



Maternal consumption of organic trace minerals alters calf systemic and neutrophil mRNA and microRNA indicators of inflammation and oxidative stress

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ABSTRACT

Organic trace mineral (ORG) supplementation to dairy cows in substitution of sulfate (INO) sources has been associated with improvement in immune function during stressful states such as the periparturient period. However, the effect of supplemental ORG during pregnancy on the neonatal calf is unknown. Therefore, our aim was to investigate the effects of ORG supplementation during late pregnancy on the immune system and growth of the neonatal calf. Of specific interest was the evaluation of inflammation-related microRNA (miRNA) and target gene expression in blood neutrophils as indicators of possible nutritional programming. Forty multiparous cows were supplemented for 30 d prepartum with 40 mg/kg of Zn, 20 mg/kg of Mn, 5 mg/kg of Cu, and 1 mg/kg of Co from either organic (ORG) or sulfate (INO) sources (total diet contained supplemental 75 mg/kg of Zn, 65 mg/kg of Mn, 11 mg/kg of Cu, and 1 mg/kg of Co, and additional Zn, Mn, and Co provided by sulfates), and a subset of calves ($n = 8/\text{treatment}$) was used for blood immunometabolic marker and polymorphonuclear leukocyte (PMNL) gene and miRNA expression analyses. Samples were collected at birth (before colostrum feeding), 1 d (24 h after colostrum intake), and 7 and 21 d of age. Data were analyzed as a factorial design with the PROC MIXED procedure of SAS. No differences were detected in BW, but maternal ORG tended to increase calf withers height. Calves from INO-fed cows had greater concentrations of blood glucose, GOT, paraoxonase, myeloperoxidase, and reactive oxygen metabolites. Antioxidant capacity also was greater in INO calves. The

PMNL expression of toll-like receptor pathway genes indicated a pro-inflammatory state in INO calves, with greater expression of the inflammatory mediators *MYD88*, *IRAK1*, *TRAF6*, *NFKB*, and *NFKBIA*. The lower expression of miR-155 and miR-125b in ORG calves indicated the potential for maternal organic trace minerals in regulating the PMNL inflammatory response at least via alterations in mRNA and miRNA expression. Overall, these results indicate that maternal nutrition with organic trace minerals could alter the neonatal innate immune response at least in part via changes in gene and miRNA expression. Further studies involving inflammatory challenges during the neonatal period should be performed to determine the functional benefit of maternal organic trace minerals on the neonatal immune response.

Key words: epigenetics, fetal programming, nutrition, transcriptomics

INTRODUCTION

Trace mineral elements such as Cu, Cr, and Zn have important roles in the health and immunity of periparturient dairy cows (Spears and Weiss, 2008). Minerals have been commonly supplemented to cattle in the form of inorganic salts, preferably as sulfates; however, the development of organic forms of trace minerals, such as minerals complexed with AA, minimize the risk of mineral antagonism and enhance absorption efficiency (Swecker, 2014). Compared with a sulfate source, supplementing lactating cows with organic Zn resulted in greater immune response as well as improved milk yield (Wang et al., 2013).

The implications of trace mineral deficiency or impaired placental transfer of these minerals to fetal and neonatal ruminant metabolism have been studied for more than 30 yr (Hidiroglou, 1980). For instance, dairy calves supplemented with an injectable trace mineral

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complex containing Se, Cu, Zn, and Mn experienced an increase in neutrophil (PMNL) and glutathione peroxidase activity, and a reduction in the incidence of diarrhea, pneumonia, and otitis (Teixeira et al., 2014). These constitute an example of the innate immune response of the animal, one in which cells such as PMNL are partly regulated via signaling pathways and changes in mRNA expression. An important inflammation-responsive pathway in the animal that connects the innate and adaptive immune response is the toll-like receptor (TLR). Its activation, and that of its target genes, leads to stimulation of several intermediate molecules and culminates with the synthesis of classical pro-inflammatory cytokines and chemokines (Wolowczuk et al., 2008; Chen et al., 2015).

Although changes in mRNA expression are known to partly control adaptations in PMNL due to inflammation, more recent studies have concluded that epigenetic modifications through microRNA (miRNA) are an important part of the regulation of several cellular processes (Aguilera et al., 2010) that modulate PMNL function including regulation of senescence, differentiation, adherence capacity, and cytokine production (Gantier, 2013). These observations are of interest in the context of dairy cow nutrition because epigenetic markers are candidates for bearing the memory of specific intrauterine nutritional exposure causing alterations in long-term gene expression, and consequently inducing developmental adaptations in physiology and metabolism (Attig et al., 2010).

Mature miRNA are non-protein-coding small RNA (~20 nucleotides length) that repress translational activity, promote destabilization of target mRNA, and regulate the abundance of mRNA target genes; however, these mechanisms remain under debate (Eulalio et al., 2008). It was reported in vitro using mouse P19 embryonal carcinoma cells that miR125-b can regulate mammalian neuronal differentiation by downregulating both translational efficiency and mRNA abundance of *lin-28* (Wu and Belasco, 2005). Using a microarray approach with miR-transfected HeLa cells (miR-1 and miR-124), it was demonstrated that miRNA could reduce the levels of many of their target transcripts, not just the amount of protein derived from these transcripts (Lim et al., 2005).

The general hypothesis of the present study was that maternal supplementation with organic trace minerals would improve neonatal calf metabolism and immune function reflected in the profiles of systemic blood biomarkers, mRNA, and microRNA in PMNL, and measures of growth and performance from birth through weaning. The possible epigenetic regulation through the action of miRNA in the pro-inflammatory signaling pathway also was evaluated.

MATERIALS AND METHODS

All the procedures for this study were conducted in accordance with the protocol approved by the Institutional Animal Care and Use Committee of the University of Illinois (protocol #12097).

Maternal Treatments

The experiment was conducted as a randomized complete blocked design with 40 multiparous Holstein cows blocked according to parity, previous lactation milk yield, and expected day of parturition. All cows received a common lactation diet (1.76 Mcal/kg of DM and 16.7% CP) during the last 60 d of lactation before dry-off, and a common early-dry period diet (1.1 Mcal/kg of DM, 14.5% CP) from -50 to -30 d relative to parturition. Both diets were supplemented at 100% of NRC (2001) requirements with Zn, Mn, Cu, and Co in the form of an inorganic trace mineral mix. All cows received the same diet (1.5 Mcal/kg of DM, 15% CP) from -30 d to parturition (close-up period).

