Maternal late-gestation metabolic stress is associated with changes in immune and metabolic responses of dairy calves

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ABSTRACT

Metabolic stress in periparturient dairy cows is characterized by excessive lipid mobilization, inflammation, and oxidative stress that is associated with immune dysfunction. Thus, metabolic stress around the time calving is linked to the development of various early-lactation health disorders. Maternal status during late pregnancy can have carryover effects on several health and production variables of neonatal calves. However, the effects of metabolic stress during gestation on metabolic and immune responses of newborn calves remain unknown. Thus, we aimed to investigate whether metabolic stress in late-gestation dairy cows is associated with changes in the metabolic and immune responses of their offspring during the first month of life. Holstein-Friesian cows ($n = 12$) were blood sampled at 28 and 15 d before expected calving. The average between these 2 sampling points in the serum concentrations of nonesterified fatty acids (NEFA), haptoglobin (Hp), and oxidant status index (OSi)—defined as the ratio between reactive oxygen and nitrogen species and total antioxidant potential—were calculated as indicators of the degree of lipid mobilization, inflammation, and oxidant status (OS), respectively. Calves were subsequently divided into groups ($n = 6$ each) according to their dams' high or low degree of lipid mobilization, inflammation, and OS. The metabolic responses of calves in each of these groups were compared weekly throughout their first month of life by assessing serum concentration of NEFA, Hp, and OSi. Additionally, whole blood was obtained from calves at each sampling period and subjected to a lipopolysaccharide (LPS)-stimulated tumor necrosis factor- α (TNF- α) production assay to assess cell-mediated innate immunity against induced inflammatory responses, using high (5 $\mu\text{g/mL}$ of blood)

and low (10 ng/mL) concentrations of LPS. Calves born to cows with higher NEFA or OSi showed lower body weight at birth and throughout the study, whereas no association between any of the maternal groups and average daily gain at 4 wk of age was identified. Serum concentrations of reactive oxygen and nitrogen species were higher in calves exposed to higher maternal NEFA concentrations or OSi when compared with calves born to cows with lower values of these biomarkers. Calves exposed to high maternal OS also had higher circulating concentrations of Hp and TNF- α , indicating greater basal inflammatory responses when compared with calves born to cows with a lower OSi. In contrast, LPS-induced inflammatory responses were less robust in calves exposed to higher maternal biomarkers of inflammation or OS, suggesting compromised immune responses to microbial agonists. Collectively, these data suggest that prenatal exposure to maternal parameters of metabolic stress may adversely affect some metabolic and inflammatory responses of the offspring that could influence disease susceptibility.

Key words: calf health, dairy cow, lipid mobilization, oxidative stress, transition period

INTRODUCTION

Dairy cows experience metabolic stress during the transition period when they fail to physiologically adapt to the profound increase in nutrient requirements associated with fetal growth and milk production (Sordillo and Mavangira, 2014). Metabolic stress is characterized by excessive lipid mobilization, oxidative stress, and inflammatory dysfunction (Abuelo et al., 2015). The negative effect of metabolic stress on the immune function, health, and production of dairy cattle during this period is well established (Kehrli et al., 1989; Sordillo and Aitken, 2009). Metabolic stress starts several weeks before calving (Grummer, 1993; Sordillo and Raphael, 2013), and therefore can potentially affect the fetus.

Evidence exists in other nonruminant species that maternal stress during gestation influences fetal devel-

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opment and exerts carryover effects on the offspring (McMillen and Robinson, 2005; Merlot et al., 2008). Studies in humans and murine models demonstrated that suboptimal intrauterine conditions during critical periods of development lead to changes in tissue structure and function (Fowden et al., 2006), which may have long-term consequences on offspring physiology and disease susceptibility (McMillen and Robinson, 2005; Merlot et al., 2008). Studies in dairy cattle have also demonstrated that exposure to heat stress and restricted or excessive energy intake during late gestation affect the immune and metabolic function of the offspring (Gao et al., 2012; Tao et al., 2012, 2014; Osorio et al., 2013). Moreover, Monteiro et al. (2016) demonstrated that the detrimental effects of in utero exposure to heat stress on milk yield and reproductive performance extend to at least the first lactation of offspring. Thus, prenatal conditions have the potential of significantly affecting the productivity and health status of replacement heifers.

The first month of life is a time when the greatest neonatal morbidity and mortality rates are observed on dairy farms (Windeyer et al., 2014); hence, maternal carryover effects can have a significant effect on calf health during this time. Unfortunately, no information exists on how metabolic stress in the dam can affect immune responses in the newborn calf when they are at risk for succumbing to health disorders. Therefore, we hypothesized that calves born to cows experiencing increased metabolic stress will exhibit altered metabolic status and immune responses in the first month of life. The objective of our preliminary observational study was to compare metabolic status and LPS-induced whole blood tumor necrosis factor- α (TNF- α) release between calves born to cows that experienced different degrees of maternal metabolic stress. As an index for quantification of metabolic stress is yet to be established (Sordillo and Mavangira, 2014; Abuelo et al., 2015), the carryover effects of the 3 components of metabolic stress triad (lipid mobilization, inflammation, and oxidative stress) were separately assessed.

