timing of SCH (Caixeta et al., 2017; McArt and Neves, 2019); such that transient hypocalcemia was unrelated to health metrics, whereas persistent and delayed hypocalcemia were associated with detrimental outcomes. These different hypocalcemia “types” may explain why inconsistencies exist regarding the association between hypocalcemia and transition cow health and performance (Martinez et al., 2012; Jawor et al., 2012; Gidd et al., 2015; Venjakob et al., 2017). Although it is becoming evident that not all cases of SCH are

equivalent, it remains unclear what underlying factors explain the different hypocalcemia classifications.

Aside from the transition period, we and others have repeatedly demonstrated that experimental immune activation (via LPS infusion) markedly reduces circulating Ca in lactating cows (Griel et al., 1975; Waldron et al., 2003; Kvidera et al., 2017; Horst et al., 2018, 2019; Al-Qaisi et al., 2020) and the response is conserved across species (Tennant et al., 1973; Cardenas-Rivero et al., 1989; Elsasser et al., 1996; Carlstedt et al., 2000; Dias et al., 2013). Immune insults are prevalent in the transition period and it is likely that nearly all cows experience repeated challenges leading up to parturition (Trevisi and Minuti, 2018) and the frequency of these challenges likely relates to the magnitude of inflammation (Trevisi and Minuti, 2018). Interestingly, immune activation was originally hypothesized by early investigators to be involved with milk fever (Thomas, 1889; Hibbs, 1950), but only a few studies in recent literature have considered it a contributing factor (Aiumlamai et al., 1992; Eckel and Ametaj, 2016). It is of interest to fully understand the relationship between immune activation and Ca homeostasis and how providing intravenous Ca may alter animal health and productivity as this likely has practical implications to the transition period and farm profitability.

Recently, we demonstrated that oral Ca (in combination with live yeast) prior to and following LPS administration ameliorates hypocalcemia and improves production performance in lactating cows (Al-Qaisi et al., 2020). However, because the oral bolus contained both Ca and live yeast we were unable to isolate how each component influenced the response. Yeast has previously been shown to be immunomodulatory (Yuan et al., 2015) and improve production performance (Desnoyers et al., 2009; Zaworski et al., 2014; Broadway et al., 2015), however, some inconsistencies exist (Swartz et al., 1994; Robinson et al., 1997; Yuan et al., 2015). Given

the marked changes in Ca homeostasis that occur following LPS administration and Ca's role in regulating processes such as gastrointestinal motility (Daniel, 1983), metabolism (Martinez et al., 2012, 2014), and immune function (Lewis, 2001; Kimura et al., 2006) we hypothesized that Ca was the primary mediator of the beneficial effects observed by Al-Qaisi et al. (2020). Thus, study objectives were to evaluate the effects of maintaining eucalcemia following LPS administration on production parameters, inflammatory biomarkers, and PMN function in lactating cows.

In agreement with others (Waldron et al., 2003; Kvidera et al., 2017; Horst et al., 2018; Al-Qaisi et al., 2020) we observed marked and sustained hypocalcemia (in the LPS-Con cows) in response to LPS administration. Furthermore, we successfully maintained eucalcemia for 12 h post-LPS administration via continuous intravenous Ca infusion in the LPS-Ca treatment. The decrease in milk yield following LPS infusion allowed us to calculate the amount of Ca not going towards milk synthesis (milk Ca deficit). The milk Ca deficit was 10.4 and 6.5 g for LPS-Con and LPS-Ca cows, respectively. In LPS-Con cows the milk Ca deficit makes up the entirety of the total Ca deficit calculation. In LPS-Ca cows the amount of Ca infused during the 12 h eucalcemic-clamp was 13.7 g which provides a total Ca deficit of 20.2 g in LPS-Ca cows. Even though we and others have repeatedly demonstrated LPS-induced hypocalcemia, little is known regarding where the 20.2 g of Ca is going and what mechanisms are regulating the disappearance.

Circulating Ca is normally under tight homeostatic control, however, during inflammation key Ca homeostatic regulators are markedly altered to favor hypocalcemia. For example, although not always consistent (Toribio et al., 2005; Merriman et al., 2017), hypoparathyroidism (Nielsen et al., 1997; Holowaychuk et al., 2012), increased circulating calcitonin and procalcitonin (Müller et al., 2000; Bonelli et al., 2018), and decreased 1,25-

(OH)₂D concentrations (Waldron et al., 2003; Holowaychuk et al., 2012) are observed in immunoactivated states. Cytokines released during infection upregulate the parathyroid Ca sensing receptor, reducing the threshold necessary for suppression of PTH secretion (Hendy et al., 2015; Klein, 2018), resulting in hypocalcemia concurrently with hypoparathyroidism. Even if hormonal regulation favors hypocalcemia during immune activation, the acute decrease in Ca is likely too rapid to be explained by these mechanisms (Waldron et al., 2003). Rather it is probably mediated by changes in cellular/tissue uptake and/or systemic clearance.

Extracellular Ca uptake by leukocytes is a key initial feature of activation and is necessary for cell proliferation and function (Lewis, 2001; Br  chard and Tschirhart, 2008). Leukocyte stimulation (i.e., interaction with antigen) triggers a signaling cascade resulting in Ca influx from the endoplasmic reticulum (**ER**) to the cytosol. This increase in cytosolic Ca signals influx from the extracellular space via Ca²⁺ release-activated Ca²⁺ channels (Lewis, 2001). Given the large gradient between extra- and intracellular Ca stores (extracellular concentrations 800-fold greater than intracellular; Goff, 1999) and the relatively small number of leukocytes in circulation (Ronald et al., 2014) it is unlikely that increased cellular Ca uptake contributes to decreased circulating concentrations (Waldron et al., 2003). Urinary Ca clearance is another probable source of Ca loss, however, reports in rodents demonstrated enhanced renal Ca conservation in response to LPS administration (Proksch et al., 1996; Ikeda et al., 2014; Meurer and H  cherl, 2019), whether the same is true in ruminants remains to be fully elucidated. Interestingly, milk became more Ca concentrated (50%) following LPS administration and this effect was most pronounced in the LPS-Ca cows, however, the marked milk yield reduction eliminated the mammary gland as a meaningful Ca sink. Interestingly, increased Ca accumulation in ascites and liver has been observed following endotoxin administration in pigs

(Carlstedt et al., 2000). Whether Ca sequestration at these sites explains the magnitude of plasma Ca reduction observed and whether it occurs similarly in ruminants is not fully understood and warrants further investigation, especially considering its practical importance to transition cows.

Decreased production performance (i.e., milk yield and DMI) is a typical response during infection and the magnitude of milk yield and DMI reduction observed herein agrees with previous LPS bolus studies (Waldron et al., 2003; Kvidera et al., 2017; Horst et al., 2018). Interestingly, and in contrast to expectations, we observed an exacerbated decrease in milk synthesis and a delayed return to euphagia when eucalcemia was maintained. Pernicious phenotypic responses were surprising considering improved productivity occurred when oral Ca was provided prior to and following LPS administration (Al-Qaisi et al., 2020). Reasons for the aforementioned discrepancies are unclear, but may be explained by administration route, effects of secondary signals, or the confounding effects of yeast. Intravenous Ca is apparently detrimental to hormonal regulation of Ca when compared to oral delivery, and studies suggest it should not be utilized to treat SCH (Wilms et al., 2019). However, because Ca was purposely infused to avoid hypercalcemia, it is unlikely that signaling pathways controlling Ca status were meaningfully altered herein. The release of secondary secretagogues in response to oral Ca is an intriguing explanation for the improved performance observed by Al-Qaisi et al. (2020). For example, Ca-sensing receptors initiate a plethora of signaling pathways regulating appetite, gut motility, metabolism, immune function, and inflammation (Brennan et al., 2014; Rehfeld et al., 2017; Liu et al., 2018). It remains unclear whether the absence of these secondary mediators with intravenous Ca can explain the marked differences between our former (Al-Qaisi et al., 2020) and current experiment. Future research investigating the mechanisms of oral vs. intravenous Ca administration following immunoactivation is warranted.

Successful immune activation was induced herein as indicated by changes in leukocyte dynamics and function, mild hyperthermia, and a marked increase in acute phase proteins (APP). In response to LPS-administration we observed a biphasic response in circulating neutrophils with initial neutropenia followed by neutrophilia which agrees with past reports (Bannerman et al., 2008; Horst et al., 2018). Reduced circulating neutrophil counts likely represent leukocyte endothelial adherence, tissue migration, or increased appearance in the marginated pool (Hoedemaker et al., 1992; Lang et al., 1992; Walther et al., 2000). Interestingly, neutropenia was more pronounced in Ca-infused cows from 9 to 12 h postbolus compared to LPS-Con cows. Although not entirely clear, this may indicate increased neutrophil extravasation or reduced production from bone marrow. Calcium is well-known for its roles in regulating leukocyte function (i.e., migration, endothelial adherence, phagocytosis, etc.; Cohen, 1994; Immler et al., 2018). Although not always consistent (Miltenburg et al., 2018), decreased circulating Ca concentrations have been associated with reduced leukocyte activity in cows (Ducusin et al., 2003; Kimura et al., 2006; Martinez et al., 2012; Zhang et al., 2019) and increasing Ca concentrations improved leukocyte activity (Vieira-Neto et al., 2017). Surprisingly, despite severe hypocalcemia in response to LPS we observed either no change or actually improved neutrophil function (i.e., MPO activity and oxidative burst). Additionally, Ca infusion had no effect on neutrophil function which agrees with others (Kehrli and Goff, 1989; Miltenburg et al., 2018). Although our model is acute and is not accompanied with periparturient physiological changes, our results challenge the tenet that hypocalcemia is the primary cause of post-calving immunosuppression (Ducusin et al., 2003; Kimura et al., 2006; Martinez et al., 2014). It is likely that the proposed transition cow immunosuppression is multifactorial and cannot be explained by a single factor.

Interestingly, cows maintained at eucalcemia post-LPS administration had significantly increased LBP and numerically increased SAA when compared to cows allowed to develop hypocalcemia. This contradicts our previous report in LPS-infused cows (Al-Qaisi et al., 2020) as well as reports in inflamed transition cows (Benzaquen et al., 2015) provided oral Ca. Reasons for observing differences in APP are not entirely clear, but may be explained by Ca's proposed role in LPS detoxification and its downstream effects on the inflammatory response (Skarnes and Chedid, 1964). During hypocalcemia, LPS aggregation is inhibited allowing LBP to transfer LPS monomers to cluster of differentiation 14 and eventually to acute-phase high density lipoproteins (**ap-HDL**) for biliary excretion by the liver. Formation of ap-HDL is mediated by SAA displacement of apolipoprotein from normal HDL (as reviewed by Eckel and Ametaj, 2016). When eucalcemia is maintained LPS monomers remain aggregated and this prevents its transfer to lipoproteins (Skarnes and Chedid, 1964). Maintaining extracellular Ca levels increases chemokine and cytokine release by circulating leukocytes thereby intensifying the inflammatory response (Rossol et al., 2012; Klein et al., 2016). For these reasons, LPS-induced hypocalcemia was opined to serve as a protective strategy during immune activation (Malcolm et al., 1989; Collage et al., 2013; Klein, 2018). This tenet is supported by the fact that Ca administration in septic states increases rates of organ failure and mortality (Malcolm et al., 1989; Hastbacka and Pettila, 2003; Dias et al., 2013). Thus, increased LBP observed herein may represent impaired lipoprotein mediated LPS detoxification. While Ca infusion may have prevented LPS disaggregation, the numerical increase in SAA concentrations from LPS-Ca cows may suggest formation of the ap-HDL molecule was not disrupted. Whether the changes in APP we observed with Ca infusion reflect an improved capacity to clear infection via pro-inflammatory mechanisms or an overly exaggerated immune response remain unclear, but the production

responses (i.e., decreased milk yield and delayed return to baseline DMI) and heightened febrile response suggests the latter. Reasons why oral Ca administration in LPS-administered cows did not alter APP production are unclear, but may be explained by the quantity of Ca appearing in circulation and the rate at which it entered. Further investigation into Ca's role in inflammation and how ameliorating hypocalcemia may influence animal health and wellbeing is of obvious interest.

The energetic requirements of immunoactivation are substantial and supporting the response requires marked alterations in nutrient partitioning (Johnson, 2012; Kvidera et al., 2017; Horst et al., 2018). The metabolic and hormonal changes implemented in response to LPS have been detailed in many of our recent publications (Kvidera et al., 2017; Horst et al., 2018; Horst et al., 2019). In agreement with our previous work, LPS administration increased circulating insulin, glucagon, and NEFA, but the patterns did not differ when eucalcemia was maintained. Not observing differences in circulating insulin was surprising considering Ca's role in insulin secretion from pancreatic β cells (Rorsman and Trube, 1986). Additionally, others have previously demonstrated hypoinsulinemia when administering the Ca chelator ethylene glycol tetraacetic acid in cows (Martinez et al., 2014). Interestingly, we observed increased circulating BUN in LPS-Ca cows, which agrees with changes in MUN, and as aforementioned may be connected to the changes observed in circulating APP as skeletal muscle proteolysis is an important source of amino acids needed for APP synthesis (Johnson, 2012). Circulating BHB was decreased with Ca administration, however the biological significance of this decrease is unclear. The mechanisms driving decreased BHB concentrations during infection remain largely unexplained, however, increased peripheral utilization (Zarrin et al., 2014; Horst and Baumgard, unpublished) and reduced production from the rumen epithelium (Pennington, 1952) due to

decreased substrate are possible contributors. Interestingly, the return to baseline BHB concentrations occurred prior to the return of euphagia which suggests reduced rumen epithelial production is not the primary reason for decreased circulating ketones.

Conclusion

Our results validate LPS administration as an effective model for inducing hypocalcemia. In contrast to oral Ca supplementation, we have demonstrated potential negative consequences (i.e., decreased milk yield and slower return to baseline DMI) of intravenous Ca infusion during immunoactivation and demonstrated little to no benefit of maintaining eucalcemia on leukocyte function. Additionally, Ca infusion increased circulating LBP concentrations which may suggest an impaired capacity to detoxify LPS via non-inflammatory routes. Future work should focus on the direct impact of Ca on lipoprotein mediated LPS detoxification and how it may impact an inflammatory response.

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Tables and Figures

Table 5.1. Ingredients and composition of diet¹

Ingredient	% of DM ²
Corn Silage	21.4
Alfalfa Hay	4.1
Baleage	16.4
Ground Corn	29.7
Corn Gluten Feed	9.4
Soy Plus ³	2.6
Soybean Meal	5.9
Whole Cottonseed	3.6
Molasses	1.8
Crude Protein Mix	5.1
Chemical analysis, % of DM	
Starch	27.9
CP	17.2
NDF	29.2
ADF	18.4
NE _L Mcal/kg DM	1.64

¹Values represent an average of ration nutrient summary reports collected throughout the trial. Diet moisture averaged 51.8%.

²Average nutrient levels: 4.86% Fat, 0.95% Ca, 0.43% P, 0.36% Mg, 0.23% S, 1.38% K, 0.49% Na, 0.65% Cl, 88.06 mg/kg of Zn, 51.31 mg/kg of Mn, 4.06 mg/kg of Fe, 15.64 mg/kg of Cu, 0.87 mg/kg of Co, 0.19 mg/kg Se, 0.87 mg/kg of I, 6,810.1 IU/kg of vitamin A, 643.6 IU/kg of vitamin D, and 18.9 IU/kg of vitamin E.

