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Gene Regulation and Systems Biology

Overfeeding Dairy Cattle During Late-Pregnancy Alters Hepatic PPARα-Regulated Pathways Including Hepatokines: Impact on Metabolism and Peripheral Insulin Sensitivity

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ABSTRACT: Hepatic metabolic gene networks were studied in dairy cattle fed control (CON, 1.34 Mcal/kg) or higher energy (overfed (OVE), 1.62 Mcal/kg) diets during the last 45 days of pregnancy. A total of 57 target genes encompassing PPARα-targets/co-regulators, hepatokines, growth hormone (GH)/insulin-like growth factor 1 (IGF-1) axis, lipogenesis, and lipoprotein metabolism were evaluated on −14, 7, 14, and 30 days around parturition. OVE versus CON cows were in more negative energy balance (NEB) postpartum and had greater serum non-esterified fatty acids (NEFA), β-hydroxybutyrate (BHBA), and liver triacylglycerol (TAG) concentrations. Milk synthesis rate did not differ. Liver from OVE cows responded to postpartal NEB by up-regulating expression of PPARα-targets in the fatty acid oxidation and ketogenesis pathways, along with gluconeogenic genes. Hepatokines (fibroblast growth factor 21 (*FGF21*), angiopoietin-like 4 (*ANGPTL4*)) and apolipoprotein A-V (*APOA5*) were up-regulated postpartum to a greater extent in OVE than CON. OVE led to greater blood insulin prepartum, lower NEFA:insulin, and greater lipogenic gene expression suggesting insulin sensitivity was not impaired. A lack of change in *APOB*, *MTTP*, and *PNPLA3* coupled with upregulation of *PLIN2* postpartum in cows fed OVE contributed to TAG accumulation. Postpartal responses in NEFA and FGF21 with OVE support a role of this hepatokine in diminishing adipose insulin sensitivity.

KEYWORDS: nutrition, nuclear receptor, obesity, lactation

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Background

During late-pregnancy through the first 3-week of lactation, dairy cows are most susceptible to metabolic disorders associated with negative energy balance (NEB).[1] The NEB leads to mobilization of long-chain fatty acids (LCFA) stored in adipose tissue, causing a striking increase in blood concentration of non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHBA).[1] Although NEFA can be utilized as an energy source by other tissues, the liver is the most important site for its removal from the bloodstream.[1] In liver, NEFA can be oxidized via β-oxidation to produce adenosine triphosphate (ATP) and ketone bodies (eg, BHBA) or be esterified to triacylglycerol (TAG), which if excessive can become a potential burden for proper liver function.[1]

In non-ruminants, several transcription factors (TF) and their target enzymes/proteins control lipid metabolism in liver, and numerous studies clearly indicate that peroxisome proliferator-activated receptor α (PPARα, gene symbol *PPARA*) is one of the key hepatic TF, particularly during high-fat feeding or under-nutrition which leads to NEB.[2,3] Among the most important metabolic functions coordinated by PPARα are LCFA uptake, intracellular activation, oxidation, and ketogenesis.[3] Similar to the expression of its target genes involved in lipid metabolism (*CPT1A*, *ACOX1*, *HMGCS2*, *ACADVL*),[4]

the expression of *PPARA* in bovine liver often increases from late-pregnancy to early lactation.[5] Some studies have also reported up-regulation after calving of *PPARA* target genes without any change in *PPARA* expression.[6,7] Data from non-ruminants have demonstrated that activation of PPAR does not necessarily involve an increase in its mRNA but rather that of its target genes. Thus, they can serve as proxy for the function of PPAR in tissues such as liver.

A novel role of PPARα uncovered in recent years is the induction of fibroblast growth factor 21 (FGF21),[8] which appears to be essential for activation of hepatic ketogenesis. In non-ruminants, this "hepatokine" along with angiopoietin-like 4 (ANGPTL4)[9] are secreted from liver as a result of PPARα activation during fasting[10] and high-fat feeding.[7] In *β*-klotho (KLB)-expressing tissues such as liver and white adipose tissue,[11] the signaling response to FGF21 requires a functional KLB as a co-receptor to augment its binding to FGF receptors (FGFR1–4). Additional functions of FGF21 in non-ruminants are to block growth hormone (GH) signaling in liver, thus decreasing insulin-like growth factor 1 (IGF-1)[12] and compromising cellular growth[13] specifically by decreasing the phosphorylation of signal transducers and activators of transcription 5 (STAT5) and the expression of *IGF1*.[12] FGF21 also can diminish insulin sensitivity in adipose tissue.[14]