MATERIALS AND METHODS

Animals

The Charles Sturt University Animal Care and Ethics Committee approved all procedures involving animals before the commencement of the study (Protocol 15/120). During October and November 2016, 12 multiparous Holstein-Friesian dairy cows and their calves were monitored for the last month of gestation and the first month of life, respectively. Given the lack of previous studies investigating the effect of maternal

metabolic stress on the calves immune and metabolic status, this sample size was selected to create 2 groups ($n = 6$) for comparison. Cows entering their 2nd to 4th lactation were selected based on the proximity of their calving dates from a 180-head commercial pasture-based dairy farm located in Ladysmith, New South Wales, Australia, with an average 305-d normalized milk production of 8,513 kg/cow.

Further selection criteria included a variation in BCS lower or equal to 0.5 points during the last month of gestation for cows and adequate passive transfer of immunoglobulins in the calves. For this, cows were body condition scored to the nearest quarter point using the 5-point (1 = lean, 5 = obese) system described by Edmonson et al. (1989) at each sampling point and within the first week after calving. To assess passive transfer of immunity, the serum sample collected in the first week of age was analyzed for IgG using a radioimmunoassay (Bovine IgG test, Triple J Farms, Bellingham, WA). The first 12 cow/calf pairs met both criteria, with all calves being singletons born from an unassisted eutotic calving and having an IgG concentration higher than the 10 g/L cutoff commonly used to define failure of passive transfer of immunity (Godden, 2008). Hence, no further animals were enrolled. Additionally, all animals underwent a weekly veterinary clinical exam throughout the study period to rule out any clinical disease. In calves, the exam included assessment of appetite, rectal temperature, heart and respiratory rate, umbilical and joint swelling, and the presence or absence of diarrhea. None of the animals enrolled in the study showed clinical signs of disease; hence, the data from all cows and calves was included in the statistical analyses. Calf weights were estimated with a heart girth tape (Holstein Calf Weigh Tape; The Coburn Company Inc., Whitewater, WI) placed vertically at the point of the elbow (Heinrichs et al., 2007).

Cows and calves in the study were each managed under identical conditions. Cows were housed outdoors on pasture, and calves were housed in individual calf shelters for the first 2 wk of life and then housed in a straw-bedded shed in groups of 6 to 8 calves. Cows were dried off 60 d before the expected calving date. During the first 4 wk of the dry period, cows grazed a ryegrass and clover pasture with an estimated supply of 2.5 kg of DM/d per cow and were offered oaten hay *ad libitum*. For the last 30 d of the dry period, cows received 2 kg of concentrate and 1 kg of anion salts once daily in addition to the same supply of pasture and *ad libitum* access to oaten hay. Calves were removed from dams within 12 h after birth. Calves received 4 L of colostrum from their respective dams within the first 12 h of life and then twice daily for the first 2 d at a rate of 10% BW/d. Thereafter, they received 2.5 L of

unpasteurized whole milk every 12 h until the end of the study period. After the first week of age, calves had ad libitum access to Lucerne hay and a commercial calf pelletized concentrate (Veanavite No. 2 Calf Pellets, Rivalea Australia Pty Ltd., Corowa, NSW, Australia). The chemical composition of the feedstuffs given to cows and calves is presented in Supplemental Tables S1 and S2 (<https://doi.org/10.3168/jds.2017-14038>).

Sample Collection

Blood samples from cows were collected via coccygeal venipuncture at 28 and 15 d before the expected calving date (23 ± 1.8 and 11 ± 1.6 d before actual calving date; mean $\pm$ SD). Calves were blood sampled via jugular venipuncture weekly for the first month of life (7 ± 0.8 , 14 ± 1.0 , 20 ± 0.8 , and 29 ± 0.8 d of age). Sampling time was kept consistent throughout the study, with cows being sampled 1 h before the administration of concentrate and anion salts, and calves 3 h after the morning milk feeding. Blood was collected into plain vacuum tubes (BD Vacutainer; Becton, Dickinson and Company, Plymouth, UK) from cows and calves. Blood was allowed to clot at room temperature and sera were subsequently harvested after centrifugation at $2,000 \times g$ for 20 min at 4°C , aliquoted, and stored at -20°C pending analysis within 3 mo of collection. Three 9-mL EDTA vacuum tubes (Vacuette K3EDTA; Greiner Bio-One GmbH, Kremsmünster, Austria) were also collected from each calf, immediately stored on crushed ice, and transported to the laboratory.

Analytical Determinations

Haptoglobin (Hp) was determined as a biomarker of inflammation, whereas the concentration of nonesterified fatty acids (NEFA) was employed to indicate the degree of lipid mobilization. Commercially available assays were used to determine serum NEFA (Wako NEFA Assay, Wako Diagnostics, Mountain View, CA) and Hp (Phase Haptoglobin Assay TP-801, Tridelata Development Limited, Maynooth, Ireland) concentrations, following the manufacturer's instructions (http://www.trideltaltd.com/includes/tng/pub/tNG_download4.php?LINK=haptoglobin-main-page&KT_download1=191122c2263dd494980e70d3f8ff938f.) The EDTA-collected blood was submitted to the Charles Sturt University Veterinary Diagnostic Laboratory (Wagga Wagga, NSW, Australia) for differential blood cell counting using a Cell Dyn 3500 automated hematology analyzer (Abbott Diagnostics, Lake Forest IL).