The close-up diet was partially supplemented with an inorganic trace mineral mix of Zn, Mn, and Cu to supply 35, 45, and 6 mg/kg, respectively, of the total dietary minerals. Cows were randomly assigned to an oral administration of a bolus once daily at the time of feeding the TMR. This contained a mix of either inorganic (INO, n = 20) or organic (AvailaZn Zn AA complex, AvailaMn Mn AA complex, AvailaCu Cu AA complex, and CoPro cobalt glucoheptonate; Zinpro Corporation, Eden Prairie, MN; ORG, n = 20) Zn, Mn, Cu, and Co to achieve 75, 65, 11, and 1 mg/kg, respectively, in diet DMI. After birth, calves were fed a common diet and managed similarly. Hence, any observed treatment effects are attributed to maternal nutrition during the last 30 d of gestation.

Animal Management and Calf Enrollment Criteria

During the dry period, cows were housed in a ventilated, sand-bedded free-stall barn, with a photoperiod of 8 h of light and 16 h of dark. Diets were fed for ad libitum intake as a TMR once daily, between 0600 and 0800 h, using an individual gate feeding system (American Calan, Northwood, NH). As cows began demonstrating signs of impending parturition, they were moved to an individual maternity pen bedded with straw. After parturition, cows were moved within 2 h to an individual chute and then milked with a porta-milker vacuum pump (catalog no. Z15664N, Nasco, Fort Atkinson, WI). Colostrum volume was recorded and IgG content was estimated based on specific gravity with a bovine colostrometer (catalog no. C10978N, Nasco).

A subset of calves was selected ($n = 8/\text{group}$) if they and their dams fulfilled all the following criteria: (1) single calf, (2) calving difficulty score <3 , (3) colostrum quality assessed by a bovine colostrometer of >60 mg/L of IgG, (4) dam produced at least 3.8 L of a good quality first colostrum, and (5) heifer calf birth weight >36 kg (Johnson et al., 2007). On d 7 and 21, calves were bled ~ 3 h after the morning feeding. Although a power test was not performed, based on previous work (e.g., papers cited in Hammon et al., 2012; Osorio et al., 2012), a total of 8 calves per group were deemed adequate.

After birth, calves were weighed, had the navel disinfected with a 7% tincture of iodine solution (First Priority Inc., Elgin, IL), vaccinated with TSV II (Pfizer Inc., New York, NY) via nostril application, and received 3.8 L of first milking colostrum from the respective dam within 2 h after birth. Animals did not receive supplemental vitamin A or D, Fe, or Se. Calves were offered first milking colostrum again on the second feeding at 4 h after birth if colostrum intake had not reached the 3.8 L required. Calves were housed in individual outdoor hutches bedded with straw, fed twice daily with a milk replacer (Advance Excelerate, Milk Specialties, Carpentersville, IL; 28.5% CP, 15% fat; from 1 to 10 d of age: 520 g/d; 11 to 20 d of age: 680 g/d; 21 to 35 d of age: 840 g/d; and 36 to 42 d of age: 420 g/d in a single feeding) and had ad libitum access to a starter grain mix (19.9% CP, 13.5% NDF). Intake was recorded daily. Health checks including fecal score were recorded daily until weaning [scale 1–4, 1: firm, well formed (not hard); 2: soft, pudding-like; 3: runny, pancake batter; 4: liquid, splatters; Osorio et al., 2012], and rectal temperature was recorded daily until 21 d of age. Growth performance including BW and withers height (**WH**) were recorded weekly. Calves were weaned at 42 d of age.

Sample Collection

Calf blood samples were collected from a jugular vein using 20-gauge BD Vacutainer needles (Becton Dickinson, Franklin Lakes, NJ) before receiving colostrum (baseline), 24 h after receiving colostrum, and 7 and 21 d after birth. At each time point, a total of 120 mL of total blood were collected in vacutainer tubes (10 mL, BD Vacutainer, Becton Dickinson) containing serum clot activator (10 mL), sodium heparin (10 mL), or solution A of trisodium citrate, citric acid, and dextrose (**ACD**; 100 mL). After blood collection, tubes with ACD and sodium heparin were placed on ice and tubes with clot activator were kept at room temperature until centrifugation (~ 30 min). Serum and plasma were obtained by centrifugation of clot activa-

tor and sodium heparin tubes, respectively, at $1,900 \times g$ for 15 min at 4°C . Serum and plasma were aliquoted and stored at -80°C until further analysis according to the manufacturer's specifications for determination of blood metabolites, oxidative stress biomarkers, and acute-phase proteins (**APP**). The RNA from PMNL was harvested from 100 mL of blood collected in ACD vacutainer tubes. It is estimated that calves in the present study lost 240 mL of blood during the first 2 samplings, which is substantially below the limit they could tolerate (i.e., 10–15 mL/kg of BW; Rosenberg, 1979). Furthermore, no visual or clinical signs of health problems were observed after collection of the blood, and the biomarkers analyzed did not indicate any alteration in blood homeostasis. All calves had a normal growth rate, as expected.

Blood Metabolites, APP, and Oxidative Stress Biomarkers

Blood samples were analyzed within 3 mo of collection for several biomarkers of energy metabolism and liver function, inflammation, oxidative stress and antioxidant capacity, and minerals. These included albumin (catalog no. 0018250040), cholesterol (catalog no. 0018250540), bilirubin (catalog no. 0018254640), creatinine (catalog no. 0018255540), urea (catalog no. 0018255440), glutamic-oxaloacetic transaminase (**GOT**; catalog no. 0018257540), γ -glutamyltransferase (**GGT**; catalog no. 0018257640), and glucose (catalog no. 0018250840) using the IL Test purchased from Instrumentation Laboratory Spa (Werfen Co., Milan, Italy) in the ILAB 600 clinical auto-analyzer (Instrumentation Laboratory, Lexington, MA). Haptoglobin was analyzed using the method described in Skinner et al. (1991), whereas ceruloplasmin was determined following a minor modification of the method proposed by Sunderman and Nomoto (1970), where the acetate buffer was changed to 0.8 M, pH 6.4, and contained 0.31% Na-EDTA. Reactive oxygen metabolites (**ROM**) were analyzed with the d-ROMs-test (cod. MC002), purchased from Diacron (Grosseto, Italy). Antioxidant potential was assessed as ferric reducing antioxidant power (**FRAP**) using the colorimetric method of Benzie and Strain (1996). Nitric oxide (**NOx**) and constituents [nitrite (NO_2^-) and nitrate (NO_3^-)] and paraoxonase (**PON**) were determined according methods previously described by Trevisi et al. (2013). Myeloperoxidase (**MPO**) was determined as previously described (Bionaz et al., 2007), via colorimetry based on the reaction of MPO contained in the plasma sample with hydrogen peroxide, which forms H_2O and O^- ; the O^- dianisidine dihydrochloride, and electron donor, reacts with the O^- , releasing H_2O and a colored compound. Nonesteri-

