**ABSTRACT**

Most immunometabolic research utilizes mid-lactation (ML) cows. Cows in early lactation (EL) are in a presumed state of immune suppression/dysregulation and less is known about how they respond to a pathogen. Study objectives were to compare the production and metabolic responses to i.v. lipopolysaccharide (LPS) and to differentiate between the direct effects of immune activation and the indirect effects of illness-induced hypophagia in EL and ML cows. Cows in EL (n = 11; 20 ± 2 d in milk) and ML (n = 12; 131 ± 31 d in milk) were enrolled in a 2 × 2 factorial design containing 2 experimental periods (P). During P1 (3 d), cows were fed ad libitum and baseline data were collected. At the initiation of P2 (3 d), cows were randomly assigned to 1 of 2 treatments by lactation stage (LS): (1) EL (EL-LPS; n = 6) or ML (ML-LPS; n = 6) cows administered i.v. a single bolus of 0.09 µg LPS/kg of body weight; *Escherichia coli* O55:B5 or (2) pair-fed (PF) EL (EL-PF; n = 5) or ML (ML-PF; n = 6) cows administered i.v. saline. Administering LPS decreased dry matter intake (DMI) and this was more severe in EL-LPS than ML-LPS cows (34 and 11% relative to baseline, respectively). By design, P2 DMI patterns were similar in the PF groups compared with their LPS counterparts. Milk yield decreased following LPS (42% on d 1 relative to P1) and despite an exacerbated decrease in EL-LPS cows on d 1 (25% relative to ML-LPS), remained similar between LS from d 2–3. EL-LPS had increased milk fat content, but no difference in protein and lactose percentages compared with ML-LPS cows. Further, cumulative ECM yield was increased (21%) in EL-LPS compared with ML-LPS cows. During P2, EL-LPS cows had a more intense increase in milk urea nitrogen (MUN) and blood urea nitrogen (BUN) than ML-LPS and EL-PF cows. Administering LPS did not cause hypoglycemia in either EL-LPS or ML-LPS cows, but glucose was increased (33%) in EL-LPS compared with EL-PF. Hyperinsulinemia occurred post-LPS, and insulin was further increased in ML-LPS than EL-LPS cows (2.2-fold at 12 h peak). During P2, circulating glucagon increased only in EL-LPS cows (64% relative to all other groups). Both EL groups had increased NEFA at 3 and 6 h post-LPS from baseline (56%), but NEFA in EL-LPS cows gradually returned to baseline thereafter and were reduced relative to EL-PF until 36 h (50% from 12 to 24 h). Alterations in β-hydroxybutyrate (BHB) did not differ between ML groups, but EL-LPS had reduced BHB compared with EL-PF from 24 to 72 h (51%). Results indicate that there are distinct LS differences in the anorexic and metabolic responses to immune activation. Collectively, EL cows are more sensitive to the catabolic effects of LPS than ML cows, but these exacerbated metabolic responses appear coordinated to fuel an augmented immune system while simultaneously supporting milk synthesis.

Keywords: lactation stage, immune activation, endotoxin

**INTRODUCTION**

Healthy periparturient dairy cows initiate homeorhetic adjustments to support the dominant physiological state of lactation and this is primarily characterized by hypoinsulinemia (coupled with reduced peripheral insulin sensitivity), increased circulating nonesterified fatty acids (NEFA), and hyperketonemia (Bauman and Currie, 1980). The evolutionary metabolic strategy for milk production is to spare glucose for the mammary gland, since lactose is the primary osmotic regulator of milk synthesis (Neville, 1990). Although highly conserved among species (Bauman and Currie, 1980; Horst et al., 2021), these metabolic adjustments are sometimes interpreted as pathological because they are thought to cause immune suppression or impaired immune function (Targowski and Klucinski, 1983; Goff and Horst, 1997; Sordillo and Raphael, 2013), which is commonly suspected as causal for the increased inci-
dence of health disorders (e.g., mastitis, metritis, etc.) in early lactation (EL; Hill et al., 1979; Lloyd, 1983; LeBlanc, 2008).

Following pathogen detection, an activated immune system utilizes an enormous amount of amino acids and glucose (Johnson, 2012; Kvidera et al., 2017) and this occurs concurrently with infection-induced inappetence, an evolutionarily preserved response among species (Ashley and Wingfield, 2012). To compensate for decreased nutrient intake and to energetically support the immune system, acutely-inflamed animals coordinate hormonal and metabolic adjustments to increase hepatic glucose output and minimize extra-immune system glucose use; a strategy to “spare glucose” for the immune system (Beisel and Wannemacher, 1980; Lang and Dobrescu, 1991; Lang, 1993).