Oxidant status (OS) was assessed as previously described (Abuelo et al., 2016). A commercially available fluorometric assay (OxiSelect In Vitro ROS/RNS As-

say Kit; Cell Biolabs Inc., San Diego, CA) was used following the manufacturer's instructions (<https://www.cellbiolabs.com/sites/default/files/STA-347-in-vitro-ros-rns-assay-kit.pdf>) to measure reactive oxygen and nitrogen species (RONS) in serum as a marker of oxidant production. In brief, a specific probe was added to and reacted with free radicals within the sample, yielding a fluorescent product. Fluorescence of the dichloro-fluorescent dye was measured at 480 nm of excitation and 530 nm of emission. The fluorescence of blank samples was subtracted from sample measurements to eliminate background fluorescence. A standard dichloro-fluorescent dye curve was included to ensure that the dye could be detected at various concentrations. The reported values represent relative fluorescent units normalized per microliter of sample.

The serum antioxidant potential (AOP) was measured using the Trolox equivalent antioxidant capacity, as previously described (Re et al., 1999). Briefly, the AOP of a sample was expressed as the equivalence of a known Trolox (synthetic vitamin E analog) standard (Sigma Aldrich, St. Louis, MO) concentration, resulting in a similar reduction of the generated radical 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (Sigma Aldrich) determined using the standard curve. Both parameters of the redox balance were assessed together as the ratio of pro-oxidant to total antioxidant defenses (RONS/AOP), namely oxidant status index (OSi), as it accurately detects changes in oxidant status during the transition period (Abuelo et al., 2013). An increase in the ratio indicates a higher risk for oxidative stress due to an increase in pro-oxidant production or defensive antioxidant depletion.

LPS Stimulation of Whole Blood

At weekly intervals during the first month of life, calf EDTA blood samples were subjected to an ex vivo cytokine release assay to estimate the innate immunologic response to external stimuli (Damsgaard et al., 2009). Stimulation of the whole blood samples with LPS (*Escherichia coli* 0111:B4, Sigma Aldrich) was performed within 2 h of collection, as described previously (O'Boyle et al., 2006) with slight modifications. Briefly, the 3 EDTA tubes collected from the same calf were mixed, and three 5-mL aliquots were stimulated for 3.5 h at 37°C with a high ($5 \mu\text{g/mL}$ of blood) or low (10 ng/mL of blood) concentration of LPS or endotoxin-free Dulbecco's Modified Eagle Medium (Sigma Aldrich) as a negative control. Blood was handled in sterile, pyrogen-free, tubes (SuperClear Centrifuge Tubes; Labcon, Petaluma, CA). Following incubation, samples were centrifuged at $1,300 \times g$ for 10 min at 4°C . Plasma samples were collected

on crushed ice, stored at -80°C , and assayed in duplicate for TNF- α concentrations using a bovine-specific ELISA assay (Bovine TNF α ELISA; Thermo Scientific, Waltham, MA) with a detection limit of 0.1 ng/mL. The intra- and interassay coefficients of variation were 6.2 and 9.4%, respectively.

Statistical Analysis

The average between the 2 sampling points of the dams was calculated for the serum Hp (marker of inflammation) and NEFA (marker of lipid mobilization) concentrations and OSi (marker of OS). The offspring of cows with a mean below and above the median for each of the above variables were placed in low ($n = 6$) and high ($n = 6$) groups, respectively. All statistical analyses were performed with SPSS Statistics for Windows version 20.0 software (IBM Corp., Armonk, NY). The Mann-Whitney U-test was used to compare adult cow data between groups and correlation analyses among adult cattle variables were performed with the Spearman's test. Generalized linear mixed models with repeated measures were built for the calf variables BW, NEFA, Hp, RONS, AOP, OSi, basal TNF- α concentration, TNF- α concentration following 10 ng/mL of LPS stimulation, TNF- α concentration following 5 $\mu\text{g/mL}$ of LPS stimulation, TNF- α percentage increase from basal concentration following 10 and 5 $\mu\text{g/mL}$ of LPS stimulation, and the different blood cells populations (total leukocytes, neutrophils, lymphocytes, monocytes, eosinophils, and basophils). Fixed effects included the sampling week (1, 2, 3, or 4), calf groups (high/low) of maternal NEFA, maternal Hp, and maternal OSi, as well as the different time $\times$ group inter-

actions. Calf identification was the random effect. For repeated measures, 5 covariance structures were tested (unstructured, autoregressive 1, variance components, compound symmetry, and Toeplitz), and the 1 resulting in the lowest Akaike information criterion was chosen. The degrees of freedom were approximated with the Kenward-Roger method. Model assumptions were assessed by evaluation of homoscedasticity and normality of residuals. To satisfy this assumption, data of NEFA and RONS were natural log-transformed and resulting least squares means estimates were subsequently back transformed and presented as the geometric mean. Similar models without repeated measures were built for the variables birth weight and ADG. These models included the different calf groups as fixed effects and calf sex as random effect. All P -values given are those controlled for multiple comparisons with Bonferroni's test. Statistical significance was declared at $P < 0.05$.

RESULTS

The mean (SD) concentration of the cow biomarkers is presented by group and sampling point in Table 1. Only the classifying variables differed significantly between the low and high groups of each biomarker. Similarly, no strong or significant correlations were identified among maternal variables apart from the ones between the OSi and its constituent variables RONS and AOP (Table 2).