fied fatty acids and BHBA were measured using kits from Wako Chemicals and Randox Laboratories Ltd., respectively, following the procedures described previously (Bionaz et al., 2007; Trevisi et al., 2012; Osorio et al., 2013). Serum amyloid A (SAA) concentration was assessed with a commercial ELISA immunoassay kit (Tridelta Development Ltd., Maynooth, Co. Kildare, Ireland). Total antioxidants were assessed through the oxygen radical absorbance capacity (ORAC) assay. This method measures a fluorescent signal from a probe (fluorescein) that decreases in the presence of radical damage (Cao and Prior, 1999). Retinol and tocopherol were determined as previously described (Bionaz et al., 2007). Bovine IL-6 plasma concentration was determined by a colorimetric sandwich ELISA using a Bovine IL-6 Screening Set, using pretitered, matched pairs of coating and detection antibodies and a calibrated recombinant protein standard (#ESS0029 Endogen, Pierce, Rockford, IL).

Minerals were extracted adding 0.3 mL of trichloroacetic acid (10% vol/vol) to 0.3 mL of plasma, sample was mixed and centrifuged at $3,500 \times g$ for 10 min at 4°C. A 0.3 mL of supernatant was added to 2.7 mL of Millipore water and mixed. Copper, Fe, Mn, and Zn in final solution were determined by inductively coupled plasma optical emission spectrometry (ICP-OES 5100, Agilent Technologies, Victoria, Australia) fitted with cyclonic chamber in which samples were introduced with SeaSpray nebulizer (Agilent Technologies). Trichloroacetic acid solution at the same concentration of the samples was used as a blank and to prepare the calibration curve diluting external standard (Merck, Darmstadt, Germany) to 5, 10, 50, and 100 ppb. Instrumental detection limits were 0.35, 0.10, 0.06, 0.25 ppb for Cu, Fe, Mn, and Zn, respectively. Accuracy of results was verified using a mineralized solution of SRM 1577b (National Institute of Standards and Technology).

PMNL Isolation and Viability Analysis

Complete details of PMNL isolation and viability analysis are included in the Supplemental File (<http://dx.doi.org/10.3168/jds.2015-9359>). Briefly, neutrophils were isolated from whole blood (100 mL) collected in ACD-containing vacutainer tubes within 1 h of sample collection. An aliquot (20 μ L) obtained during the PMNL isolation process was used for PMNL quantification and viability using a granulocyte primary antibody (CH138A, Veterinary Microbiology and Pathology, Washington State University, Pullman, WA) followed by a second antibody (Goat Anti-Mouse IgM, Human ads-PE, Southern Biotech, Birmingham, AL). Cells were fixed with 150 μ L of 4% paraformaldehyde

(Sigma-Aldrich, St. Louis, MO) and preserved at 4°C until flow cytometry reading (LSR II, Becton Dickinson, San Jose, CA). This procedure was performed to ensure good quality samples. All samples harvested and used for analysis contained more than 80% neutrophils and had at least 90% viability.

mRNA and miRNA Isolation

For the PMNL extraction, the miRNeasy kit (Qiagen, Hilden, Germany) was used following the manufacturer's protocols, allowing the recovery of both mRNA and miRNA for measuring the expression of the desired targets (Table 1). Samples were treated on-column with DNaseI (Qiagen); quantification was accessed using the NanoDrop ND-1000 (NanoDrop Technologies, Rockland, DE), and RNA quality was measured using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). All samples had an RNA integrity number factor greater than 6. Information required by the minimum information for publication of quantitative real-time PCR experiments (Bustin et al., 2009) guidelines are provided in the Supplemental Materials (<http://dx.doi.org/10.3168/jds.2015-9359>).

Target Gene cDNA Synthesis and Quantitative PCR

All the details for cDNA synthesis, quantitative PCR, and primer design can be found in the supplementary material published by Osorio et al. (2013). For this study, *GOLGA5*, *SMUG1*, and *OSBPL2* were used as internal control genes, which have been previously used to normalize PMNL gene expression data (Moyes et al., 2010; Seo et al., 2013). The geometric mean of the internal control genes was used to normalize the expression data. The stability of the normalization factor was assessed with geNorm software (Vandesompele et al., 2002) with a favorable final pairwise variation of 0.20.

miRNA Procedures

For cDNA synthesis, we used the qScript miRNA cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD). Each reaction started with 500 ng of total RNA, mixed with 2 μ L of Poly (A) Tailing Buffer (5 \times), 1 μ L of Poly (A) Polymerase, and 7 μ L of RNase/DNase-free water. The mixture was incubated at 37°C for 20 min and then 70°C for 5 min. A second mix containing 9 μ L of miRNA cDNA Reaction Mix and 1 μ L of qScript Reverse Transcriptase was added and incubated at 42°C for 20 min and then 85°C for 5 min. A pool of cDNA samples was used to prepare the 6-point standard curve (dilution 1:4). Then cDNA samples were also diluted 1:4 with DNase/RNase-free water. A combination of 1

Table 1. Genes and microRNA selected for transcript profiling in bovine neutrophils

Gene name	HUGO gene symbol
Interleukin 1, β	<i>IL1B</i>
Interleukin-1 receptor-associated kinase 1	<i>IRAK1</i>
Myeloperoxidase	<i>MPO</i>
Myeloid differentiation primary response gene (88)	<i>MYD88</i>
Nuclear factor (erythroid-derived 2)-like 2	<i>NFE2L2</i>
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	<i>NFKB1</i>
Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, α	<i>NFKBIA</i>
Nitric oxide synthase 2, inducible	<i>NOS2</i>
Selectin L	<i>SELL</i>
Superoxide dismutase 1, soluble	<i>SOD1</i>
Toll-like receptor 2	<i>TLR2</i>
Toll-like receptor 4	<i>TLR4</i>
Tumor necrosis factor- α	<i>TNF</i>
TNF receptor-associated factor 6	<i>TRAF6</i>
Z-DNA binding protein 1	<i>ZBP1</i>
MicroRNA miR-125b	<i>MIR125b</i>
MicroRNA miR-146a	<i>MIR146a</i>
MicroRNA miR-155	<i>MIR155</i>
MicroRNA miR-223	<i>MIR223</i>
MicroRNA miR-9	

μ L of diluted cDNA with 9 μ L of the mix containing of 5.25 μ L of Perfecta SYBR Green Fast Mix (Quanta Biosciences), 0.45 μ L each of 10 μ M sequence-specific forward primer and Universal PCR Primer (Quanta Biosciences), and 3.85 μ L of DNase/RNase-free water were added to each well of a MicroAmp Optical 384-Well Reaction Plate (Applied Biosystems, Grand Island, NY). The reactions were performed in an ABI Prism 7900 HT SDS instrument (Applied Biosystems) under the following thermocycler conditions: 95°C for 2 min, and 40 cycles of 95°C for 5 s and 60°C for 30 s, followed by a dissociation curve step (95°C for 15 s, 60°C for 15 s, and 95°C for 15 s). The miR-let7a, miR-103 and miR-191 were used as internal controls, and their geometric mean was used to normalized the miRNA expression data.