Although few data on the functional importance of PPAR activation in ruminants are available, a recent review underscored the importance that the PPARα signaling network might help in coordinating metabolic adaptations in bovine liver during the transition from late-pregnancy to lactation.[15] Furthermore, one of the "peculiarities" of the ruminant liver is that it is not a lipogenic tissue, but earlier data demonstrated that the activity of some lipogenic enzymes could be increased in response to high dietary carbohydrate.[16]

Our hypothesis was that prepartal dietary energy affects energy balance (EBAL) status and liver and tissue biomarkers of EBAL with a consequent change in the hepatic gene expression of metabolic genes and particularly PPARα-regulated targets. The specific objectives were to measure concentrations of metabolites and hormones in blood, lipid composition in liver tissue, and gene expression of 57 target genes encompassing PPARα-targets and co-regulators, hepatokines, GH/IGF-1 axis-related proteins, lipogenic proteins, receptors involved in FGF21 signaling, and proteins involved in TAG synthesis and lipoprotein metabolism.

Methods

Animals and treatments. All procedures were performed under protocols approved by the University of Illinois Institutional Animal Care and Use Committee (protocol 06145). Details of the experimental design have been published previously.[17,18] Briefly, 12 (n = 6/dietary group) out of 40 Holstein cows in their second or greater lactation were selected for this study. One group of cows was assigned to a control (CON) high wheat-straw diet, ie, ~41.9% of total ingredients, that was fed for ad libitum intake to supply at least 100% of calculated net energy for lactation (NE_L, 1.34 Mcal/kg of dry matter (DM); Supplementary Table 1). Another group of cows was fed a moderate-energy diet (overfed (OVE), 1.62 Mcal/kg of DM) with corn silage as the major dietary component, ie, ~50.3% of total, to supply >140% of calculated NE_L requirements during the entire dry period (~45 days). Diets were fed as a total mixed ration (TMR) once daily (0600 hours) using an individual Calan (American Calan, Northwood, NH, USA) gate feeding system during the dry period or in open individual managers during lactation. From parturition to 30 days in lactation, all cows were fed the same diet.[16,17] Calculation of EBAL, sampling of feed ingredients, TMR for composition analyses, housing of cows pre- and postpartum, and also details on measurements of body weight (BW), body condition score, milk production, and milk composition were as described previously.[17,18]

Blood biomarkers. Blood was sampled from the coccygeal vein or artery on days (±3) –14, –5, –2, –1, 0, 1, 2, 5, 7, 10, 14, and 21 relative to parturition. Samples were collected at 1200 hours into evacuated serum tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) containing either EDTA or lithium heparin for plasma and a clot activator for serum. After blood collection, tubes with EDTA and lithium heparin were placed on ice whereas tubes with clot activator were kept at ~37 °C until centrifugation (~30 minutes). Serum and plasma were obtained by centrifugation at 1,900 × *g* for 15 minutes. Aliquots of serum and plasma were frozen (–20 °C) until further analysis. Measurements of NEFA, BHBA, glucose, and insulin were performed using commercial kits in an auto-analyzer or by radioimmunoassay (RIA) at the University of Illinois Veterinary Diagnostic Laboratory (Urbana, IL, USA). TAG was measured using a commercial kit (LabAssay™ Triglyceride, Wako Chemicals Inc.) following the manufacturer's protocol. FGF21 was determined using a commercial ELISA kit validated for bovine (BioVendor Research and Diagnostic Products). Details of the validation of the FGF21 ELISA kit for bovine are reported in the supplementary file (Supplementary Figure 1).

Liver tissue. Liver was sampled via puncture biopsy, as described by Dann et al.,[19] from cows under local anesthesia at approximately 0700 hours on days (±3) –14 and 7, 14, and 30 relative to parturition. Liver tissue was frozen immediately in liquid nitrogen and stored until further analysis for contents of total lipids and TAG[19] or used for RNA extraction.

RNA extraction, quantitative PCR (qPCR), and primer design. Details of these procedures are reported in the supplementary file. Briefly, RNA samples were extracted from frozen liver tissue using established protocols in our laboratory.[9] For qPCR, cDNA was synthesized using 100 ng RNA, 1 μg dT18 (Operon Biotechnologies, Huntsville, AL, USA), 1 μL 10 mmol/L dNTP mix (Invitrogen Corp., CA, USA), 1 μL random primer $p(dN)_6$ (Roche®, Roche Diagnostics GmbH, Mannheim, Germany), and 10 μL DNase/RNase free water. qPCR was performed using

Table 1. Genes selected for transcript profiling in bovine liver.

