Prepartal Energy Intake Alters Blood Polymorphonuclear Leukocyte Transcriptome During the Peripartal Period in Holstein Cows

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ABSTRACT: In the dairy industry, cow health and farmer profits depend on the balance between diet (ie, nutrient composition, daily intake) and metabolism. This is especially true during the transition period, where dramatic physiological changes foster vulnerability to immunosuppression, negative energy balance, and clinical and subclinical disorders. Using an Agilent microarray platform, this study examined changes in the transcriptome of bovine polymorphonuclear leukocytes (PMNLs) due to prepartal dietary intake. Holstein cows were fed a high-straw, control-energy diet (CON; NE_L = 1.34 Mcal/kg) or overfed a moderate-energy diet (OVE; NE_L = 1.62 Mcal/kg) during the dry period. Blood for PMNL isolation and metabolite analysis was collected at −14 and +7 days relative to parturition. At an analysis of variance false discovery rate <0.05, energy intake (OVE vs CON) influenced 1806 genes. Dynamic Impact Approach bioinformatics analysis classified treatment effects on Kyoto Encyclopedia of Genes and Genomes pathways, including activated oxidative phosphorylation and biosynthesis of unsaturated fatty acids and inhibited RNA polymerase, proteasome, and toll-like receptor signaling pathway. This analysis indicates that processes critical for energy metabolism and cellular and immune function were affected with mixed results. However, overall interpretation of the transcriptome data agreed in part with literature documenting a potentially detrimental, chronic activation of PMNL in response to overfeeding. The widespread, transcriptome-level changes captured here confirm the importance of dietary energy adjustments around calving on the immune system.

KEYWORDS: Transition cow, gene expression, neutrophil, nutrigenomics

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Introduction

The transition into lactation is a volatile period in dairy cow health, largely due to the shifts in energy demands and nutrient partitioning that occur after calving.[1,2] Traditionally, a depression in dry matter (DM) intake is observed postpartum, although cow energy requirements increase dramatically to support milk production. The result is a negative energy balance (NEB) that can precede a host of health complications.[3] Appropriate nutritional management throughout the lactation cycle, especially preceding calving (ie, the dry period), is therefore essential to ensuring short-term and long-term herd health and productivity.

In the past, nutritional strategies to alleviate postpartum NEB included overfeeding by increasing prepartum dietary energy.[3,4] Although prepartum energy intake of dairy cows does influence postpartum health, recent studies have revealed that it tends to have a negative rather than positive impact. Overfeeding energy prepartum can deepen NEB and increase fat mobilization and deposition in liver, ketone production, and incidence of metabolic disorders, whereas controlling energy intake improves these outcomes.[5–7] Some studies also suggest that milk yield[8] and reproductive measures[4,9] following calving are enhanced when prepartum energy is restricted. Prepartal dietary intake may even yield more substantial metabolic effects than dietary composition.[10]

In addition, recent studies demonstrate that transition period adaptations can compromise the immune system, further exposing cows to postpartum disorders and increased susceptibility to disease.[11] Diversion of maintenance energy in favor of lactation, as well as increasing cortisol and estrogen levels, may drive immunosuppression near calving, leaving cows susceptible to pathogens.[12,13] Therefore, the importance of researching the immune system in peripartal cows cannot be understated. As first responders of the innate immune system and coordinators of the adaptive immune response,[14] polymorphonuclear leukocytes (PMNLs) are representative immune cells. Understanding their behavior during the transition period may provide insights into cow health status.

Although some transcriptome-level work evaluating bovine PMNL has already been conducted,[12,15,16] these have largely focused on the effect of parturition or parturition-related conditions, eg, glucocorticoid levels. As proposals for new transition cow management strategies arise, it is necessary to evaluate the PMNL transcriptome in context of nutrition and management

trials, such that immunologic health is maintained or improved with shifting practices. The present data were therefore generated from PMNL in cows fed higher or control-energy diets to illustrate the impacts of current dry period diets (ie, greater energy intake) versus emerging recommendations (lower energy intake) on immunity during transition. We hypothesized that adaptation of PMNL in overfed cows would consist of significant transcription-level changes.

Materials and Methods

Animals and treatments

Cows used for this study were from the overfeeding study of Khan et al, and details of animal care and treatment have been outlined in previous publications.[17,18] Briefly, 8 cows were assigned to 1 of the 2 groups which differed in dietary energy levels fed throughout the 45-day dry period. One group received at least 100% calculated NE_L (CON; 1.34 Mcal/kg DM) from a diet high in wheat straw, whereas the other received over 140% calculated NE_L (OVE; 1.62 Mcal/kg DM) from a corn silage–based diet. Both diets were fed once daily (0600 hours) using an individual gate feeding system (American Calan, Northwood, NH, USA). All cows were housed in a ventilated, enclosed barn for the entire dry period. After calving, cows were fed a common lactation diet (NE_L = 1.69 Mcal/kg DM) as total mixed ration once daily (0600 hours) and housed in a tie-stall barn. Milking occurred twice daily (0400 and 1600 hours).

PMNL isolation and RNA isolation

The specific details of these procedures are included in the Supplemental File.

Microarrays

Complementary RNA synthesis and labeling, fragmentation, hybridization, and slide scanning. The specific details of these procedures are included in the Supplemental File. Array data are publicly available in the Gene Expression Omnibus database (Series ID: GSE95677).

Statistical analysis

Gene analysis. Microarray statistics were analyzed using SAS (SAS Institute Inc., Cary, NC, USA). Data from the 8 microarrays were adjusted using Lowess normalization and array centering to account for dye and array effects. A mixed model with repeated measures was fitted to the normalized $\log_2$-transformed adjusted ratios using PROC MIXED. The model included fixed effects of time (−14 and +7 days), diet (CON and OVE), and interaction of time × diet. Cow was a random effect. Raw *P* values were adjusted to account for multiple comparisons using Benjamini and Hochberg's false discovery rate (FDR).[19] Significant differences in gene expression were considered at FDR < 0.05. Only genes with degree of freedom ⩾7 were considered. The signal intensity (average between days −14 and +7) of the differentially expressed genes (DEGs) for each animal was represented as a heat map (Figure 1). Genes were clustered using the average linkage method, with Pearson coefficient as distance measurement. Results below will focus on transcriptome differences due to the main effect of diet.

Pathway analysis. The Dynamic Impact Approach (DIA) was used to analyze Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways based on DEGs between dietary groups (OVE vs CON). Complete DIA methodology has already been reported,[20] but overall, the model illustrates impact (relevance) and flux (direction of impact) for KEGG pathways. Full data including Entrez Gene ID, Oligo Gene ID, FDR (<0.05), *P* value (<.05), and fold change (FC) were entered into the DIA. Information for pathways was only provided when ⩾30% of annotated genes were covered by the data set.

Results of the final pathway analysis are compiled in Tables 1 and 2 and Figures 2 and 3.

Results and Discussion

Due to the abundance of literature on transition period (ie, time effect) adaptations, discussion here will focus on the effect of treatment, ie, overfeeding versus feeding to meet requirements in the close-up dry period on PMNL gene expression. We chose to analyze the samples collected at −14 and 7 days relative to parturition to avoid interference of parturition by sampling too close to it prepartum and to observe transcriptomic changes in the first week of lactation when cows are most susceptible to diseases.[21] Data on 43 target genes measured via reverse transcription-polymerase chain reaction (RT-PCR) at −14, 7, and 14 days relative to parturition are reported in Zhou et al.[18]

Overall, 1806 DEGs were entered into the DIA for pathway analysis. There were 1000 upregulated and 806 downregulated genes. Of these, 49 upregulated genes had an FC > +3 (see Table 1) and 90 downregulated genes had an FC < −3 (see Table 2).