All primers, PCR products, and PCR efficiencies are reported in the Supplementary Material, Tables S1 to S5 (<http://dx.doi.org/10.3168/jds.2015-9359>). The model of the TLR pathway under miRNA control is illustrated in the Supplementary Material.

Statistical Analysis

Data were analyzed with the PROC MIXED procedure of SAS 9.4 (SAS Institute Inc., Cary, NC). Fixed effects in the model were treatment, day, or week (for BW, WH, rectal temperature, fecal score, and starter intake), and their interaction [treatment (**T**) \times day (**D**), **T** \times week (**W**)]. Random effect was calf within diet. The exponential correlation covariance structure SP for repeated measures was used for analysis of blood metabolites and relative gene and miRNA expression.

Blood biomarkers and gene/miRNA expression results were \log_2 -scale transformed if needed to comply with normal distribution of residuals. Least squares means separation was performed using the PDIF statement. Statistical significance was declared at $P \leq 0.05$ and tendencies at $P \leq 0.10$. For ease of interpretation, the least squares means reported in Tables 2 and 3 and Supplementary Tables S7 and S8 (<http://dx.doi.org/10.3168/jds.2015-9359>) are the \log_2 back-transformed means that resulted from the statistical analysis.

RESULTS

Growth Performance and Health

Colostrum IgG was not affected ($P = 0.87$) by diet and averaged 96.7 ± 6.2 mg of IgG/mL. Similarly, apparent efficiency of absorption was not affected ($P = 0.93$) by maternal diet, averaging $35.9 \pm 3.1\%$.

Maternal supplementation with ORG did not affect ($P > 0.05$) BW at birth or throughout the experiment. However, regardless of treatment, a linear increase in BW ($P < 0.001$) occurred in both groups (Figure 1). In contrast, WH tended to be greater overall ($P = 0.063$) in ORG calves than INO. An overall increase ($P < 0.001$) across time was observed for WH (Figure 1).

No treatment effect ($P > 0.05$) was observed for starter intake despite the increase in consumption through time ($P < 0.001$) in both groups (Supplementary Table S6; <http://dx.doi.org/10.3168/jds.2015-9359>). Rectal temperature decreased ($P = 0.032$) from birth to 2 wk in both groups; however, it tended to be lower ($P = 0.069$) in ORG calves (Supplementary Table S6; <http://>

Table 2. Blood immunometabolic biomarkers in calves born to dams fed a conventional diet supplemented with only inorganic (INO) or inorganic plus organic (ORG) trace minerals during the last 30 d prepartum

Item ¹	Treatment	Day of age				SEM ²	P-value ³		
		0	1	7	21		T	D	T × D
Energy metabolism and liver function									
Glucose, mmol/L	ORG	2.06 ^{B,c}	8.03 ^a	6.12 ^b	5.58 ^b	0.37	0.001	<0.001	0.017
	INO	4.09 ^{A,c}	8.95 ^a	5.86 ^b	6.39 ^b	0.34			
NEFA, mmol/L	ORG	1.00	0.32	0.40	0.17	0.08	0.808	<0.001	0.781
	INO	1.10	0.30	0.39	0.16	0.07			
BHBA mmol/L	ORG	0.04	0.06	0.08	0.11	0.01	0.361	<0.001	0.112
	INO	0.04	0.06	0.08	0.15	0.01			
Creatinine (log2), µmol/L	ORG	7.63	6.78	6.64	6.42	0.08	0.445	<0.001	0.756
	INO	7.77	6.79	6.71	6.43	0.07			
Urea, mmol/L	ORG	4.53 ^a	3.25 ^b	3.21 ^b	3.96 ^{ab}	0.36	0.968	<0.001	0.039
	INO	3.79 ^b	3.67 ^b	2.85 ^c	4.58 ^a	0.34			
Bilirubin, µmol/L	ORG	4.03	4.28	3.16	2.20	0.20	0.170	<0.001	0.756
	INO	4.07	4.34	3.56	2.46	0.19			
GOT, U/L	ORG	5.40	6.72	5.38	5.77	0.13	0.015	<0.001	0.611
	INO	5.61	6.79	5.81	6.02	0.12			
GGT, U/L	ORG	3.87	11.22	8.74	6.88	0.29	0.422	<0.001	0.444
	INO	3.78	11.40	9.16	7.37	0.29			
Cholesterol, mmol/L	ORG	0.74	1.00	1.58	2.58	0.13	0.268	<0.001	0.345
	INO	0.79	1.02	1.69	3.00	0.12			
Albumin, g/L	ORG	29.7	25.9	29.2	31.5	0.6	0.113	<0.001	0.192
	INO	31.2	26.6	30.4	32.8	0.6			
Inflammation									
Ceruleplasmin, µmol/L	ORG	0.03	0.86	2.76	2.66	0.26	0.345	<0.001	0.468
	INO	0.29	1.24	2.91	2.60	0.22			
Haptoglobin, g/L	ORG	0.40	0.40	0.51	0.39	0.07	0.894	0.251	0.768
	INO	0.46	0.41	0.47	0.33	0.07			
Paraoxonase, U/mL	ORG	6.3 ^c	12.1 ^{bc}	17.2 ^b	42.5 ^{B,ab}	2.6	<0.001	<0.001	0.053
	INO	10.2 ^c	15.6 ^c	23.7 ^b	56.9 ^{A,ab}	2.7			
SAA, µg/mL	ORG	41.94	243.9	267.2	149.6	22.56	0.398	<0.001	0.652
	INO	51.49	270.5	307.6	141.9	24.24			
IL-6, pg/mL	ORG	275.7	453.3	300.2	285.6	97.04	0.796	0.056	0.607
	INO	133.5	493.9	275.2	294.8	103.2			
Oxidative stress and antioxidants									
Myeloperoxidase (log2), U/L	ORG	ND ⁴	4.62	5.04	5.86	0.38	0.003	0.013	0.822
	INO	ND ⁴	5.38	5.83	6.28	0.27			
ROMt (log2), mg H ₂ O ₂ /100 mL	ORG	ND ⁴	0.09	2.08	2.22	0.26	0.083	<0.001	0.712
	INO	ND ⁴	0.40	2.57	2.40	0.21			
FRAP, µmol/L	ORG	256	198	165	165	19	0.043	<0.001	0.746
	INO	293	220	218	213	19			
NOx, µmol/L	ORG	14.44	20.89	16.95	26.54	5.49	0.229	0.006	0.852
	INO	15.03	28.05	22.04	27.65	4.71			
NO ₂ ⁻ , µmol/L	ORG	6.07	8.91	6.62	11.98	2.87	0.216	0.016	0.857
	INO	6.92	13.51	9.65	11.75	2.45			
NO ₃ ⁻ , µmol/L	ORG	8.35	11.97	9.67	14.59	2.67	0.228	0.002	0.712
	INO	8.70	16.11	12.38	15.81	2.27			
ORAC, TE mol/L	ORG	8.89	10.59	11.57	11.04	0.58	0.109	<0.001	0.382
	INO	8.96	11.55	12.56	12.79	0.58			
Retinol, µg/100 mL	ORG	5.45	10.12	13.75	15.64	3.38	0.335	<0.001	0.529
	INO	6.18	14.61	18.52	24.78	3.31			