There is an increasing awareness that all transition cows (in the period 3 weeks before and after parturition; Drackley, 1999) are inflamed and it is only the magnitude and resolution of it that differs among cows (Bertoni et al., 2008; Trevisi et al., 2015; Trevisi and Minuti, 2018). To model peripartal immune activation, immunometabolic research normally uses healthy cows in established lactation (Vernay et al., 2012; Zarrin et al., 2014; Horst et al., 2020b) to reduce variability in the physiological, metabolic, and inflammatory responses - systems that are notoriously variable during the periparturient period (Bell, 1995; LeBlanc, 2020). However, like all models, key limitations exist in employing the ML cow in immunometabolic research. During established lactation, milk synthesis is highly sensitive to LPS and milk production is acutely and severely decreased following immune activation (Kvidera et al., 2017; Horst et al., 2019a, 2020a); a key mechanism by which cows spare glucose (and amino acids) for the immune system (Horst et al., 2021). However, forfeiting milk synthesis in EL may not be evolutionarily favorable when the neonate predominantly relies on their mother’s milk for survival. Thus, the EL hyper-inflamed cow has an energetic conundrum as it needs to nourish its young (spare glucose for milk synthesis) while congruently partitioning nutrients to the immune system (spare glucose for leukocyte consumption) during inflammation-induced anorexia (Horst et al., 2021).

Periparturient cows likely have increased exposure to LPS since multiple epithelia (uterine, mammary, and intestinal epithelial) are at heightened risk of permeability from stressors encountered in early lactation (dystocia, lactogenesis, and dietary changes; Eckel and Ametaj, 2016; Horst et al., 2021). Thus, the immune system may develop LPS tolerance – or the dampening of immune activation, as repeated exposure occurs (Beeson, 1947). Reduced LPS immunogenicity in transition cows might cause considerable discrepancies between EL and ML production and metabolism following an immune insult. Therefore, objectives were to characterize the metabolic and production responses to an acute i.v. LPS challenge in EL and ML dairy cows. We hypothesized EL cows would have reduced LPS responsiveness indicated by attenuated production loss relative to ML cows. However, we expected the changes in metabolites and hormones in EL cows to reflect characteristic homeorhetic adjustments cows engage when attempting to maximize milk synthesis (i.e., hypoinsulinemia, hypoglycemia, increased NEFA, and hyperketonemia), which would differ from ML cows (e.g., typical responses to LPS in ML cows: hyperinsulinemia and reduced NEFA and ketones).

**MATERIALS AND METHODS**

**Experimental Design**

A detailed description of the experimental design can be found in the companion paper (Opgenorth et al., 2024). All procedures were approved by the Iowa State University Animal Care and Use Committee. Twenty-three non-pregnant, multiparous EL (20 ± 2 DIM; 52 ± 6 kg daily milk yield; 2.5 ± 0.8 parity; n = 11) and ML (131 ± 31 DIM; 52 ± 5 kg daily MY; 2.8 ± 0.9 parity; n = 12) Holstein cows were enrolled in a 2 × 2 factorial experiment in 2 replicates and moved to individual box-stalls (4.57 × 4.57 m) at the Iowa State University Dairy Farm (Ames, IA). Cow selection criteria included clinically healthy cows with < 250,000 SCC on last test day with no reported clinical disease in the current lactation. Regardless of lactation stage (LS), all cows were fed a diet formulated to meet or exceed the predicted requirements of energy, protein, minerals, and vitamins for early lactation cows (NRC, 2001; Table 1). Cows were given 4 d to acclimate, and bilateral jugular catheters were implanted as previously described (Horst et al., 2020b). Following acclimation to their new surroundings, the study included 2 experimental periods (P). During P1 (3 d) cows were fed ad libitum and baseline production data were obtained. At the initiation of P2 (3 d), cows were randomly assigned to receive 1 of 2 treatments by LS: (1) EL (EL-LPS) or ML (ML-LPS) administered 0.09 µg/kg BW *Escherichia coli* O55:B5 in 4 mL sterile saline (approximately 15.3 µg/mL), i.v. or (2) EL control pair-fed (EL-PF) or ML (ML-PF) to LPS treatments of their respective LS and i.v. administered 4 mL sterile saline. The LPS dose (0.09 µg/kg BW) was selected in an attempt to prevent maximum immune activation and allow potential LS differences to be exposed. In an effort to differentiate between the direct effects of immune stimulation and LPS-induced hypophagia, feed...
intake of EL-LPS and ML-LPS was recorded every 2 h through 72 h post-bolus. Cows in the EL-PF and ML-PF groups were PF to their respective EL-LPS and ML-LPS counterparts by individually calculating the percent reduction in feed intake of LPS cows from P1 to P2. The reduction in feed intake was then applied to PF cows such that feed intake of EL and ML cows were similar between treatments every 2 h. To allow time for PF calculations, the experimental timeline of PF cows began 2 d after LPS cows.