KEGG analysis

Data for pathways within human diseases categories are omitted from analysis results. Full summary of impact and flux for KEGG categories and subcategories can be reviewed in Figure 2. The top 20 affected pathways, ranked by impact in Figure 3, are discussed by category below.

Metabolism

Energy metabolism. It is known that PMNLs primarily depend on glycolysis to produce energy.[22] Therefore, it is surprising that the oxidative phosphorylation pathway was upregulated in OVE cows. However, regardless of glucose availability, as examined in guinea pig PMNLs, an increase in oxygen uptake

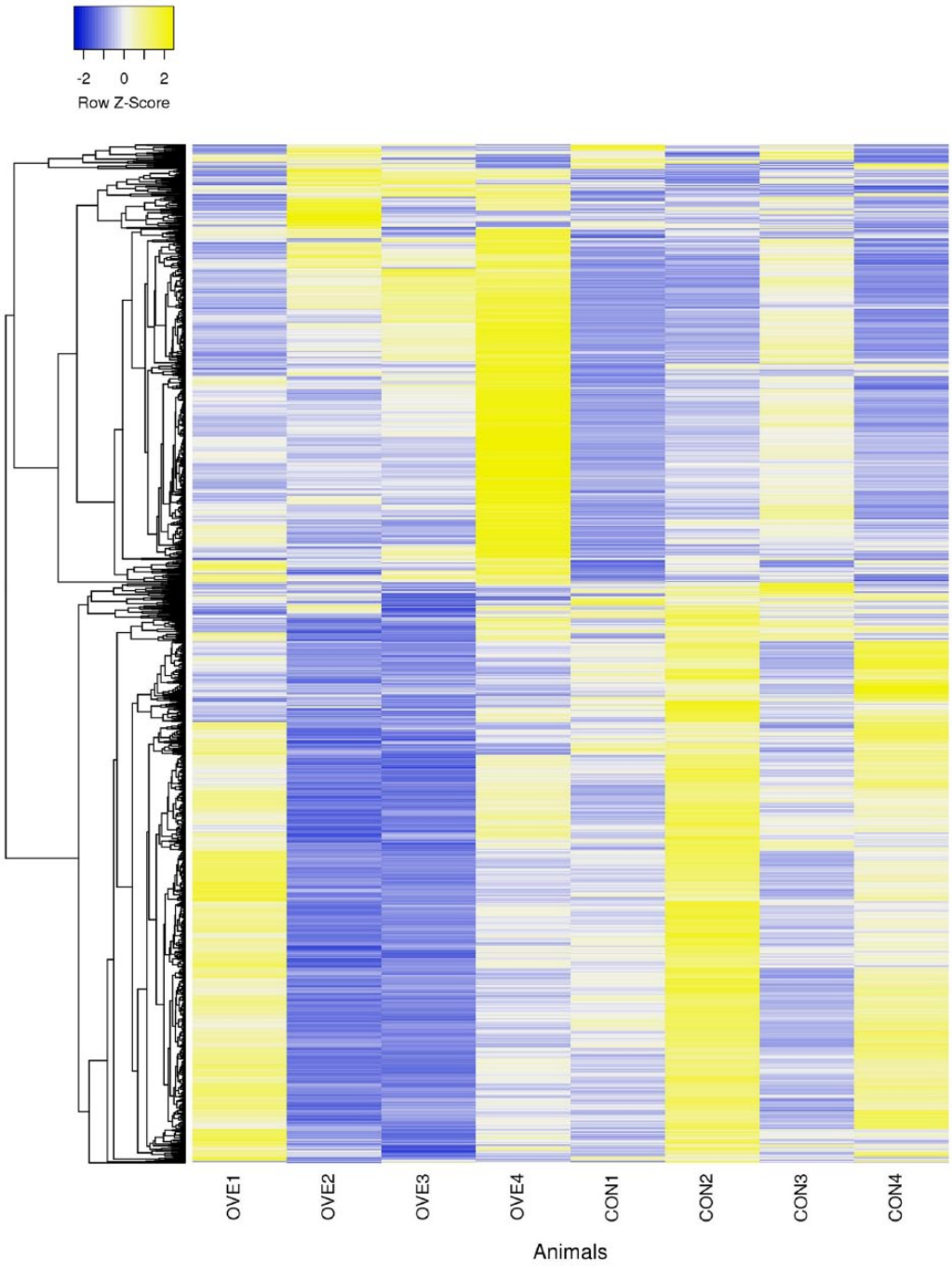

Figure 1. Heat-map representation of signal intensity (average days –14 and +7) for differentially expressed genes (DEGs) in polymorphonuclear leukocytes of individual cows overfed energy (OVE) versus control fed (CON). The DEGs were clustered using the average linkage method, with distances calculated as Pearson coefficients.

and respiration occurs in phagocytic cells compared with those in the resting state.[23,24] Increases in respiration during phagocytosis were also detected in chicken PMNLs.[25] This indicates that, across species, oxygen utilization helps promote PMNL phagocytosis. In addition, in anaerobic environments, glucose consumption or glycogen breakdown (ie, retrieval of glucose for glycolysis in either substrate-rich or substrate-poor environments) increases for phagocytic cells compared with an aerobic environment.[24] In other words, alternate sources of energy from oxidative phosphorylation may be available under aerobic conditions. In anaerobic environments, however, glycolysis is primarily responsible for meeting the increased energy demand.

Taken together, the above results demonstrate a potential preference for oxidative phosphorylation during phagocytosis. This preference may correspond to the so-called oxidative burst that allows PMNLs to eliminate phagocytosed particles via reactive oxygen species (ROS). This is supported by findings of Fossati et al[26] that complex V of the mitochondrial respiratory chain, or adenosine triphosphatase, is involved in functions

Table 1. Differentially expressed genes with fold change (FC) greater than +3 in cows overfed a moderate-energy diet (OVE) versus fed to maintenance with a control-energy diet (CON).

GENE SYMBOL	GENE NAME	FC
MRPL24	Mitochondrial ribosomal protein L24	15.76
TLR5	Toll-like receptor 5	11.28
SURF6	Surfeit 6	9.38
CHPF	Chondroitin polymerizing factor	9.12
CEBPG	CCAAT/enhancer-binding protein gamma	8.36
RNF19B	Ring finger protein 19B	7.47
GADD45B	Growth arrest and DNA-damage-inducible, beta	7.28
HMG20A	High-mobility group 20A	7.03
SNCAIP	Synuclein alpha interacting protein	6.44
CLIP1	CAP-Gly domain–containing linker protein 1	5.53
ANAPC16	Anaphase-promoting complex subunit 16	5.30
ATP5G1	ATP synthase, H+ transporting, mitochondrial Fo complex subunit C1 (subunit 9)	5.28
ACTL6A	Actin-like 6A	5.18
LSM7	LSM7 homolog, U6 small nuclear RNA, and mRNA degradation associated	4.84
DAZAP2	DAZ-associated protein 2	4.83
IGFBP5	Insulinlike growth factor binding protein 5	4.76
DCK	Deoxycytidine kinase	4.70
SELL	Selectin L	4.45
RPL37	Ribosomal protein L37	4.41
COPS6	COP9 signalosome subunit 6	4.38
MOCS3	Molybdenum cofactor synthesis 3	4.37
MRI1	Methylthioribose-1-phosphate isomerase 1	4.36
C7H19orf25	Chromosome 7 open reading frame, human C19orf25	4.33
EEF1B2	Eukaryotic translation elongation factor 1 beta 2	3.99
EIF4B	Eukaryotic translation initiation factor 4B	3.88
ATP5E	ATP synthase, H+ transporting, mitochondrial F1 complex, epsilon subunit	3.88
MRPL22	Mitochondrial ribosomal protein L22	3.77
MUM1	Melanoma-associated antigen (mutated) 1	3.68
LCP2	Lymphocyte cytosolic protein 2	3.66
ATP5A1	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle	3.65
COL18A1	Collagen type XVIII alpha 1	3.58
FNDC3B	Fibronectin type III domain containing 3B	3.53
AGPAT5	1-acylglycerol-3-phosphate O-acyltransferase 5	3.45
SARS	Seryl-tRNA synthetase	3.44
ATP5G3	ATP synthase, H+ transporting, mitochondrial Fo complex subunit C3 (subunit 9)	3.43
SOX15	SRY-box 15	3.39