Continued

Table 2 (Continued). Blood immunometabolic biomarkers in calves born to dams fed a conventional diet supplemented with only inorganic (INO) or inorganic plus organic (ORG) trace minerals during the last 30 d prepartum

Item ¹	Treatment	Day of age						P-value ³		
		0	1	7	21	SEM ²	T	D	T × D	
Tocopherol, µg/mL	ORG	0.58 ^b	1.67 ^b	2.75 ^a	3.31 ^a	0.43	0.119	<0.001	0.086	
	INO	0.67 ^b	1.36 ^b	1.24 ^b	3.08 ^a	0.46				
Minerals										
	Cu, µmol/L	3.13	5.38	11.40	11.51	0.86	0.952	<0.001	0.624	
Mn, µmol/L	ORG	2.93	6.25	11.40	11.05	0.89				
	INO	0.12	0.13	0.10	0.11	0.01	0.722	0.678	0.147	
Fe, µmol/L	ORG	0.10	0.11	0.12	0.13	0.01				
	INO	20.3	20.5	11.5	14.2	2.5	0.478	<0.001	0.127	
Zn, µmol/L	ORG	16.8	25.2	14.4	17.5	2.7				
	INO	15.3 ^a	8.7 ^b	18.6 ^a	19.6 ^a	2.7	0.633	<0.001	0.091	
		12.2 ^c	15.7 ^{bc}	16.9 ^b	22.6 ^a	2.7				

^{A,B}Differences ($P < 0.05$) between treatments within a given time point when the interaction was significant.

^{a-c}Differences ($P < 0.05$) between time points within treatment when the interaction was significant.

¹SAA = serum amyloid A; ROMt = total reactive oxygen metabolites; FRAP = ferric reducing antioxidant power; ORAC = oxygen radical absorbance capacity; TE = Trolox equivalents.

²Greatest SEM is shown.

³P-value for treatment (T), day (D), or their interaction (T × D).

⁴Not detectable.

dx.doi.org/10.3168/jds.2015-9359). Although no overall treatment effect ($P = 0.69$) was found, INO calves had greater (T × W, $P = 0.060$) fecal score than ORG calves at 4 wk of age. Regardless of treatment effect, calves experienced the greatest ($P < 0.001$) fecal score during wk 1 of age, followed by a gradual decrease ($P < 0.001$) until 7 wk (Supplementary Table S6; <http://dx.doi.org/10.3168/jds.2015-9359>). In addition, all calves always consumed all the milk replacer that was offered.

Blood Immunometabolic Biomarkers

Main effects of diet, time, and interactions for blood immunometabolic biomarkers are reported in Table 2. Supplementary Table S7 (<http://dx.doi.org/10.3168/jds.2015-9359>) reports effects due to day. Overall, calves born to dams supplemented with ORG had lower glucose concentrations ($P = 0.001$) and this was particularly evident at birth (T × D, $P = 0.017$). Subsequently, change in glucose concentrations were similar with a rapid increase from birth to 1 d of life followed by stabilization after 1 wk of age ($P < 0.001$).

The NEFA and creatinine decreased from birth to 1 d of age ($P < 0.001$), with no treatment effect ($P > 0.05$), whereas BHBA concentration increased over time ($P < 0.001$). Urea decreased from birth to 7 d of age ($P < 0.001$) in both groups. The ORG calves had an abrupt decrease in urea from birth to 1 d of age, and INO calves had a gradual decrease until 7 d of age (T × D, $P = 0.039$). No difference were observed from birth to 1 d of age in bilirubin concentrations ($P > 0.05$), and a gradual decrease regardless of treatment was observed until 21 d ($P < 0.001$).

The hepatic enzymes GOT and GGT had a marked increase from birth to 1 d of age, followed by a gradual decrease ($P < 0.001$). An overall treatment effect ($P = 0.015$) was observed in GOT associated with lower concentration in ORG calves, which had a more pronounced decrease from 1 to 7 d of age.

Compared with INO, ORG calves had lower para-oxonase ($P < 0.001$), MPO ($P = 0.003$), and FRAP ($P = 0.043$) concentrations, and tended to have lower ROM ($P = 0.083$) and ORAC ($P = 0.109$). No treatment effect was observed for albumin, ceruloplasmin, cholesterol, haptoglobin, SAA, and IL-6 ($P > 0.05$). However, ceruloplasmin and cholesterol increased over time ($P < 0.001$), and SAA and IL-6 had a marked increase from birth to 1 d of age ($P < 0.001$ and $P = 0.056$, respectively). Concentration of ORAC, retinol, tocopherol, NOx, and constituents (NO_2^- and NO_3^-) increased over time ($P < 0.05$).

No effect ($P > 0.05$) was present of maternal diet on the evaluated blood mineral concentrations. At birth,

concentrations of all minerals were numerically greater in ORG than INO. A time effect was observed for Cu ($P < 0.001$) and Zn ($P < 0.001$) concentrations because of an increase over time, whereas concentration of Fe decreased ($P < 0.001$).