Regarding the offspring, high maternal lipid mobilization during late gestation was associated with significantly higher serum OSi and Hp in the calves (Table 3). In addition, calves born to cows with a higher OSi during late gestation had a significantly higher serum

Table 1. Mean (SD) of the maternal variables categorized by group and sampling point¹

Maternal variable	Days before expected calving	Group					
		NEFA		Hp		OSi	
		Low	High	Low	High	Low	High
BCS	28	3.0 (0.36)	3.0 (0.41)	3.2 (0.14)	2.9 (0.15)	3.2 (0.25)	3.0 (0.51)
	15	3.1 (0.31)	3.1 (0.41)	3.2 (0.31)	3.1 (0.32)	3.1 (0.34)	3.2 (0.37)
NEFA (mmol/L)	28	0.20 (0.037)	0.35 (0.099)*	0.26 (0.101)	0.28 (0.116)	0.24 (0.106)	0.31 (0.126)
	15	0.25 (0.029)	0.50 (0.114)*	0.37 (0.193)	0.37 (0.131)	0.34 (0.116)	0.40 (0.177)
Hp (g/L)	28	0.21 (0.151)	0.14 (0.086)	0.09 (0.024)	0.30 (0.179)*	0.12 (0.083)	0.24 (0.161)
	15	0.24 (0.186)	0.18 (0.098)	0.12 (0.033)	0.29 (0.182)*	0.16 (0.094)	0.28 (0.184)
RONS (RFU/ μL)	28	32.6 (5.84)	32.6 (3.88)	30.9 (5.05)	34.3 (4.76)	23.1 (3.52)	42.2 (5.43)*
	15	33.5 (5.73)	28.8 (3.58)	32.5 (4.55)	29.7 (5.14)	26.5 (4.57)	45.8 (4.30)*
AOP (μM)	28	39.3 (4.52)	40.5 (5.36)	34.7 (5.08)	45.16 (4.83)	40.1 (4.19)	38.9 (7.53)
	15	38.9 (3.06)	41.7 (4.18)	40.2 (4.09)	44.9 (3.64)	44.7 (4.47)	40.4 (3.23)
OSi (arbitrary units)	28	0.81 (0.274)	0.89 (0.16)	0.93 (0.144)	0.78 (0.126)	0.58 (0.104)	1.20 (0.127)**
	15	0.87 (0.154)	0.75 (0.25)	0.83 (0.123)	0.70 (0.149)	0.67 (0.122)	0.98 (0.166)*

¹At each sampling point, groups were compared statistically with the Mann-Whitney U-test. NEFA = nonesterified fatty acids; Hp = haptoglobin; RONS = reactive oxygen and nitrogen species; RFU = relative fluorescence units; AOP = antioxidant potential; OSi = oxidant status index. * $P < 0.05$; ** $P < 0.01$.

Table 2. Correlation analyses among maternal biomarkers of metabolic stress (shown as Spearman ρ correlation coefficient)¹

Variable	NEFA	Hp	RONS	AOP	OSi
NEFA	—	0.105	0.007	0.130	−0.04
Hp	0.105	—	0.062	−0.034	0.07
RONS	0.007	0.062	—	0.104	0.75**
AOP	0.130	−0.034	0.104	—	−0.58**
OSi	−0.04	0.07	0.75**	−0.58**	—

¹NEFA = nonesterified fatty acids; Hp = haptoglobin; RONS = reactive oxygen and nitrogen species; AOP = antioxidant potential; OSi = oxidant status index.

* $P < 0.05$; ** $P < 0.01$.

concentration of RONS and Hp. Calves born to cows with higher NEFA or OSi showed significantly lower BW at birth and throughout the study period, whereas no association between any of the maternal metabolic stress groups and ADG at 4 wk of age was identified (Table 4; Figure 1). No differences were observed in the differential blood count results between low and high groups of any of the studied variables (Table 5).

Exposure to higher OSi during late gestation was associated with higher basal concentrations of TNF- α , as well as significantly higher TNF- α plasma concentrations after stimulation with either 10 ng/mL or 5 μ g of LPS (Tables 6 and 7). Conversely, calves born to cows with a higher serum Hp concentration during late gestation showed a lower TNF- α plasma concentration after stimulation with either LPS concentrations (Tables 6 and 8). The responses in TNF- α concentration observed were proportional to the concentration of LPS used for the stimulation. When the TNF- α response following LPS-stimulation was quantified as the percentage increased from the basal TNF- α concentration, calves born to cows that suffered higher OS

or inflammation showed a lower fold increase in TNF- α after LPS stimulation (Figure 2).

DISCUSSION

We estimated the in utero exposure to metabolic stress during the last month of gestation because this is the time when periparturient immunosuppression starts (Goff and Horst, 1997; Nonnecke et al., 2003), concurrently with the fastest proliferation of immune cells in the bovine fetuses (Higgins et al., 1983). For this, we measured biomarkers of the 3 components of the metabolic stress triad (Abuelo et al., 2015) in the dam's serum and classified their offspring according to the degrees of maternal lipid mobilization, inflammation, and OS experienced. The lack of correlation among the maternal biomarkers (Table 2) demonstrated the independency of each biomarker.

Calves exposed to high maternal lipid mobilization during late gestation had a significantly higher serum OSi and Hp concentrations. To our knowledge, the effect of maternal NEFA concentration during late ges-

Table 3. Least squares means of the offspring sera biomarkers of oxidant status, lipomobilization, and inflammation according to the degree of maternal stress experienced in late gestation¹

Variable	Maternal NEFA		Maternal Hp		Maternal OSi		SE	P-value						
	Low (n = 6)	High (n = 6)	Low (n = 6)	High (n = 6)	Low (n = 6)	High (n = 6)		Maternal groups			Group $\times$ time			
								NEFA	Hp	OSi	Time	NEFA	Hp	OSi
NEFA (mmol/L)	0.19	0.16	0.18	0.17	0.15	0.19	0.026	0.35	0.90	0.35	0.12	0.42	0.47	0.76
Hp (g/L)	0.11	0.17*	0.13	0.15	0.11	0.20*	0.014	0.036	0.29	0.013	0.73	0.76	0.91	0.11
RONS (RFU/ μ L)	22.3	16.8	18.7	20.4	15.0	24.1*	2.20	0.19	0.68	0.041	0.24	0.67	0.67	0.22
AOP (μ M)	44.8	38.3	45.8	37.3	40.0	43.2	5.09	0.41	0.29	0.68	0.14	0.09	0.48	0.75
OSi	0.24	0.78*	0.43	0.59	0.42	0.61	0.091	0.026	0.27	0.062	0.044	0.08	0.39	0.054

¹NEFA = nonesterified fatty acids; Hp = haptoglobin; RONS = reactive oxygen and nitrogen species; RFU = relative fluorescence units; AOP = antioxidant potential; OSi = oxidant status index.