GENE NAME	HUGO GENE SYMBOL
ATP-binding cassette, sub-family D (ALD), member 1	*ABCD1*
Abhydrolase domain containing 5	*ABHD5*
Acetyl-CoA carboxylase-α	*ACACA*
Acyl-CoA dehydrogenase, very long chain	*ACADVL*
Acyl-CoA oxidase 1, palmitoyl	*ACOX1*
Angiopoietin-like 4	*ANGPTL4*
Apolipoprotein A-V	*APOA5*
Apolipoprotein B	*APOB*
Coactivator-associated arginine methyltransferase 1	*CARM1*
Carnitine palmitoyltransferase 1 A	*CPT1A*
Carnitine O-acetyltransferase	*CRAT*
Carnitine O-octanoyltransferase	*CROT*
Citrate synthase	*CS*
Cytochrome b5 type A (microsomal)	*CYB5A*
Diacylglycerol O-acyltransferase homolog 1	*DGAT1*
Diacylglycerol O-acyltransferase homolog 2	*DGAT2*
Electron-transfer-flavoprotein, beta polypeptide	*ETFB*
Electron-transferring-flavoprotein dehydrogenase	*ETFDH*
Fatty acid binding protein 1	*FABP1*
Fibroblast growth factor 21	*FGF21*
Fibroblast growth factor receptor 1	*FGFR1*
Fibroblast growth factor receptor 2	*FGFR2*
Fibroblast growth factor receptor 3	*FGFR3*
Fibroblast growth factor receptor 4	*FGFR4*
Growth hormone receptor	*GHR*
Glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1	*GPIHBP1*
3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	*HMGCS2*
Insulin-like growth factor 1 (somatomedin C)	*IGF1*
Insulin-like growth factor binding protein, acid labile subunit	*IGFALS*
Insulin-like growth factor binding protein 1	*IGFBP1*
Klotho beta	*KLB*
Lipin 1	*LPIN1*
Malate dehydrogenase 2, NAD (mitochondrial)	*MDH2*
Malonyl-CoA decarboxylase	*MLYCD*
Mediator complex subunit 1	*MED1*
Microsomal triglyceride transfer protein	*MTTP*
Methylmalonyl-CoA mutase	*MUT*
Nuclear receptor coactivator 1	*NCOA1*
Nuclear receptor coactivator 3	*NCOA3*
Nuclear receptor corepressor 2	*NCOR2*
Nuclear receptor interacting protein 1	*NRIP1*
Patatin-like phospholipase domain containing 3	*PNPLA3*
Pyruvate dehydrogenase (lipoamide) alpha 1	*PDHA1*
Pyruvate dehydrogenase kinase, isozyme 4	*PDK4*
Perilipin 2	*PLIN2*
Peroxisome proliferator-activated receptor alpha	*PPARA*
Peroxisome proliferator-activated receptor delta	*PPARD*
Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	*PPARGC1A*
Phosphoenolpyruvate carboxykinase 1 (soluble)	*PCK1*
Propionyl CoA carboxylase, alpha polypeptide	*PCCA*
Pyruvate carboxylase	*PC*
RAR-related orphan receptor A	*RORA*
Retinoid X receptor, alpha	*RXRA*
Stearoyl-CoA desaturase	*SCD*
Sterol regulatory element binding transcription factor 1	*SREBF1*
THRSP thyroid hormone responsive	*THRSP*
Trimethylguanosine synthase 1	*TGS1*

4 μL diluted cDNA (dilution 1:4) combined with 6 μL of a mixture composed of 5 μL 1 × SYBR Green master mix (Applied Biosystems, CA, USA), 0.4 μL each of 10 μM forward and reverse primers, and 0.2 μL DNase/RNase free water in a MicroAmp™ Optical 384-Well Reaction Plate (Applied Biosystems, CA, USA). Primers (Supplementary Table 2 and Table 3) for the genes selected (Table 1) were designed using Primer Express 2.0 or 3.0 with minimum amplicon size of 80 bp (when possible amplicons of 100–150 bp were chosen) and limited 3′ G+C (Applied Biosystems, CA, USA). Efficiency of PCR amplification for each gene was calculated using the standard curve method ($E = 10^{(-1/\text{slope})}$) (Supplementary Table 4). The final data were normalized using the geometric mean ($V2/3 = 0.20$) of ubiquitously expressed transcript (*UXT*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and ribosomal protein S9 (*RPS9*).

Statistical analysis. The MIXED procedure of SAS (SAS Institute, Inc., Cary, NC, USA) was used for statistical analysis. The fixed effects included treatment (CON or OVE), day (–14 and 7, 14, and 30 relative to parturition), and interaction of day and treatment. The covariate structure used was AR(1). Data were normalized by logarithmic transformation before statistical analysis. All means were compared using the PDIFF statement of SAS (SAS Institute, Inc., Cary, NC, USA). Significant difference was declared at $P < 0.05$ and tendency at $P < 0.10$.