³Mechanically-processed soybean meal, Dairy Nutrition Plus, Ralston, IA

Table 5.2. iSTAT parameters from cows allowed to develop hypocalcemia vs. cows maintained at eucalcemia following lipopolysaccharide (LPS) infusion during P2¹

Parameters	Treatment ²		SEM	P		
	LPS-Con	LPS-Ca		Treatment	Time	Treatment × Time
Base excess, mmol/L	2.14	3.92	0.53	0.03	<0.01	0.15
HCO ₃ , mmol/L	26.29	27.85	0.47	0.03	<0.01	0.12
Potassium, mmol/L	3.88	3.93	0.05	0.47	<0.01	0.12
Sodium, mmol/L	137.91	138.04	0.48	0.87	<0.01	0.09
PCO ₂ , mmHg	39.08	39.56	0.54	0.55	<0.01	0.10
pH	7.43	7.46	0.01	<0.01	0.06	0.50
PO ₂ , mmHg	37.12	33.20	1.86	0.16	0.07	0.67
sO ₂ , %	68.05	65.19	1.93	0.32	0.01	0.16
TCO ₃ , mmol/L	27.46	29.04	0.46	0.02	<0.01	0.14

¹Data presented as an average value from samples collected during the 4 d of P2²LPS-Con = LPS bolus; LPS-Ca = LPS bolus, calcium infused**Table 5.3.** Milk composition from cows allowed to develop hypocalcemia vs. cows maintained at eucalcemia following lipopolysaccharide (LPS) infusion during period 2 (P2)¹

Parameters	Treatment ²		SEM	P		
	LPS-Con	LPS-Ca		Treatment	Time	Treatment × Time
Fat, %	4.38	4.80	0.20	0.18	<0.01	0.12
Lactose, %	4.64	4.67	0.06	0.73	<0.01	<0.01
Protein, %	3.39	3.45	0.09	0.67	<0.01	0.06
MUN, mg/dL	10.87	12.02	0.52	0.14	<0.01	0.45
Somatic Cell Count, ×1000	401	244	91	0.24	0.53	0.58

¹Data presented as an average value from samples collected during the 4 d of P2²LPS-Con = LPS bolus; LPS-Ca = LPS bolus, calcium infused**Table 5.4.** Circulating leukocytes from cows allowed to develop hypocalcemia vs. cows maintained at eucalcemia following lipopolysaccharide (LPS) infusion during period 2 (P2)¹

Parameters	Treatment ²		SEM	P		
	LPS-Con	LPS-Ca		Treatment	Time	Treatment × Time
White Blood cells, ×10 ³ /μL	6.71	6.49	0.58	0.80	<0.01	0.93
Monocytes, ×10 ³ /μL	0.21	0.17	0.05	0.57	<0.01	0.73
Lymphocytes, ×10 ³ /μL	3.11	2.94	0.20	0.55	<0.01	0.57

¹Data presented as an average value from samples collected during the 4 d of P2²LPS-Con = LPS bolus; LPS-Ca = LPS bolus, calcium infused

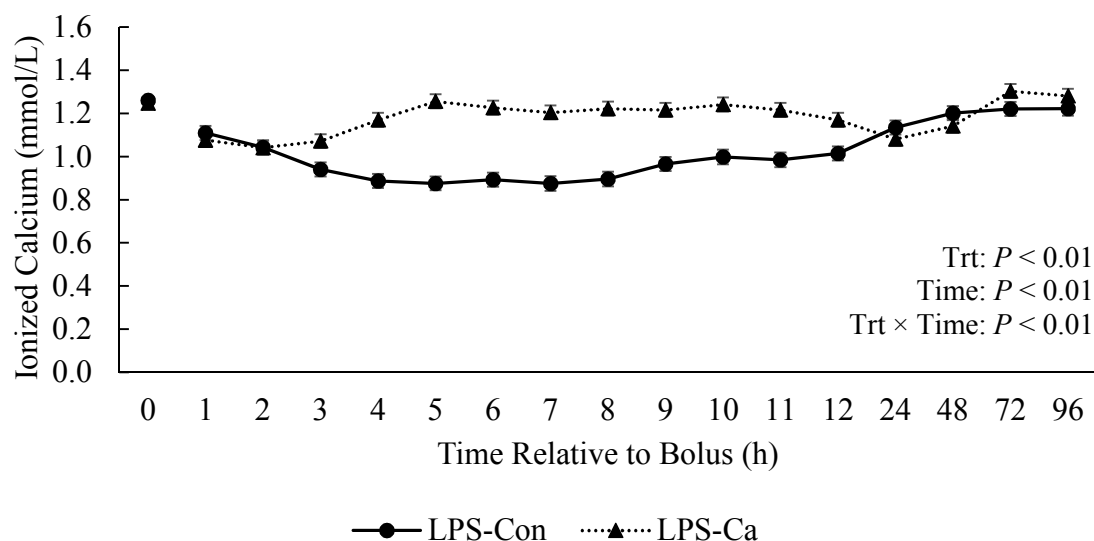


Figure 5.1. Ionized calcium concentrations from cows allowed to develop hypocalcemia vs. cows maintained at eucalcemia following lipopolysaccharide (LPS) infusion. Horizontal dashed lines represent $\pm 10\%$ of baseline. Data were analyzed using PROC MIXED and included fixed effects of treatment, hour, and their interaction. Hour 0 represents an average of measurements obtained prior to LPS administration (-0.5 and 0 h) and was utilized as a covariate. Data are represented as least squares means \pm standard error of the mean and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$. * represents significant differences between treatments. † represents a tendency for a difference between treatments.

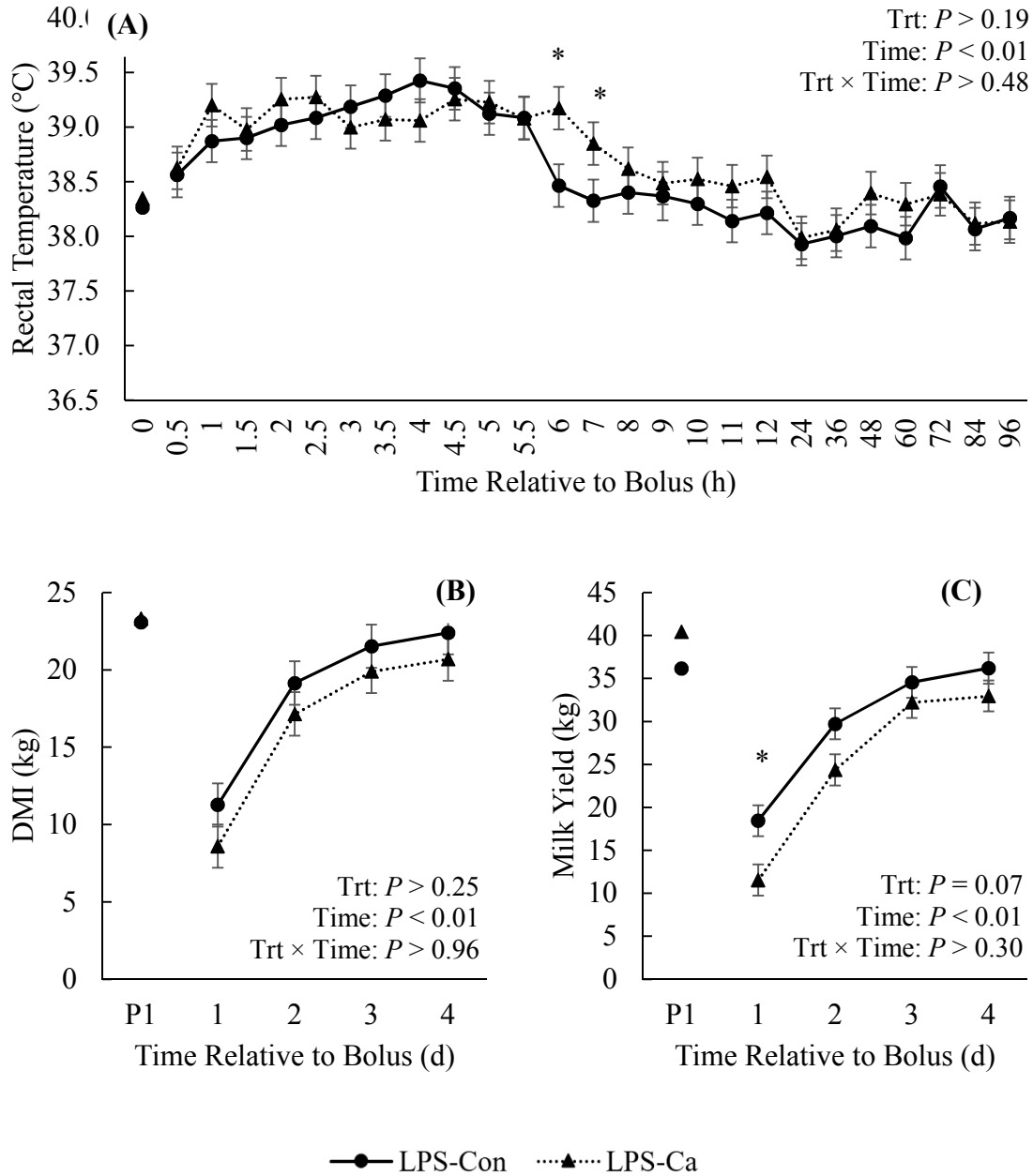


Figure 5.2. (A) Rectal temperature, (B) DMI, and (C) milk yield from cows allowed to develop hypocalcemia vs. cows maintained at eucalcemia following lipopolysaccharide (LPS) infusion. Data were analyzed using PROC MIXED and included fixed effects of treatment, time (h or d), and their interaction. Hour 0 represents an average of measurements obtained prior to LPS administration (-0.5 and 0 h) and was used as a covariate for rectal temperature. P1 represents an average of measurements obtained during the 3 d of P1 and was utilized as a covariate for DMI and milk yield. Data are represented as least squares means \pm standard error of the mean and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$. * represents significant differences between treatments. † represents a tendency for a difference between treatments.

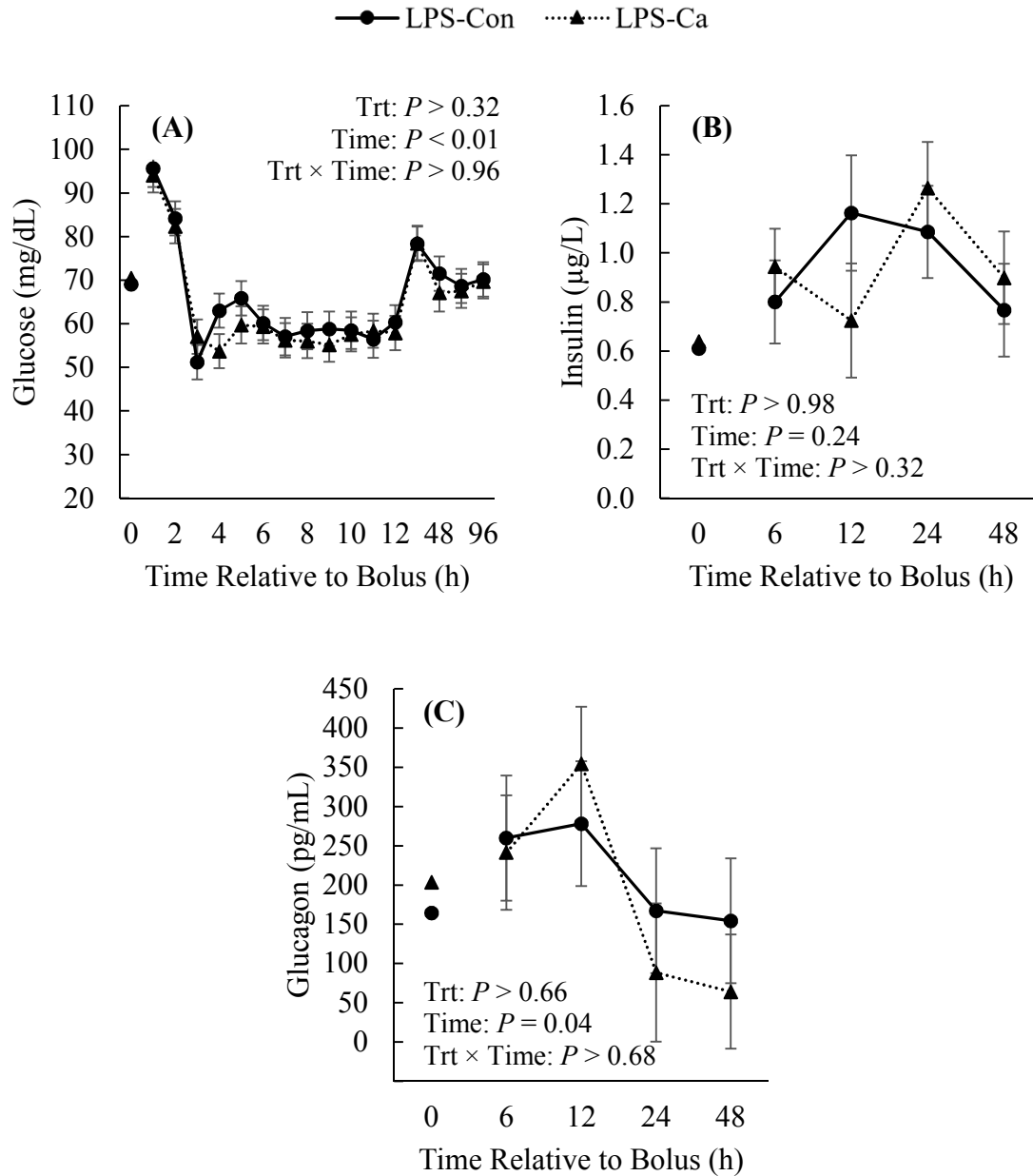


Figure 5.3. Circulating (A) glucose, (B) insulin, and (C) glucagon from cows allowed to develop hypocalcemia vs. cows maintained at eucalcemia following lipopolysaccharide (LPS) infusion. Data were analyzed using PROC MIXED and included fixed effects of treatment, hour, and their interaction. Hour 0 represents an average of measurements obtained prior to LPS administration (-0.5 and 0 h) and was utilized as a covariate. Data are represented as least squares means \pm standard error of the mean and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$. † represents a tendency between treatments.

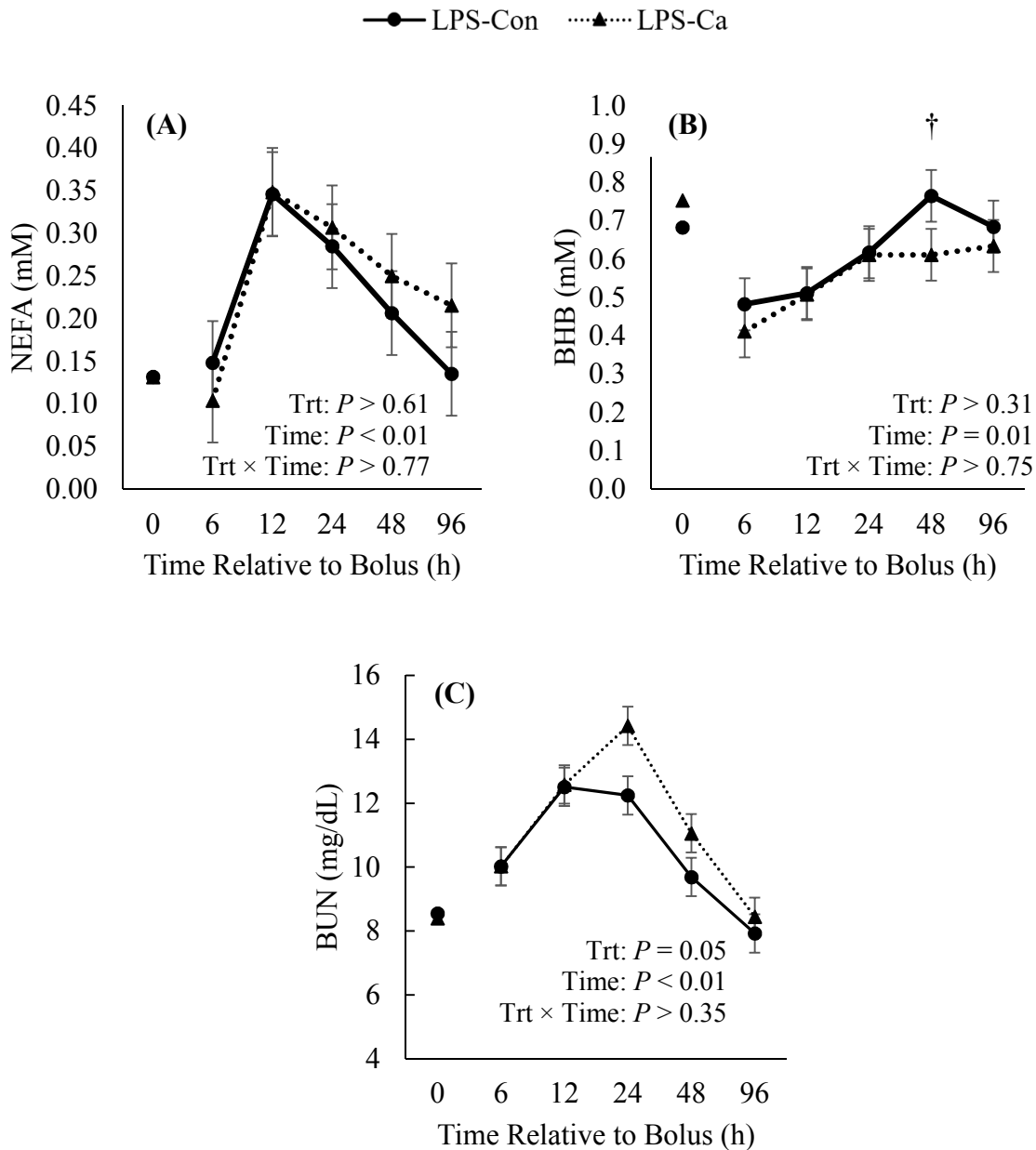


Figure 5.4. Circulating (A) non-esterified fatty acids, (B) BHB, and (C) BUN from cows allowed to develop hypocalcemia vs. cows maintained at eucalcemia following lipopolysaccharide (LPS) infusion. Data were analyzed using PROC MIXED and included fixed effects of treatment, hour, and their interaction. Hour 0 represents an average of measurements obtained prior to LPS administration (-0.5 and 0 h) was utilized as a covariate. Data are represented as least squares means \pm standard error of the mean and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$. * represents significant differences between treatments. † represents a tendency for a difference between treatments.