Table 1. (Continued)

GENE SYMBOL	GENE NAME	FC
SDS	Serine dehydratase	3.39
SNRPG	Small nuclear ribonucleoprotein polypeptide G	3.37
COX11	COX11 cytochrome c oxidase assembly homolog (yeast)	3.37
DNAJC19	DnaJ heat shock protein family (Hsp40) member C19	3.35
MPG	*N*-methylpurine DNA glycosylase	3.33
ZNF143	Zinc finger protein 143	3.30
MRPL52	Mitochondrial ribosomal protein L52	3.21
ANAPC10	Anaphase-promoting complex subunit 10	3.20
NR1H3	Nuclear receptor subfamily 1 group H member 3	3.14
MTFMT	Mitochondrial methionyl-tRNA formyltransferase	3.09
CPO	Carboxypeptidase O	3.09
EBNA1BP2	EBNA1-binding protein 2	3.02
SMARCE1	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily e, member 1	3.01

Table 2. Differentially expressed genes with fold change (FC) less than –3 in cows overfed a moderate-energy diet (OVE) versus fed to maintenance with a control-energy diet (CON).

GENE SYMBOL	GENE NAME	FC
TMEM192	Transmembrane protein 192	–7.12
C15H11orf31	Chromosome 15 open reading frame, human C11orf31	–6.39
RFNG	RFNG O-fucosylpeptide 3-beta-*N*-acetylglucosaminyltransferase	–6.25
ACTA2	Actin, alpha 2, smooth muscle, aorta	–6.11
ATG2A	ATG2 autophagy-related 2 homolog A (*Saccharomyces cerevisiae*)	–6.02
ARID4A	AT-rich interaction domain 4A	–5.46
TMEM220	Transmembrane protein 220	–5.29
FER	FER tyrosine kinase	–5.11
MGC139164	Uncharacterized LOC509649	–5.03
TPST1	Tyrosylprotein sulfotransferase 1	–4.81
ZMAT3	Zinc finger, matrin-type 3	–4.81
SNRPA	Small nuclear ribonucleoprotein polypeptide A	–4.79
DGAT2	Diacylglycerol O-acyltransferase 2	–4.79
GPBAR1	G protein–coupled bile acid receptor 1	–4.75
PARD6G	Par-6 family cell polarity regulator gamma	–4.73
ENPP5	Ectonucleotide pyrophosphatase/phosphodiesterase 5 (putative)	–4.67
EIF2S2	Eukaryotic translation initiation factor 2 subunit beta	–4.66
PI4K2A	Phosphatidylinositol 4-kinase type 2 alpha	–4.64
NRG4	Neuregulin 4	–4.62

(Continued)

Table 2. (Continued)

GENE SYMBOL	GENE NAME	FC
LOC100196900	Uncharacterized LOC100196900	−4.60
SKP2	S-phase kinase-associated protein 2, E3 ubiquitin protein ligase	−4.49
DCTPP1	dCTP pyrophosphatase 1	−4.43
FAM45A	Family with sequence similarity 45 member A	−4.37
LDLRAD3	Low-density lipoprotein receptor class A domain containing 3	−4.36
DEDD2	Death effector domain containing 2	−4.30
ARHGAP25	Rho GTPase activating protein 25	−4.27
PDIA2	Protein disulfide isomerase family A member 2	−4.23
RPL9	Ribosomal protein L9	−4.18
SLC40A1	Solute carrier family 40 (iron-regulated transporter), member 1	−4.10
IL12B	Interleukin 12B	−4.07
DYNLRB1	Dynein, light chain, roadblock-type 1	−4.05
ZNF7	Zinc finger protein 7	−4.03
UTP18	UTP18 small subunit processome component	−3.95
MRPL35	Mitochondrial ribosomal protein L35	−3.92
CYLD	Cylindromatosis (turban tumor syndrome)	−3.90
SERPINB7	Serpin peptidase inhibitor, clade B (ovalbumin), member 7	−3.90
POLR2G	Polymerase (RNA) II (DNA-directed) polypeptide G	−3.87
CREBRF	CREB3 regulatory factor	−3.85
EFL1	Elongation factor–like GTPase 1	−3.83
TXNDC15	Thioredoxin domain containing 15	−3.74
BTBD6	BTB (POZ) domain containing 6	−3.73
MARK2	MAP/microtubule affinity-regulating kinase 2	−3.68
CEP78	Centrosomal protein 78 kDa	−3.67
LOC619061	60S ribosomal protein L9 pseudogene	−3.62
CYP51A1	Cytochrome P450, family 51, subfamily A, polypeptide 1	−3.61
KLF11	Kruppel-like factor 11	−3.61
LYPLAL1	Lysophospholipase-like 1	−3.59
LOC523083	Olfactory receptor 2S2	−3.57
TMOD3	Tropomodulin 3	−3.54
SLC10A2	Solute carrier family 10 (sodium/bile acid cotransporter), member 2	−3.52
LRP10	LDL receptor–related protein 10	−3.50
SLC1A6	Solute carrier family 1 (high-affinity aspartate/glutamate transporter), member 6	−3.49
SLC2A3	Solute carrier family 2 (facilitated glucose transporter), member 3	−3.49
PRKACA	Protein kinase, cAMP-dependent, alpha catalytic subunit	−3.48
VAMP3	Vesicle-associated membrane protein 3	−3.46
TOX2	TOX high-mobility group box family member 2	−3.43

Table 2. (Continued)

GENE SYMBOL	GENE NAME	FC
SLC30A1	Solute carrier family 30 (zinc transporter), member 1	–3.42
TMEM119	Transmembrane protein 119	–3.41
BNC1	Basonuclin 1	–3.41
FDPS	Farnesyl diphosphate synthase	–3.41
HIPK3	Homeodomain-interacting protein kinase 3	–3.38
FAM117A	Family with sequence similarity 117 member A	–3.34
LOC618070	Putative olfactory receptor 2B8	–3.34
HSD17B11	Hydroxysteroid (17-beta) dehydrogenase 11	–3.34
CCDC8	Coiled-coil domain containing 8	–3.32
TUBA1B	Tubulin, alpha 1b	–3.27
CTNNBL1	Catenin beta–like 1	–3.25
RPS27L	Ribosomal protein S27 like	–3.22
CHCHD2	Coiled-coil-helix-coiled-coil-helix domain containing 2	–3.22
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	–3.22
STRADA	STE20-related kinase adaptor alpha	–3.22
YME1L1	YME1-like 1 ATPase	–3.19
PHB	Prohibitin	–3.16
METTL2A	Methyltransferase-like 2A	–3.16
POLR3D	Polymerase (RNA) III (DNA-directed) polypeptide D, 44 kDa	–3.16
PSMG2	Proteasome (prosome, macropain) assembly chaperone 2	–3.15
CYSTM1	Cysteine-rich transmembrane module containing 1	–3.15
NPVF	Neuropeptide VF precursor	–3.14
PSMA7	Proteasome subunit alpha 7	–3.13
HOXC11	Homeobox C11	–3.12
SOBP	Sine oculis binding protein homolog	–3.11
PRDX3	Peroxiredoxin 3	–3.10
TGFBR2	Transforming growth factor beta receptor II	–3.10
PDLIM7	PDZ and LIM domain 7	–3.08
CCDC137	Coiled-coil domain containing 137	–3.07
EIF1AX	Eukaryotic translation initiation factor 1A, X-linked	–3.05
LOC100126544	Uncharacterized LOC100126544	–3.03
CASP8AP2	Caspase 8–associated protein 2	–3.02
KIF3C	Kinesin family member 3C	–3.02
RPL9	Ribosomal protein L9	–3.02

related to cell death regulation and respiratory burst. Inhibition of this unit by oligomycin inhibited respiratory burst and chemotaxis. Therefore, upregulation of the oxidative phosphorylation pathway here might indicate higher phagocytic activity of PMNLs, ie, a more activated immune system. This also agrees with earlier quantitative PCR findings that OVE cows had