Throughout the experiment, cows were milked 4 × daily at 0600, 1200, 1800, and 0000 h, and yields were compiled to a daily sum. Milk samples were collected at each milking for composition analysis throughout P1 and P2. 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) to quantify milk component concentrations. Daily milk component concentrations are reported as averages, whereas yields are reported as a daily sum. Blood samples were collected from the jugular catheter at 0, 3, 6, 9, 12, 24, 36, 48, and 72 h relative to bolus administration in tubes containing K2EDTA (BD, Franklin Lakes, NJ) to harvest plasma for circulating metabolite and hormone analysis. Additionally, 3 mL of whole blood designated for glucagon analysis was collected into a separate K2EDTA tube containing 150 µL of aprotinin to protect stability before analysis (BP2503–10; Thermo Fisher Scientific, Fair Lawn, NJ). Plasma was harvested after centrifugation at 1,500 × g for 15 min at 4°C before storing at −20°C until analysis. Whole blood was also collected in tubes containing heparin (BD, Franklin Lakes, NJ) to analyze glucose with an iSTAT hand-held machine and cartridge (CG8+; Abbott Point of Care, Princeton, NJ).

Plasma NEFA, BHB, BUN, glucagon, and insulin concentrations were determined with commercially available kits (NEFA, Wako Chemicals USA Inc., Richmond, VA; BHB, Pointe-Scientific Inc., Canton, MI; BUN, Teco Diagnostics, Anaheim, CA; glucagon, R&D Systems, Minneapolis, MN; insulin, Mercodia AB, Uppsala, Sweden). Samples were re-analyzed when coefficients of variation exceeded 15%. The inter- and intra-assay coefficients of variation for NEFA, BHB, BUN, glucagon, and insulin were: 3.3 and 4.1%, 9.1 and 7.0%, 5.1 and 4.9%, 3.2 and 4.8%, and 5.2 and 5.6%, respectively.

### Statistical Analysis

Data were analyzed with the MIXED procedure in SAS 9.4 (SAS Institute Inc., Cary, NC). Fixed effects of (LPS vs. PF), LS (EL vs. ML), time, their interactions (i.e., group × LS, group × time, LS × time, and group × LS × time), and replicate were analyzed with a spatial power covariance structure for blood parameters and an autoregressive covariance structure for production parameters. Time relative to LPS administration served as a repeated measure and included a subject of cow within group. Baseline P1 data with no repeated measures (i.e., circulating metabolites and hormones) were not analyzed with a repeated measure but rather included a random effect of cow within group. Data from P1 and P2 were analyzed separately from each other to increase lucidity. To improve clarity, MUN and BUN were additionally analyzed by comparing the magnitude of change (%) from each individual cow’s baseline concentrations. Pre-planned contrasts were analyzed to isolate group and LS (i.e., EL-LPS vs. ML-LPS, EL-PF vs. EL-LPS, and ML-PF vs. ML-LPS) treatment comparisons. Data are reported as least squares means ± standard error of the mean and considered significant if P ≤ 0.05 and a tendency if 0.05 < P ≤ 0.10. Statistical differences between EL-LPS and ML-LPS, EL-PF and EL-LPS, and ML-PF and ML-LPS are additionally conveyed in tables and figures to isolate differences by

### Table 1. Ingredients and composition of diet1

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<thead>
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<th>Item</th>
<th>Parameter5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredient (% of DM)</td>
<td>1.28% K, 0.21% S, 0.28 mg/kg Se, 5524.00 IU/kg vitamin A, 1104.80 IU/kg vitamin D, and 22.10 IU/kg vitamin E.</td>
</tr>
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<tr>
<td>Corn gluten feed</td>
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<td>Soy Plus2</td>
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</tr>
<tr>
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<td>3.6</td>
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<tr>
<td>VTM mix3</td>
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<tr>
<td>NFR4 analysis (% of DM)</td>
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<tr>
<td>Starch</td>
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</tr>
<tr>
<td>CP</td>
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<tr>
<td>NDF</td>
<td>22.3</td>
</tr>
<tr>
<td>ADP</td>
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</tr>
</tbody>
</table>

1Values represent an average of ration nutrient summary reports collected throughout the trial. Dry matter averaged 54%.
2Mechanically processed soybean meal (Dairy Nutrition Plus,Ralston, IA).
3Vitamin and trace mineral (VTM) mix (% of diet DM); 2.17% soybean meal, 1.35% CaCO3, 1.11% Palmit 80 (Global Agri-trade Corporation, Rancho Dominguez, CA), 0.99% blood meal, 0.96% Na sesquicarbonate, 0.83% pork meat and bone meal, 0.60% distillers grains, 0.40% salt, 0.26% MgO, 0.21% urea, 0.16% rice hulls, 0.06% Smartantine M (Adisseo Inc., Antony, France), 0.06% choice white grease, 0.02% ZnSO4 36%, and < 0.01% of vitamin ADE premix, MnSO4 32%, Se 0.16%, organic Se yeast 2000, Rumensin 90.7 (Elanco Animal Health, Greenfield, IN), CuSO4, biotin 2%, CaCO3 46%, and organic I EDDI-99%.
4NIR = near infrared refractometry.
5Average nutrient levels: 4.56% fat, 0.94% Ca, 0.42% P, 0.37% Mg, 1.28% K, 0.21% S, 0.28 mg/kg Se, 5524.00 IU/kg vitamin A, 1104.80 IU/kg vitamin D, and 22.10 IU/kg vitamin E.
Feed Intake and Production