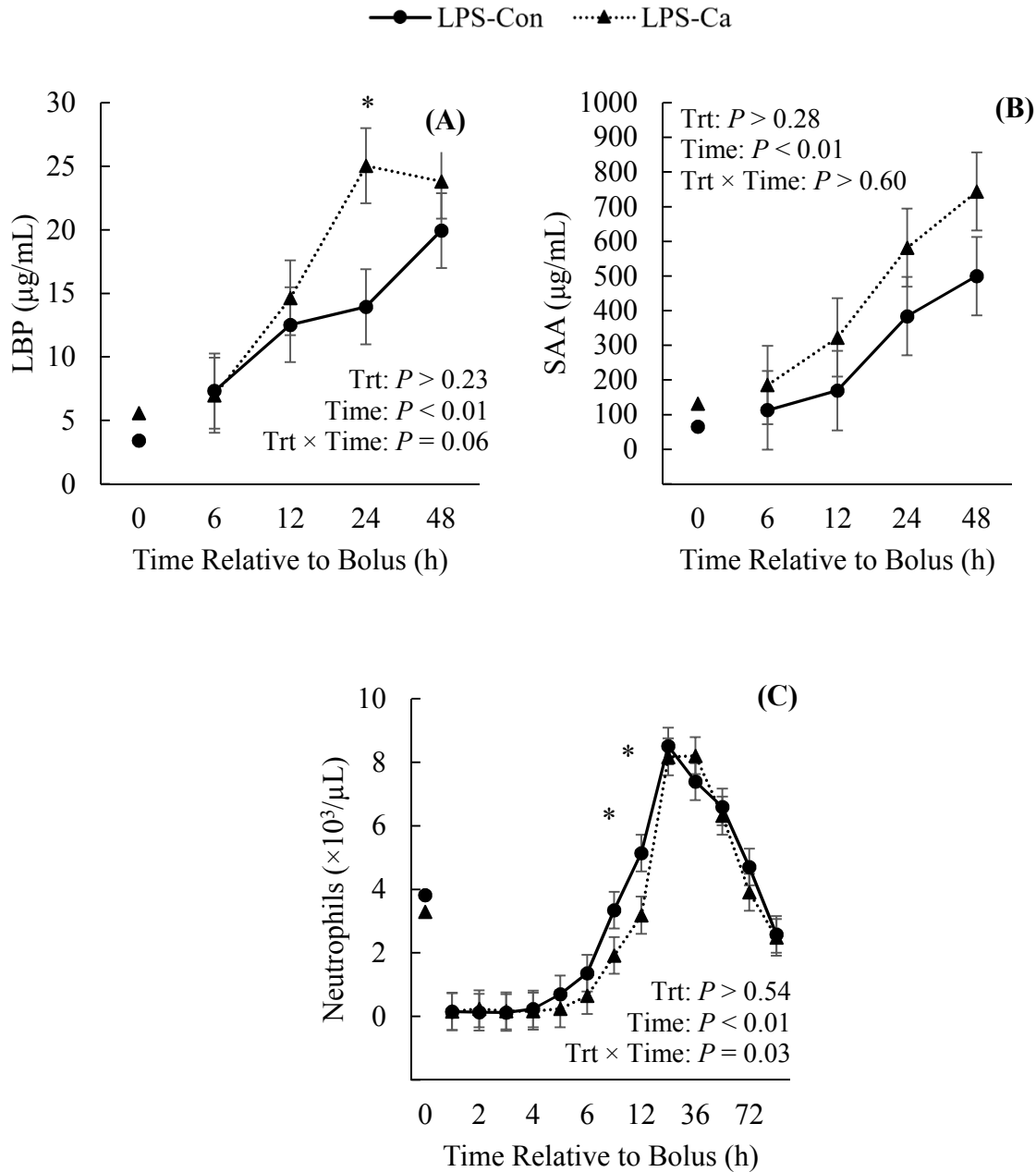


Figure 5.5. Circulating (A) LPS-binding protein, (B) serum amyloid A, and (C) neutrophil counts from cows allowed to develop hypocalcemia vs. cows maintained at eucalcemia following lipopolysaccharide (LPS) infusion. Data were analyzed using PROC MIXED and included fixed effects of treatment, hour, and their interaction. Hour 0 represents an average of measurements obtained prior to LPS administration (-0.5 and 0 h) and was utilized as a covariate. Data are represented as least squares means \pm standard error of the mean and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$. * represents significant differences between treatments. † represents a tendency for a difference between treatments.

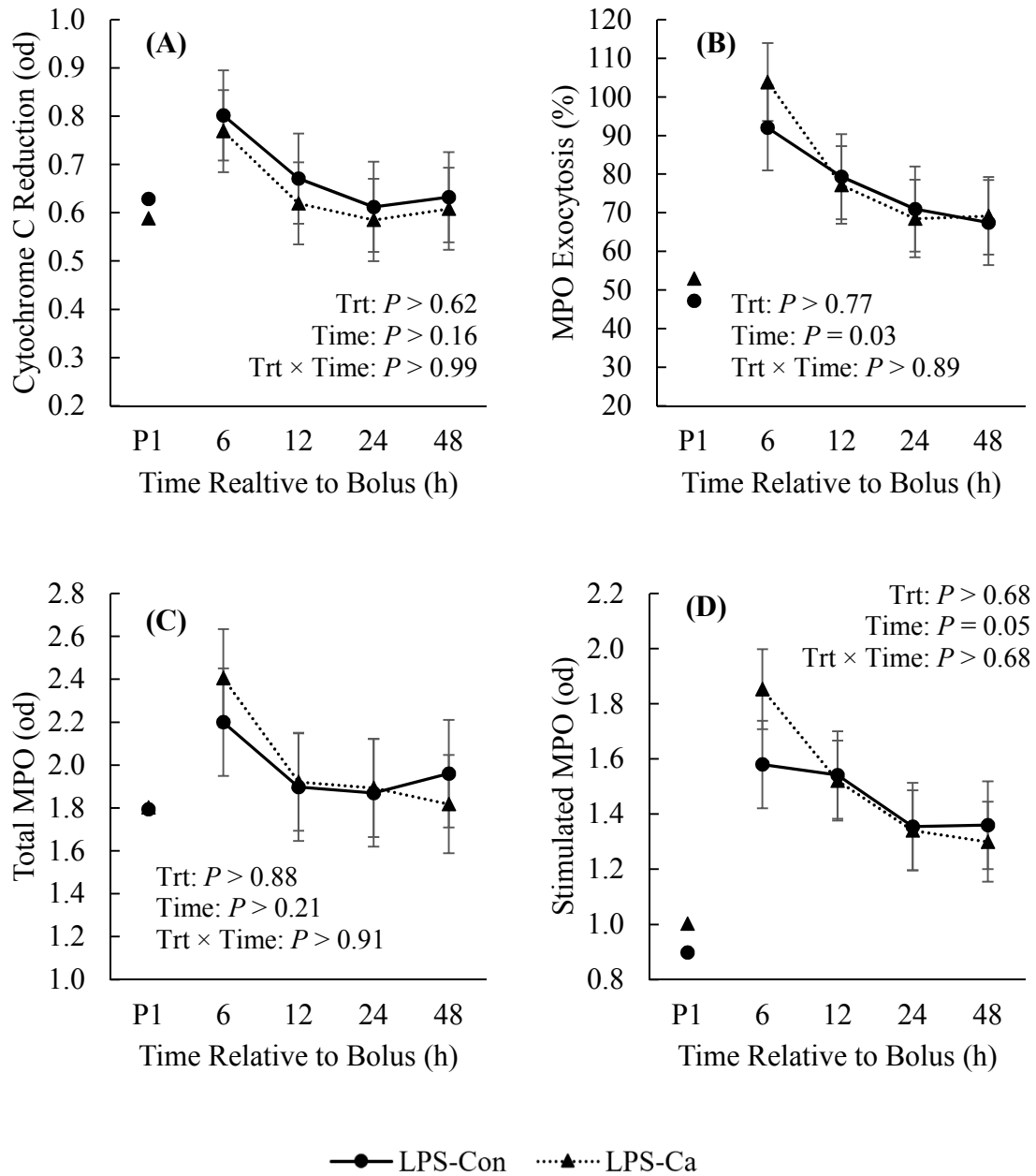


Figure 5.6. Neutrophil (A) cytochrome C reduction, (B) myeloperoxidase (MPO) exocytosis, (C) total MPO, and (D) stimulated MPO release from cows allowed to develop hypocalcemia vs. cows maintained at eucalcemia following lipopolysaccharide (LPS) infusion. Data were analyzed using PROC MIXED and included fixed effects of treatment, hour, and their interaction. P1 represents an average of measurements obtained during the 3 d of P1 and was utilized as a covariate. Data are represented as least squares means \pm standard error of the mean and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$.

CHAPTER 6. EVALUATING ACUTE INFLAMMATION'S EFFECTS ON HEPATIC TRIGLYCERIDE CONTENT IN EXPERIMENTALLY-INDUCED HYPERLIPIDEMIC DAIRY COWS IN LATE LACTATION

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Abstract

Inflammation appears to be a predisposing factor and key component of hepatic steatosis in a variety of species. Objectives were to evaluate effects of inflammation (induced via intravenous lipopolysaccharide [LPS] infusion) on metabolism and liver lipid content in experimentally-induced hyperlipidemic lactating cows. Cows (765 ± 32 kg BW; 273 ± 35 d in milk) were enrolled in 2 experimental periods (P); during P1 (5d), baseline data were obtained. At the start of P2 (2d), cows were assigned to 1 of 2 treatments: 1) intralipid + control (IL-CON; 3mL of saline; n=5) or 2) intralipid + LPS (IL-LPS; $0.375 \mu\text{g LPS/kg of BW}$; n=5). Directly following intravenous bolus (saline or LPS) administration, IL (20% fat emulsion) was intravenously infused continuously (200 mL/h) for 16h to induce hyperlipidemia during which feed was removed. Blood samples were collected at -0.5, 0, 4, 8, 12, 16, 24, and 48 h relative to bolus administration and liver biopsies were obtained on d1 of P1 and at 16 and 48h postbolus. By experimental design (feed was removed during the first 16h of d1), DMI decreased in both treatments on d1 of P2, but the magnitude of reduction was greater in LPS cows. Dry matter intake of IL-LPS remained decreased on d2 of P2, while IL-CON cows returned to baseline. Milk yield decreased in both treatments during P2, but the extent and duration was longer in LPS

infused cows. Administering LPS increased circulating LPS-binding protein (2-fold) at 8h postbolus, after which it markedly decreased (84%) below baseline for the remainder of P2. Serum amyloid A concentrations progressively increased throughout P2 in IL-LPS cows (3-fold, relative to controls). Lipid infusion gradually increased non-esterified fatty acids and triglycerides in both treatments relative to baseline (3- and 2.5-fold, respectively). Interestingly, LPS infusion blunted the peak in non-esterified fatty acids, such that concentrations peaked (43%) higher in IL-CON compared to IL-LPS cows, and heightened the increase in serum triglycerides (1.5-fold greater relative to controls). Liver fat content remained similar in IL-LPS relative to P1 at 16h, however, hyperlipidemia alone (IL-CON) increased liver fat (36% relative to P1). No treatment differences in liver fat were observed at 48h. In IL-LPS cows, circulating insulin increased markedly at 4h postbolus (2-fold relative to IL-CON), and then gradually decreased during the 16h of lipid infusion. Inducing inflammation with simultaneous hyperlipidemia altered the characteristic patterns of insulin and LPS-binding protein, but did not cause fatty liver.

Keywords: lipopolysaccharide, NEFA, fatty liver

Introduction

Early lactation cows are typically unable to consume enough energy to meet the requirements of both maintenance and milk production. As a result, cows enter into a state of negative energy balance and marked alterations in whole-body carbohydrate and lipid metabolism are employed to spare glucose for milk synthesis. In particular, hypoinsulinemia and reduced skeletal muscle and adipose tissue insulin sensitivity are key components of this homeorhetic adaptation (Bauman and Currie, 1980). Although these normal strategies are crucial for ensuring a successful lactation initiation, metabolic maladaptation and subsequent hepatic steatosis and subclinical ketosis occur in a large percentage of cows (Drackley, 1999; Bobe et al.,

2004). Ketosis is traditionally thought to result from excessive adipose tissue mobilization (Baird, 1982; Drackley, 1999). The liver takes up non-esterified fatty acids (**NEFA**) in proportion to blood concentrations (Herdt, 1988), kinetics which presumably causes fatty liver and excessive ketone body synthesis (Grummer, 1993).

In non-ruminants, hepatic steatosis is commonly observed during intestinal hyperpermeability pathologies (Ilan, 2012; Hamarneh et al., 2017). Hepatocyte and Kupffer cell recognition of gut-derived lipopolysaccharide (LPS) triggers inflammatory cytokine production, which negatively impacts hepatic lipid trafficking (Lanza-Jacoby and Tabares, 1990; Endo et al., 2007; Stienstra et al., 2010). In laboratory models, inflammation's role on hepatic lipid metabolism is confirmed by LPS and cytokine recognition interference experiments that ameliorate liver fat accumulation (Endo et al., 2007; Jin et al., 2017; Jia et al., 2018). In non-ruminants it is generally believed that increased hepatic NEFA delivery and inflammation must coincide for fatty liver disease progression; a concept known as the two-hit hypothesis (Day and James, 1998; Csak et al., 2011). Regardless, it appears that hepatic steatosis is more complex than historically assumed.

A systemic inflammatory response is observed in most periparturient dairy cows (Humblet et al., 2006), even in seemingly healthy ones (Bionaz et al., 2007; Bertoni et al., 2008; Graugnard et al., 2012). The magnitude and persistency of inflammation appears dependent on the frequency and severity of immune insults (Bertoni et al., 2008; Trevisi and Minuti, 2018) and is predictive of fatty liver (Ohtsuka et al., 2001; Ametaj et al., 2005, 2010). In agreement with the rodent literature, administering tumor necrosis factor-alpha (**TNF- α** ; an inflammatory cytokine) in late lactation cows increased liver triglyceride (**TG**) content (Bradford et al., 2009). In contrast, we were unable to recreate hepatic steatosis in mid-lactation cows administered LPS

to induce systemic inflammation (Horst et al., 2019b). A possible explanation for the absence of hepatic steatosis in our aforesaid inflammation model may have been the lack of an endogenous increase in NEFA comparable to transition cows. The early lactation cow would be the ideal experimental model of periparturient inflammation because they are naturally hyperlipidemic. However, a host of other physiological events occurring during early lactation (including endocrine, metabolic and inflammatory) are highly variable; sequela which hinders our capacity to intensely investigate the effect of a periparturient systemic inflammation on lipid liver accumulation. For this reason, we chose to use a model of mid-lactation cows whose production and metabolic parameters are in a relative “steady-state” combined with continuous infusion of intralipid (IL) to sustain increased NEFA concentrations as described previously (Chelikani et al., 2003; Caixeta et al., 2017; Lamp et al., 2018). Therefore, our objective was to evaluate the effects of inflammation on metabolism and liver lipid by simultaneously recreating hyperlipidemia and inflammation in cows during established lactation. We hypothesized that acute systemic inflammation and hyperlipidemia would exacerbate liver lipid accumulation similarly to that observed in poorly transitioning dairy cows.

Materials and Methods

Animals and Experimental Design

All procedures were approved by the Iowa State University Institutional Animal Care and Use Committee. Ten non-pregnant lactating Holstein cows (765 ± 32 kg BW; 273 ± 35 DIM; parity 3 ± 0.4) were housed in individual box-stalls (4.57×4.57 m) at the Iowa State University Dairy Farm (Ames) in August 2018. Cows were allowed 3 d to acclimate during which they were implanted with bilateral jugular catheters. In brief, cows were restrained in a head gate, the neck was cleaned with alternating betadine surgical scrub and 70% ethanol 3 times. The jugular vein was occluded and the vein was localized with a 14 G, 3.75 cm introducer (MILA

International, Erlanger, KY) using a percutaneous technique. Once in the vein, sterilized tygon tubing was passed through the introducer, the introducer was removed, and the tubing was sutured to the skin using a tape butterfly. A tubing adapter was fitted to the external end of the tubing which was flushed with 5 cc heparinized saline. Cows were fed ad libitum once daily (0600 h) with a diet formulated to meet or exceed the predicted requirements (NRC, 2001) of energy, protein, minerals, and vitamins (Table 6.1). Cows were milked twice daily (0600 and 1800 h) throughout the experiment and yield was recorded. A sample for composition analysis was obtained at each milking and stored at 4°C with a preservative (bronopol tablet; D & F Control System, San Ramon, CA) until analysis by Dairy Lab Services (Dubuque, IA) using AOAC approved infrared analysis equipment and procedures (AOAC International, 1995). Rectal temperature (**Tr**) was recorded after each milking using a digital thermometer (GLA M700 Digital Thermometer, San Luis Obispo, CA).