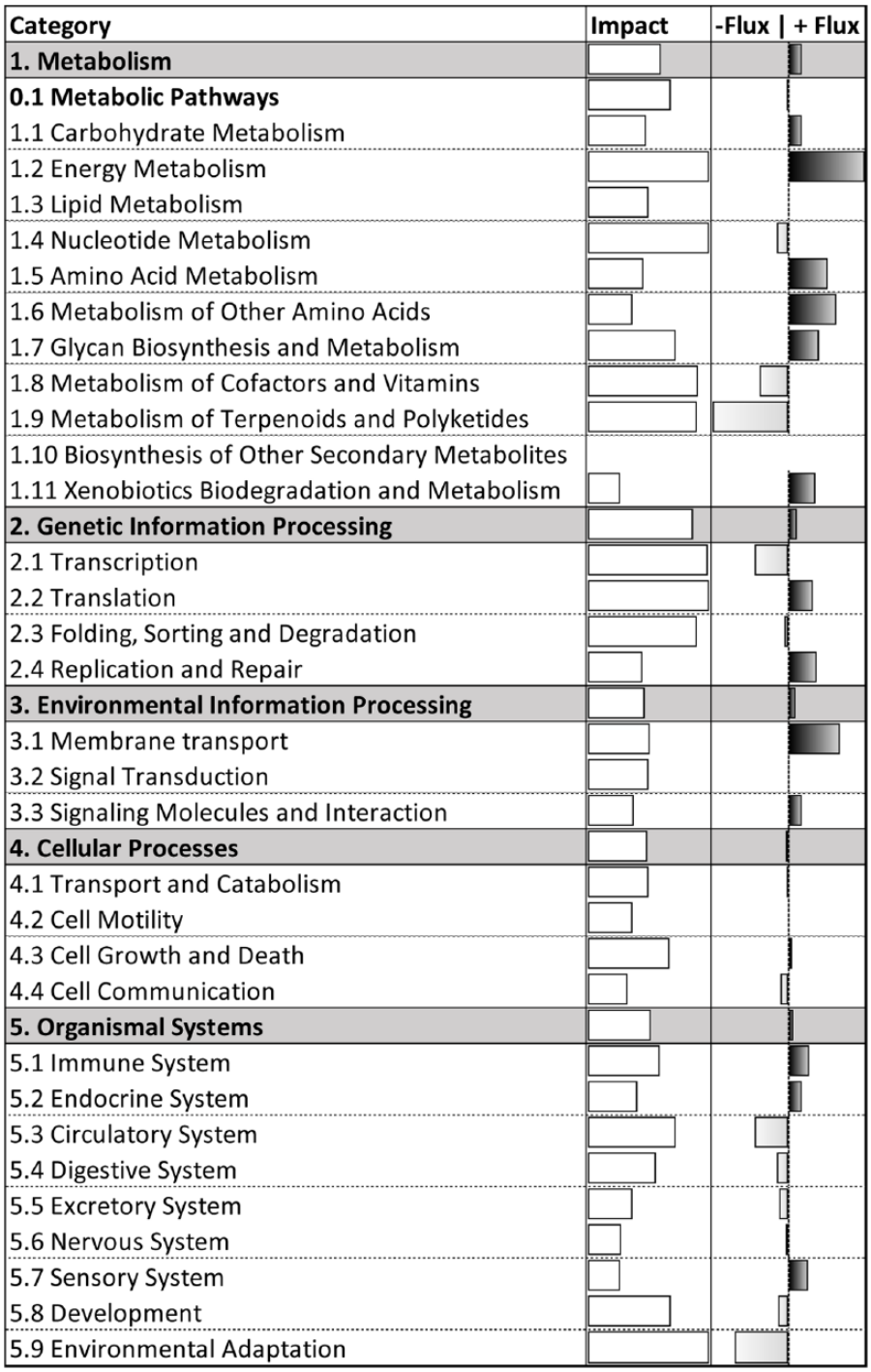

Figure 2. Summary of treatment effects on all Kyoto Encyclopedia of Genes and Genomes pathways in polymorphonuclear leukocytes of overfed (OVE) versus control-fed (CON) cows, divided by category and subcategory. The second column of white bars indicates impact on a relative scale of 0 to 50. The third column of gray and black bars indicates flux, or direction of impact, on a scale of –25 to +25, where gray bars represent negative flux (–25 to 0) and black bars represent positive flux (0 to +25).

higher expression of genes associated with adhesion, migration, and phagocytosis.[18]

Another possibility remains open; that is, that adenosine triphosphate (ATP) produced in the mitochondria via oxidative phosphorylation is not formed for cellular consumption but rather for signaling purposes. Purinergic signaling (ie, through ligands such as ATP, adenosine, and other purine metabolites) via $P2Y_2$ receptors has been implicated in neutrophil chemotaxis.[27,28] In fact, it has recently been suggested that the extracellular ATP which fuels chemotaxis in human neutrophils is produced specifically by PMNL mitochondria.[29] Chemical uncoupling of mitochondria prevented ATP release from stimulated neutrophils, although it did not change the intracellular ATP levels. In the same study, inhibiting mitochondrial ATP also reduced ROS production, demonstrating that oxidative phosphorylation also participates in oxidative burst as

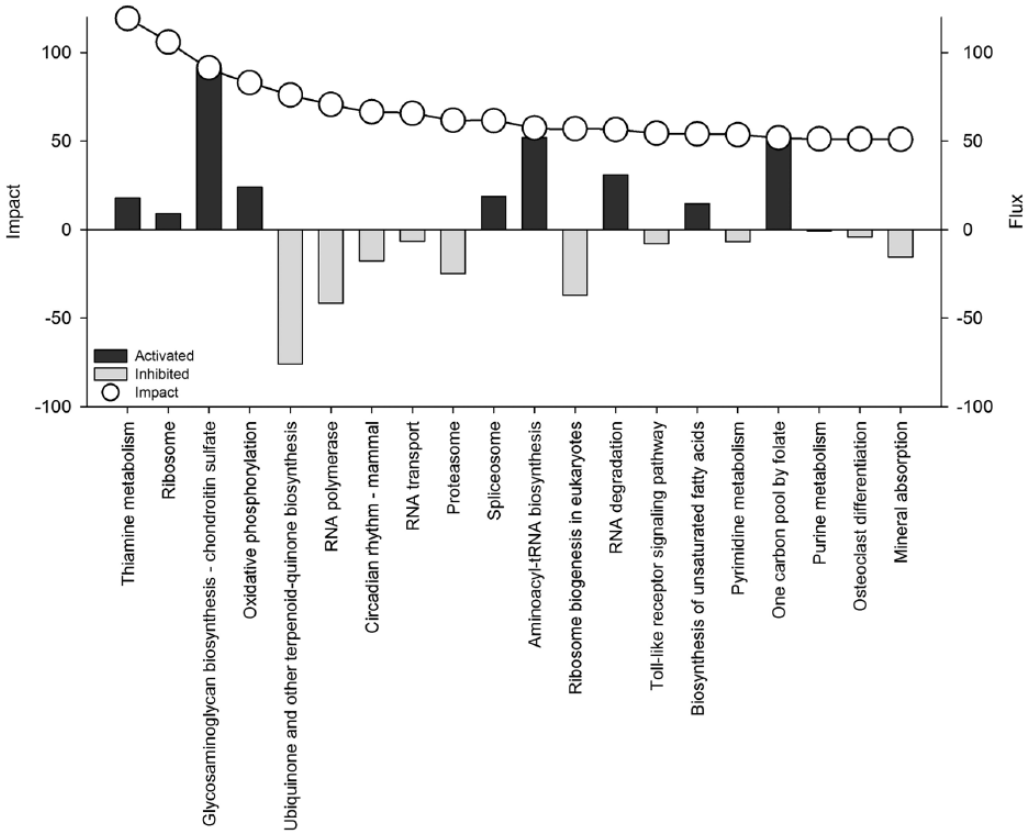

Figure 3. Top 20 affected Kyoto Encyclopedia of Genes and Genomes pathways in polymorphonuclear leukocytes of overfed (OVE) versus control-fed (CON) cows, ranked as such.

