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Multiomic analysis highlights differences between negative and positive energy balance in Holstein cows

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Introduction

Negative energy balance (NEB) in the transition from gestation to lactation, a consequence of inadequate feed intake and increased energy demands, results in an elevated risk of metabolic disease (eg ketosis) and reduced reproductive performance in dairy cows (LeBlanc *et al.,* 2006). However, it is not feasible to generate a large training population where NEB is measured directly. Multiple studies have demonstrated that multi-omics data can be used to rank variants according to their impact on complex traits (Xiang *et al*., 2019 and 2025) and validated that these variants increase the accuracy of genomic predictions (eg. Xiang *et al*., 2019, Khansefid *et al*., 2020) or explain large proportions (~70%) of the heritability of complex dairy traits (Xiang *et al*., 2023). As regulation of gene expression is dynamic and often tissue specific, increased effectiveness is expected from multi-omic datasets using samples that express the phenotype of interest. Therefore, this study contrasted large numbers of multi-omic measurements, as traits correlated with NEB, to gain biological insight into transition cow health and identify genomic variants associated with NEB.

Materials & Methods

Sample collection and Multi-omics. Research was approved by the DEECA Animal Ethics Committee (application 2022-04). 374 spring-calving Holstein cows were enrolled over 2 years. Stage of lactation was used as a proxy for energy balance. Blood samples were taken at 5-14 days in milk (DIM), to represent the period of greatest NEB and 65-80 DIM, where all cows were expected to be in positive energy balance (PEB) but not yet pregnant. Cows were aged: 2-11 years; parity: 1-9; average daily milk production: 33 L. Blood samples were collected via coccygeal venipuncture into 1 serum and 1 EDTA tube (BD Vacutainer System). Serum was separated by centrifugation and frozen at -80°C. Whole blood was added to RNAprotect animal blood tubes (Qiagen) and frozen at -80°C. *Biochemistry:* Serum samples were analysed for biomarkers previously associated with animal health; bilirubin, glucose, non-esterified fatty acids (NEFA), total protein, blood urea nitrogen (BUN), cholesterol, triglycerides, beta-hydroxybutyrate (BHBA), albumin (A), calcium and magnesium on a ChemWell® 2910 Automated EIA and Chemistry Analyzer (Awareness Technology, Inc.). Globulin (G) concentration was the difference between albumin and total protein concentrations, and the A:G ratio calculated. *Transcriptomics:* For 327 NEB and 296 PEB samples RNA was extracted from blood using RNeasy Protect Animal Blood Kit (Qiagen). Individually barcoded RNA sequence libraries prepared using NEXTFLEX Rapid Directional RNA-Seq Kit 2.0 (Revvity) were sequenced on a DNBSEQ-G400 (MGI Tech, 2x150bp) DNA sequencer generating ~50M reads per library. Adapter sequences trimmed and reads aligned (STAR v2 dual-pass mode, Dobin *et al.,* 2013) to bovine reference genome (ARS-UCD2.0, Rosen *et al.,* 2020). Duplicate reads removed using Picard MarkDuplicates. Gene counts

generated with featureCount function of SubReads (Liao *et al*., 2014). *Metabolomics:* 100ul of serum was combined with MeOH (300uL), 0.25ug/mL L-valine, 10ug/mL D-sorbitol and 5ug/mL succinic acid internal standard, vortexed, centrifuged (13,000 rcf 10 min 4°C) and an aliquot transferred to a HPLC vial. LC-MS parameters were as per Vasiliadis *et al*. (2019) except extracts were analysed in positive and negative electrospray ionization mode. Using Genedata Expressionist® 18.0.1 MS Refiner chromatogram chemical noise subtraction, grid, chromatogram RT alignment, chromatogram peak detection, chromatogram isotope clustering and singleton filtering was undertake to obtain a cluster list of features. *Lipidomics:* Serum sample preparation, lipid identification and quantification was described by Liu *et al*. (2025). Milk sample preparation, lipid identification and quantification was described by Liu *et al.* (in press).