The trial consisted of 2 experimental periods; period 1 (**P1**) lasted 5 d and served as the baseline (data generated for covariate analysis). During period 2 (**P2**), which lasted 2 d, animals were randomly assigned to 1 of 2 intravenous treatments: 1) IL infusion + control (**IL-CON**; IL infusion and 3 mL sterile saline; n = 5) or 2) IL infusion + LPS (**IL-LPS**; IL infusion and 0.375 µg LPS/kg of BW; n=5). Lipopolysaccharide (*Escherichia coli* O55:B5; Sigma Aldrich, St. Louis, MO) was dissolved in sterile saline at a concentration of 65.1 µg/mL and passed through a 0.2 µm sterile syringe filter (Thermo Scientific; Waltham, MA). The total volume of LPS solution administered was approximately 4 mL. Each respective treatment bolus was administered immediately following the a.m. milking and 0 min blood sample collection. Immediately (within 30 sec) following bolus (saline or LPS) administration, cows in both treatments were continuously i.v. infused with 20% IL solution (Fresenius Kabi, Deerfield, IL;

product #0338 0519-13; consisting primarily of C18:2, C18:1, C16:0, C18:3, C18:0) at a rate of 200 mL/h for 16 h using calibrated infusion pumps (Deltec 3000, Deltec Inc., St. Paul, MN) as previously described (Caixeta et al., 2017). Cows were tethered during the 16 h infusion period (but allowed to stand up and lay down) to allow for frequent sampling and constant connection with the infusion pump. Water was provided ad libitum and feed was removed from all cows ~0.5 h prior to bolus administration. Animals remained fasted during the 16 h data collection period to eliminate the confounding effect of dissimilar nutrient intake.

Rectal temperature was obtained at -0.5 and 0 h relative to LPS administration, every 0.5 h for the first 8 h, and every 4 h for the next 8 h. Plasma and serum samples were collected daily following the a.m. milking during P1 and at -0.5, 0, 4, 8, 12, 16, 24, and 48 h relative to bolus administration (and the start of IL infusion) during P2. Serum samples were allowed to clot at room temperature for 1 h prior to centrifugation. Plasma and serum were harvested following centrifugation at $1500 \times g$ for 15 min at 4°C and were subsequently frozen at -20°C until analysis. Samples for complete blood count (**CBC**) analysis were collected on d 1 and 3 of P1 and at 4, 8, 12, 16, 24, 32, and 48 h post-LPS administration. A 3 mL plasma sample was obtained from the catheter and stored at 4°C for approximately 12 h before submission to the Iowa State University's Department of Veterinary Pathology for analysis. Samples for evaluating blood ionized calcium were measured using an iSTAT hand-held machine and cartridge (CG8⁺; Abbott Point of Care, Princeton, NJ) and were obtained on d 5 of P1 and at 12, 24, and 48 h post-LPS administration.

Tissue Collection and Liver TG Content Analysis

Liver biopsies were collected from all animals on d 1 of P1 following the morning milking and at 16 and 48 h relative to LPS administration (following blood sampling) during P2 as previously described (Rhoads et al., 2010). Briefly, biopsy sites were shaven, scrubbed with

betadine, and sprayed with 70% alcohol. The area was locally anesthetized using 2% lidocaine (MWI Veterinary Supply Co., Glendale, AZ) before performing a percutaneous biopsy with a trocar. All tissue samples were snap frozen in liquid nitrogen and stored at -80°C until analysis. Hepatic TG content was measured as previously described (Morey et al., 2011). Briefly, approximately 20 mg of each liver sample was homogenized with 500 μ L chilled PBS. The homogenate was then centrifuged at $8,000 \times g$ for 2 min at 4°C. Free glycerol was immediately determined using 10 μ L of supernatant via enzymatic glycerol phosphate oxidase method (Sigma-Aldrich, St. Louis, MO). An additional 300 μ L of supernatant was removed and incubated with 75 μ L lipase (MP Biomedicals, Solon, OH) at 37°C for 16 h before determining total glycerol using the same method. Free glycerol (before lipase digestion) was subtracted from total glycerol (after lipase digestion) in order to determine TG content, and this was expressed as a percentage of wet weight of the original sample. The intra-assay coefficients of variation for free glycerol and total glycerol were 6.1 and 2.5%, respectively.

Measurement of Serum and Plasma Parameters

Serum cortisol and TG and plasma insulin, glucagon, NEFA, BHB, LPS-binding protein (**LBP**), serum amyloid A (**SAA**), BUN, and glucose concentrations were determined using commercially available kits according to manufacturers' instructions (cortisol, Enzo Life Sciences Inc., Farmingdale, NY, catalog #AD1-900-071; TG, Sigma, Saint Louis, MO; insulin, Mercodia AB, Uppsala, Sweden, catalog #10-1201-01; glucagon, RD Systems Inc., Minneapolis, MN, catalog #DGCG0; NEFA, Wako Chemicals USA, Richmond, VA, catalog #276-76491; BHB, Pointe Scientific Inc., Canton, MI, catalog #23-666-471; LBP, Hycult Biotech, Uden, the Netherlands, catalog #HK503; SAA, Tridelata Development Ltd., Kildare, Ireland, catalog #TP802; BUN, Teco Diagnostics Anaheim, CA, catalog #B549-150; glucose, Wako Chemicals USA Inc., Richmond, VA, catalog #997-03001). All assays were analyzed in duplicates and a

pooled sample was utilized on each plate to assess inter-assay variation. The inter- and intra-assay coefficients of variation for glucose, NEFA, BHB, BUN, insulin, glucagon, LBP, SAA, and cortisol were 6.7 and 4.4%, 1.2 and 3.5%, 1.4 and 8.2%, 4.3 and 4.6%, 7.9 and 6.1%, 34.0 and 13.2%, 0.2 and 8.2%, 4.9 and 8.2%, and 16.7 and 11.4% respectively. The inter- and intra-assay coefficient of variations for serum free and total glycerol were 13.2 and 11.7% and 5.9 and 12.5%, respectively.

Statistical Analysis

The sample size calculation was determined using PROC POWER and was based on the effect of inflammation on liver TG content using 90% power and a 99% confidence level. The sample size of 5 cows per group was based on a detection difference of 1.2 ± 0.33 % (mean \pm SD) in liver TG. Each animal's respective parameter was analyzed using repeated measures with an autoregressive covariance structure (equally spaced time points) for DMI, milk yield and composition and a spatial power law covariance structure (unequal spacing) for blood metabolites, inflammatory biomarkers, CBC, ionized calcium, and rectal temperature data. The repeated effect was time (h or d) relative to LPS administration. Each specific variable's pre-bolus values (i.e., P1 average or 0 h timepoint) served as a covariate for analysis of P2. Effects of treatment, time (h or d relative to bolus administration), and treatment \times time interactions were assessed as a completely randomized design using PROC MIXED (SAS Inst. Inc., Cary, NC). In order to evaluate effects of IL infusion, a separate analysis was used to make statistical comparisons with baseline in which the average P1 value (DMI, milk yield, rectal temperature, liver TG content) or 0 h (blood variables) was included as an additional timepoint. Data are reported as least squares means and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$.

Results

There were no treatment differences in evaluated metrics (other than LBP and milk yield) during P1. Administering LPS induced mild pyrexia (0.8°C relative to IL-CON cows) for ~ 5.5 h postbolus ($P = 0.01$; Figure 6.1A). By experimental design (i.e., feed intake was removed for 16 h post-LPS administration), DMI decreased in both treatments on d 1 of P2 relative to P1 (34 and 80% in IL-CON and IL-LPS cows, respectively; $P < 0.01$). However, the degree of DMI reduction on d 1 of P2 was greater (68%) with LPS infusion relative to control cows ($P < 0.01$; Figure 6.1B). Dry matter intake from IL-LPS remained decreased on d 2 of P2 (25% from baseline; $P = 0.01$), while in IL-CON cows it returned to baseline.

During P2, milk yield from IL-LPS cows decreased relative to baseline and control cows (74 and 64%, respectively, $P < 0.01$; Figure 6.1C). Milk yield from IL-CON cows decreased on d 1 (11% relative to P1; $P < 0.01$), but did not differ from baseline on d 2 ($P > 0.30$). Administering LPS decreased milk lactose (15%; $P = 0.02$) and increased milk fat and protein percentages (68 and 35%, respectively; $P \leq 0.01$) relative to control cows (Table 6.2). Hyperlipidemia alone did not alter these milk composition variables. Overall MUN was increased in IL-LPS relative to IL-CON cows ($P = 0.04$; Table 6.2). Somatic cell count did not differ by treatment during P2 ($P > 0.39$; Table 6.2).

Administering LPS increased circulating LBP 2-fold at 8 h postbolus relative to controls, after which it markedly decreased below baseline (84%; $P < 0.01$; Figure 6.2A). Overall, SAA concentrations increased (3-fold) in LPS administered cows relative to controls throughout P2 ($P < 0.01$; Figure 6.2B). Circulating LBP and SAA increased in IL-CON at 48 h relative to P1 ($P \leq 0.02$). Cortisol levels from LPS-administered cows markedly increased (8-fold) for the first 8 h postbolus, whereas in IL-CON cows it was unchanged ($P < 0.01$; Figure 6.2C). Relative to controls, circulating WBC from IL-LPS cows decreased (62%) for 12 h postbolus and then

gradually increased (32%) throughout P2 ($P < 0.01$; Figure 6.2D). Circulating neutrophils (which make up the majority of WBC) mirrored the WBC pattern following LPS administration ($P < 0.01$; Table 6.3). Relative to IL-CON cows, patterns of circulating lymphocytes, monocytes, and basophils reflected each other with initial leukopenia (84, 102, 94%, respectively) followed by a gradual return to baseline post-LPS administration ($P < 0.01$; Table 6.3). Administering LPS decreased circulating platelets (68%) relative to controls throughout P2 ($P < 0.01$; Table 6.3). Hyperlipidemia alone did not influence circulating leukocyte patterns ($P > 0.10$).

Non-esterified fatty acid concentrations gradually increased in both treatments during the first 12 h of IL infusion (3-fold, relative to baseline; $P \leq 0.04$); however, infusing LPS blunted the NEFA increase at 16 h ($P = 0.01$; Figure 6.3A). After cessation of lipid infusion, NEFA concentrations from LPS cows remained increased at 24 h postbolus (2-fold relative to baseline; $P = 0.01$) while in IL-CON cows NEFA returned to baseline. During lipid infusion, serum TG increased in both treatments relative to baseline ($P < 0.01$), and LPS administration exacerbated the increase (1.5-fold increase relative to IL-CON; $P < 0.01$; Figure 6.3B). Relative to baseline, IL infusion tended to increase liver TG content in IL-CON cows at 16 h (36%; $P = 0.09$), but no difference was observed in cows previously exposed to LPS ($P > 0.50$). Overall during P2, liver fat content from IL-LPS cows tended to be decreased (25%) relative to controls ($P = 0.06$), and the largest difference was observed at 16 h of lipid infusion (34% decrease relative to controls; $P = 0.03$; Figure 6.3C).

A treatment by time interaction was observed for circulating glucose such that concentrations from LPS cows decreased (17% relative to controls) at 8 h postbolus then gradually increased, while in control cows concentrations remained similar ($P = 0.02$; Figure 6.4A). Transient hyperinsulinemia was observed following LPS administration; concentrations

peaked at 4 h postbolus (2- and 4-fold relative to P1 and controls, respectively) then gradually declined reaching P1 values at 16 h postbolus ($P < 0.01$). At 24 h, insulin concentrations from LPS cows were decreased (46%) relative to controls ($P < 0.01$; Figure 6.4B). Overall, circulating glucagon did not differ due to treatment ($P = 0.34$; Figure 6.4C), but when just the 8th through 12th hour were evaluated, glucagon increased (3-fold) in LPS cows relative to controls ($P = 0.03$). Blood urea nitrogen increased in both treatments during the first 24 h of P2, after which it remained elevated (36%) in LPS cows while in controls it returned to baseline ($P < 0.01$; Figure 6.4D). During the first 8 h of lipid infusion, BHB concentrations tended to decrease in both treatments relative to baseline (26%; $P \leq 0.07$; Figure 6.4E). Administration of LPS markedly decreased ionized calcium (43%) at 12 h postbolus relative to control cows, after which it gradually returned to baseline ($P < 0.01$; data not shown). No effects of IL infusion were observed on ionized Ca from IL-CON cows ($P > 0.70$; data not shown).

Discussion

Excessive adipose tissue mobilization has traditionally been considered the causative factor leading to ketosis and fatty liver in poorly transitioning cows (Grummer, 1993; Drackley, 1999; Oetzel, 2004). A cornerstone of this dogma is that ruminants have a limited capacity to export TG as very low density lipoproteins (Kleppe et al., 1988). However, increasing evidence suggests that inflammation may be an etiological prerequisite for these disorders (Ametaj et al., 2005; Bertoni et al., 2006; Eckel and Ametaj, 2016); a scenario supported by human and rodent models indicating that inflammation causes hepatic steatosis (Endo et al., 2007; Ilan, 2012; Ceccarelli et al., 2015). Almost all transition cows experience some degree of inflammation immediately postpartum (Humbler et al., 2006; Bertoni et al., 2008) and the magnitude and persistency of the inflammatory state is predictive of transition cow performance (Ohtsuka et al., 2001; Ametaj et al., 2005; Abuajamieh et al., 2016). The severity of the ensuing inflammatory

response is ostensibly dependent on the frequency and type of immune insults encountered (Bertoni et al., 2008; Bradford et al., 2015; Trevisi and Minuti, 2018). Increased liver fat accumulation has been previously observed in late and early lactation cows in response to intravenous TNF- α (Bradford et al., 2009) and intramammary LPS infusion (Graugnard et al., 2013; Minuti et al., 2015). However, we were unable to reproduce fatty liver in inflamed mid-lactation cows (Horst et al., 2019b). A limitation to our previous model is the lack of a biologically meaningful and simultaneous increase in circulating NEFA (and the corresponding hepatic NEFA uptake) that naturally occurs in the periparturient cow. Therefore, this study aimed to create a more appropriate model of periparturient inflammation by inducing hyperlipidemia immediately following LPS administration in mid-lactation cows. Alternative models would have been to achieve a steady state of hyperlipidemia prior to immune activation or a pleatued inflammatory state prior to lipid infusion, but the appropriate chronological sequences of the model remains in flux.

Administering LPS induced a systemic inflammatory response herein, as indicated by mild hyperthermia, altered immune cell dynamics, hypercortisolemia, increased acute phase proteins, and decreased production performance. Furthermore, NEFA concentrations increased in both control and immune activated cows during the 16 h of continuous IL infusion, indicating experimental treatments were successfully implemented. Although IL infusion increased NEFA, the pattern observed in both treatments differed from previous lipid infusion studies, which observed a rapid increase in NEFA followed by a “steady-state” within approximately 3 h (Burdick Sanchez et al., 2015; Caixeta et al., 2017; Lamp et al., 2018). Discrepancies in the NEFA pattern may be explained by differences in energetic state, as all cows were fasted during the lipid infusion period herein. As expected, we observed a mild increase in hepatic TG content

in IL-CON cows after 16 h of IL infusion, but, it was moderate when compared to previous reports in non-lactating cows (Caixeta et al., 2017; Rico et al., 2018) and remained below the threshold of “mild fatty liver” (1-5% TG; Bobe et al., 2004). During lactation, the mammary gland is an alternative sink of blood TG and NEFA, which may partially explain why we did not detect a similar increase in the magnitude of liver lipid as was previously reported in dry cows (Caixeta et al., 2017). Regardless and in stark contrast to our hypothesis, acute systemic inflammation did not exacerbate liver fat accumulation compared to controls; which is ostensibly explained by insufficient hepatic NEFA delivery (other potential explanations are discussed below).

Circulating NEFA from LPS cows reached “steady-state” concentrations of approximately 0.5 mmol/L at 12 h post-lipid infusion start. Although NEFA concentrations exceeded levels observed in our previous LPS reports (Kvidera et al., 2017; Horst et al., 2018, 2019a,b), they did not reach levels typically observed in periparturient cows experiencing fatty liver (Bobe et al., 2004; Ametaj et al., 2005). Potential factors contributing to the blunted response may include less endogenous NEFA and reduced circulating triglyceride hydrolysis. Administering LPS increases circulating NEFA, but the response is delayed and dampened in comparison to non-inflamed animals on the same plane of nutrition (Horst et al., 2018); therefore, contribution of adipose tissue mobilization to circulating NEFA was presumably less than in controls. Reasons for blunted adipose tissue lipolysis are most likely explained by LPS-induced immediate reduction in milk synthesis and LPS-induced hyperinsulinemia. Lastly, LPS may have reduced hydrolysis of TG in the infused lipid emulsion. The IL solution contains TG-rich particles similar in size to chylomicrons (Samra et al., 1998) and these particles are hydrolyzed to glycerol and free fatty acids by lipoprotein lipase (LPL). However, LPL activity is

decreased in response to LPS in rodents and pigs (Sakaguchi and Sakaguchi, 1979; Kawakami and Cerami, 1981; Feingold et al., 1992); a metabolic scenario leading to hypertriglyceridemia. We observed increased serum TG in both treatments in response to lipid infusion, however, the magnitude of increase was 1.5-fold greater in LPS administered cows. This suggests a greater proportion of the lipid administered in LPS cows was not hydrolyzed to NEFA, consequently, rates of hepatic NEFA uptake were presumably less than controls. It is of interest for future studies to evaluate the chronic effects of hyperlipidemia or direct NEFA infusion on hepatic lipid accumulation during immune activation in order to get the increase necessary to alter liver fat trafficking.