Results

Dry matter intake, milk production, and EBAL. Results of dry matter intake (DMI), milk production, and EBAL are shown in Figure 1. We observed a gradual decrease

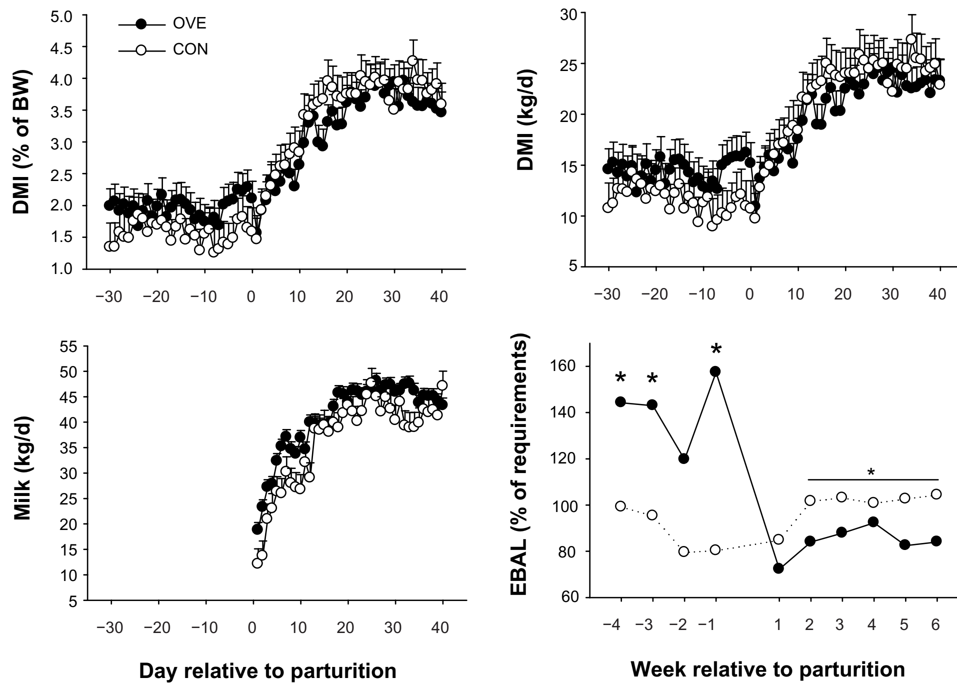

Figure 1. Pattern of daily dry matter intake (DMI) as a percentage of body weight (BW) or kg per day, milk yield, and estimated weekly energy balance (EBAL) in cows (n = 6/diet) fed a control diet (CON; 1.34 Mcal/kg of DM) or a moderate energy diet (OVE; 1.62 Mcal/kg of DM) during the entire dry period.
Notes: *D × T $P < 0.05$.

(day $P < 0.05$) in DMI during the last week prepartum and then a consistent increase after the first week postpartum ("calving") (OVE and CON). Cows in OVE exceeded calculated energy requirements during the prepartal period (−4 to −1 weeks relative to parturition). During this period, the OVE group was in significantly greater [day × treatment ($D \times T$) $P < 0.05$] EBAL (~150%) compared with the CON group (~80%) (Fig. 1). However, both groups were in NEB one week after calving with a large drop in the OVE group. The CON group was able to meet ~100% EBAL by about two weeks post-calving with a gradual increase in DMI but the OVE group remained in NEB even after six weeks postpartum. Milk production was recorded up to five weeks after parturition at which point both groups were producing similar amounts of milk (Fig. 1).

Blood biomarkers and liver tissue composition. Serum NEFA and BHBA concentrations increased ($D \times T$ $P < 0.05$) in the OVE group around parturition and remained high through one week postpartum (Fig. 2). Compared with the OVE group, no changes

Table 2. mRNA expression of FGF receptors and growth hormone signaling-related genes in cows (n = 6/treatment) fed a control diet (CON; 1.34 Mcal/kg of DM) or a moderate-energy diet (OVE; 1.62 Mcal/kg of DM) during the entire dry period.

		DAY RELATIVE TO PARTURITION					*P*-VALUE		
GENE	DIET	−14	7	14	30	SEM	D	T	D × T
FGFR1	OVE	0.21	−0.36	−0.17	−0.14	0.34	0.01	0.13	0.23
	CON	−0.14	−1.25	−0.81	−0.67				
FGFR2	OVE	0.25	−0.08	−0.04	−0.04	0.22	0.01	0.11	0.08
	CON	−0.13	−0.64	−0.59	−0.09				
FGFR3	OVE	0.39	0.18	0.30	0.42	0.30	0.46	0.20	0.63
	CON	0.06	−0.09	−0.16	−0.20				
FGFR4	OVE	−0.11[a]	−1.27[b]	−1.21[b]	−1.22[b]	0.27	0.01	0.18	0.01
	CON	−1.06	−1.01	−1.48	−1.62				
KLB	OVE	0.12[a]	0.18[a]	0.04[ab]	−0.23[b]	0.20	0.29	0.73	0.01
	CON	−0.17[a]	0.08[ab]	0.24[b]	0.24[b]				

Notes: D = day effect. T = treatment effect. D × T = day × treatment interaction. [a–c]Means within a row with different superscripts differ ($P < 0.05$).

were observed in NEFA and BHBA concentrations in the CON group during the transition period. It is noteworthy that the serum concentrations of TAG and FGF21 were greater ($D \times T$ $P < 0.05$) in the CON group prepartum but decreased just after parturition although there was no change ($P > 0.05$) observed in the OVE group over time.