* $P < 0.05$ compared with the low group.

Table 4. Least squares means of the offspring birth weight and ADG during the first 4 wk of life according to the degree of maternal stress experienced in late gestation¹

Variable							SE	P-value		
	Maternal NEFA		Maternal Hp		Maternal OSi			Maternal groups		
	Low	High	Low	High	Low	High		NEFA	Hp	OSi
	(n = 6)	(n = 6)	(n = 6)	(n = 6)	(n = 6)	(n = 6)				
Birth weight (kg)	40.6	38.7*	40.1	39.2	40.5	39.0*	0.52	0.040	0.24	0.045
ADG (kg/d)	0.61	0.58	0.59	0.57	0.60	0.56	0.018	0.24	0.75	0.12

¹Generalized linear mixed models were built for the variables birth weight and ADG with the fixed effects of the calf groups and the random effect of calf sex. NEFA = nonesterified fatty acids; Hp = haptoglobin; OSi = oxidant status index.

* $P < 0.05$ compared with the low group.

tation on the oxidant and inflammatory status of the offspring in postnatal life has not yet been investigated. The mechanism by which prenatal exposure to high maternal NEFA concentrations in late gestation can be associated with an elevated OSi is unknown. However, in vitro studies demonstrate that fatty acids may induce cellular RONS production directly (Inoguchi et al., 2000; Listenberger et al., 2001) and indirectly by stimulating inflammatory pathways (Ohtsu et al., 2017). Furthermore, in vitro studies in bovine oocytes and blastocysts demonstrate that elevated NEFA concentrations alter gene expression related to oxidative metabolism (Van Hoeck et al., 2013, 2015). Osorio et

al. (2013) did not find changes in the expression of some genes related to redox and inflammation homeostasis in PMNL of calves from dams fed prepartum diets differing in energy content. However, it is yet to be determined whether prenatal exposure to higher maternal NEFA concentrations during late gestation influences the expression of such genes. Further research is required to determine whether offspring exposed to high maternal NEFA concentrations during late gestation are at increased risk of diseases associated with oxidative stress and inflammation, such as diarrhea, pneumonia, and mastitis (Ranjan et al., 2006; Lykkesfeldt and Svendsen, 2007), later in life.

Figure 1. Body weight of the calves throughout the first 4 wk of age according to the degree of maternal oxidant status, inflammation, or lipid mobilization during late gestation. Results expressed as LSM $\pm$ SE. OSi = oxidant status index; Hp = haptoglobin; NEFA = nonesterified fatty acids. * $P < 0.05$.

Maternal OS during late gestation was positively associated with the serum concentration of RONS in the offspring. However, it was not associated with differences in their serum AOP or OSi. Antioxidants are produced endogenously and supplied by the diet (Abuelo et al., 2015). Thus, it is possible that the supply of diet-derived antioxidants was sufficient to offset the higher serum RONS concentration in calves exposed to higher maternal OS during late gestation. As the OS is determined by the ratio of oxidants to antioxi-

dants, the clinical significance of an elevated level of RONS in the absence of an increased OSi is unknown. Given the small sample size of the current study, these results cannot rule out a positive relationship between maternal OS during gestation and the offspring's OSi in postnatal life. Nonetheless, the elevated OS observed in calves may have contributed to their diminished immune responsiveness and elevated level of basal inflammation, as oxidative stress may lead to immune and inflammatory dysfunction (Sordillo and Mavangira,

Table 5. Least squares means of the calves' white blood cell differential counts (10^9 cells/L) according to week of age and group¹