Hypertriglyceridemia is a well-characterized response to infection in monogastrics that develops as a result of reduced TG clearance and increased hepatic TG production (Blackburn, 1977; Grunfeld and Feingold, 1992; Memon et al., 1992). Increased circulating TG allow for LPS detoxification via an anti-inflammatory pathway (Read et al., 1995; Vreugdenhil et al., 2003; Barcia and Harris, 2005). Although hypertriglyceridemia has been well-described in monogastrics, it remains largely unexplored in immune activated ruminants and the limited results that do exist have been inconsistent with both increased (Steiger et al., 1999; Ballou et al., 2008; Graugnard et al., 2013) and decreased (Wang et al., 2017) circulating TG being reported. Discrepancies may be explained by sampling time as the response appears to be short-lived (Steiger et al., 1999; Ballou et al., 2008; Graugnard et al., 2013). It is of interest to further investigate if infection-induced hypertriglyceridemia occurs in ruminants and whether clearance rate or production (or both) is responsible. Whether or not hypertriglyceridemia would naturally occur in a ruminant, our results suggest lipid infusion in LPS-administered cows may mimic this immune-metabolic phenomenon. Therefore, we presume that many of the metabolic and

inflammatory effects we observed (discussed below) are a consequence of increased TG-mediated LPS detoxification.

Perhaps one of the most intriguing results of our study was the markedly different pattern in circulating LBP in response to LPS. In contrast to previous LPS infusion studies which demonstrate a continuous increase in LBP for ~24 h (Horst et al., 2018, 2019a, 2020), we observed an abrupt decrease below baseline values at 12 h post-LPS infusion. Interestingly, this decrease occurred concurrently with the NEFA plateau and peak TG concentrations. Presumably, TG-rich lipoproteins bound LPS monomers (with the help of LBP and soluble cluster of differentiation 14) and prevented LPS binding to leukocyte toll-like receptor (TLR)-4 receptors, as has been previously demonstrated *in vitro* (Vreugdenhil et al., 2003). In rodents, LBP and SAA facilitate the formation of lipoprotein-LPS complexes, a process described in detail by Berbée et al. (2005). In brief, LBP transfers LPS monomers to acute-phase lipoproteins which are formed after the displacement of apolipoproteins by SAA. Lipoprotein-LPS complexes are taken up by the liver for biliary excretion and this process may be an additional factor leading to LPS-induced increases in hepatic TG (Eckel and Ametaj, 2016). If IL infusion increased the formation of lipoprotein-LPS complexes it is reasonable to assume that this would have increased liver TG content, however, the rate at which these complexes are cleared by the liver is not well understood and differs between different lipoprotein classes (Vreugdenhil et al., 2003). Regardless, the unusual and dramatic reduction in LBP most likely represents a decrease in unbound LBP (i.e., not bound to LPS) levels (Pearce et al., 2014) as a result of increased association with lipoproteins. Little literature exists on the effects of hypertriglyceridemia on SAA and LBP concentrations, however, lipoprotein infusion reduces cytokine concentrations in mice (Levine et al., 1993), rabbits (Hubsch et al., 1995; Casas et al., 1996), dogs (Quezado et al.,

1995), and steers (Burdick Sanchez et al., 2015) and increases survivability to endotoxin in rats (Read et al., 1995). Thus, TG infusion may represent a potential therapeutic approach to mitigate the negative consequences of infection.

Across species, several well-conserved metabolic alterations are employed during immune activation to meet the energetic requirements of leukocytes (Filkins, 1978; Lanza-Jacoby et al., 1998; McGuinness, 2005) and these have been described in detail in our previous ruminant publications (Kvidera et al., 2017; Horst et al., 2018, 2019a,b, 2020). In brief, a biphasic response in circulating glucose is observed in response to LPS with an initial period of hyperglycemia followed by hypoglycemia (Kvidera et al., 2017; Horst et al., 2018, 2019a). During the hyperglycemic phase, glucose appearance from glycogenolysis and gluconeogenesis outweighs leukocyte glucose consumption, but, within ~2 h leukocyte glucose utilization predominates and hypoglycemia develops. Because it was not a primary objective, we did not collect samples during what would be the initial hyperglycemic phase; however, we did observe decreased glucose concentrations post-LPS administration. Upon activation, leukocytes increase their reliance on insulin as the driving force for glucose uptake (Calder et al., 2007), which may partially explain the marked hyperinsulinemia that develops in response to LPS (Waldron et al., 2003; Zarrin et al., 2014; Kvidera et al., 2017). Furthermore, LPS may act directly on the pancreas to increase insulin secretion as we have recently described (Baumgard et al., 2016). Interestingly, the pattern of circulating insulin in response to LPS was markedly different from our previous reports (Kvidera et al., 2017; Horst et al., 2018), specifically it peaked earlier and declined faster during the first 16 h post-LPS. This peculiar pattern may be explained by a dampened immune response as a result of lipoprotein LPS detoxification. It is of interest to further evaluate the connection between increased lipid, LPS concentrations, and insulin

secretion. In agreement with previous experiments (Horst et al., 2019a), LPS increased circulating glucagon which is a potent stimulator of glycogenolysis and gluconeogenesis, thereby (presumably) providing additional glucose for leukocyte use. Furthermore, we observed a continued increase in circulating BUN in the LPS cows following termination of IL infusion relative to controls. Muscle catabolism is a critical strategy for providing gluconeogenic precursors under fasting conditions. However, in immune activated animals amino acid mobilization is exaggerated due to increased glucose requirements of activated leukocytes (Kvidera et al., 2017; Horst et al., 2018) and the synthesis of acute phase proteins (Klasing and Austic, 1984).

As alluded to earlier, many of the metabolic changes described in response to LPS closely resemble observations in poorly transitioning cows. However, a key dissimilarity between these two situations is the absence of hyperketonemia in the LPS administered cow. In response to LPS, circulating ketones are markedly decreased in ruminants (Waldron et al., 2003; Graugnard et al., 2013; Kvidera et al., 2017) and the mechanisms mediating this decrease remain poorly understood. In rodents, LPS administration reduces hepatic ketogenesis (Takeyama et al., 1990; Maitra et al., 2009), but in ruminants LPS had no effect (Waldron et al., 2003), however this was evaluated in mid-lactation cows which lacked high NEFA concentrations; one variable required for ketogenesis. Lipid infusion has been shown to increase BHB concentrations in previous studies (Chelikani et al., 2003; Caixeta et al., 2017), however, the NEFA load administered herein was presumably insufficient to increase ketones; in fact, we observed decreased BHB concentrations in both treatments during the infusion period. Because feed was removed from both treatments during the infusion period, the response probably resulted from a decrease in ketone production by the rumen epithelium which is the primary source of ketones in

the well-fed ruminant (Pennington, 1952). Regardless, a better understanding of inflammation's role in ketone metabolism has obvious implications to the transition dairy cow.

Although we believe that insufficient hepatic NEFA delivery may partially explain why inflammation did not exacerbate liver fat accumulation herein, other potential explanations need consideration. First, transition cows are exposed to chronic inflammation which can persist for several weeks (Bionaz et al., 2007; Bertoni et al., 2009; Abuajamieh et al., 2016), whereas our study modeled an acute inflammatory response. Throughout the transition period, cows are exposed to a myriad of inflammation inducing events of both biotic and abiotic origin and repeated exposures interfere with inflammation resolution and hepatic phenotypes (Trevisi and Minuti, 2018). A single LPS bolus or even continuous LPS infusion (accompanied by tolerance) may not surpass the inflammatory threshold necessary to lead to the dysregulated response. A second important consideration is differences in systemic vs. compartmentalized inflammation. Intestinally derived endotoxin, which escapes gut defense mechanisms, enters the portal vein for delivery to the liver, the major LPS-detoxifying organ (as reviewed by Guerville and Boudry, 2016). If the liver's capacity to neutralize endotoxin is not overwhelmed endotoxemia is prevented, but systemic inflammation still occurs (Ogden et al., 2020). In this scenario, LPS is prevented from binding to extra-splanchnic tissues, including mammary epithelial cells which contain TLR-4 receptors (Ibeagha-Awemu et al., 2008). Pro-inflammatory cytokines (at least TNF- α) do not appear to meaningfully reduce milk synthesis (Bradford et al., 2009; Martel et al., 2014). Thus, alimentary tract and hepatic immune activation might not directly inhibit milk synthesis and thus the mammary gland's nutrient requirement remains robust. In contrast, LPS administered via the jugular vein obviously causes acute systemic endotoxemia and LPS binding to mammary epithelial cell TLR-4 receptors causes an immediate decrease in milk synthesis in

vitro (Liu et al., 2015) and in vivo (Kvidera et al., 2017; Horst et al., 2018). This proposed mechanism may partially explain why adipose tissue mobilization from LPS administered cows is blunted when compared to healthy cows on the same plane of nutrition (i.e., the metabolic demands of milk synthesis markedly differ).

Conclusion

In conclusion, experimentally-induced hyperlipidemia (induced by IL infusion) during acute systemic inflammation did not exacerbate fatty liver in late lactation cows as we hypothesized. However, infusing lipid following LPS administration markedly altered the characteristic patterns of circulating insulin and LBP; changes we believe are reflective of “dampened” immune activation. Developing a more accurate model of transition cow inflammation (i.e., chronic response) is a prerequisite to understanding how (or if) the immune system and immunometabolism contribute to hepatic steatosis.

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Tables and Figures

Table 6.1. Ingredients and composition of diet¹

Ingredient	% of DM ²
Corn Silage	30.1
Alfalfa Hay	3.4
Baleage	19.9
Ground Corn	18.9
Corn Gluten Feed	10.4
Soy Plus ³	4.3
Soybean Meal	1.7
Whole Cottonseed	3.5
Molasses	0.9
Mineral and Protein Mix	6.9
Chemical analysis, % of DM	
Starch	21.1
CP	15.8
NDF	37.1
ADF	26.6
NE _L Mcal/kg DM	1.54

¹Values represent an average of ration nutrient summary reports collected throughout the trial. Diet moisture averaged 57.0%.

²Average nutrient levels: 4.78% Fat, 0.80% Ca, 0.45% P, 0.35% Mg, 0.21% S, 1.26% K, 0.44% Na, 0.51% Cl, 77.12 mg/kg of Zn, 43.86 mg/kg of Mn, 3.47 mg/kg of Fe, 13.37 mg/kg of Cu, 0.75 mg/kg of Co, 0.32 mg/kg Se, 0.75 mg/kg of I, 6052.8 IU/kg of vitamin A, 1210.6 IU/kg of vitamin D, and 24.2 IU/kg of vitamin E.

³ Soy plus: Mechanically processed soybean meal, Dairy Nutrition Plus, Ralston, IA

Table 6.2. Effects of saline or lipopolysaccharide (LPS) infusion on milk composition in experimentally-induced hyperlipidemic cows during P2

Parameters	Treatment ¹		SEM	<i>P</i>		
	IL-CON	IL-LPS		Treatment	Time	Treatment × Time
Fat, %	4.12	6.92	0.50	<0.01	0.01	0.11
Lactose, %	4.59	3.88	0.16	0.02	0.01	0.05
Protein, %	3.09	4.16	0.21	0.01	<0.01	0.08
MUN, mg/dL	13.72	16.99	0.93	0.04	<0.01	<0.01
SCC, ×1000	89	620	348	0.39	0.34	0.35

¹IL-CON = intralipid infused, saline; IL-LPS = intralipid infused, LPS bolus

Table 6.3. Effects of saline or lipopolysaccharide (LPS) infusion on complete blood count parameters in experimentally-induced hyperlipidemic cows during P2

Parameters	Treatment ¹		SEM	<i>P</i>		
	IL-CON	IL-LPS		Treatment	Time	Treatment × Time
Neutrophils, ×10 ³ /μL	3.42	4.22	0.19	0.02	<0.01	<0.01
Platelets, ×10 ³ /μL	336	148	17	<0.01	0.29	0.21
Monocytes, ×10 ³ /μL	0.29	0.14	0.02	0.01	<0.01	0.02
Lymphocytes, ×10 ³ /μL	2.98	1.76	0.22	<0.01	0.02	<0.01
Basophils, ×10 ³ /μL	0.05	0.03	0.01	0.12	<0.01	<0.01

¹IL-CON = intralipid infused, saline; IL-LPS = intralipid infused, LPS bolus

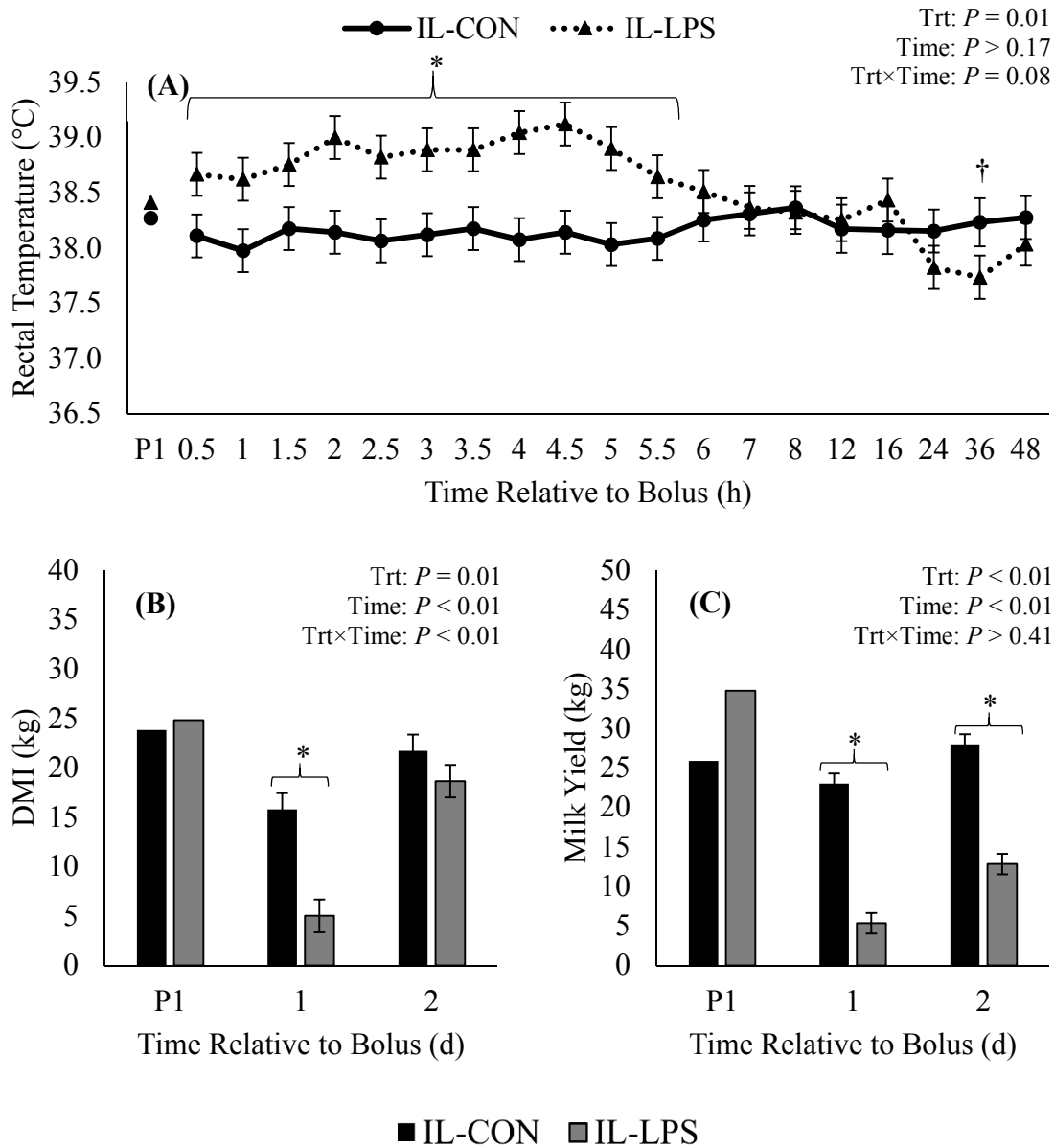


Figure 6.1. (A) Rectal temperature, (B) DMI, and (C) milk yield from hyperlipidemic (induced via intralipid infusion) late lactation cows administered saline (CON) or a lipopolysaccharide (LPS) bolus. Data were analyzed using PROC MIXED and included fixed effects of treatment, time (h/d), and their interaction. P1 represents an average of measurements obtained during the 5 d of P1 and was utilized as a covariate. Data are represented as least squares means \pm standard error of the mean and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$. *represents significant differences between treatments. †represents a tendency for a difference between treatments.