IGF-1 was greater ($D \times T$ $P < 0.05$) in the OVE group from two weeks prepartum to the day of calving. Concentrations remained low postpartum than prepartum in both OVE and CON.

Day relative to parturition

Figure 2. Concentrations of non-esterified fatty acids (NEFA), β-hydroxybutyrate (BHBA), triacylglycerol (TAG), FGF21, GH, IGF-1, glucose, insulin, and NEFA:insulin ratio in cows (n = 6/diet) fed a control diet (CON; 1.34 Mcal/kg of DM) or a moderate energy diet (overfed, OVE; 1.62 Mcal/kg of DM) during the entire dry period.

Notes: *D × T $P < 0.05$.

We observed an abrupt increase ($D \times T\ P < 0.05$) in GH concentration in blood for CON and OVE between –14 and 2 days before parturition after which concentration in both groups decreased by two days postpartum. However, GH increased between two and seven days postpartum in the OVE group (Fig. 2).

No effect of $D \times T$ was observed for blood glucose concentration although glucose decreased (time $P < 0.05$) slightly, as expected, in early lactation compared to the dry period. There was a $D \times T$ observed for blood insulin concentration primarily because of the markedly greater concentration on day –14 in cows fed OVE (Fig. 2). There was a gradual decrease (time $P < 0.05$) in insulin concentration after parturition regardless of diet, and concentrations were similar between treatments as early as –2 days around parturition.

The concentration of lipid and TAG in liver tissue is included in Figure 3. For both parameters, greater concentrations were observed in the OVE group on day 14 ($D \times T$ $P < 0.05$), at which point concentrations were more than two-fold greater compared with the CON group. By 30 days postpartum, the concentration of lipid and TAG was similar between groups (Fig. 3).

PPAR and liver fatty acid metabolism. The expression of *PPARA* was not affected by the interaction of $D \times T$ ($P > 0.05$), and in both groups, its expression decreased around parturition and gradually increased just after calving (Fig. 4). We observed a greater expression ($D \times T\ P < 0.05$) of *RXRA*, the heterodimer of *PPARA*, before and after two weeks of parturition in the OVE group but a gradual increase in expression was observed in the CON group from day –14 to 30 (Fig. 4). The greater expression ($D \times T\ P < 0.05$) of *PPARGC1A* in the OVE group on day 7 was because of the marked increase from –14 to 7 days in these cows; however, the CON group had an increase in expression from 7 to 30 days postpartum (time $P < 0.05$) (Fig. 4).

It is noteworthy that the expression of *PPARD* was affected by the interaction of $D \times T$ ($P < 0.05$) namely because of the gradual decrease in expression with OVE and the concomitant increase with CON such that on day 30 postpartum, the expression was lower in cows fed OVE (Fig. 5). Another nuclear receptor affected by an interaction was RAR-related orphan receptor A (*RORA*), which despite the decrease in expression between –14 and 7 days was greater on both days in cows fed OVE than CON.

In addition to the significant interaction of $D \times T$ ($P < 0.05$) for *RXRA* and *PPARD*, we also detected greater expression at 7–14 days postpartum of the PPARα target genes *CPT1A*, *ACADVL*, *ACOX1*, *HMGCS2*, *CROT*, carnitine acetyltransferase (*CRAT*), and malonyl-CoA decarboxylase (*MLYCD*) in cows fed OVE (Fig. 4). Those responses were driven mainly by the marked increase in expression between –14 and 7 days postpartum. However, in some cases (*ACOX1*, *HMGCS2*, *CROT*), the interaction effect also was associated with a down-regulation in expression between –14 and 7 days in cows fed CON. It is noteworthy that in cows fed CON, the expression of *ACOX1*, *HMGCS2*, *CROT*, and *CRAT* (all involved in fatty acid oxidation) increased ($D \times T\ P < 0.05$) between 7 and 14 days postpartum (Fig. 4).