Data Analysis. Missing metabolite values were imputed with the k-nearest neighbour method (Runmin *et al*., 2018). All measurements were log-transformed to normalize. Gene counts were normalised for read depth using Edge R (Chen *et al*., 2025). Differential abundance (DA) was undertaken for biomarker, gene, metabolite and lipid counts contrasting time point 1 (NEB) and 2 (PEB) fitting fixed effects of parity, age, cohort, haemolysis and fibrin clot in samples with Limma (Ritchie *et al*., 2015) where significance thresholds were false discovery rate <0.05 and fold change > 0.2. All animals were genotyped using a custom 50K Beadchip (Illumina Inc) and imputed to whole genome sequence using Beagle v5.2 following methodology of Nguyen *et al.* (2025). 49,114,602 variants were imputed across autosomes and the non-pseudoautosomal region of the X chromosome. Genotypes with Beagle imputation accuracy ≥ 0.9 and minor allele frequency > 0.05 were retained. Genome Wide Association Study (GWAS) were undertaken for each molecular phenotype using phenotypes from all animals at 1) time point 1 (n=351, NEB), 2) time point 2 (n=351, PEB), 3) using the difference between time point 1 and 2 for each animal (n=351, CH). GWAS were performed using mlma function of GCTA (Yang *et al.,* 2011) fitting parity, age, cohort, haemolysis and formation of fibrin clot as fixed effects. A five-fold cross validation prediction of subclinical ketosis (BHBA) using each serum and milk lipid (corrected for fixed effects) at NEB was conducted using a simple linear regression model using 4 folds as training to predict 5th fold. Significant ($p<0.05$) predictors in 3 of 5 folds were combined into a single prediction. A five-fold Lasso multiple regression was also used to select serum and milk lipids using NEB and PEB as outcome variables selecting lipids significant in 3 of 5 folds. Accuracy of prediction was the correlation (r) between actual and predicted BHBA.

Results & Discussion

Differential abundance analyses revealed which molecular phenotypes differed in abundance between cows in NEB and PEB stages of lactation. This in turn indicated which cellular processes are being affected by (consequence), or affecting (cause) energy balance. GWAS tested which of the millions of genetic variants in the bovine genome were associated with, and possibly regulating, the expression of these molecular phenotypes. All datasets were able to differentiate samples in NEB and PEB. Having analysed over 22,000 molecular phenotypes we have found more than 11,000 were significantly differentially abundant (Figure 1). Among all NEB, PEB and CH phenotypes 32,675 had h^2 estimates >0.1 and 5,237 were associated with genetic variation in the bovine genome (Table 1).

Six of 13 health biomarkers were significant (adj.p<0.05) DA (Table 1). Results indicate the selected time points did in fact correspond to NEB (time point 1) and PEB (time point 2). Cholesterol, NEFA, and BHBA are directly related to the mobilization of fat, metabolism and energy homeostasis, while Bilirubin is a prominent indicator of liver function. NEFA are converted to BHBA in the liver to use as an energy source during NEB. Decreased levels of

NEFA and BHBA at the 2nd time point indicates that cows were no longer in NEB. This combined with the increase in triglyceride level and low levels of bilirubin confirm that the cows were in fact in PEB at the 2nd time point.

Table 1. Summary of the molecular phenotypes tested in differential and GWAS analysis listing the number of phenotypes tested for each molecule type, significantly differentially abundant between NEB and PEB, with h^2 greater than 0.1 and significant QTL detected in negative (QTL_{NEB}) or positive (QTL_{PEB}) energy balance stages as well as with significant $QTL_{CH,}$ where the phenotype is the change in abundance between NEB and PEB. The number of phenotypes that were DA and had QTL_{NEB} QTL_{PEB} and QTL_{CH} are also reported.

Molecule type	Biomarker	Gene	Metabolite	Serum Lipid	Milk Lipid
Phenotypes	13	14,104	6,505	165	164
Differentially abundant	6	3,406	3,100	145	145
h^2 >0.1 NEB	8	8,484	3,200	162	140
h^2 >0.1 PEB	7	10,027	3,656	164	124
h^2 >0.1 CH	3	4,745	1,745	142	68
QTL_{NEB}	1	1,583	381	146	0
QTL_{PEB}	1	2,193	289	60	285
QTL_{CH}	0	33	151	13	103
DA and with QTL	1	1,012	591	188	83