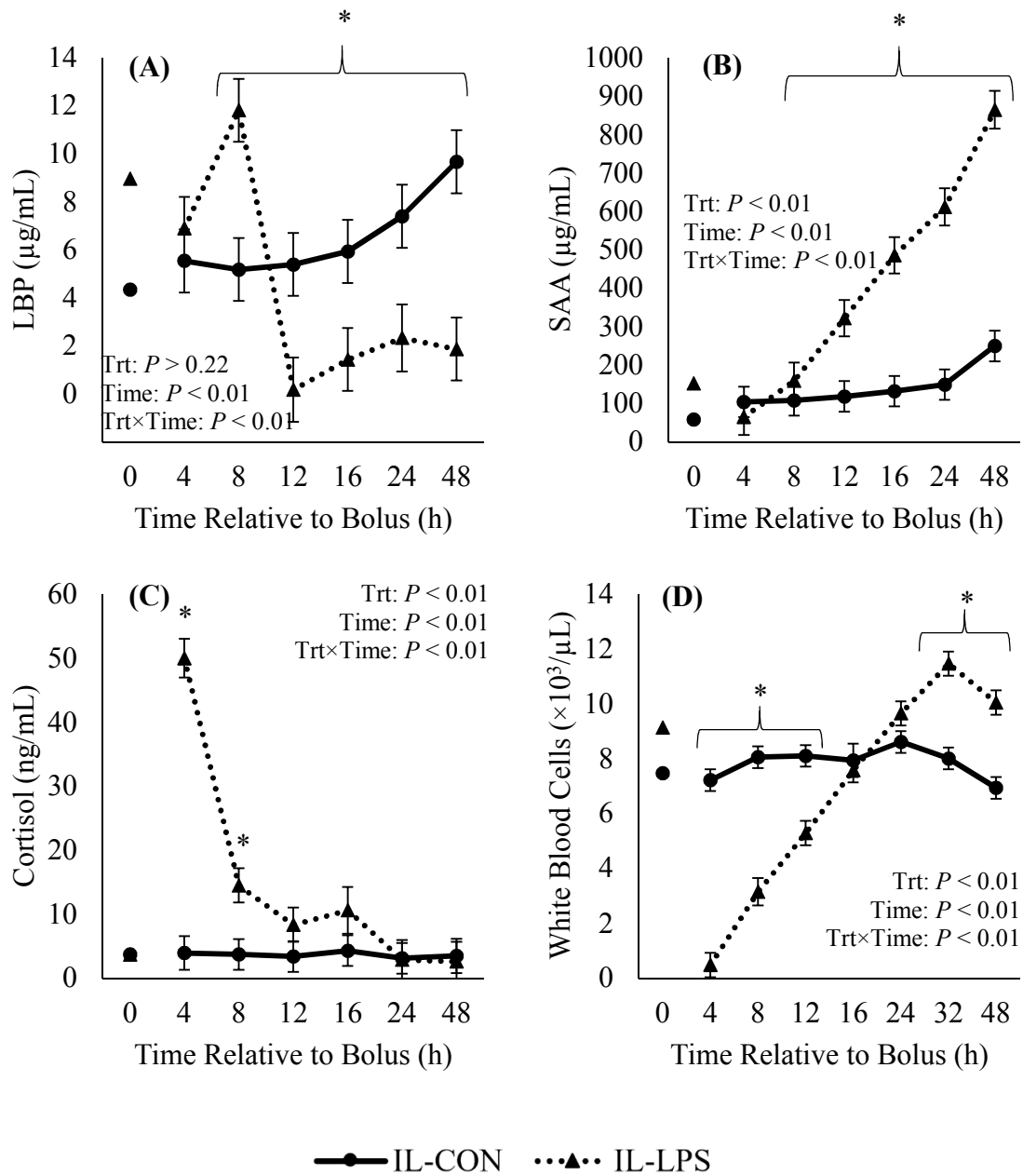


Figure 6.2. Circulating (A) LPS-binding protein (LBP), (B) serum amyloid A (SAA), (C) cortisol, and (D) white blood cell counts from hyperlipidemic (induced via intralipid infusion) late lactation cows administered saline (CON) or a lipopolysaccharide (LPS) bolus. Data were analyzed using PROC MIXED and included fixed effects of treatment, hour, and their interaction. Hour 0 represents an average of measurements obtained prior to LPS administration (-0.5 and 0 h) and was utilized as a covariate. Data are represented as least squares means \pm standard error of the mean and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$. *represents significant differences between treatments.

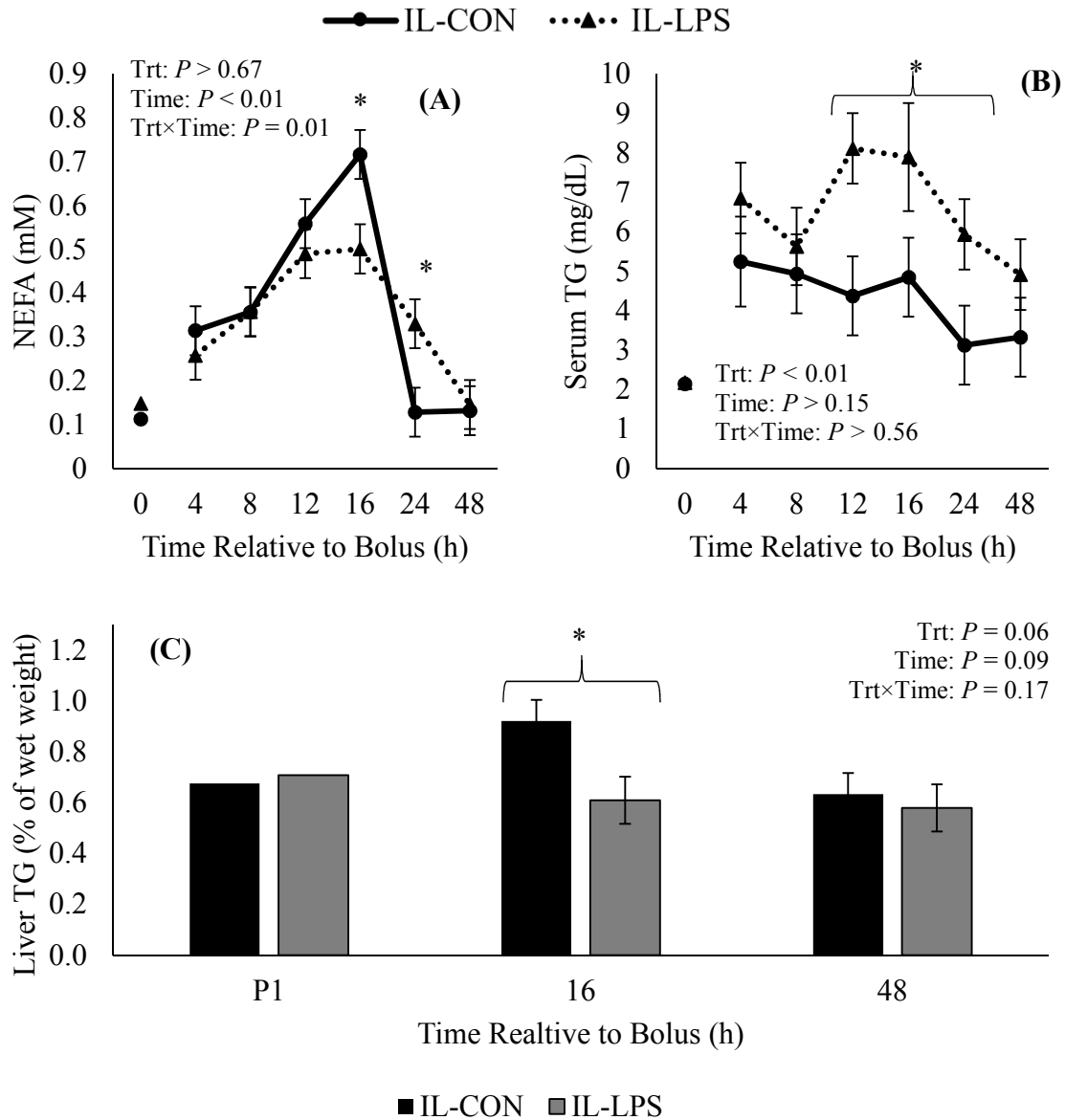


Figure 6.3. (A) Circulating NEFA, (B) serum triglycerides (TG), and (C) liver TG content from hyperlipidemic (induced via intralipid infusion) late lactation cows administered saline (CON) or a lipopolysaccharide (LPS) bolus. Data were analyzed using PROC MIXED and included fixed effects of treatment, hour, and their interaction. Hour 0 represents an average of measurements obtained prior to LPS administration (-0.5 and 0 h) and was utilized as a covariate for NEFA and triglycerides. P1 represents the liver TG on d 1 of P1 and was utilized as a covariate. Data are represented as least squares means \pm standard error of the mean and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$. *represents significant differences between treatments.

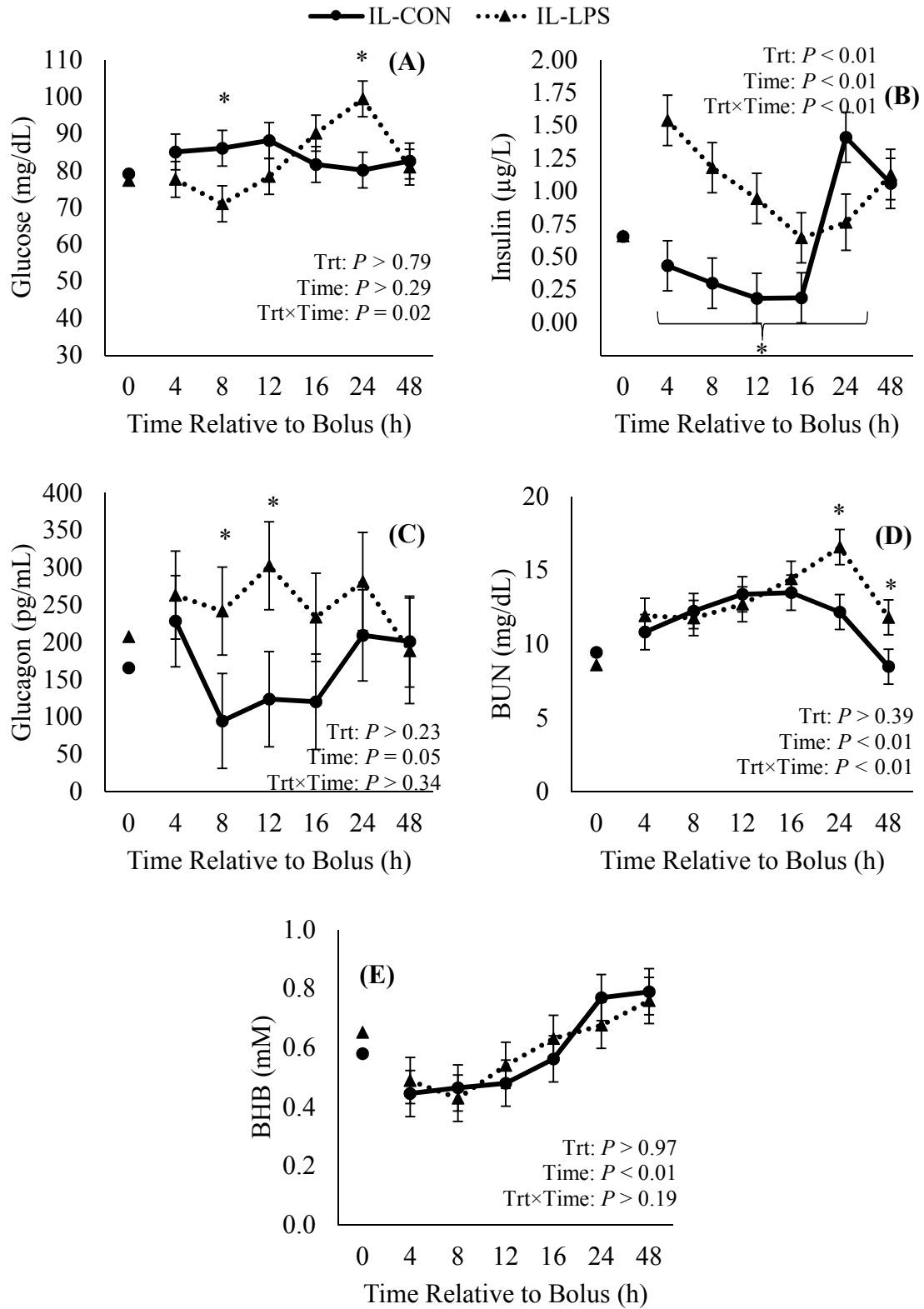


Figure 6.4. Circulating (A) glucose, (B) insulin, (C) glucagon, (D) blood urea nitrogen (BUN) and (E) β -hydroxybutyrate (BHB) from hyperlipidemic (induced via intralipid infusion) late lactation cows administered saline (CON) or a lipopolysaccharide (LPS) bolus. Data were analyzed using PROC MIXED and included fixed effects of treatment, hour, and their interaction. Hour 0 represents an average of measurements obtained prior to LPS administration (-0.5 and 0 h) and was utilized as a covariate. Data are represented as least squares means \pm standard error of the mean and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$. *represents significant differences between treatments.

CHAPTER 7. GENERAL CONCLUSION

Dairy cows are exposed to a myriad of stressors between dry off and the early postpartum period that permit endotoxin translocation into systemic circulation and thereby initiate an inflammatory response (see Chapter 1 “Sources of LPS in the Transition Cow”). Obvious sites of LPS infiltration include the uterus (metritis) and the mammary gland (mastitis). Additionally, increasing evidence points to the gastrointestinal tract as a potential source of LPS-induced inflammation as many situations such as rumen acidosis, heat stress, weaning, and psychological stress can compromise intestinal barrier integrity (Moeser et al., 2007; Khafipour et al., 2009; Baumgard and Rhoads, 2013; Zhang et al., 2013). Furthermore, the short-term feed restriction (FR) which accompanies these situations independently affects intestinal permeability (Zhang et al., 2013; Pearce et al., 2015; Kvidera et al., 2017a,c). Dietary strategies that strengthen the epithelial barrier and reduce the risk of immune activation are currently of great interest.

Zinc is essential for proper epithelial barrier function and it regulates damaged epithelium regeneration (Alam et al., 1994). Previous studies have demonstrated improved intestinal integrity with supplementation of more bioavailable Zn sources (Sanz-Fernandez et al., 2014; Pearce et al., 2015; Abuajamieh et al., 2016). Therefore, objectives of Chapter 2 were to corroborate FR’s impact on intestinal permeability and to assess dietary Zn hydroxychloride’s ability to mitigate intestinal dysfunction caused by FR. In addition, it was of interest to evaluate the temporal changes in intestinal permeability using the *in vivo* paracellular permeability marker Cr-EDTA. In agreement with our past report (Kvidera et al., 2017a), FR altered villus morphology and initiated an acute phase protein response, and this was mirrored by reduced milk yield and altered metabolism. Furthermore, FR increased plasma Cr appearance following ruminal Cr-EDTA administration (indicating intestinal hyperpermeability) and effects were more

pronounced during the acute phase of FR (d 2). Dietary Zn hydroxychloride improved villus morphology and reduced circulating acute phase proteins. Thus, in this study we concluded that Zn hydroxychloride is a promising strategy to improve barrier integrity and reduce the risk of pathogen infiltration during periods of stress.

Chapter 2 successfully demonstrated increased inflammatory biomarkers, altered metabolism, and reduced productivity during intestinal dysfunction. The objectives of the remaining chapters in this dissertation were to investigate how immune activation and inflammation impact energetic and Ca homeostasis and how these modified homeostatic systems are relevant to transition cow biology. In addition, it was of interest to evaluate potential dietary strategies capable of ameliorating the negative consequences of infection. Following activation, leukocytes undergo a metabolic shift from oxidative phosphorylation to aerobic glycolysis and begin utilizing copious amounts of glucose (Palsson-McDermott and O'Neil, 2013). In an effort to facilitate glucose uptake, immune cells become more insulin sensitive (Maratou et al., 2007), whereas peripheral tissues become insulin resistant (Spitzer et al., 1980; Lang et al., 1990). Furthermore, metabolic adjustments including: hyperglycemia or hypoglycemia (depending on the stage and severity of infection), increased insulin and glucagon, increased skeletal muscle catabolism, hypertriglyceridemia, and hypoketonemia (Lanza-Jacoby et al., 1998; McGuinness, 2005; Zarrin et al., 2014; Kvidera et al., 2017b) are employed to ensure adequate glucose delivery to activated leukocytes. Quantifying the leukocyte energetic demand is difficult due to the ubiquitous and migrating nature of the immune system. Therefore, our group developed a technique, the LPS-euglycemic clamp, to quantify this requirement (Kvidera et al., 2017b) and we estimated that the intensely activated immune system utilized more than 1 kg of glucose in a

12 h period. This substantial glucose requirement is of particular relevance to the dairy industry because glucose is crucial for supporting copious milk synthesis.