speculated above.[29] If this mechanism holds true for bovine, it may help reconcile the high levels of oxidative phosphorylation found here in OVE cows, with previous (and current) evidence of glycolysis as the primary energy production pathway in PMNLs. Because purine metabolism was also altered in this study, purinergic signaling is further explored in the "Nucleotide Metabolism" section below.

Lipid metabolism. Biosynthesis of unsaturated fatty acids was induced in OVE cows compared with CON. This can be partly explained by significant upregulation of stearoyl-CoA desaturase (*SCD*), the enzyme responsible for transforming stearic acid into oleic acid.[30] Once in the circulation, eg, from adipose tissue lipolysis, oleic acid may stimulate degranulation[31] and superoxide production in PMNLs.[32] This indicates that oleic acid signaling could provoke a greater level of PMNL activation in OVE cows, despite abundant evidence in other species that it can be both pro- and anti-inflammatory.[33] More research is needed on signaling action of oleic acid in bovine PMNLs. Further research would also be beneficial in understanding the effects of *SCD* in bovine PMNLs as in rodent liver *SCD* plays a role in lipid oxidation and accumulation.[34]

The upregulation of this pathway could also help describe the increased expression of *ALOX5AP* and *PLA2G4A* postcalving in OVE cows.[18] These enzymes are responsible for freeing arachidonic acid from membrane phospholipids and, further, deriving leukotriene B_4 (LTB4) from the polyunsaturated fatty acid intermediate.[35] Leukotriene B_4 enhances inflammatory processes, including PMNL migration[36] and adhesion,[37] eosinophil recruitment,[38] and generation of ROS.[39] It is noteworthy, however, that LTB4 can also exert prosurvival effects in PMNLs through activation of nuclear factor κB (NF-κB).[40] In this group of cows, *NF-κB* followed a similar gene expression pattern to *ALOX5AP* and *PLA2G4A* through the peripartal period.[18] Therefore, it is possible that unsaturated fatty acids produce more eicosanoids and drive persistent PMNL activation and survival (ie, chronic inflammation) in OVE cows postcalving, even when no clinical disease is present.

Nucleotide metabolism. Both purine metabolism and pyrimidine metabolism were downregulated in OVE versus CON cows. As mentioned above, changes to purine metabolism may be related to the processing of ATP as a chemotactic signal.[27] Because release of ATP is a signal of cellular distress or death, it can induce ROS production, signaling, and migration of immune cells.[41] Both ATP[42] and adenosine[43] have been implicated in delaying neutrophil apoptosis. In this study, suppression of purine metabolism could be an adaptation to conserve ATP and other purinergic signals, feeding the inflammatory state. The affected genes in this pathway support this hypothesis.

Among the downregulated genes are ectonucleotide pyrophosphate (*ENPP1*) and nucleotide triphosphatase (*NTPCR*), both enzymes that hydrolyze purine metabolites (ie, adenosine monophosphate [AMP] to adenosine and ATP

to adenosine diphosphate, respectively) and regulate their signaling capacity.[44] Upregulation of deoxycytidine kinase (*DCK*) and adenosine kinase (*ADK*) is consistent with this immune profile. Deoxycytidine kinase is a salvage enzyme for deoxynucleosides which allows for DNA repair in nonproliferating immune cells. This enzyme is normally less active in PMNLs than in monocytes or lymphocytes,[45] such that its more than 4-fold upregulation in OVE versus CON cows indicates that PMNLs may have had greater DNA repair and survival capacity. In the future, this could be verified by measuring protein expression and/or enzyme activity alongside gene expression.

Adenosine kinase can also affect PMNL function through purine metabolism; it is responsible for creating AMP by phosphorylating adenosine.[46] Therefore, higher expression of *ADK* may indicate reduced adenosine concentrations. Because adenosine has historically been reported as an anti-inflammatory molecule,[47] lower adenosine concentrations would again point to aggravated inflammation. Specifically, the interaction of *ADK* and adenosine has implications in PMNL adhesion and accumulation.[46,48] Although this may seem incongruous with its antiapoptotic effect in PMNLs, Walker et al[43] suggested that apoptosis can be regarded as a neutrophil function.

Pyrimidines may also participate in immune function and signaling, and in this study, pyrimidine metabolism followed a similar pattern to that of purines. The release of uridine triphosphate (UTP) from PMNL and other cell types can stimulate PMNL through the same $P2Y_2$ receptor to a lesser extent than ATP. When added in combination with chemoattractants to human neutrophils, UTP has been shown to augment intracellular calcium and ROS levels, as well as cell aggregation.[49,50] Therefore, it could be expected that, as with ATP, genes that help maintain UTP levels would increase. This appeared to be the case in OVE cows by upregulation of uridine-cytidine kinase (*UCK2*). If OVE PMNLs are, in fact, hyperactivated, production of ROS would damage DNA. Maintaining a pool of both purines and pyrimidines would aid DNA repair and cell survival. This effect is illustrated by upregulated *DCK*, as in purine metabolism, and downregulated deoxycytidine triphosphate pyrophosphatase (*DCTPP*) and ectonucleoside triphosphate diphosphohydrolase (*ENTPD8*).

The above changes indicate a potential accumulation of repair nucleotides and precursors by conserving phosphorylated forms of the bases.[51] Further evidence of potentially increased ROS and DNA damage is indicated by the downregulation of thymidine kinase (*TK2*), a mitochondrial DNA salvage enzyme whose activity is known to be downregulated during oxidative stress.[52] Based on these results, more research on purine and pyrimidine metabolism could be beneficial to clarify the relationship between PMNL activation and survival.

Glycan biosynthesis and metabolism. Chondroitin sulfate (CS) is one of the several glycosaminoglycan chains which modify proteins through glycosylation. After alteration, proteoglycan products are either secreted by the cell or retained in the plasma membrane.[53] Recent evidence revealed that CS in both the extracellular matrix and the membrane may contribute to neutrophil activation.[53]

Recently, the role of free CS on neutrophils has been tested. Incubation of PMNL with CS amplified ROS production in response to interleukin 8 (IL-8).[54] Thus, there is potential that OVE PMNL with increased CS biosynthesis secretes the proteoglycan as a pro-inflammatory autocrine signal. This molecule is also involved in neutrophil-activating pathways from the membrane. The carbohydrate portion of CS forms binding sites on the receptor for platelet factor 4 (PF4), an α-chemokine that promotes exocytosis, even independently of IL-8 or calcium mobilization.[55] In contrast, the sulfate portion of CS has been identified as a binding site for human leukocyte elastase and cathepsin G.[56] Both of these proteases activate cytokines and coordinate neutrophil responses once released from PMNL granules.[57] This study underscores a relationship between CS and the immune status of the cow, such that increased CS biosynthesis corresponded to more activated neutrophils. However, the specific mechanisms of action for CS-moderated responses in bovine neutrophils need further investigation.