Item	Leukocytes	Neutrophils	Monocytes	Lymphocytes	Eosinophils	Basophils
Wk 1						
NEFA						
Low	9.6	5.2	0.80	3.7	0.056	0.019
High	7.4	6.3	0.56	3.3	0.044	0.021
Hp						
Low	7.6	6.1	0.48	3.3	0.041	0.012
High	8.3	5.5	0.58	3.6	0.032	0.013
OSi						
Low	7.9	4.1	0.69	3.1	0.047	0.036
High	7.4	5.4	0.58	3.8	0.059	0.031
Wk 2						
NEFA						
Low	7.5	3.4	0.49	3.6	0.020	0.026
High	7.9	3.4	0.56	3.8	0.029	0.031
Hp						
Low	7.3	3.6	0.45	3.2	0.019	0.027
High	8.0	3.3	0.60	4.1	0.030	0.032
OSi						
Low	7.4	3.1	0.64	3.8	0.019	0.027
High	7.9	3.7	0.60	3.6	0.030	0.022
Wk 3						
NEFA						
Low	8.7	3.1	0.57	3.14	0.024	0.036
High	7.4	2.9	0.81	3.68	0.031	0.041
Hp						
Low	8.0	3.4	0.75	3.6	0.022	0.026
High	7.2	3.4	0.67	4.1	0.018	0.022
OSi						
Low	6.8	2.6	0.93	3.2	0.021	0.019
High	7.3	3.1	0.84	3.3	0.018	0.022
Wk 4						
NEFA						
Low	7.3	3.0	0.76	3.86	0.034	0.039
High	8.1	4.2	0.90	4.2	0.038	0.038
Hp						
Low	8.9	4.3	0.72	3.8	0.052	0.031
High	9.3	3.9	0.88	3.9	0.048	0.045
OSi						
Low	9.2	4.5	1.1	3.8	0.053	0.031
High	8.7	4.0	1.0	4.3	0.049	0.029
SE	2.50	1.08	0.321	1.50	0.0034	0.0020
P-value						
Group effect						
NEFA	0.65	0.40	0.46	0.38	0.35	0.86
Hp	0.76	0.18	0.10	0.33	0.29	0.86
OSi	0.98	0.22	0.58	0.69	0.35	0.16
Time	0.40	0.04	0.13	0.90	0.38	0.43
Group × time						
NEFA	0.11	0.65	0.49	0.11	0.09	0.34
Hp	0.89	0.72	0.45	0.94	0.32	0.22
OSi	0.45	0.34	0.69	0.31	0.09	0.84

¹NEFA = nonesterified fatty acids; Hp = haptoglobin; OSi = oxidant status index. Each group (low or high) was composed of 6 calves.

Table 6. Least squares means of offspring tumor necrosis factor (TNF)- α plasma concentration (ng/mL) after LPS stimulation for the first 4 wk of life according to the degree of maternal NEFA, Hp, and OSi during late gestation¹

LPS stimulation	P-value													
	Maternal NEFA		Maternal Hp		Maternal OSi		SE	Maternal groups				Group × time		
	Low (n = 6)	High (n = 6)	Low (n = 6)	High (n = 6)	Low (n = 6)	High (n = 6)		NEFA	Hp	OSi	Time	NEFA	Hp	OSi
Basal	2.33	2.01	1.47	1.88	1.10	2.23*	0.353	0.078	0.46	0.037	0.86	0.56	0.46	0.60
10 ng/mL of LPS	2.89	2.36	2.93	2.32*	1.73	3.02*	0.292	0.10	0.036	0.017	0.71	0.099	0.25	0.47
5 µg/mL of LPS	4.98	6.55	6.28	4.02*	3.94	6.40*	0.695	0.084	0.013	0.010	0.18	0.68	0.39	0.55

¹NEFA = nonesterified fatty acids; Hp = haptoglobin; OSi = oxidant status index.

**P* < 0.05 compared with the low group.

2014). Further research is required to determine the clinical effect of OS during the first months of life. This is of particular interest because oxidative stress is involved in the pathogenesis of prevalent diseases that affect dairy calves, such as diarrhea and pneumonia (Svensson et al., 2003; Ranjan et al., 2006; Lykkesfeldt and Svendsen, 2007). Furthermore, previous studies have shown that preweaning neonatal calves experience higher OS than periparturient dairy cows (Abuelo et al., 2014). Hence, increasing our understanding of the implications of OS in neonatal health could lead to management practices that result in reduced morbidity and mortality rates in neonates via nutritional manipulation.

In the current study, calves exposed to high maternal OS during late gestation had significantly higher serum Hp and basal TNF- α concentration. These data suggest that prenatal exposure to elevated maternal OS is associated with an increased level of inflammation. Similar results were also reported in a recent human study where maternal lipid peroxides and malondialdehyde (a product of lipid oxidation) were positively associated with the expression of proinflammatory cytokines in the offspring (Hernández-Trejo et al., 2017). As oxidants are intrinsically involved in the activation of several inflammatory pathways (Mittal et al., 2014), the significantly higher plasma concentration of RONS in calves exposed to high maternal OS may contribute to their elevated level of basal inflammation. For example, RONS activates nuclear factor kappa B, which enhances inflammation by increasing the expression of proinflammatory cytokines (Blaser et al., 2016) and adhesion molecules (Mittal et al., 2014). Furthermore, RONS also activate mitogen-activated protein kinases pathways, which play an essential role in intracellular signaling pathways that are involved in the regulation of cellular activities during inflammation (Son et al., 2011). In vitro studies performed in bovine endothelial cells also demonstrate that OS-induced peroxidation is

associated with an increased inflammatory phenotype in these cells (Weaver et al., 2001; Sordillo et al., 2008).

The clinical significance of elevated TNF- α and Hp concentrations during the first month of life is unknown. Further research is warranted to determine whether an elevated basal inflammatory status during this period is associated with adverse health and production outcomes. Murray et al. (2014) reported that serum Hp concentrations in dairy calves during the first week of life are positively associated with morbidity and mortality during the first 4 mo of life. Furthermore, elevated TNF- α concentrations contribute to impaired metabolic function in dairy cattle (McCarthy et al., 2016) and may adversely affect growth rates by inhibition of IGF-1 (Suda et al., 2003; Borghetti et al., 2009). The relationship between the amount of basal inflammation during the neonatal period and the development of dysregulated inflammatory responses later in life is also unknown. This is of particular interest because dysregulated inflammation in dairy cattle is highly implicated in the pathogenesis of metabolic stress during the transition period and inflammatory-based diseases, such as mastitis and endometritis (LeBlanc, 2014; Sordillo and Mavangira, 2014).