Of particular interest are the phenotypes that are both DA but are also heritable and associated with variation in the genome. Such molecules provide biological insight to improve management of NEB and their associated genetic variants an opportunity to develop genomic predictions to improve transition cow health. Cholesterol was differentially abundant and NEB and PEB levels were associated with a QTL on chromosome 11 (67-81 MB, Figure 1A). This QTL region contains *ApoB*, which encodes part of a lipoprotein complex and helps transport cholesterol from the liver to tissues. A mutation has been reported in this gene that was associated with low cholesterol levels in serum (Gross *et al*., 2019) and increased juvenile mortality (Kipp *et al*., 2016). 1,012 genes, 591 metabolites, 188 serum and 83 milk lipids were also DA and had significant QTL (Table 1). Half of the DA lipids belonged to the lipid class phosphatidylcholine which are an integral part of cellular and organelle membranes and have a crucial role in homeostasis of cholesterol and other lipids (Lagace 2015). They are also involved in regulation of inflammatory response (Treede *et al.,* 2007) and the inflammatory process during the peri-parturient period can result in reduced feed intake (Kuhla 2020) which contributes to NEB in dairy cows. Phosphatidylcholine phosphate (PC-P:33:2) was DA with 1.5-fold increased abundance at NEB. At NEB it had a h^2 of 0.27 (SD 0.11) and a QTL was detected on chromosome 18 (Figure 1B). Located within this QTL region is *FCAR*, a gene involved in regulation of inflammatory response (Mkaddem *et al*., 2019). The gene *PRDM16* was DA with 3-fold increase in abundance at PEB and h2 of 0.52 (SD 0.15). It is a transcription factor involved in numerous cellular processes including lipid metabolism, regulation of mitochondrial dynamics, glucose homeostasis, and control of oxidative stress (Jiang *et al.,* 2020) and has been flagged as a promising therapeutic for obesity and diabetes in human because of it's key role in adipose tissue metabolism. While the gene is located on chromosome 1 it has suggestive (p<9x10e-6) trans QTL_{NEB} on chromosome 8 and QTL_{PEB} on chromosome 5 in line with it's transcription factor function.

Using all serum and milk lipids individually to predict subclinical ketosis (BHBA) resulted in 65 serum and 69 milk lipids with significant predictability in the simple linear regression while

17 lipids were significant with Lasso selection. Combining all significant lipids in a single prediction resulted in an accuracy to 0.46 (SD 0.05) for serum and 0.35 (SD 0.05) in milk. With Lasso selected lipids the accuracy was 0.28 (SD 0.07) in serum and 0.41 (SD 0.07) in milk. Though only moderate, further health and fertility traits (preferably clinical disease) need to be evaluated as well as other molecular phenotypes.

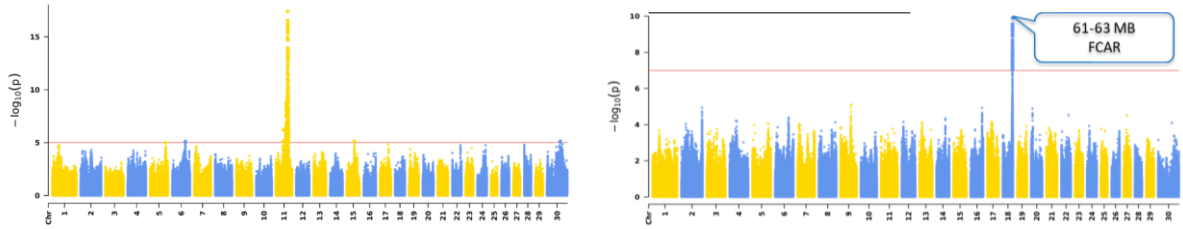

Figure 1. Manhattan plots from GWAS of A) Cholesterol at NEB, B) Phosphatidylcholine Phosphate (PC-P:33:2) at NEB. Red line indicates significance threshold ($p<10^{-7}$).

Conclusions

With more than 11,000 phenotypes DA between NEB and PEB stages of lactation these results indicate this is a rich dataset for the identification of molecules involved in mobilization of fat, metabolism and energy homeostasis. More than 1800 phenotypes are also heritable and associated with genetic variation. Further work is required to validate findings and test the utility of these novel phenotypes in improving transition cow health as predictors of transition disease and their associated genetic variants for improving genomic predictions for health and fertility traits.

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