The genes involved in electron transport, electron-transfer-flavoprotein, beta polypeptide (*ETFBI*) and electron-transferring-flavoprotein dehydrogenase (*ETFDH*), had a significant interaction ($D \times T\ P < 0.05$) on day 7 with greater expression in the OVE group for both genes. For *ETFB*, this effect was because of the marked decrease in CON cows from –14 to 7 days. However, the pattern of expression for *ETFB* between groups differed from 7 through 30 days such that in cows fed CON it increased gradually but in cows fed OVE it decreased by 30 days. At this point, cows fed CON had greater ($D \times T\ P < 0.05$) expression than those of OVE (Fig. 4).

Hepatokines. The expression of *ANGPTL4* had a marked increase from –14 to early postpartum in the OVE group leading to an interaction effect ($D \times T\ P < 0.05$) because of greater expression on days 7 and 14 postpartum compared with cows fed CON (Fig. 5). The expression of *FGF21* followed a similar response ($D \times T\ P < 0.05$), with expression being lower in OVE than CON cows at –14 days and increasing by 14 days at which point expression was greater in OVE than CON cows (Fig. 5).

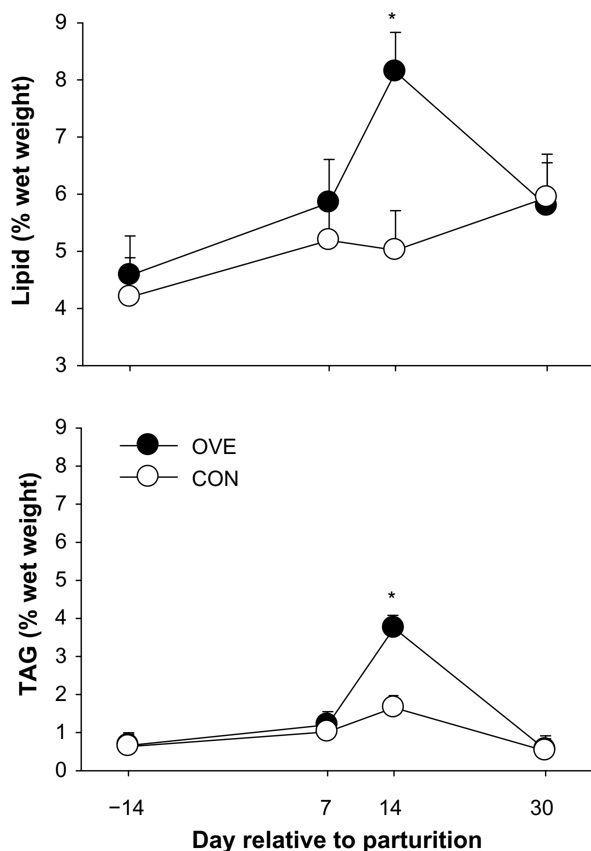

Figure 3. Liver lipid and triacylglycerol (TAG) concentration in cows (n = 6/diet) fed a control diet (CON; 1.34 Mcal/kg of DM) or a moderate energy diet (overfed, OVE; 1.62 Mcal/kg of DM) during the entire period.
Notes: *D × T $P < 0.05$.