Relative to P1, DMI decreased at 24 h following LPS (68 and 32% in EL-LPS and ML-LPS, respectively; \( P < 0.01 \); Figure 1A) and returned to baseline for ML-LPS but not EL-LPS by 3 d post-bolus. By design, PF cows shared similar DMI patterns with LPS-infused cows. During P1, milk yield did not differ between group or LS (Figure 1B). Administering LPS caused an abrupt decrease in milk yield regardless of LS (37% relative to PF groups on P2 d 1; \( P < 0.01 \)), and EL-LPS experienced a more severe decrease on P2 d 1 relative to ML-LPS (25%; \( P = 0.04 \)). However, cows recovered such that milk yield did not differ between EL-LPS and ML-LPS on P2 d 2 or 3. Further, the cumulative 3-d milk yield between EL-LPS and ML-LPS cows did not differ (data not shown). The PF regimen had a surprisingly large effect on milk production in EL cows as milk yield did not differ from EL-LPS and EL-PF on d 2 and 3, and this contrasted the relationship of ML-LPS and ML-PF (\( P = 0.02 \)). Energy-corrected milk was increased in EL compared with ML cows during P1 (19%; \( P < 0.01 \); Figure 1C), decreased after LPS, and did not statistically differ in quantity between EL-LPS and ML-LPS (\( P > 0.15 \)). Overall, cumulative P2 ECM was actually increased in EL-LPS compared with ML-LPS cows (21%; \( P = 0.02 \); data not shown).

All P1 data for milk component parameters are reported in Supplementary Table 1 whereas all corresponding P2 results are reported in Table 2. Milk fat yields were decreased post-LPS but remained increased in EL-LPS compared with ML-LPS cows (37%; \( P < 0.01 \); Table 2). Daily milk protein yields decreased with LPS relative to PF groups (15%; \( P = 0.02 \)), but EL-LPS and ML-LPS did not differ overall during P2 (\( P > 0.13 \); Table 2). Likewise, daily lactose yield decreased following LPS compared with PF (13%; \( P = 0.04 \)), and the decrease in EL-LPS was more severe compared with ML-LPS cows on P2 d 1 (30%; \( P = 0.02 \); data not shown) but did not differ overall during P2 (Table 2). During P2, the net change in MUN from P1 was increased (\( P < 0.01 \)) in EL-LPS above ML-LPS (31.9 vs. 5.7%) and EL-PF cows (31.9 vs.10.2%), but ML-LPS and ML-PF did not differ. Somatic cell score was unaffected by group during P2.

Metabolism

Following LPS administration, glucose concentrations were minimally perturbed regardless of LS. However, EL-PF cows became hypoglycemic over time compared with EL-LPS, ML-LPS and ML-PF cows (30%; \( P < 0.01 \); Figure 2A). Basal circulating insulin was increased in ML compared with EL cows (2.1-fold; \( P = 0.01 \); Figure 2B), increased after LPS compared with PF (3.7-fold; \( P < 0.01 \)), was overall elevated in ML-LPS relative to EL-LPS (92%; \( P < 0.01 \)), and post-hoc analysis revealed insulin increased 2.3-fold in ML-LPS compared with EL-LPS cows during h 6 and 12 post-challenge (\( P = 0.05 \); data not shown). Circulating glucagon acutely increased in EL-LPS compared with all other groups (64% during P2; \( P < 0.01 \); Figure 2C). Interestingly, glucagon did not change in ML-LPS compared with ML-PF.