Metabolism of cofactors and vitamins. Thiamine metabolism was the most affected pathway in this study. Although thiamine has functions in energy metabolism, eg, as a cofactor in carbohydrate catabolism, it also has antioxidant capacity. The active metabolite of thiamine, thiamine pyrophosphate, is known to protect against oxidative stress in liver,[58,59] cardiac muscle,[60] and ovaries[61] in rats. Thiamine also exerts antioxidant activity directly on neutrophils by inhibiting the peroxidase/H_2O_2/halide system. These data, rather than denoting an inhibition of neutrophil function, are indicative that thiamine increases neutrophil motility by promoting chemoattraction over antimicrobial signals.[62] Thiamine also prevents oxidation of components of PMNL membranes, eg, sulfhydryl groups, by inhibiting the peroxidase system.[63] We speculate that the reduction in cell activation and damage in favor of cell migration through thiamine could be a regulatory mechanism that contributes to PMNL longevity and more chronic inflammation in OVE cows.

One carbon pool by folate was also upregulated in OVE cows. The upregulated genes driving this change encode mitochondrial proteins, methylene tetrahydrofolate dehydrogenase 2 (*MTHFD2*), and mitochondrial methionyl-tRNA formyltransferase (*MTFMT*), indicating that the diet impact on folate metabolism was localized to the mitochondria. *MTHFD2* is instrumental in converting methylene tetrahydrofolate (THF) to formate in the mitochondria. Mitochondrial formate can be transported to the cytosol and used in purine synthesis, thus sparing cytosolic pools of THF for use in transferring 1-carbon groups, the other main function accomplished by cytosolic counterpart *MTHFD1*.[64,65] As discussed above, purine synthesis (and metabolism) is important in modulating the immune response of PMNLs. However, the present data

reveal that OVE PMNLs may experience more 1-carbon transfer through the methionine cycle.[66] This could have broad effects, although it has been speculated that methionine availability in PMNL enhances immune function.[67]

The enzyme encoded by *MTFMT* catalyzes the formylation of methionyl-tRNA, which corresponds to the start codon in mitochondrial protein synthesis.[68] Because formylated methionyl-tRNA is used to initiate synthesis of nearly all mitochondrial proteins, it is hard to correlate the increment in *MTFMT* with a particular outcome. However, it may hint at a more active mitochondria, and therefore, a more active cell. This argument could be strengthened by a deeper analysis of mitochondrial protein expression and activity.

The last cofactor pathway, ubiquinone and other terpenoid-quinone biosynthesis, was downregulated in response to OVE. After some disagreement as to whether ubiquinone is involved in PMNL ROS production,[69,70] it has been demonstrated that ubiquinone, or coenzyme Q_2 is associated with neutrophil granules[71] and can, in fact, have an inhibitory effect on superoxide production via membrane signaling.[72] At the same time, ubiquinone is required in the electron transport chain of mitochondria, which produces ATP and can produce ROS as by-products.[73] Therefore, the inhibition of ubiquinone biosynthesis could have dual effects on superoxide formation. This is further complicated by the fact that oxidative phosphorylation was increased in OVE cows, leading to an expectation that, overall, elements of the respiratory chain would be upregulated, not downregulated. Nevertheless, the affected genes of this pathway are primarily related to production of ubiquinone for oxidative phosphorylation. These contrasting results indicate that more research is needed to evaluate ubiquinone synthesis and use during periods of elevated mitochondrial activity in PMNLs.

Genetic information processing

Transcription and translation. The instinctive relationship between transcription and translation, formed by the central dogma, DNA to RNA to protein, allows for a better view of cellular function when the 2 processes are evaluated together. Therefore, the results of relevant pathways will be interpreted together in the following section.

First, the RNA polymerase pathway was downregulated in OVE cows. Although RNA polymerases are responsible for the transcription of DNA to RNA,[74] this result does not necessarily mean that there was less overall transcription. In eukaryotes, there are 3 distinct RNA polymerases (each with a particular function regarding the different types of RNA, and each of these was affected in this study). Simplistically, polymerase I transcribes ribosomal RNA (rRNA), polymerase II transcribes messenger RNA (mRNA), and polymerase III transcribes transfer RNA (tRNA).[75] Furthermore, these transcription complexes are composed of heterogeneous subunits, which can individually affect the transcription complex; decreases in the availability of any one subunit can prevent complex assembly.[76] Some subunits are conserved between polymerases and/or across species, whereas others contribute to their differential functions.[77] Because the downregulation of the RNA polymerase pathway in this study was driven by changes to the expression of subunit-encoding genes, the specific roles of those genes, and consequences for downstream pathways, will be expanded when possible. Highly affected pathways upstream of RNA polymerase will also be considered when relevant.

Thus far, it seems that the DEG in OVE cows support greater ROS production and immune activation. Under such conditions, these cells may experience a degree of oxidative stress,[78] and previous inquiries into PMNL gene expression revealed transcription-level differences to this effect.[18] Polymerase I activity (ie, synthesis of rRNA) is known to be downregulated under oxidative stress.[79] The only polymerase I gene altered in this pathway was *POLR1D*, which encodes the smaller of 2 α subunits in polymerase I cores.[80] This subunit is essential to polymerase assembly and function.[81] In fact, mutation of this gene in humans causes a disturbance to rRNA transcription and ribosomal biogenesis, characterized by craniofacial defects.[82] Therefore, downregulation of the gene in OVE cows could certainly inhibit function of polymerase I. This is supported by a corresponding downregulation of the ribosome biogenesis in eukaryotes pathway.

Ribosome biogenesis is particularly important for growing and dividing cells to perform efficient protein synthesis.[76] Because neutrophils are terminally differentiated with normally short life spans,[83] this process may simply be less biologically important than in other cell types. However, this does not account for differential expression of relevant genes between OVE and CON cows. Another conflict regarding this idea is that in OVE cows, ribosome biogenesis was downregulated, but the ribosome pathway itself was upregulated. This discordance could reflect a distinction between the activity of cytoplasmic and mitochondrial ribosomes, which are believed to have evolved different functions.[84] Recall that genes from the folate pathway pointed to an increase in formylated methionyl-tRNA, which initiates most mitochondrial protein synthesis.[68] Potentially, mitochondrial proteins or functions (eg, oxidative phosphorylation) become more important in the adaptation to overfeeding, whereas more basic cellular proteins or functions are suppressed.

An analysis of each affected mitochondrial and cytoplasmic gene in the ribosome pathway is beyond the scope of this article. However, one result that stands out is a 15-fold upregulation in mitochondrial ribosomal protein L24 (*MRPL24*). There is currently little research dedicated to the gene or protein it encodes; however, due to the dramatic change in expression brought on by overfeeding, significance of this gene to PMNLs and/or bovine cells should be further investigated. In addition, another noteworthy mitochondrial ribosomal protein, *MRPL18*, was upregulated. It has recently been uncovered that *MRPL18*

induces heat shock protein translation under stressful conditions.[84] Because this could potentially support the hypothesis that OVE PMNLs have increased cell survival, more in-depth research appears warranted.

As argued above, assuming that OVE PMNLs produce more ROS and are exposed to greater oxidative stress, cellular protein synthesis could be downregulated to an extent.[85] In this scenario, subunits of polymerase II (responsible for mRNA transcription) would likely experience some level of transcriptional control themselves. If less mRNAs are being translated into proteins, it would be a cellular waste to continue producing transcription machinery. Upstream mechanisms of this regulation could be multifactorial, but one possibility is that polymerase I repression (represented here by downregulation of *POLR1D*) contributed to feedback signals on polymerase II activity, as Laferte et al[86] discovered.