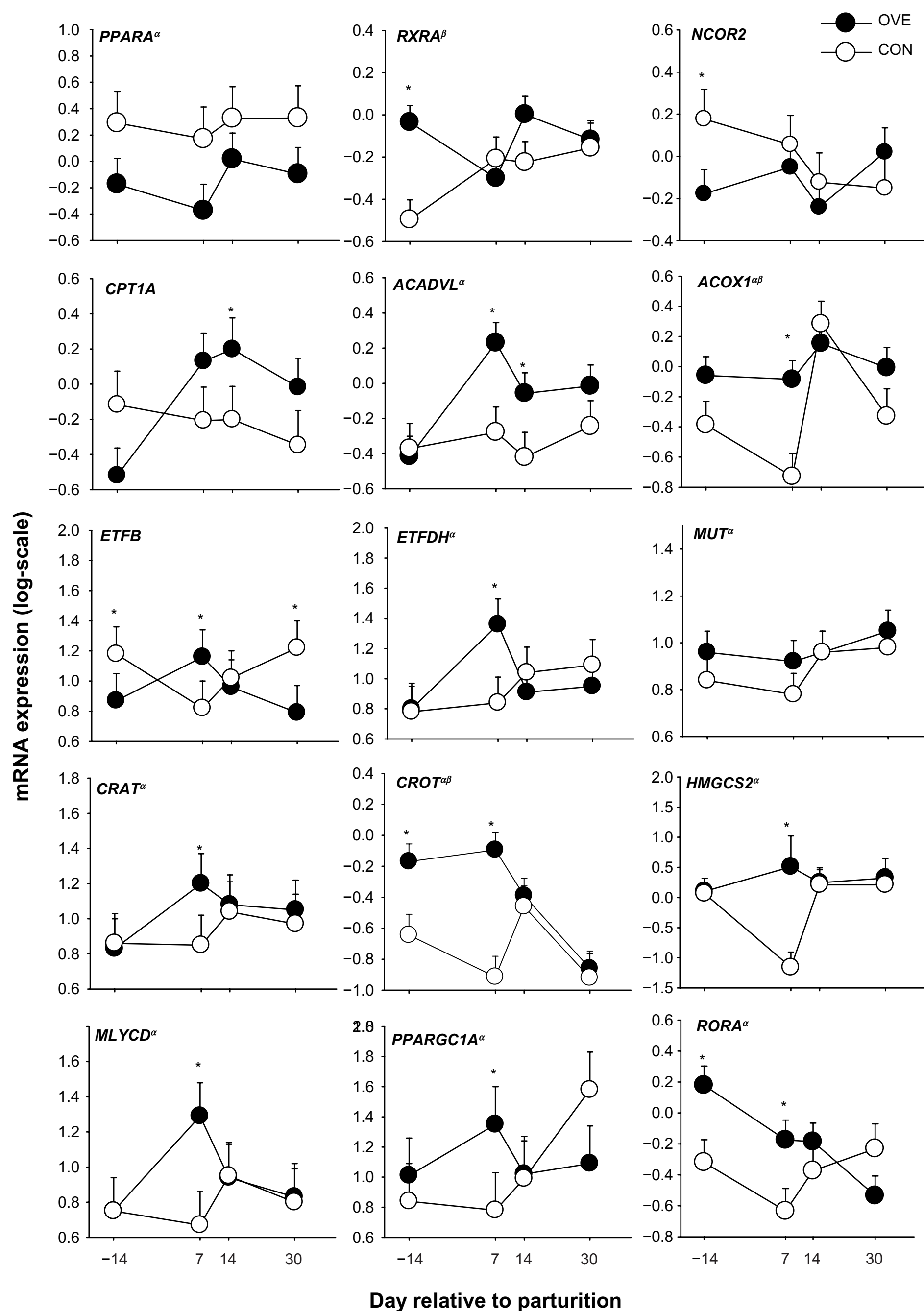

Figure 4. mRNA expression of PPARα, co-regulators, several target genes, and electron transport chain proteins in liver of cows (n = 6/diet) fed a control diet (CON; 1.34 Mcal/kg of DM) or a moderate energy diet (overfed, OVE; 1.62 Mcal/kg of DM) during entire dry period.
Notes: *D × T ($P < 0.05$). $^{\alpha}$Day $P < 0.05$ and $^{\beta}$Treatment $P < 0.05$.

Our results revealed that the expression of *FGFR4* and *KLB* was affected by the interaction of $D \times T$ ($P < 0.05$) (Table 2). In the case of *FGFR4*, feeding OVE resulted in a marked decrease in expression between −14 and 7 days postpartum after which expression remained lower. No change in expression over time was observed in cows fed CON. In contrast, the expression of *KLB* in cows fed OVE did not change between −14 and 14 days postpartum but then decreased ($D \times T$ $P < 0.05$) at 30 days. The opposite response was apparent in cows fed CON, which had greater expression of *KLB* at 14 and 30 days compared with −14 days (Table 2).

Propionate and carbohydrate metabolism. Compared with CON cows, those fed OVE had a lower expression ($D \times T$ $P < 0.05$) of propionyl-CoA carboxylase (*PCCA*) at −14, 7, 14, and 30 days. The enzyme encoded by this gene is involved in propionate metabolism. The expression of the mitochondrial enzyme methylmalonyl-CoA mutase (*MUT*), involved in metabolism of propionate to succinyl-CoA, increased in expression in the postpartum period in both groups (time $P < 0.05$).