In the current dissertation (Chapter 3 and 4), we utilized the LPS-euglycemic clamp model to evaluate the effects of two different mineral supplements on metabolism, inflammation, and leukocyte glucose requirements following immune activation. Chromium is a trace-mineral well-known for its role in augmenting insulin action (Davis and Vincent, 1997) and has been shown to improve cellular glucose uptake (Chen et al., 2006). Previous studies have demonstrated improved leukocyte function with Cr supplementation (see Chapter 1 Table 2). Results of Chapter 3 demonstrated that dietary Cr decreased LPS-induced hyperinsulinemia and increased circulating neutrophil numbers but did not alter the leukocyte glucose requirement. In chapter 4, we evaluated effects of Zn amino acid complex (**AvZn**). In addition to its role in maintaining epithelial integrity, Zn plays a crucial role in regulating leukocyte formation, maturation and differentiation, activation, cellular function, and apoptotic rate (Haase and Rink, 2014; Wessels and Cousins, 2015). Partial replacement of Zn sulfate with AvZn did not appear to alter the total glucose deficit. However, AvZn reduced the hyperglycemic phase and decreased circulating glucagon and insulin, demonstrating a dampened metabolic requirement following immune activation. In addition, AvZn increased SAA, reduced the febrile response, and decreased circulating cortisol and somatic cell counts. These results demonstrate well-coordinated alterations in metabolic and inflammatory parameters with complexed Zn supplementation during immune activation. Altogether, results of Chapters 3 and 4 demonstrate the unique metabolic alterations employed to fuel the immune response and suggest that Cr and AvZn may be promising dietary strategies to modulate this response.

Aside from disrupting energetic homeostasis, immune activation causes a marked and unexplainable decrease in circulating Ca and the response is conserved across species (Carlstedt et al., 2000; Waldron et al., 2003; Holowaychuk et al., 2012; Dias et al., 2013). Objectives of Chapter 4 were to evaluate the effects of preventing hypocalcemia on production parameters, inflammatory biomarkers, and neutrophil function in immune activated dairy cows. In contrast to our expectations, results revealed that maintaining eucalcemia (via i.v. Ca infusion) following LPS administration caused a more intense inflammatory response (i.e., increased acute phase proteins and rectal temperature) and impaired production performance. Even though surprising, results actually agree with the sepsis literature. Septic humans are typically hypocalcemic (Kelly and Levine, 2013) and early reports indicate that Ca administration to septic patients increased the incidence of organ failure and mortality (Malcolm et al., 1989). It is now hypothesized that sepsis-induced hypocalcemia serves as a protective strategy and should not be considered pathologic. Results of this work validate the role immune activation plays in hypocalcemia and challenges many long held dogma's (that periparturient hypocalcemia is a pathology needing prevention) in animal physiology and nutrition. Based upon the literature and our supporting work we suggest that post-calving hypocalcemia can be explained, at least partially, by immune activation, a concept previously proposed by early investigators (Thomas, 1889; Hibbs, 1950).

Hypocalcemia is not the only undesirable postpartum phenotype that may be explained by immune activation (see Chapter 1 "LPS/Inflammation and Transition Cow Performance"). In non-ruminants, inflammatory cytokines produced in response to LPS recognition, can negatively impact hepatic lipid trafficking resulting in fatty liver (Lanza-Jacoby and Tabares, 1990; Endo et al., 2007; Stienstra et al., 2010) and a recent report suggests the same may be true in ruminants (Bradford et al., 2009). However, we were unable to recreate fatty liver in mid-lactation cows

administered LPS to induce systemic inflammation (Horst et al., 2019). A possible explanation for the absence of hepatic steatosis in our aforesaid inflammation model may have been the lack of an endogenous increase in NEFA comparable to transition cows. The early lactation cow would be the ideal experimental model of periparturient inflammation because they are naturally hyperlipidemic. However, a host of other physiological events occurring during early lactation (including endocrine, metabolic and inflammatory) are highly variable; sequela which hinders our capacity to intensely investigate the effect of a periparturient systemic inflammation on lipid liver accumulation. Therefore, objectives of Chapter 6 were to evaluate the effects of inflammation on metabolism and liver lipid content by simultaneously recreating hyperlipidemia in cows in established lactation. However, in contrast to our hypothesis inflammation did not exacerbate liver fat accumulation. As discussed in detail in Chapter 6 there were several limitations to our experimental model that may explain our conflicting results including; an insufficient NEFA load, acute vs. chronic inflammation, and systemic vs. compartmentalized inflammation. Future work is required to evaluate inflammation's influence on fatty liver in ruminants.

In conclusion, results of this dissertation demonstrate the marked alterations in energetic and Ca homeostasis that occur in response to immune activation and its relevance to transition cow health and performance. Furthermore, it demonstrated that dietary Cr and Zn are two plausible nutritional strategies with potential to improve barrier integrity and ameliorate the negative consequences of immune activation.

Future Directions

Results of this dissertation provide foundational knowledge for inflammation's potential role in transition cow disorders, however, key knowledge gaps remain. First, a more appropriate inflammatory model is needed. Although bolus LPS administration is a reliable approach, it

likely falls short in accurately mimicking the inflammatory response observed in transition cows. Cows often encounter numerous insults between dry off and the early postpartum period and the frequency of these insults presumably determines the magnitude and persistency of the inflammatory response. Therefore, a repeated or stacked stressors model (e.g., LPS administration and feed restriction) may be more appropriate and will improve our understanding of how transition period inflammation influences metabolism and leukocyte function. Following development of a chronic model, it will be of interest to evaluate inflammation's effect on hepatic TG synthesis, ketogenesis, and peripheral tissue ketone oxidation. Assessment of ketone metabolism should be evaluated using ketone tracer technology and ketone tolerance tests. Furthermore, evaluating changes in leukocyte function and metabolism during chronic inflammation is warranted as this will improve our understanding of the purported "immune suppression" observed in the transition cow. Lastly, it is imperative that we gain a better understanding of the mechanism by which Ca influences the inflammatory response. This mechanistic research will improve our understanding of transition cow disease and is a prerequisite to identifying strategies to improve health and performance in the transition period. In addition to the mechanistic research, studies evaluating the temporal changes in inflammatory mediators, production parameters, and metabolites between dry off and the early postpartum period are needed to improve our understanding of the sequence of events leading up to transition cow disorders.

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APPENDIX. KETOSIS, FEED RESTRICTION, AND AN ENDOTOXIN CHALLENGE DO NOT AFFECT CIRCULATING SEROTONIN (5-HT) IN LACTATING DAIRY COWS

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Abstract

Circulating serotonin (5-HT) appears associated with various energetic disorders and hypocalcemia during the transition period. Objectives were to evaluate the effect of ketosis, feed restriction (FR), or endotoxin challenge (models where energetic and calcium metabolism are markedly altered) on circulating 5-HT in lactating Holstein cows. Blood samples were obtained from three separate experiments and circulating β -hydroxybutyrate (BHB), non-esterified fatty acids (NEFA), and glucose were measured in all three while ionized calcium was only measured in the endotoxin challenge. In the ketosis study, blood samples from cows clinically diagnosed with ketosis ($n = 9$) or classified as healthy ($n = 9$) were obtained from a commercial dairy farm at d -7 , 3 , and 7 relative to calving. Ketosis was diagnosed using a urine based test starting at 5 d in milk. There was no effect of health status on circulating 5-HT nor an association between 5-HT and BHB, NEFA, or glucose, however, 5-HT concentrations progressively decreased following calving. In the FR experiment, mid-lactation cows were either fed ad libitum ($n = 3$) or restricted to 20% of their ad libitum intake ($n = 5$) for 5 d. There were no FR effects on circulating 5-HT nor was it correlated with energetic metabolites. In the immune activation model, mid-lactation cows were either i.v. challenged with lipopolysaccharide (LPS; $1.5 \mu\text{g/kg}$ BW; $n = 6$) or sterile saline (CON; $n = 6$). Administering LPS decreased (56%) blood ionized calcium (Ca^{2+}), but had no effect on circulating 5-HT, nor was there a correlation between

circulating 5-HT and NEFA, BHB, or ionized Ca^{2+} . Circulating 5-HT tended to be positively correlated ($r = 0.54$) with glucose in LPS-administered Holstein cows. In summary, in contrast to expectations, circulating 5-HT was unaffected in models of severely disturbed energetic and Ca^{2+} homeostasis.

Keywords: periparturient, milk fever, calcium

Short Communication

Serotonin (5-hydroxytryptamin, **5-HT**) is a neurotransmitter derived from tryptophan and it is synthesized by the central nervous system (**CNS**) and peripherally including enterochromaffin cells of the gastrointestinal tract (Gershon et al., 1965) and mammary epithelial cells (Berger et al., 2009; El-Merahbi et al., 2015). In fact, in laboratory animals the majority (>90%) of 5-HT is produced by enterochromaffin cells and carried by platelets which release it following activation (Berger et al., 2009). Serotonin is involved in multiple organ and physiological systems (Berger et al., 2009) and has gained considerable attention for its apparent role in lactation (Pai and Horseman, 2008; Collier et al., 2012; Hernandez et al., 2008, 2018). In particular, 5-HT was first identified as a key homeostatic regulator of lactation and involution (Matsuda et al., 2004; Collier et al., 2012). Additionally, 5-HT is related with periparturient calcium levels (**Ca**; Laporta et al., 2015; Weaver et al., 2016; Hernández-Castellano et al., 2017a,b) and energetics (Laporta et al., 2013a; Laporta and Hernandez, 2015; Moore et al., 2015). Although less established in livestock species (Hernández-Castellano et al., 2017c), 5-HT seems to also act as an immune modulator (Baganz and Blakely, 2012; Duerschmied et al., 2013). Consequently, 5-HT has practical implications to multiple variables in the periparturient dairy cow.

The transition into lactation is characterized by significant hormonal, metabolic, mineral and immunological changes, which are necessary (and normal) to support the new dominant

physiological state (Bauman and Currie, 1980; Baumgard et al., 2017). However, maladaptation to lactation increases the risk of metabolic disorders (i.e., ketosis, hypocalcemia) and these acutely and chronically compromise profitability (Coleman et al., 1985; McArt et al., 2012). It is of interest to identify strategies which may improve animal health and farm profitability during this period. Therefore, study objectives were to investigate if circulating 5-HT concentrations are altered in models of severe metabolic and mineral perturbation, as these results may determine whether it could serve as a potential target molecule with therapeutic utility.

All procedures were approved by the Iowa State University Institutional Animal Care and Use Committee. Subsets of cows were chosen from 3 separate studies. Experiment 1 was an observational study in which periparturient multiparous Holstein cows (parity 3.5 ± 0.9 ; housed on a commercial dairy) were retrospectively categorized into 1 of 2 groups consisting of: 1) healthy cows not diagnosed with any illnesses or 2) clinically diagnosed ketotic cows with no other overt health disorder (Abuajamieh et al., 2016). Nine cows were diagnosed for ketosis (> 15 mg/dL urine acetoacetate) using a urine-based test (Ketostix®, Bayer Health Care LLC, Mishawaka, IN) starting at 5 DIM. Each of the ketotic cows were paired with 9 healthy cows calving at approximately the same time. Serum samples were collected via coccygeal venipuncture into silicone-coated vacutainers containing a silica act clot activator (BD Vacutainer, Franklin Lakes, NJ) on d -7, 3, and 7 (± 3 DIM) relative to calving.

Experiment 2 utilized 8 lactating Holstein cows (157 ± 9 DIM, 713 ± 10 kg BW, parity 2 to 4) which were housed in individual box-stalls (4.57×4.57 m) at the Iowa State University Dairy Farm (Kvidera et al., 2017b). Following 5 d of period 1 (**P1**; baseline data collection), cows were allocated to 1 of 2 feed restriction (**FR**) treatments: 1) ad libitum feed intake (**AL**; $n = 3$) or 2) 20% of P1 feed intake (**FR**; $n = 5$). Daily feed intake during period 2 (**P2**) was

determined by applying the desired percentage (100 or 20%) of FR to each cow's mean daily intake during P1. The calculated amount of feed was divided into 3 equal portions during P2 (0800, 1300, and 1800 h) in order minimize metabolic variation due to gorging. Blood samples were collected on d 4 of P1 and d 5 of P2 at 1730 h prior to milking and feeding, and were distributed equally between a tube containing 50 μ L sterile heparin (Sagent Pharmaceuticals, Schaumburg, IL) and an empty glass tube (for serum collection).

Lastly, experiment 3 utilized 12 Holstein cows (718 ± 16 kg; 169 ± 7 DIM; parity 2 or 3) which were housed at the Iowa State University Dairy Farm as previously described (Kvidera et al., 2017a). Cows were randomly assigned to 1 of 2 i.v. bolus treatments: 1) control (**CON**; 3 mL of sterile saline; $n = 6$) or 2) LPS-administered (**LPS**; 1.5 μ g LPS/kg of BW; $n = 6$). Feed was removed ~ 1 h prior to treatment administration and animals remained fasted during the 720 min data collection period. Blood samples were obtained 0, 360, and 720 min post-LPS infusion and were distributed equally between a tube containing K₂EDTA (BD®, Franklin Lakes, NJ; for plasma collection) and an empty glass tube (for serum collection). Blood ionized Ca²⁺ was measured using an i-STAT hand-held machine and cartridge (CG8⁺; Abbott Point of Care, Princeton, NJ).

Serum and plasma from all 3 experiments were harvested following centrifugation at $1,500 \times g$ for 15 min at 4°C and were subsequently frozen at -20°C until analysis. Analysis descriptions of non-esterified fatty acids (**NEFA**), glucose, and BHB are detailed in each respective paper. Serum serotonin was determined using a commercially available kit according to manufactures' instructions (Serotonin; Immunotech, Beckman Coulter, Marseille Cedex 9, France) as previously described (Laporta and Hernandez, 2015; Moore et al., 2015); inter- and intra-assay coefficients of variation were 9.1 and 7.2%.

Each animal's respective parameter was analyzed using repeated measures autoregressive covariance or spatial power law structure. The repeated effect was time (day for the transition cow study and minute endotoxin challenge study) and the random effect was cow. Effects of treatment, time, and treatment by time were assessed as a completely randomized design using PROC MIXED (SAS INC., Cary, NC). Correlation analysis was evaluated using PROC CORR for 5-HT vs. NEFA, glucose, BHB, and ionized calcium (iCa; LPS study only) during P2.

In the transition cow study, compared to prepartum levels, circulating 5-HT progressively decreased postpartum (57 and 62% on d 3 and 7 postpartum; $P = 0.01$; Figure A.1A), however, despite the stark difference in metabolic state, there were no health status (ketotic vs. healthy) differences in 5-HT concentrations ($P > 0.74$; Figure A.1A). In the FR experiment, circulating 5-HT was not altered following 5 d of severe FR ($P > 0.96$; Figure A.1C). In the endotoxin trial, LPS administration induced severe hypocalcemia (46%), but there was no LPS effect on circulating 5-HT concentrations ($P > 0.56$; Figure A.1B). No correlations were observed between 5-HT and NEFA or BHB, in any of the experiments or iCa in the LPS infusion trial (Figure A.2A, B, C and Figure A.3A, C, D). However, there was a tendency for a positive correlation between glucose and 5-HT in LPS-administered cows ($P = 0.07$; $r = 0.54$; Figure A.3B).

Evidence suggests 5-HT functions as a homeostatic regulator of lactation. Serotonin concentrations change dynamically throughout lactation and are thought to govern seemingly distinctly different biological processes including mammary gland involution, energy balance, and Ca homeostasis (Matsuda et al., 2004; Hernandez et al., 2011; Laporta et al., 2013a,b, 2015; Laporta and Hernandez, 2015; Moore et al., 2015). In agreement with previous literature (Laporta and Hernandez, 2015; Moore et al., 2015), we observed a temporal change in

circulating 5-HT following parturition, ostensibly reflecting the dual manner in which 5-HT regulates mammary epithelial cell tight junction permeability during lactation and involution (Stull et al., 2007; Pai and Horseman, 2008). High concentrations of 5-HT in cell culture are thought to inhibit milk protein synthesis and induce mammary epithelial cell apoptosis (Pai and Horseman, 2008, 2011; Hernandez et al., 2011); a scenario contributing to milk synthesis cessation. Further, increased circulating 5-HT concentrations postpartum is associated with lower milk yield and increased SCC (Kessler et al., 2018a,b). Therefore, the concentration and temporal pattern of circulating 5-HT are thought to largely influence its function within the mammary gland.