Concentrations of BUN increased during P2 and returned to baseline over time in all groups (\( P < 0.01 \); Figure 3A). Cows in ML-LPS and ML-PF groups did not have different BUN concentrations throughout P2. However, the magnitude of increase in BUN from baseline was intensified in EL compared with ML cows (35 relative to 9%, respectively; \( P < 0.01 \); Figure 3B) and in EL-LPS above EL-PF and ML-LPS (44 compared with 26 and 10%, respectively; \( P < 0.02 \)) where BUN peaked at 24 h and gradually returned to baseline thereafter. During P1, cows in EL had elevated NEFA in comparison with ML cows (2.5-fold; \( P < 0.01 \); Figure 3C). Following LPS, NEFA concentrations increased in EL-LPS cows from 3 to 6 h (44% relative to P1) and gradually returned to baseline thereafter. Circulating NEFA were decreased in EL-LPS relative to EL-PF cows overall during P2 (23%; \( P = 0.01 \)). Plasma BHB were increased in EL compared with ML cows throughout P2 (2.9-fold; \( P < 0.01 \); Figure 3D) but was reduced in EL-LPS relative to EL-PF from 24 to 72 h (51%; \( P < 0.01 \)).
Early lactation cows prioritize milk synthesis through healthy homeorhetic adjustments that optimize glucose sparing for the mammary gland (Baumgard et al., 2017). However, the metabolic and mineral footprints (i.e., increased NEFA, hyperketonemia, and hypocalcemia) that dominate the periparturient period have been assumed causal toward immune suppression and the ensuing increased disease susceptibility (Curtis, 1983; Goff and Horst, 1997; Ingvartsen and Moyes, 2013). For multiple reasons (i.e., parturition, lactogenesis, dietary changes), postpartal cows are presumably at increased risk of encountering epithelial barrier dysfunction and the accompanying pathogen-associated molecular pattern insult (i.e., LPS; Eckel and Ametaj, 2016). Frequent LPS infiltration can lead to immune desensitization that reduces host responsiveness and promotes immunosuppression (i.e., ameliorated cytokines and neutrophil adhesion; Beeson, 1947; Ziegler-Heitbrock, 1995; Biswas and Lopez-Collazo, 2009). To evaluate the metabolic and inflammatory response to immune activation, ML cows are often employed as a model for peripartal cows to minimize physiological, metabolic, and disease variability associated with transition cows. However, due to distinctly different metabolic profiles between LS and purported immune suppression in EL cows, we sought to compare their response to i.v. LPS with ML cows. We hypothesized EL cows would be less responsive toward LPS due to their previous LPS exposure during and following parturition, and that this would be reflected by attenuated loss in milk production and exaggerated metabolic adjustments EL cows use to prioritize milk synthesis.

Immune activation in response to LPS caused substantial losses in production metrics. Dry matter intake was acutely reduced in LPS cows, corroborating other LPS models (Horst et al., 2019a; Al-Qaisi et al., 2020; Gross et al., 2020). Although cows in both LS were hypophagic, the decrease in DMI was more severe in EL-LPS than ML-LPS cows, and this disagrees with our hypothesis as it suggests a heightened responsiveness to LPS in EL cows. However, the differential LS effects herein agree with a previous report indicating EL cows developed more severe anorexia from experimentally induced mastitis than ML cows (Hill et al., 1979). Immune activation causes inappetence across species (Ashley and Wingfield, 2012; Li et al., 2021), and the more severe DMI deficit in EL-LPS than ML-LPS cows, and this disagrees with our hypothesis as it suggests a heightened responsiveness to LPS in EL cows. However, the differential LS effects herein agree with a previous report indicating EL cows developed more severe anorexia from experimentally induced mastitis than ML cows (Hill et al., 1979). Immune activation causes inappetence across species (Ashley and Wingfield, 2012; Li et al., 2021), and the more severe DMI deficit in EL-LPS relative to ML-LPS cows corroborates the corresponding increase in immune activation biomarkers (i.e., tumor necrosis factor [TNF]-α, IL-6, Hp, and LBP) and febrile response in EL-LPS cows (discussed in our companion paper; Opgenorth et al., companion paper). Overall, results
herein agree with previous studies demonstrating that LPS administration reduces DMI; however, EL cows are especially sensitive to the anorexic consequences of immune activation.

Similar to DMI, milk yield also decreased in both LS, but the magnitude of decrease in the EL-LPS cows on d 1 was slightly more than the ML-LPS cows. Importantly, milk synthesis recovered quickly in the EL-LPS cows such that milk production on d 2 and 3 did not differ between LPS groups and overall (d 1–3) milk production was similar in EL-LPS and ML-LPS cows. In fact, EL-LPS cows produced almost 26 kg more (P < 0.05) ECM than ML-LPS. The overall milk yield response to LPS in EL vs. ML cows is perplexing considering the EL-LPS cows had a prolonged febrile response, increased cytokines, acute phase proteins, marked hypocalcemia (Opgenorth et al., companion paper), and experienced a more pronounced decrease in DMI. However puzzling, our results agree with others demonstrating that transition cows have a similar or less severe of a reduction in milk synthesis following stressors such as LPS, heat stress, nutrient restriction, and clinical diseases than cows in later lactation (Maust et al., 1972; Perera et al., 1986; Lehtolainen et al., 2003; Bjerre-Harpøth et al., 2012; Habel and Sundrum, 2023; Kennedy and Kuhla, 2023). During established lactation, LPS and its inflammatory milieu downregulate nutrient transporter gene expression in mammary epithelial cells in varies species (Kobayashi et al., 2013; Gross et al., 2015; Tsugami et al., 2021). However, it is unclear if immune activation influences substrate transporter expression differently by LS in dairy cows. Our results suggest the mammary gland in EL cows is less perturbed by heightened systemic immune activation since milk yield recovers similarly or even quicker than in ML cows. The use of homeorhetic mechanisms to quickly recover milk synthesis in EL-LPS cows despite the staggering nutrient expense of reduced feed intake and heightened immune activation implies EL physiology continues to prioritize milk production (likely an evolutionary adaptation to ensure neonatal calf survival accomplished through an intensely catabolic, but coordinated, metabolic response (described below)).