In this study, 2 polymerase II genes, *POLR2G* and *POLR2L*, were downregulated in OVE cows. The subunit from *POLR2G* participates in the complex which induces a conformational change in polymerase structure, underscoring its importance to transcription initiation.[87] Similarly, the protein encoded by *POLR2L* has been identified as a key protein to yeast cell viability for its role in transcription.[88] If downregulation of this gene can also negatively affect the viability of bovine PMNLs, it may be of interest to consider how and to what extent it can affect the life span of PMNLs, which otherwise seemed to be increased in OVE cows.

As a logical response to lower polymerase II gene expression, OVE cows also experienced suppression of the RNA transport pathway. RNA transport allows mRNA transcribed in the nucleus to be processed and translated later in the cytoplasm.[89] With lower mRNA transcription, subsequent processing steps obviously become less important. Multiple genes downregulated in RNA transport are also eukaryotic translation initiation factors, without which proper assembly of the translation initiation complex is not possible.[90] Along with lower ribosome biogenesis, the flux of this pathway introduces the idea that both transcription and translation are inhibited in OVE cows.

Polymerase III was also critically affected in OVE cows by changes to 2 subunits used in initiation of tRNA transcription: *POLR3C* and *POLR3G*. They comprise 2 parts of a trimer that confers stability to the preinitiation complex and is essential to recruitment of the polymerase to segments of DNA.[91,92] Unlike other changes in RNA polymerase gene expression, these subunits were upregulated in OVE cows. Not surprisingly, there was a corresponding increase in aminoacyl-tRNA biosynthesis. Surprisingly, although these findings are consistent with each other, they are inconsistent with other transcription and translation data.

Cherkasov et al[93] have recently published findings regarding yeast cell cytoplasmic stress granules. Interestingly, some of the findings from stress granules display similarities to the neutrophils in OVE cows, including increases in aminoacyl-tRNA synthetases, as well as proteins involved in ribosome biogenesis and translation. However, it should be kept in mind that sequestering these proteins in heat-stressed yeast makes them less available for cellular functions. Therefore, OVE PMNLs resemble those stressed eukaryotic cells, in that ribosome biogenesis and translation initiation were inhibited (as mentioned above), yet, differ regarding tRNA. Where yeast accumulated more tRNA synthetases in stress granules, and therefore could create fewer tRNA for protein synthesis, OVE PMNLs had increased tRNA synthetase expression, which should increase protein synthesis. Another level of complexity is added when one considers that other transcription and translation-related molecules, eg, polymerase II subunits and initiation factors, were downregulated in OVE cows. It is not clear why regulation of polymerase III and tRNA biosynthesis were not coordinated with other genetic information processing pathways. Whether this could be attributable to differences in mitochondrial and cytoplasmic translation requirements, as postulated for the ribosome pathway, requires further research.

By the same token, upregulation of the spliceosome pathway in OVE cows indicates greater pre-mRNA processing that should facilitate translation. Many small nuclear ribonucleoproteins, Sm proteins, LSm proteins, and 1 heat shock protein, *HSPA1A*, were among the genes upregulated in OVE cows. These components are recruited to form the spliceosome on pre-mRNA molecules, assist in removing introns and ligating exons, then disassemble for recycling.[94] The spliceosome is important for cell viability, an idea which has been demonstrated by studies in human cancer research,[95,96] and fits with the idea that OVE PMNLs have enhanced survival. Nonetheless, it is a surprising result given that mRNA transcription, transport, and even translation seemed suppressed. It is striking that these changes mirror those surrounding ribosome; the reason(s) why major ribonuclear units such as the ribosome and spliceosome are induced in OVE cows, yet closely related pathways do not support their functions, is not known.

Folding, sorting, and degradation. Alternatively, 1 pathway that did concur with spliceosome was RNA degradation. Although increased RNA degradation may normally cause concern for the validity of microarray results derived from RNA, it should be noted that more than 1 gene upregulated in this pathway is an LSm gene, which has already been identified as a spliceosome component.[94] Other upregulated genes such as exoribonuclease *DIS3* act mainly in the nucleus, and, thus, may also play a role in processing premature mRNA.[97] Then, it is reasonable to suggest that RNA degradation may simply account for the cleavage and breakdown of introns or unstable nuclear mRNA, rather than untranslated exons. The high impact on this pathway serves as a reminder that the transcriptome should not be strictly interpreted as actual biological activity without

the supporting proteomic work because many changes occur between the expression of genes and proteins.[98]

Posttranslationally, the cellular profile can be partly controlled by the ubiquitin-proteasome system. Highly specific ubiquitination marks target proteins for degradation by the proteasome to invoke quality control and normal turnover.[99] Because the proteasome can be localized to many cellular locations and is responsible for degrading proteins involved in all aspects of cell growth, signaling, and death,[100] changes in its activity do not speak to one end result. Despite this, the proteasome pathway (as well as the lower affected ubiquitin-mediated proteolysis pathway) was downregulated in OVE cows, which at least suggests that these cells required greater protein conservation. This would be especially important if OVE PMNLs were experiencing decreased rates of protein synthesis. The process of ubiquitination and proteolysis via this system is also ATP-requiring[99,101]; thus, reduced expression of proteasome subunits may occur partly to conserve ATP, an effort that we also detected in nucleotide metabolism pathways in OVE PMNLs.

The impact on proteasome may also relate with previous observations and hypotheses of increased mitochondrial activity and protein synthesis in the PMNL. It was previously thought that individual proteases were more relevant in the mitochondria than the proteasome.[102] Instead, recent experiments by Lehmann et al[103] reveal a high level of ubiquitination on mitochondria-specific proteins and presence of multiple proteasomal components within the mitochondria. If the proteasome does govern more protein turnover in the mitochondria, its downregulation may corroborate the activating effect of OVE on PMNL that leads to prioritization of mitochondrial function.

Organismal systems

Immune system and development. Although it is counterintuitive that osteoclast differentiation should be highly affected in terminally differentiated PMNL, this can be explained by examining the group of genes implicated. The KEGG pathways are species specific but not tissue specific. Many of the osteoclast differentiation genes are involved in cell cycle or growth and differentiation signaling but are also shared by the toll-like receptor (TLR) signaling pathway, where their functions are primarily tied to immunity. Due to the high proportion of similar genes, both pathways had the same overall direction of impact. For this reason, these 2 pathways and their KEGG categories have been combined and will mostly be discussed within context of TLR signaling.

This article has so far emphasized the activation of OVE PMNL, which makes it surprising that the TLR signaling pathway, typically associated with immune activation, was downregulated. Toll-like receptors are transmembrane proteins that recognize pathogen-associated molecular patterns and initiate cascades of pro-inflammatory cytokine signals.[104] In OVE cows, 2 specific TLR genes were downregulated—*TLR4*, which recognizes lipopolysaccharide of gram-negative bacteria[105] and *TLR5*, which recognizes bacterial flagellin.[106] Given the evidence above that OVE PMNLs are more activated, the downregulation of *TLR4/5* implies that this response is pathogen independent and therefore inappropriate. Lower expression of TLR and related signaling molecules may also cause these PMNLs to later be less responsive toward real pathogens, such as the gram-negative bacteria that can cause acute, environmental mastitis, among other diseases.[107] Other pro-inflammatory signaling molecules, downstream of TLR or related networks, were downregulated in both the TLR signaling and osteoclast differentiation pathways. Phosphatidylinositol-4,5-bisphosphate 3-kinase (*PI3KCG*), signal transducer and activator of transcription (*STAT1*), and mitogen-activated protein kinase kinase kinase 7 (*MAP3K7* or *TAK1*) were all dually reported.

PI3KCG encodes a subunit of PI3K that is critical for PIP_3 signaling, chemokinesis, neutrophil accumulation, and ROS production.[108] It is interesting that this key gene is downregulated, impairing inflammation, although the PMNL generally seemed activated. Other pathways that have similar activation but run parallel to PI3K, such as Jak/STAT and MAPK,[109] should be examined as alternate means to achieve PMNL activation. In fact, the PI3K/Akt and Jak/STAT pathways have similar primary functions: inflammation and neutrophil survival,[110] and both alternate pathways are well-represented within the DEG.