Gene and miRNA Gene Expression

TLR Pathway. Data on mRNA and miRNA in PMNL over time are reported in Table 3. Despite the

overall greater expression of *TLR2* in ORG calves ($P = 0.018$), an overall decrease occurred of several genes that participate in the pro-inflammatory cytokine response. The calves in ORG had lower expression of *IRAK1* ($P = 0.050$), *NFKB1* ($P = 0.031$), and *NFKBIA* ($P = 0.047$) and tended to have lower *MYD88* ($P = 0.059$) and *TRAF6* ($P = 0.063$) expression. Although only a trend for treatment effect was observed for *IL1B* ($P = 0.103$), the expression of *TNF* had an interaction (T \times D, $P = 0.056$) namely due to lower ($P = 0.017$) expres-

Table 3. Relative mRNA expression of genes related to pro-inflammatory signaling cascades, cell adhesion, pathogen recognition, oxidative stress, and microRNA in calves born to dams fed a conventional diet supplemented with only inorganic (INO) or inorganic plus organic (ORG) trace minerals during the last 30 d prepartum¹

Item	Treatment	Day of age				SEM ²	P-value ³		
		0	1	7	21		T	D	Trt \times D
Gene									
<i>TLR2</i>	ORG	5.70	5.55	5.31	4.95	0.21	0.018	<0.001	0.348
	INO	5.26	5.33	5.19	4.13	0.21			
<i>TLR4</i>	ORG	5.48	5.38	5.48	5.48 ^A	0.20	0.367	0.035	0.033
	INO	5.74 ^a	5.26 ^a	5.48 ^a	4.71 ^{B,b}	0.20			
<i>MYD88</i>	ORG	5.11	5.28	5.25	5.19	0.19	0.059	0.709	0.810
	INO	5.57	5.58	5.57	5.30	0.19			
<i>IRAK1</i>	ORG	4.64	5.34	5.19	5.38	0.23	0.050	<0.001	0.232
	INO	5.31	5.60	5.49	6.24	0.23			
<i>TRAF6</i>	ORG	5.23	5.35	5.47	5.41	0.13	0.063	0.009	0.519
	INO	5.31	5.62	5.77	5.75	0.13			
<i>NFKB1</i>	ORG	4.77	5.36	5.32	5.50	0.12	0.031	<0.001	0.351
	INO	5.29	5.45	5.50	5.75	0.12			
<i>NFKBIA</i>	ORG	5.80	5.15	5.51	5.50	0.19	0.047	<0.001	0.636
	INO	6.34	5.68	5.83	5.60	0.19			
<i>IL1B</i>	ORG	4.59	5.54	5.84	6.03	0.26	0.103	<0.001	0.800
	INO	4.21	5.35	5.64	5.43	0.26			
<i>TNF</i>	ORG	4.10 ^b	5.67 ^a	4.80 ^b	5.22 ^{B,ab}	0.46	0.299	<0.001	0.056
	INO	4.32 ^c	5.60 ^b	5.31 ^b	6.90 ^{A,a}	0.46			
<i>SELL</i>	ORG	4.65	5.12	5.43	5.30	0.18	0.057	<0.001	0.720
	INO	4.97	5.24	5.91	5.47	0.18			
<i>ZBP1</i>	ORG	1.51	3.39	5.77	4.57	0.51	0.185	<0.001	0.992
	INO	2.15	3.98	6.43	4.98	0.51			
<i>NFE2L2</i>	ORG	5.41 ^b	5.52 ^{ac}	5.47 ^{A,bc}	5.35 ^{A,c}	0.20	0.025	0.002	0.017
	INO	5.10 ^b	5.34 ^a	5.02 ^{B,a}	4.21 ^{B,c}	0.20			
<i>MPO</i>	ORG	4.16 ^{B,b}	5.16 ^a	4.62 ^{ab}	4.26 ^b	0.40	0.206	0.003	0.026
	INO	5.77 ^{A,a}	6.02 ^a	4.01 ^b	4.54 ^b	0.40			
<i>SOD1</i>	ORG	4.66	5.05	5.91	5.91	0.34	0.264	<0.001	0.661
	INO	4.72	5.38	6.38	6.66	0.34			
<i>NOS2</i>	ORG	4.85	5.09	4.62	4.49	0.47	0.361	0.005	0.113
	INO	6.04	6.17	4.23	4.20	0.47			
miRNA									
miR-155	ORG	0.57 ^{B,c}	0.64 ^c	1.82 ^a	1.01 ^{B,b}	0.36	0.024	<0.001	0.027
	INO	0.89 ^{A,c}	0.79 ^c	2.67 ^a	2.60 ^{A,b}	0.36			
miR-125b	ORG	0.88	0.88	1.08	0.83	0.16	0.036	0.893	0.712
	INO	1.25	1.13	1.28	1.30	0.16			
miR-146a	ORG	0.62	1.33	1.37	1.34	0.33	0.893	0.003	0.541
	INO	0.93	1.44	1.45	0.89	0.33			
miR-223	ORG	1.57	1.11	1.07	0.99	0.17	0.707	0.164	0.702
	INO	1.18	1.15	1.11	0.91	0.17			
miR-9	ORG	0.96	1.56	2.54	2.12	0.46	0.804	<0.001	0.353
	INO	1.34	0.97	1.91	1.88	0.46			

^{A,B}Differences ($P < 0.05$) between treatments within a given time point when the interaction was significant.

^{a-c}Differences ($P < 0.05$) between time points within treatment when the interaction was significant.

¹LSM are log₂ backtransformed.

²Greatest SEM is shown.

³P-value for treatment (T), day (D), or their interaction (T \times D).

sion in ORG calves at 21 d of age. An overall increase from birth to 1 d of life was observed for the expression of *IRAK1* ($P = 0.007$), *TRAF6* ($P = 0.017$), *NFKB1* ($P = 0.006$), *TNF* ($P < 0.001$), and *IL1B* ($P < 0.001$), whereas *NFKBIA* ($P < 0.001$) expression decreased during the same period (Table 3).

miRNA Related to the TLR Signaling Pathway. Maternal supplementation had an effect on miR-155 ($P = 0.024$) and miR-125b ($P = 0.036$), resulting in a downregulation in expression in ORG calves. The expression of miR-155, miR-146a, and miR-9 was up-regulated ($P < 0.004$) from birth to 7 d of age. The expression of miR-223 was not affected by maternal treatment ($P = 0.707$) or time ($P = 0.164$; Table 3).

Cell Surface Adhesion, Pathogen DNA Binding, and Oxidative Stress. Expression of *SELL* tended to be greater ($P = 0.057$) in INO calves, with a marked increase ($P < 0.001$) from birth to 7 d of life. Overall expression of *ZBP1* also increased ($P < 0.001$) during the first week of life, but no treatment effect ($P = 0.185$) was detected (Table 3).