Endotoxin administration normally results in a biphasic glucose response, characterized by 2–3 h of hyperglycemia (stemming from increased hepatic glycogenolysis and gluconeogenesis) followed by a long period (up to 24 h) of hypoglycemia (Giri et al., 1990; Yates et al., 2011; Burdick Sanchez et al., 2013). The LPS-induced hypoglycemia indicates glucose uptake by the immune system exceeds the synchronized efforts of increased hepatic output and reduced systemic tissue glucose utilization (Naylor and Kronfeld, 1985; Kvidera et al., 2017; Horst et al., 2020a). The post-LPS glucose pattern allows the LPS-Euglycemic clamp technique to roughly estimate the amount of glucose used by an activated immune system (Kvidera et al., 2017; Horst et al., 2018, 2019). Interestingly, i.v. LPS did not cause a similar hypoglycemia pattern in this experiment (in either LS) and this is likely because the dose was substantially lower than used in previous experiments. However, it is noteworthy that EL-LPS cows were able to remain euglycemic (even had brief periods of increased glucose) throughout P2, particularly because their immune and inappetence responses were so severe. Mechanistically, this was probably mediated by reduced skeletal muscle and adipose tissue glucose utilization and enhanced hepatic glucose output (based

<table>
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<tr>
<th>Parameter</th>
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<th>LPS</th>
<th>SEM</th>
<th>Group</th>
<th>LS</th>
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<th>EL-LPS vs. ML-LPS</th>
<th>EL-PF vs. EL-LPS</th>
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1During period 2 (P2), cows were assigned to a treatment group (lipopolysaccharide [LPS] or pair-fed [PF]) by lactation stage (early [EL; n = 11] or mid-lactation [ML; n = 12]).
2P-values of time and time interactions are omitted for brevity.
3Group: LPS or PF
4Calculated as the percent change relative to P1
5Values within a row with differing superscripts denote differences (P ≤ 0.05) between treatments if Group × LS P ≤ 0.05.

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upon hormonal and metabolic adjustments; including insulin, glucagon, and BUN). Regardless, whether or not maintaining euglycemia in the face of intense immune activation contributed to the mammary gland’s apparent refractoriness to immune activation is not clear as the mammary GLUT transporter’s Km are quite low (Zhao and Keating, 2007) and normally unaffected by mild hypoglycemia (McCarthy et al., 2020). However, mammary GLUT-1 expression is downregulated by LPS (Ling and Alcorn, 2010; Kobayashi et al., 2013; Gross et al., 2015), thus mammary glucose kinetics during immune activation may potentially be more dependent upon blood glucose concentrations.

Cows administered LPS developed hyperinsulinemia, and this agrees with previous reports (Calder et al., 2007; Waldron et al., 2006; Horst et al., 2019a). However, the hyperinsulinemic phase was blunted in EL-LPS relative to ML-LPS cows, corroborating prior research where cows in more severe NEBAL have a reduced insulin response to LPS (Pires et al., 2019). We did not obtain samples before the 3rd hour post-LPS, so it is unclear if the difference in insulin responses between LS is explained by differences in the hyperglycemic phase. Regardless, in healthy cows, EL hypoinsulinemia is the primary endocrine strategy to partition glucose to the mammary gland (Bauman and Currie, 1980; Baumgard et al., 2017). Thus, reduced insulin concentrations in EL-LPS cows after immune activation may indicate a homeorhetic preference to preserve the hormonal state to “spare glucose” for the mammary gland. Coupled with hypoinsulinemia, healthy EL cows are also hypo-responsive to insulin such that hepatic gluconeogenesis increases and peripheral glucose uptake decreases (Bell and Bauman, 1997). Immune activation also causes its own state of reduced insulin sensitivity in peripheral tissues to reserve glucose for the immune system (Lang, 1993). Therefore, EL homeorhetic adjustments (i.e., hypoinsulinemia and insulin insensitivity) in concert with LPS-mediated insulin resistance may have optimized glucose preservation in EL-LPS cows.