Differences in the cell-mediated innate immune response of the offspring associated with the late-gestation maternal metabolic stress biomarkers were assessed by LPS stimulation of whole blood and subsequent cytokine analysis as described previously in experiments involving adult dairy cows (Røntved et al., 2005; O'Boyle et al., 2006). Compared with stimulation of peripheral blood mononuclear cells, measuring cytokines in whole blood has the advantage of preserving the physiological proportions of all natural components of the blood (Damsgaard et al., 2009). The main limitation, however, refers to the potential difference in the release of TNF- α in animals due to differences in the number of cells present in the peripheral blood, where mononuclear cells account for the bulk of TNF- α pro-

Table 7. Results of the LPS stimulation test based on the degree of maternal oxidant status, presented by week of life [shown as tumor necrosis factor- α plasma concentration (LSM $\pm$ SE; ng/mL) after the stimulation with different concentrations of LPS]¹

LPS stimulation	Wk 1		Wk 2		Wk 3		Wk 4	
	Low OSi (n = 6)	High OSi (n = 6)	Low OSi (n = 6)	High OSi (n = 6)	Low OSi (n = 6)	High OSi (n = 6)	Low OSi (n = 6)	High OSi (n = 6)
Basal	1.01 $\pm$ 0.237	2.48 $\pm$ 1.300*	0.84 $\pm$ 0.089	1.96 $\pm$ 0.331*	0.97 $\pm$ 0.097	2.22 $\pm$ 0.377*	1.05 $\pm$ 0.076	2.08 $\pm$ 0.391*
10 ng/mL of LPS	1.82 $\pm$ 0.272	3.49 $\pm$ 0.447*	1.72 $\pm$ 0.041	2.66 $\pm$ 0.152*	1.49 $\pm$ 0.150	3.57 $\pm$ 0.793*	1.82 $\pm$ 0.107	2.89 $\pm$ 0.298*
5 μ g/mL of LPS	3.31 $\pm$ 0.414	5.52 $\pm$ 0.544*	3.06 $\pm$ 0.321	5.57 $\pm$ 0.695*	4.00 $\pm$ 0.520	7.99 $\pm$ 0.710*	4.83 $\pm$ 0.573	8.65 $\pm$ 0.887*

¹LPS = *Escherichia coli* lipopolysaccharide; OSi = oxidant status index; Low OSi = low maternal OSi; High = high maternal OSi.

**P* < 0.05 compared with the low group within week.

duction. Nevertheless, we found no differences among groups in either total or differential cell counts (Table 4), and all the samples were within the reported reference intervals. Hence, we were able to rule out differences in TNF- α production due to different numbers of mononuclear cells in blood.

Successful immune activation depends upon a rapid increase in proinflammatory cytokine production (Watkins et al., 1995), such as TNF- α . Likewise, the capacity to recover homeostasis following an acute inflammatory insult relies on a quick cytokine storm that is resolved promptly (Bradford et al., 2015). Hence, we expressed the release of TNF- α following LPS stimulation as the percentage increase from the basal concentration to reflect the signaling capacity of the immune cells against a microbial agonist. Prenatal exposure to higher maternal inflammation (assessed by serum Hp concentration) during late gestation was associated with a reduced LPS-stimulated TNF- α release during the first month of life (Table 7, Figure 2B). Studies in murine models also demonstrate that in utero programming of neonatal cell-mediated immunity is sensitive to maternal inflammation. For example, rats exposed to a proinflammatory stimulus during late gestation produced offspring with a significantly reduced TNF- α response to LPS stimulation (Hodyl et al., 2007, 2008; Beloosesky et al., 2010). Although it was beyond the scope of our study to investigate the clinical significance of these results, prenatal exposure to high maternal inflammation has been associated with increased risk of infectious disease in human neonates. For example, Morales et al. (2011) reported in humans that exposure to high maternal inflammation during gestation is positively associated with the development of wheeze and recurrent respiratory tract infections during the first 14 mo of life. Therefore, it is likely that metabolic stress in dairy cows during late gestation may also have a negative effect on the resistance of their offspring to infectious diseases.

Calves born to cows that experienced a higher degree of OS showed a higher TNF- α concentration following LPS stimulation but a significantly lower increase from the basal concentration, which could suggest a reduced signaling capacity in these animals associated with their higher proinflammatory status. This finding is compatible with research in periparturient dairy cattle, where sustained inflammation can result in an anti-inflammatory response to prevent a cytokine storm and maintain homeostasis (Bradford et al., 2015). It is unknown, however, whether the diminished fold increase in TNF- α following LPS stimulation in calves born to high OS cows is a direct carryover effect of OS exposure during gestation due to the higher basal TNF- α concentration observed concurrently or to other physiologi-

Table 8. Results of the LPS stimulation test based on the degree of maternal inflammatory status, presented by week of life [shown as the tumor necrosis factor- α plasma concentration (LSM $\pm$ SE; ng/mL) after the stimulation with different concentrations of LPS]¹