Several reports suggest a role of 5-HT in regulating glucose and lipid metabolism in rodents (Laporta et al., 2013b; Wantanabe et al., 2011), dogs (Moore et al., 2004, 2005), and ruminants (Watanabe et al., 2014; Laporta et al., 2015; Hernández-Castellano et al., 2017b). Infusing 5-HT or 5-hydroxy-L-tyrtophan (5-HTP; a serotonin precursor) has been shown to increase insulin secretion (Sugimoto et al., 1990), stimulate hepatic glycogenesis (Hampson et al., 2007), increase mRNA abundance of hepatic gluconeogenic enzymes, and increase mammary glucose transporters (Laporta et al., 2013b, 2015). In lactating cows, infusing 5-HTP altered circulating markers of energy metabolism including increased glucose and NEFA and decreased BHB (Laporta et al., 2015), although this has not been corroborated (Hernández-Castellano et al., 2017a; Weaver et al., 2017). Furthermore, endogenous 5-HT levels have also been shown to be affected by or correlated with fasting and ketosis severity, respectively (Sumara et al., 2012; Laporta et al., 2013a). The aforesaid increase in gluconeogenic capacity and mammary glucose uptake (ostensive variables associated with increased milk synthesis) following 5-HTP administration (Laporta et al., 2013b, 2015) is seemingly contradictory with

endogenous 5HT's role in inhibiting milk synthesis (Hernandez et al., 2008, 2011; Kessler et al., 2018). Regardless, and in contrast to the aforementioned reports, we observed no treatment differences in 5-HT concentrations in the ketosis or FR study, despite observing marked alterations in energetic metabolism (Abuajamieh et al., 2016; Kvidera et al., 2017b). In addition to not detecting treatment differences, we surprisingly observed no correlation between 5-HT and glucose, NEFA, or BHB in the transition cow study. The relationship between 5-HT and glucose is variable; as negative (Moore et al., 2015) and positive (Laporta and Hernandez, 2015) correlations have been observed, while others have not detected a relationship (Laporta et al., 2013a; Kessler et al., 2018a). Not observing an association between 5-HT and NEFA or BHB herein agrees with previous observations (Kessler et al., 2018a). Inconsistencies in 5-HT's influence on metabolism may be partially explained by differences in infusion vs. endogenous sources of 5-HT, as endogenous sources may be insufficient to alter circulating metabolites and this agrees with Kessler et al. (2018a). Further investigating the relationship between 5-HT and energetics without exogenous manipulation is warranted.

In agreement with the transition cow and FR studies, we observed no change in 5-HT concentrations following LPS administration, but this disagrees with previous LPS reports (Emau et al., 1986; Endo and Nakamura, 1992). Administering LPS markedly decreases circulating platelets (Kvidera et al., 2017a), which store the majority of 5-HT and release it following activation (Lesurtel et al., 2006). Following LPS administration platelets rapidly migrate to the presumed site of infection and are largely taken up by the liver, as a result circulating 5-HT levels decrease while hepatic concentrations increase (Endo and Nakamura, 1992). Reasons for not observing LPS-induced changes in 5-HT concentrations (despite observing thrombocytopenia) are not entirely clear but warrant further investigation. Besides

directly influencing the cells which store 5-HT, LPS markedly affects energetics and Ca homeostasis (Kvidera et al., 2017a), which we incorrectly hypothesized would alter 5-HT concentrations. In addition to observing no LPS effects on circulating 5-HT, no relationship between 5-HT and NEFA or BHB was detected. However, 5-HT tended to be positively correlated with glucose in the LPS-infused cows. Administering LPS induces a marked biphasic change in circulating glucose with an initial period of hyperglycemia followed by hypoglycemia (Kvidera et al., 2017a). Further assessing the impact of LPS administration on hepatic and circulating 5-HT concentrations and its relationship with hepatic glucose output has transition cow implications.

In addition to energetic metabolism, 5-HT is purportedly an important regulator of Ca homeostasis (Laporta et al., 2013c; Hernandez et al., 2012, 2018; Hernández-Castellano et al., 2017a,b). The mammary gland has a large Ca demand at the onset of lactation and relies on proper hormonal regulation (increased parathyroid hormone and decreased calcitonin action, respectfully) to maintain eucalcemia (Horst et al., 2005). However, mammary Ca uptake is so acute and extensive that it often exceeds homeostatic strategies employed to maintain eucalcemia (Goff et al., 2008) and clinical or subclinical hypocalcemia develops. Parathyroid hormone-related protein (**PTHrP**) is the primary hormone responsible for Ca mobilization during lactation (Wysolmerski et al., 1995, 2010), and several studies have observed a positive correlation between 5-HT and PTHrP (Laporta et al., 2013a) and 5-HT and Ca (Laporta et al., 2013a; Moore et al., 2015). Additionally, cows classified as having “high” 5-HT levels tended to have increased total Ca concentrations (Kessler et al., 2018a). Oral 5-HTP supplementation increases circulating PTHrP and Ca, osteoclast number, and expression of Ca transporters in the rodent mammary gland (Laporta et al., 2013c, 2014), although results in ruminants are

inconsistent (Laporta et al., 2015; Weaver et al., 2016; Hernández-Castellano et al., 2017a,b). Similar to early lactogenesis, LPS administration results in a severe and sustained hypocalcemia lasting > 24 h (Horst et al., 2018). Surprisingly, we did not observe a correlation between 5-HT and ionized Ca^{2+} concentrations in the present study. Reasons why we did not detect a relationship between severe hypocalcemia and 5-HT are unclear, but our model of immune activation is acute (<24 h) and periparturient hypocalcemia is chronic (>1-3 d).

In summary, the experiments presented herein caused marked alterations in energetic and Ca homeostasis, changes similar to a transitioning dairy cow, yet 5-HT concentrations were unaffected and no correlations were observed between 5-HT and NEFA, BHB, or ionized Ca^{2+} . The experimental models we utilized and implemented were admittedly severe and may have temporally overwhelmed 5-HT's regulatory role. Maybe less metabolically insulting and longer lasting models may have produced a different interpretation. Regardless, the effects of 5-HT on energetic and Ca metabolism is incredibly variable and its supposed causal role in common transition cow disorders needs a more thorough and critical evaluation.

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Figures

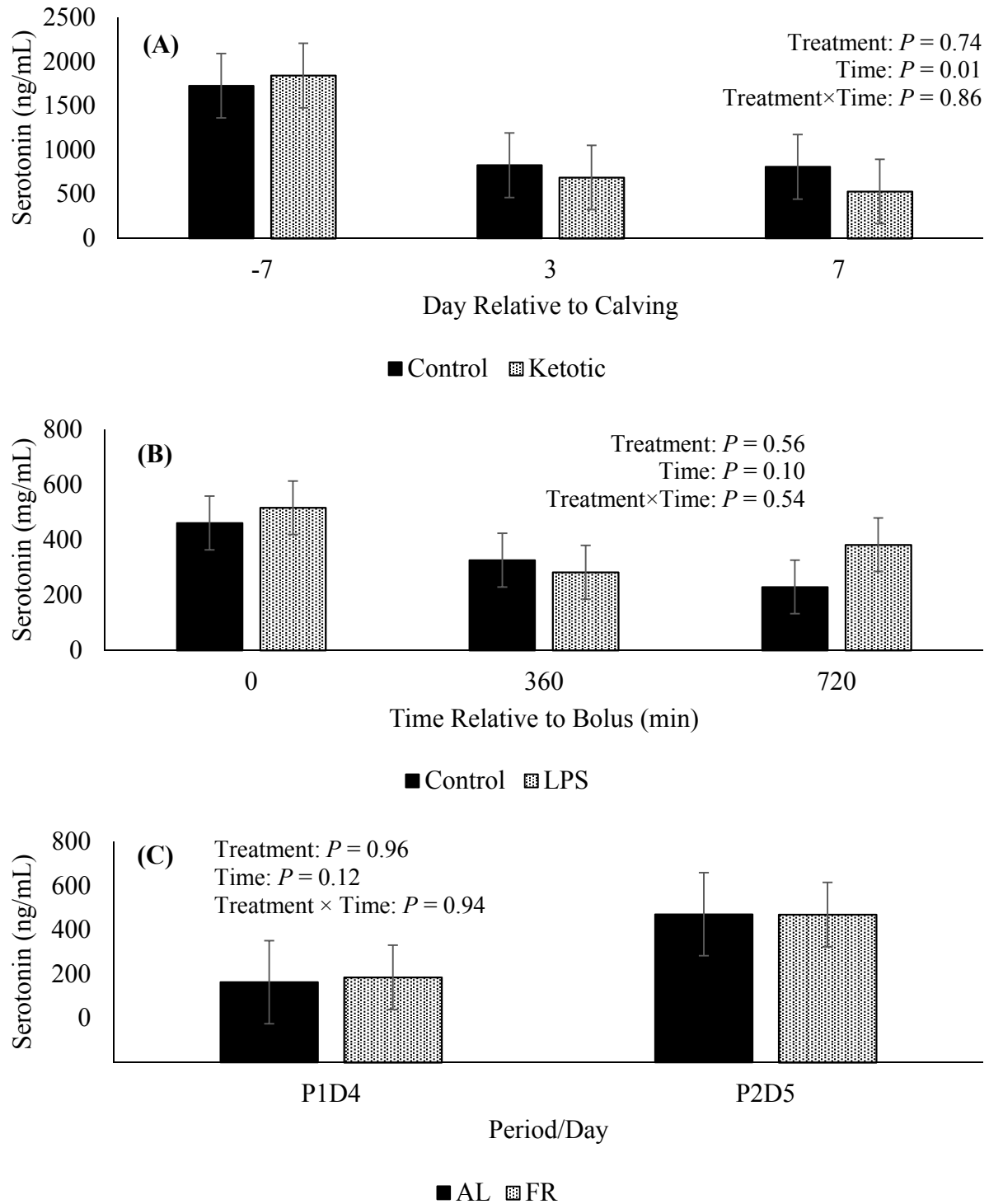


Figure A.1. Effects of ketosis (A), an LPS bolus (B), and feed restriction (C) on circulating serotonin (5-HT) in Holstein cows. Data are reported as least squares means \pm SEM.

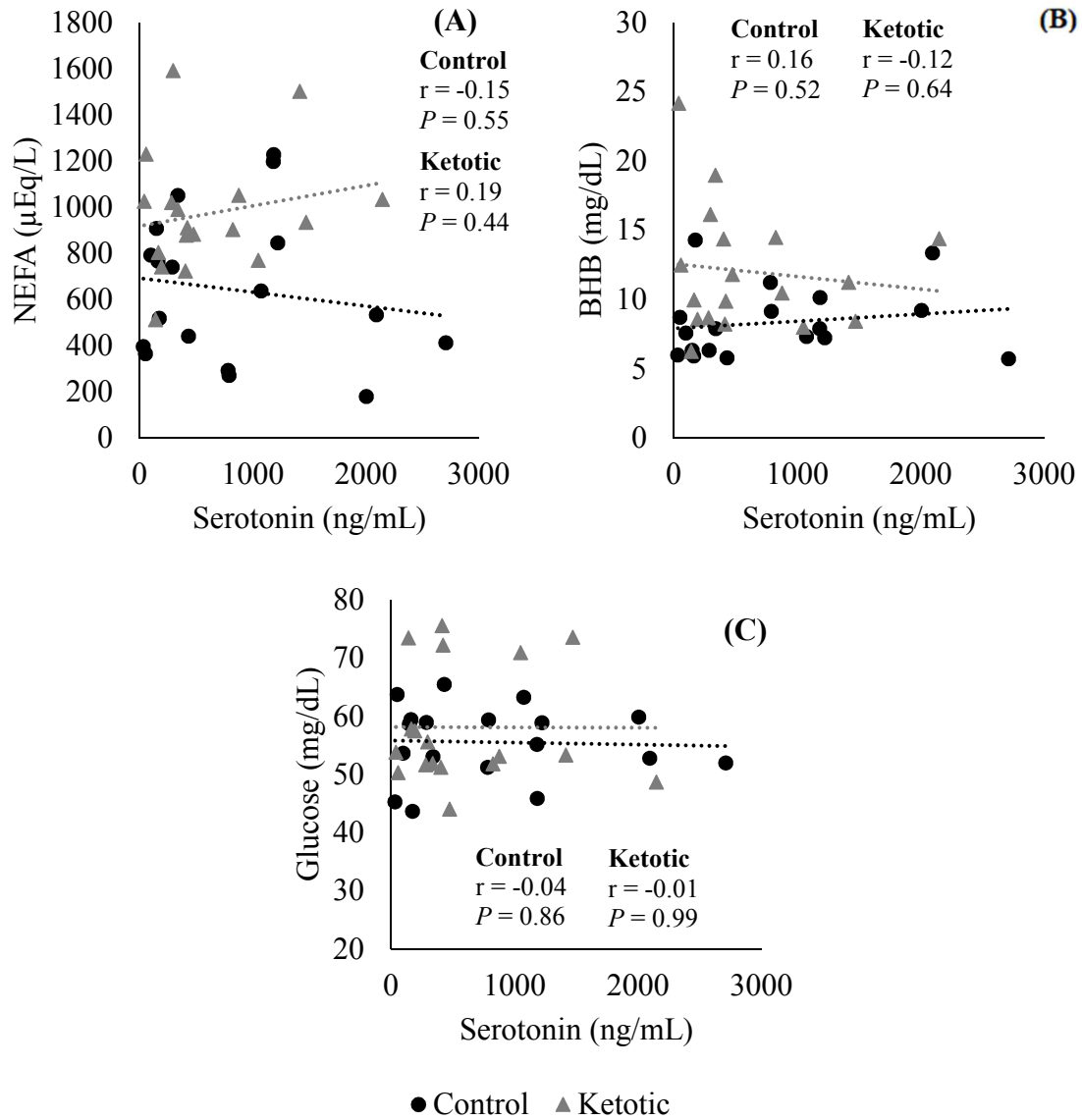


Figure A.2. Correlation of circulating serotonin (5-HT) and NEFA (A), BHB (B), and glucose (C) in clinically ketotic (solid gray triangle) or healthy (solid black circle) Holstein cows.

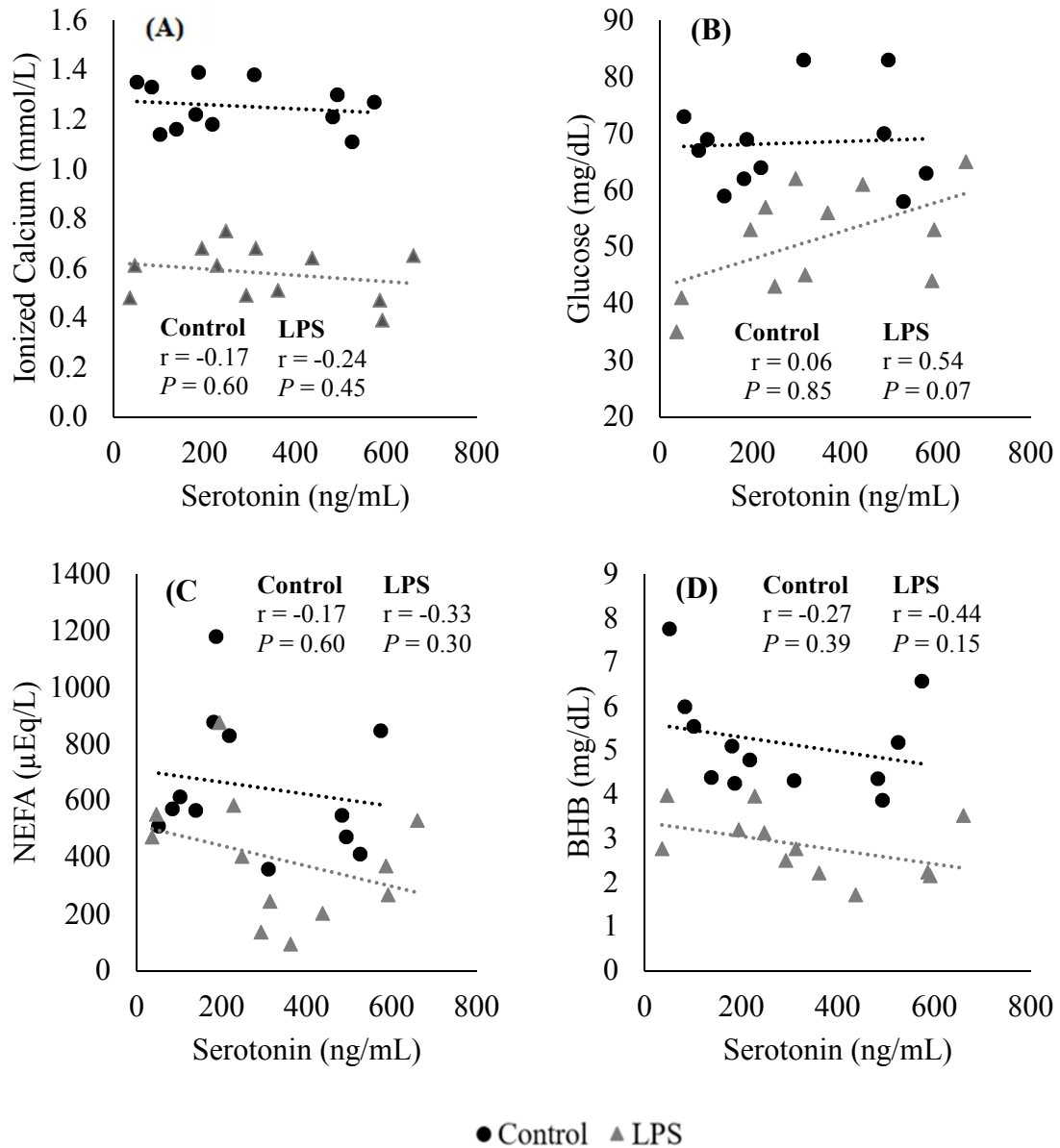


Figure A.3. Effects of a LPS (solid gray triangle) or saline bolus (solid black circle) on the correlation of circulating serotonin (5-HT) and ionized calcium (A), glucose (B), NEFA (C), and BHB (D) in lactating Holstein cows.