Hyperglucagonemia occurred following LPS, but only in EL-LPS cows. The pattern of increased glucagon in EL-LPS cows herein corroborates others and likely indicates an endocrine attempt to upregulate hepatic gluconeogenesis in response to LPS (Naylor and Kronfeld, 1985; Horst et al., 2019a). The exacerbated glucagon release in EL-LPS cows may have been mediated through increased IL-6 concentrations (described in the companion paper; Oppenorth et al., companion paper), which augments glucagon secretion during immune activation (Barnes et al., 2014). The elevated glucagon response is presumably an additional endocrine mechanism that EL cows utilize to modulate metabolism and

Figure 2. Effects of group (lipopolysaccharide [LPS] or pair-fed [PF]) and lactation stage (LS: early [EL; n = 11] or mid-lactation [ML; n = 12]) on (A) glucose, (B) insulin, and (C) glucagon during period 2 (P2). Baseline data reported before LPS represent an average of measurements obtained at 0 h or 0 when analyzing the percent change of parameters. Data are represented as LSM ± SEM and considered significant if $P \leq 0.05$ and $P < 0.05 < P \leq 0.10$. Only $P$-values $\leq 0.10$ are reported. $P$-values not reported in figures include (A) LS: $P < 0.01$; Group: $P < 0.01$; Time: $P < 0.01$; LS × Time: $P = 0.06$; Group × LS × Time: $P < 0.01$; (B) Time: $P = 0.02$; Group × Time: $P < 0.01$; post hoc analysis from 6 to 12 h revealed a difference ($P = 0.05$) between EL-LPS and ML-LPS, and (C) Group: $P < 0.01$; LS: $P < 0.01$; Time: $P < 0.01$; Group × Time: $P < 0.01$. 
simultaneously provide glucose for the immune system and milk synthesis.

Increased (in absolute and in percent change) BUN and MUN were observed in EL-LPS cows, but not ML-LPS cows relative to their PF counterparts. During immune activation, skeletal muscle is mobilized to provide AA precursors for gluconeogenic and APP synthesis (Beisel and Wannemacher, 1980; Webel et al., 1997; Iseri and Klasing, 2014). The profile of AA mobilized from muscle is dissimilar to that of APP (Reeds et al., 1994) and thus the excessive AA are deaminated (similar to that of the gluconeogenic AA), and amino groups enter into the urea cycle (Horst et al., 2019b). Several factors may have contributed to a more severe BUN response in EL-LPS cows. Glucagon (which was increased in EL-LPS cows) facilitates hepatic uptake of AA and thus increases deamination for gluconeogenesis (Brockman and Bergman, 1975; Lang et al., 1989; Flakoll et al., 1994). Additionally, EL adaptations of reduced insulin sensitivity in muscle and decreased insulin-like growth factor promote muscle catabolism (Rhoads et al., 2007; Dimitriadis et al., 2011; Baumgard et al., 2017), and

Figure 3. Effects of group (lipopolysaccharide [LPS] or pair-fed [PF]) and lactation stage (LS; early [EL; n = 11] or mid-lactation [ML; n = 12]) on (A) BUN, (B) relative to baseline, percent change in BUN, (C) NEFA, and (D) BHB during period 2 (P2). Baseline data reported before LPS represent an average of measurements obtained at 0 h or 0 when analyzing the percent change of parameters. Data are represented as LSM ± SEM and considered significant if P ≤ 0.05 and P < 0.05 < P ≤ 0.10. Only P-values ≤0.10 are reported. P-values not reported in figures include: (A) Time: P < 0.01; LS × Time: P = 0.06, and (B) Group × LS: P = 0.08; Time: P < 0.01; LS × Time: P < 0.01, (C) Group: P = 0.01; Time: P = 0.02; Group × Time: P = 0.07; LS × Time: P = 0.03, and (D) Group: P = 0.02; Time: P < 0.01; Group × Time: P < 0.01; LS × Time: P < 0.01; Group × LS × Time: P < 0.01.
this metabolic scenario may be amplified during immune activation. Overall, in response to LPS, EL cows had markedly altered metabolism (i.e., increased glucagon and urea nitrogen), but maintained metabolic signatures associated with their LS (i.e., attenuated insulin) that suggested a more catabolic response relative to ML cows, and this seemingly contributed to their ability to prioritize milk synthesis.