Expression of *NFE2L2* had a significant interaction ($T \times D$, $P = 0.017$) due to greater ($P = 0.02$) expression in ORG calves than INO at 7 and 21 d of age. An overall time effect ($P = 0.002$) also was observed mainly due to a linear downregulation in its expression from 1 to 21 d in the INO calves. No treatment effect on *MPO* expression ($P = 0.206$) was observed, but a significant interaction ($T \times D$, $P = 0.026$) occurred, where greater ($P = 0.009$) expression was observed in INO calves at birth. Expression of *SOD1* and *NOS2* was not affected ($P = 0.264$ and 0.361) by maternal treatment. Expression of *SOD1* increased from birth to 7 d ($P < 0.001$), whereas *NOS2* decreased ($P = 0.005$) during the same period (Table 3). Supplementary Table S8 (<http://dx.doi.org/10.3168/jds.2015-9359>) reports effects of day specifically for gene and miRNA expression data.

DISCUSSION

Although exact mechanisms are unknown and the human and ruminant placental structure differ, in general, the human placenta is permeable to trace minerals (Smith et al., 1992). If the trace mineral is still chelated with the AA while crossing placental membranes, at least 15 to 20 different AA transport systems are expressed on the microvillous and basal membrane of the placenta that could allow for uptake via active transport (Jones et al., 2007; Larqué et al., 2013).

The mammary gland has a remarkable capacity to strictly regulate the secretion of trace elements in milk, even avoiding an imbalance when supplemented above requirements (Lonnerdal, 2007). Anionic and neutral

AA are transported by Na^+ -dependent or independent mechanisms, whereas cationic AA transfer has not been completely elucidated. In bovine and rat mammary tissue, AA transport is not Na^+ dependent (Shennan and Peaker, 2000).

Growth Performance and Health

Direct effects of ORG supplementation in enhancing bone growth are already well-established in poultry (Bao et al., 2010; Rao et al., 2013); thus, the trend for greater WH in ORG calves could have been influenced by the same mechanisms. Intrauterine bone development is strongly affected by growth factors such as insulin-like growth factor 2, and epigenetic modifica-

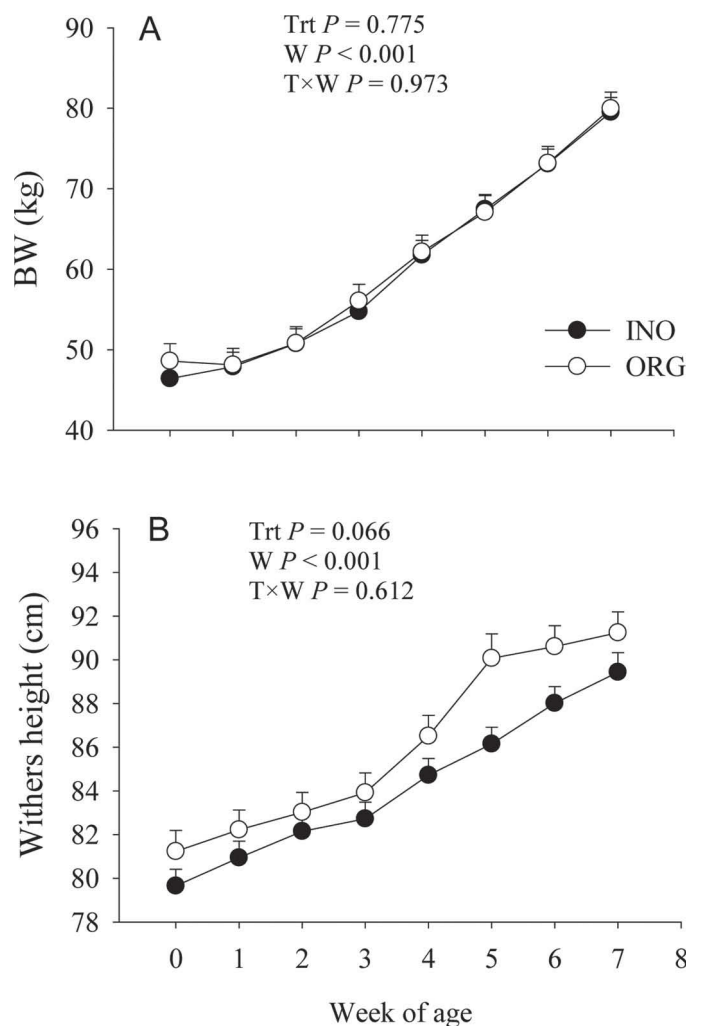


Figure 1. Body weight (A) and withers height (B) of calves born to dams fed a conventional diet supplemented with only inorganic (INO) or inorganic and organic (ORG) trace minerals during the last 30 d prepartum. Shown are P -values for main effects of treatment (Trt) and week (W), and their interaction (T \times W).

tions modulate the pathway (Constancia et al., 2002; Tabano et al., 2010). Female rats that were exposed to intrauterine growth restriction had a reduction in bone size and mineral content and strength in part due to a decrease in endochondral ossification responsiveness (Chen et al., 2013). Proper cartilage and bone formation requires Mn, as it is a cofactor for enzymes that synthesize chondroitin-sulfate side chains of proteoglycan molecules (Tomlinson et al., 2004). Whether the same mechanisms account for increasing WH in calves from cows fed ORG remains to be determined.

Although rectal temperature tended to be greater in INO than ORG calves, it did not surpass the normal physiological threshold (Scibilia et al., 1987). However, the 0.5°C increase in temperature in INO calves could be of biological significance, and as suggested by the *TNF* and *NFKB1* expression, could reflect a transient inflammatory response. As such, it could have increased maintenance energy needs, hence impairing normal bone growth (Mundy, 1993; Lacey et al., 2009) and partly explaining the greater blood glucose concentrations in INO calves. Although not specifically measured in the present study, supplementing calves with an injectable trace mineral supplement containing Se, Cu, Zn, and Mn enhanced the immune response (Teixeira et al., 2014). Thus, feeding ORG to dams could have elicited a better postnatal health status [e.g., enhancing gluconeogenic rate via Mn (Rognstad, 1981)] and sparing glucose from maintenance functions in favor of tissue growth. Clearly, differences between direct effects due to supplementation (prenatal) and the effects through maternal transfer (including colostrum) in the observed responses should be taken into account but could not be accurately discerned in the present study.