LPS stimulation	Wk 1		Wk 2		Wk 3		Wk 4	
	Low Hp (n = 6)	High Hp (n = 6)	Low Hp (n = 6)	High Hp (n = 6)	Low Hp (n = 6)	High Hp (n = 6)	Low Hp (n = 6)	High Hp (n = 6)
Basal	2.70 $\pm$ 0.505	1.39 $\pm$ 0.146*	1.60 $\pm$ 0.281	1.57 $\pm$ 0.460	2.71 $\pm$ 0.277	1.40 $\pm$ 0.228*	2.20 $\pm$ 0.320	1.27 $\pm$ 0.218*
10 ng/mL of LPS	3.75 $\pm$ 0.317	1.55 $\pm$ 0.222*	2.39 $\pm$ 0.245	1.68 $\pm$ 0.032*	3.34 $\pm$ 0.694	1.44 $\pm$ 0.121*	2.76 $\pm$ 0.174	1.788 $\pm$ 0.097*
5 μ g/mL of LPS	4.87 $\pm$ 0.564	3.13 $\pm$ 0.475*	4.01 $\pm$ 0.727	4.02 $\pm$ 0.670	5.90 $\pm$ 0.948	5.90 $\pm$ 1.310	8.60 $\pm$ 1.010	4.93 $\pm$ 0.592*

¹LPS = *Escherichia coli* lipopolysaccharide; Hp = haptoglobin; Low Hp = low maternal Hp; High Hp = high maternal Hp.

* $P < 0.05$ compared with the low group within week.

cal mechanism not controlled for in our observational study. In periparturient dairy cows, it has been established that animals with a sustained proinflammatory status show a reduced capacity to mount an effective immune response (Sordillo and Raphael, 2013). Calves exposed to higher maternal OS also showed a significantly higher plasma concentration of RONS, which may have contributed further to the reduced TNF- α release observed in these calves. Increases in the concentration of RONS may cause immunosuppression as overproduction of free radicals compromise leukocyte function (Sordillo and Aitken, 2009).

The results of our study demonstrate that prenatal exposure to higher maternal inflammation and OS are associated with a reduced fold increase in TNF- α concentration after an ex vivo LPS stimulation. However, it is unknown whether the resulting differences in cell-mediated innate immune response in these calves is substantial enough to increase their risk of infectious disease. Cell-mediated immunity plays an essential role in the elimination of intracellular pathogens (Zhu and Paul, 2008), which are highly implicated in the etiology of diarrhea and respiratory disease (Ames, 1997; Svensson et al., 2003; Izzo et al., 2011). Furthermore, morbidity during the first 3 mo of life is associated with adverse health and production later in life (Waltner-Toews et al., 1986; Warnick et al., 1997). Therefore, further research is warranted to determine whether prenatal exposure to high maternal inflammation and OS influences the offspring's disease susceptibility and future productivity.

Prenatal exposure to a higher degree of lipid mobilization and OS was associated with lower BW at birth and throughout the 4-wk study period. However, no association was identified between maternal metabolic stress and ADG at 4 wk of age. These findings are in line with previous research investigating the effect of in utero heat stress exposure, where calves born to heat-stressed cows showed compromised immune responses

from birth to weaning and decreased weights at birth and weaning but similar weaning ADG as calves born to cooled calves (Tao et al., 2012). Tao et al. (2012) also observed no differences in weight or height between heat-stressed (n = 9) and cooled (n = 12) groups from 3 to 7 mo of age, thereby indicating an effect of maternal status of fetal growth that is compensated after weaning. Hence, it will be interesting to explore in a larger study setting the long-term implications of maternal metabolic stress during late gestation in terms of immune function, susceptibility to diseases, and growth rates.

We established for the first time that the degree of metabolic stress in dairy cows during late gestation is associated with differences in the immune and metabolic responses of their offspring in postnatal life. The LPS-stimulated TNF- α release is commonly used to assess cell-mediated innate immunity, as TNF- α is produced primarily by activated macrophages. This cytokine, however, can also be secreted by other cell types, such as lymphocytes, natural killer cells, or neutrophils, among others (Parkin and Cohen, 2001); hence, an implication of adaptive immunity in the results cannot be ruled out. Consequently, the findings of our study can only conclude that maternal metabolic stress during late gestation influences some aspects of the cell-mediated immunity of the offspring in postnatal life, but not which specific immune cells populations are affected. Similarly, due to the pilot nature of our study and the reduced sample size employed, other effects not identified here cannot be ruled out. Further studies with adequate sample sizes are required to quantify the effect of the differences observed in the calves' metabolic and immune responses in terms of susceptibility to diseases and growth performance. Is also important to note that the feeding regimen of the calves in our study, still more commonly used on Australian dairy farms (Dairy Australia, 2017), allows for significantly lower growth rates than those more intense regimens typically found

Figure 2. Tumor necrosis factor (TNF)-α response of calf blood cells to an ex vivo LPS stimulation test according to the degree of maternal (A) oxidant status or (B) inflammation during late gestation. Results expressed in percentage increase from basal concentration (LSM ± SE). OSI = oxidant status index; Hp = haptoglobin. * $P < 0.05$, ** $P < 0.01$.

in North America. Hence, further studies are needed to measure the effect of maternal metabolic stress on calves under these intense rearing systems.

CONCLUSIONS

The results of this study demonstrate that metabolic stress in dairy cows during late gestation was associated with differences in the immune and metabolic responses of the offspring during the first mo of life. A higher degree of exposure to the 3 components of the metabolic stress triad in utero was associated with conditions in calves (e.g., sustained proinflammatory status, reduced TNF- α release, increased oxidant status) linked to impaired health and production in adult cows. Hence, reducing metabolic stress during the dry period could potentially result in enhanced dairy cow health and productivity and also benefit calf health. Further research is required to determine the mechanisms by which metabolic stress during late gestation affects the immune and metabolic responses of the offspring and the clinical effect of these carryover effects on the health and growth of the offspring. This will allow the development of management practices that ultimately enhance the health and production of replacement heifers.

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