Lipopolysaccharide influences lipid metabolism, and the differences denoted by LS further allude to how EL-LPS cows were able to prioritize milk yield despite being in a more catabolic and inflammatory state. Ofentimes, administering LPS decreases NEFA relative to PF or fasting controls in ruminant models (attributed to the abrupt blunting of lipolysis through LPS-induced hyperinsulinemia; Fain et al., 1966) (Waldron et al., 2006; Kvidera et al., 2017; Horst et al., 2018), which agrees with the NEFA response in the ML-LPS cows. Incidentally, this in vivo response contradicts in vitro systems indicating LPS directly causes adipocyte lipolysis (Zu et al., 2009; Chirivi et al., 2022). However, instead of immediately decreasing, NEFA in both the EL-LPS and EL-PF cows increased similarly for the first 6 h. The EL-PF cows continued to have increased NEFA (as expected), while in the EL-LPS cows NEFA began to decrease from the 6th hour onwards and was similar to baseline concentrations by the 12th hour. The transient increase in NEFA after LPS has been observed previously in some EL models (Lehtolainen et al., 2003; Graugnard et al., 2013). This initial increase in NEFA may be attributed to the blunted insulin response in the EL-LPS cows. A dampened insulin surge following LPS has meaningful metabolic advantages to EL cows; primarily characterized by increased metabolic flexibility. Having the opportunity to oxidize FFA may also be energetically conducive for mounting a febrile response (Beisel et al., 1980) as the EL-LPS cows had a much more robust fever than ML-LPS cows (Opgenorth et al., companion paper). Regardless, overall NEFA were temporally increased in EL-LPS cows and this pattern is distinctly different than the immediate decrease in NEFA typically observed in previous in vivo immune activation experiments using cows in established lactation (Waldron et al., 2006; Horst et al., 2018).

Hyperketonemia in EL provides an important alternative fuel that allows glucose sparing for lactation during NEBAL (Holtenius and Holtenius, 1996; Horst et al., 2021). Ruminants typically have reduced BHB concentrations in response to acute LPS (Steiger et al., 1996; Kvidera et al., 2017; Horst et al., 2019a), and this has been attributed to, at least in part, decreased butyrate originating from the gastrointestinal tract (GIT; Waldron et al., 2003). Herein, only EL-LPS, but not ML-LPS cows, had reduced BHB relative to PF counterparts (despite having a simultaneous increase in NEFA). This indicates that either ketogenesis was downregulated or ketone utilization was increased in EL-LPS cows. Evidence in ruminants points to maintained hepatic ketogenesis (Waldron et al., 2003) but reduced butyrate metabolism in the GIT (in response to immune-activation-induced hypophagia; Bedford et al., 2020). Further, circulating ketone clearance increases in immune-activated lactating cows (Zarrin et al., 2014). These findings indicate cows in EL may upregulate ketone utilization in peripheral tissues as alternative fuel during acute immune activation in a metabolic attempt to maintain milk yield. Although we hypothesize immune activation or its influence on feed intake may be causal to hyperketonemia in EL (Horst et al., 2021), acute experimental LPS administration clearly does not cause hyperketonemia. The inflammatory and pathogenic milieu EL cows normally encounter is complex and less abrupt than a single molecular pattern administered i.v., and it is these “stacked stressors” that may chronically introduce the metabolic conditions necessary for hyperketonemia while the cow is attempting to prioritize milk synthesis.

The unique responses to PF in EL cows deserve consideration. The concept of utilizing PF controls in immunometabolism research is to isolate differences in response to immune activation while eliminating the confounding effects of dissimilar DMI (Baumgard and Rhoads, 2013). Milk production in EL was surprisingly sensitive to reduced DMI and in fact, overall P2 milk yield did not differ between EL-LPS and EL-PF cows. Reasons why are not clear as we anticipated that EL milk synthesis would largely be refractory to brief feed reductions. However, the temporal pattern of key nutrient partitioning may provide some clues. The EL-PF cows experienced substantial hypoglycemia from the 12th hour onwards and this mirrored the decrease in NEFA. Despite decreased NEFA, hyperketonemia started at and progressed from the 12th hour onwards. The use of PF controls in an EL LPS challenge has been employed before, but only for the first 8 h of the experiment whereby PF milk yield was minimally affected (Waldron et al., 2006), and this agrees with the first day of EL-PF milk synthesis herein. However, there appeared to be lasting consequences to EL-PF milk yield after the initial loss of DMI on d 1 that prevented milk yield recovery.

CONCLUSION

The feed intake and metabolic response to LPS were more pronounced and severe in EL compared with ML cows. This was hormonally characterized by a more robust glucagon and blunted insulin responses to LPS.
in EL-LPS cows. Metabolically, LPS caused an acute increase in NEFA in EL cows, contrasting the distinctive decrease in circulating NEFA observed in cows during established lactation. The more severe catabolic and anorexic effects of LPS coincide with a much more intensely activated immune system (Opgenorth et al., companion paper) in EL cows. However, despite strong evidence demonstrating that LPS is more immunogenic in EL, milk synthesis was not as severely affected as it was in ML cows. Ergo, the EL cow appears to homeo-thermically coordinate metabolism to spare glucose for both an activated immune system and milk production; a Darwinian scenario accompanied with strong biological plausibility. Regardless of the natural selection interpretation, the physiology described herein has pragmatic implications to how both healthy and pathological periparturient metabolic adjustments are interpreted.

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