Overall, the Jak/STAT cascade seems activated. The osteoclast differentiation pathway reveals a downregulation of suppressor of cytokine signaling 3 (*SOCS3*)—a Jak inhibitor[111]—with concurrent upregulation of *JAK1*. In addition, interferon regulatory factor 9 (*IRFN9*) was upregulated. After activation by Jak1,[112] STAT1 and STAT2 complex with IRFN9 to promote pro-inflammatory transcription due to interferon signaling.[113] Transcription of a different set of immune-related genes can even occur by combination of STAT2 and IRFN9, but in the absence of STAT1,[114] which could potentially explain downregulation of *STAT1* in an activated PMNL profile.

MAP3K7 is central to another complex cascade, mediating the effects of cytokine (eg, IL-1β) and toll-like signals on pro-inflammatory gene transcription by MAPK, JNK, and NF-κB.[115] This particular gene may have a negative regulator function in mouse neutrophils, although this contrasts with data from other immune cells and human PMNL[115,116] and needs to be confirmed in bovine. However, in an inhibitory role, downregulation of *MAP3K7* would tend to fit with patterns of pro-inflammatory activation in OVE cows, including upregulated gene expression of myeloid differentiation primary response 88 (*MYD88*) upstream (in TLR signaling pathway) and partners *RELA* (in both pathways) and *NF-κB*[18] downstream.[117]

Although upregulation of *MYD88* suggests activation of the MyD88-dependent pathway, downregulation of preceding cytokines (eg, *IL1B* and *IL12B*) and receptors (eg, *TLR4,*

TLR5, interferon receptor *IFNAR2*, and transforming growth factor β receptor *TGFBR2*) may indicate alternative ways of stimulation for this pathway. Saturated fatty acids, eg, are known to stimulate pro-inflammatory signaling through TLRs,[118,119] and this mechanism is exacerbated by ROS production.[120] In OVE cows with higher serum nonesterified fatty acids and ROS,[17] saturated fatty acids could very well be a part of pathogen-independent activation.

At some point, negative feedback may act to limit inflammation and prevent stress-induced PMNL apoptosis in OVE cows.[121] Upregulation of NF-κB inhibitor (*NFKBIA*; in both pathways) could be one example of how and where this feedback influences NF-κB–induced inflammation. With downregulation of most protein-management systems such as proteasome, this feedback likely results in more important transcription-level changes, emphasizing the relevance of the transcriptome seen here.

Digestion. Mineral absorption was downregulated in OVE cows. Due to its importance in neutrophilic responses, this section will focus on calcium (Ca^{2+})-related effects. Free Ca^{2+} signaling is central to PMNL functions such as chemotaxis, degranulation, and ROS production.[122] It is also closely related to mitochondrial ATP production and purinergic signaling,[29] pathways already recognized for their importance to OVE cows.

Ca^{2+} release from intracellular stores is stimulated by chemokine and G protein–coupled receptor signaling.[123] When Ca^{2+} is released, a new influx is required to replenish stores.[124] In neutrophils, this influx occurs through the transient receptor potential family of transporters,[125] where TRPM7 is the primary transporter in mammals.[122] Downregulation of *TRPM7* and *TRPV6* indicates a lower capability to take up Ca^{2+}, and therefore, lower potential for Ca^{2+}-mediated immune responses. This contrasts with previous results, especially those of metabolic pathways, which strongly support the idea that OVE cows have more active PMNL. A couple of possibilities exist to resolve this disagreement. First, that the microarray provides a snapshot of the entire transcriptome at just 2 time points. As with any other signaling pathway, changes captured could represent activated functions of the pathway as well as negative feedback. Downregulation of Ca^{2+} channel expression could be another example of feedback meant to limit inflammation in a chronically activated system. Another possibility is that there is, in fact, greater PMNL activation before calving (as suggested by Zhou et al[18]), but that the downregulation of mineral transporters here represents a stronger postpartum effect of Ca^{2+} partitioning to the mammary gland. The demand for Ca^{2+} during early lactation is such that all intact cows developed postpartum hypocalcemia in one study comparing mastectomized versus intact cows.[126] The same study also concluded that metabolic demands of the mammary gland contributed to general loss of immune cell functions. The specific inhibitory effect of parturition (ie, induction of lactation) on Ca^{2+} signaling in monocytes has also been documented.[127] Therefore, genes relevant to Ca^{2+} signaling in PMNL may be better considered from a time effect or interaction perspective as well.

Environmental adaptation. Circadian rhythm was downregulated in OVE cows. Although this may seem peripheral to immediate immune function, it is suggested that circadian rhythm actually plays a role in the function of bovine neutrophils.[128] Thus, this pathway could help explain the changes in OVE PMNL behavior. Two key regulating factors to circadian rhythm are the cryptochrome (CRY) and period (PER) proteins. These 2 proteins dimerize and provide negative feedback for their own cyclic gene transcription.[129] The level of activity from these proteins is tightly controlled by phosphorylation, dephosphorylation, and ultimately proteolysis.[129,130] In OVE cows, cryptochrome gene *CRY1* was downregulated, whereas E3 ubiquitin ligase component *RBX1* was upregulated. This combination suggests that there was an inhibition of the circadian rhythm in the PMNL. Regulatory gene casein kinase 1ε (*CSNK1E*) was also downregulated, suggesting that there was less phosphorylation (ie, inhibition) to PER[131] and some balance to control of the system even as it was altered. However, as PER does not act without CRY, and as there are additional levels of control by phosphorylation, it is likely that the system was still shifted. Dysregulation of circadian rhythm could, thus, be one contributor to abnormal PMNL function, and it would be useful to elucidate the mechanism by which OVE interacts with this system. This potentially has great importance to the transition cow because the dry period is already associated with photoperiod manipulation, which can also influence the immune system and circadian rhythm.[129,132]

Conclusions

This study investigated whether dietary energy intake in the dry period affected immune function, as represented by the neutrophil transcriptome. Both metabolic and nonmetabolic PMNL functions were highly affected by the difference in energy level. Together, the above results portray that feeding higher energy diets in the close-up period may prolong PMNL life span and heighten nonpathogenic inflammation. With more activated and longer circulating immune cells, it is likely that OVE cows experience some degree of chronic inflammation. This confirms results from a previous target gene expression study (using RT-PCR) of the same cows, and other experiments which document poorer health outcomes in overfed transition cows.

Although no clinical disease or production differences were detected in these cows, chronic inflammation may result in subclinical or long-term health conditions that should be considered. In addition, this study has highlighted a few topics which would benefit from further examination. Of specific interest to the immune function of transition cows might be the expanding role of the mitochondria in active PMNL,

purinergic signaling, and the contrasting results for translation-related and TLR-related genes and pathways, including how they contribute to cell survival. Generating appropriate data will require more bovine-specific and neutrophil-specific work because many differences may exist between bovine PMNL and yeast or human line cells, which mostly contribute to current literature relevant to the KEGG pathways. Future research on these specific topics as well as more cow-level outcomes would confirm whether overfeeding creates any immune or metabolic disadvantage to the transition cow. If true, controlling or restricting dry period, dietary energy may be better justified, from a holistic standpoint, and nutrition trials will be useful to pinpoint more ideal energy levels.

Author Contributions

JJL designed the cow study. MJK, DEG and MVR performed the experiments, gene function and pathway analysis. AA performed gene function and pathway analysis, interpreted the data, and wrote the manuscript. SLZ performed statistical analysis of the transcriptome data. JSO and JJL participated in data interpretation and revision of the paper. All authors reviewed and approved the final version of the manuscript.

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