Blood Biomarkers of Energy, Protein, and Inflammatory Metabolism

The overall increases in GOT and GGT concentrations after colostrum intake could have been a result of intestinal absorption as immunoglobulins, as their content is higher in colostrum (Ontsouka et al., 2003; Bertoni et al., 2009), and not necessarily related to liver damage. As indices of liver damage, GOT and GGT in the newborn calf also could reflect a metabolic disorder of the dam such as subclinical acidosis (Lechowski, 1996), but based on previous work this increase appears to be a normal physiological response in the calf (Bertoni et al., 2009).

The neonatal capacity for FA oxidation and ketone body production in liver is low, but increases markedly during the first 24 h of life in response to intakes of colostrum and milk replacer (Blum, 2006). The increase

of BHBA through the time of weaning is associated with the gradual intake of solid feed, which allows for rumen development and fermentation capacity, leading to production of VFA of which butyrate can be metabolized by rumen epithelium to BHBA and acetoacetate (Naeem et al., 2012). The decrease in NEFA concentrations after birth underscored the depletion of body fat stores of the calf.

The acute-phase response is characterized by the concomitant increase in concentration of positive APP such as ceruloplasmin, haptoglobin, and SAA, and the decrease of negative APP such as paraoxonase and albumin (Eckersall and Bell, 2010). Regardless of maternal nutrition, paraoxonase concentration remained within physiological levels for healthy calves, and the increase over time agrees with a previous study (Giordano et al., 2013). The increase in IL-6 concentration right after birth is indicative of an activation of the immune system. Together, these responses could be related to maturation of hepatic metabolic pathways, immune stimulation, and colostrum intake (Orro et al., 2008).

Despite FRAP values being already close to peak values at birth, the fact that MPO and ROM were undetectable at birth but were measurable at 1 d of age regardless of maternal treatment is suggestive of an activation of the oxidative stress response. Furthermore, the lower concentrations of MPO, ROM, and FRAP in ORG calves suggest a positive effect of maternal supplementation with ORG. Antioxidant activity has been positively correlated with increased FRAP (Benzie and Strain, 1996) and ORAC; thus, the higher FRAP and ORAC concentration in INO calves could be related to a greater need for neutralizing ROS production. Whether such an effect was induced in utero or via colostrum cannot be ascertained.

PMNL Gene Expression Regulation of the Innate Immune Response

The pro-inflammatory cascade can be initiated via different pathways, but both TLR2 and TLR4 are essential for neutrophil pathogen recognition and defense, including adhesion, generation of ROM, and release of chemokines (Sabroe et al., 2005). Despite the fact that calves in the ORG treatment had greater *TLR2* expression, that response was not associated with upregulation of the pro-inflammatory pathway as indicated by the lower expression of the cytokine mediators *IRAK1*, *NFKB1*, and *NFKBIA*, a trend for lower *MYD88*, *TRAF6*, *IL1B*, the lack of change in *TNF*, and a trend for lower *SELL*. It could be possible that maternal ORG resulted in a more efficient immune

system such that calves maintained higher expression of TLR and did not need to upregulate expression of the pro-inflammatory signaling genes.

The marked increase in *SELL* expression during the first week of life, also detected previously (Osorio et al., 2013), likely reflects the potential of colostrum to trigger inflammation and prime the innate immune response. The same stimulus could be responsible for the temporal expression profile of *ZBP1*, a cytosolic pathogen DNA recognition molecule that acts as a sensor to activate the innate immune response (Takaoka et al., 2007).

The transcription factor *NFE2L2* regulates the expression of genes associated with detoxification, antioxidant, and anti-inflammatory cellular responses; hence, it plays an important protective role for the development of disease (Cardozo et al., 2013). Thus, the greater expression of *NFE2L2* in ORG calves indicates they benefitted from the antioxidant and anti-inflammatory regulation controlled via *NFE2L2*. As opposed to the blood biomarkers related to oxidative stress (MPO, ROM, and FRAP), the fact that no treatment effect was observed in the expression of *SOD1*, *MPO*, and *NOS2* demonstrates the existence of posttranscriptional regulation.

miRNA Expression and Its Relationship with the TLR Pathway

The expression of miR-155 is strongly induced by TLR pathogen sensing and pro-inflammatory cytokines (O'Connell et al., 2007), whereas miR-125b is inhibited (Tili et al., 2007). Despite the lack of statistical differences in systemic biomarkers of inflammation, the overall greater miR-155 expression in INO calves agrees with the overall greater oxidative stress status indicated by the greater concentrations of PON, ROM, MPO, and FRAP and the lower tocopherol. At the molecular level, the greater expression of miR-155 in INO calves agrees with the greater expression of most pro-inflammatory genes measured (e.g., *NFKB1*, *TNF*). The fact that both miR-155 and *TNF* expression were greater in INO calves might be explained by data demonstrating that induction of miR-155 by a pro-inflammatory stimulus such as LPS enhanced *TNF* translation (Tili et al., 2007). Thus, as the miRNA acts to repress the protein translation process, it is possible that enhanced mRNA synthesis (or greater half-life) is a compensatory response to counterbalance this inhibitory effect. Assuming that similar mechanisms of control exist in bovine, the greater overall expression of miR-125b in INO calves appears unrelated to oxidative stress or inflammatory status. Furthermore, a relationship between TLR and miR-125b in the present study is not evident

because the interaction effect for *TLR4* expression was due to a decrease in expression at 21 d in INO calves. Further studies involving pro-inflammatory challenges are required to establish more precisely mechanisms of miRNA regulation in neonatal calf immune cells.

The expression of miR-146a can be induced by bacterial activation of cell surface TLR (TLR2, TLR4, TLR5) and also by TNF and IL1B treatment in an NFkB-dependent manner (Taganov et al., 2006). The genes *IRAK1* and *TRAF6* are direct targets of miR-146a, and their downregulation over time regardless of treatment via the miRNA may function as a negative feedback regulatory mechanism (i.e., miR-146a expression may be critical to prevent uncontrolled inflammation). The increased expression of miR-9 during the first week of life was likely related to TLR4 activation in an NFkB-dependent manner, operating in a negative feedback control by exerting close control on the expression of key members of the NFkB pathway (Bazzoni et al., 2009).

The present data demonstrate the plasticity of miRNA expression in bovine neutrophils during the neonatal period, as well as how maternal diet supplementation with organic trace minerals can influence growth and the immune response. Our results indicate that calves from dams fed ORG during the last 30 d of gestation had a lower oxidative stress status during the postnatal period. Although this was not measured directly, we surmise that this effect benefitted immune function as reflected in the downregulation of inflammatory mRNA and miRNA. If systemic inflammation was indeed reduced, additional energy (e.g., glucose) would have been spared for growth, and partly explain the trend for greater WH around weaning time. Additional research in this area to clarify the mechanisms at play seems warranted.

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