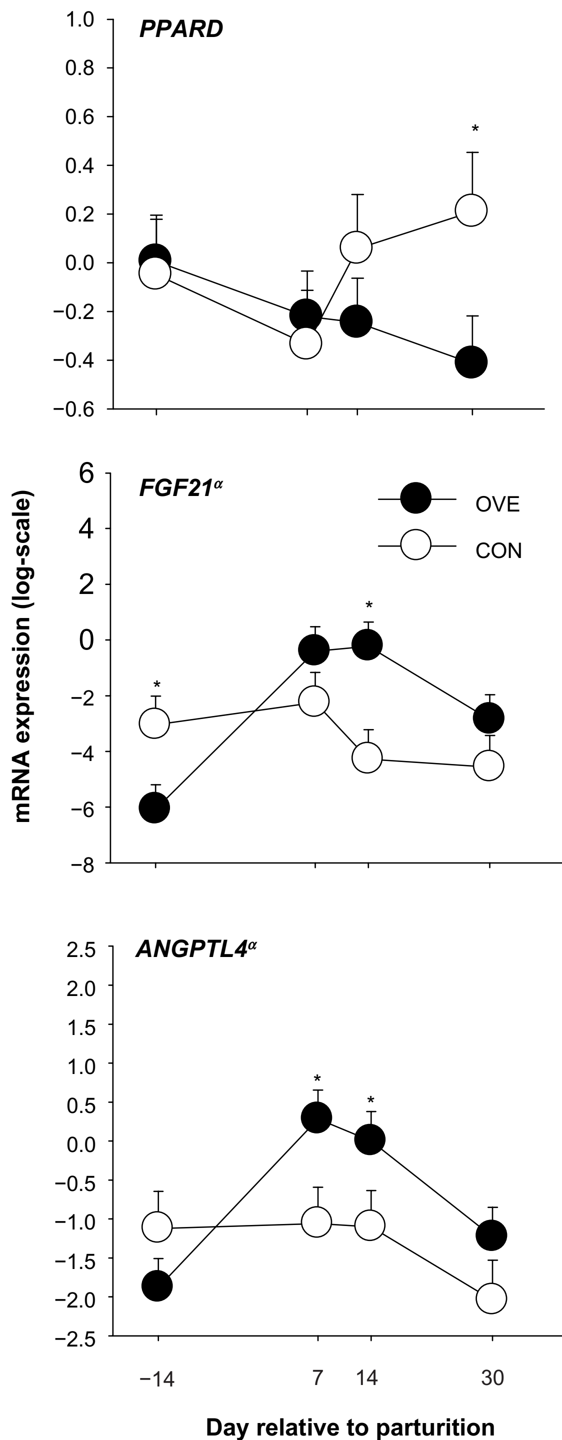

Figure 5. mRNA expression of PPARdelta and hepatokines in liver of cows (n = 6/diet) fed a control diet (CON; 1.34 Mcal/kg of DM) or a moderate energy diet (overfed, OVE; 1.62 Mcal/kg of DM) during entire dry period.
Notes: *D × T ($P < 0.05$). $^{\alpha}$Day $P < 0.05$.

The expression of pyruvate dehydrogenase (lipoamide) alpha 1 (*PDHA1*), encoding a protein that controls metabolism of pyruvate in the tricarboxylic acid cycle (TCA) cycle, increased between –14 and 7 days in both groups (time $P < 0.05$), but in OVE cows it decreased on day 14 and in CON cows it increased ($D \times T$ $P < 0.05$). Among the key gluconeogenic enzymes, cows fed OVE compared with CON had a lower expression of *PC* on –14 days ($D \times T$ $P < 0.05$); however, expression increased in OVE by 7 days (time $P < 0.05$) but did not change in CON cows (Fig. 6). There was an interaction ($D \times T$ $P < 0.05$) for both pyruvate kinase 4 (*PDK4*) and *PCK1* because of an increase in expression between –14 and 7 days in cows fed OVE compared with CON. The former encodes a kinase that inhibits pyruvate metabolism in the TCA cycle, and the latter a key cytosolic protein controlling the rate of gluconeogenesis. There was an overall time effect ($P < 0.05$) for *MDH2* (Fig. 6).

Lipogenesis. An overall treatment effect was observed for the expression of the lipogenic transcription regulator *SREBF1* and the mitochondrial enzyme *CS*, which is required for the synthesis of mitochondrial citrate. In non-ruminants, mitochondrial citrate provides acetyl-CoA in the cytosol that serves as a lipogenic substrate. Despite a modest decrease in expression from prepartum to postpartum, both *SREBF1* and *CS* were greater overall in cows fed OVE. It is noteworthy that the expression of the lipogenic enzyme *ACACA*, partly controlled by *SREBF1*, did not differ greatly but the expression of the transcription regulator *THRSP* was greater ($D \times T$ $P < 0.05$) at –14 days in cows fed OVE compared with CON (Fig. 7).

GH—IGF-1 axis and metabolism. Compared with CON, in cows fed OVE the expression pattern of *SOCS2* (Fig. 8) increased ($D \times T$ $P < 0.05$) gradually from –14 days through parturition and resulted in greater expression on day 30. Although feeding OVE resulted in greater ($D \times T$ $P < 0.05$) expression of *IGF1* and GH receptor (*GHR*) on day –14, the pattern of expression throughout the study was similar regardless of treatment ie greater expression prepartum followed by a decrease on day 7 and a subsequent increase by day 14 which was maintained on day 30 (Fig. 8).

Regardless of treatment, a marked increase in the expression of *IGFBP1* was observed from –14 to 7 days. The expression of *STAT5B*, the activator of *IGF1*, had a relatively similar expression pattern to *IGF1* and *GH* in the OVE group but there was a steady decrease ($D \times T$ $P < 0.05$) in the CON group between 7 and 30 days (Fig. 8). By the end of the study, the expression of *STAT5B* was greater in cows fed OVE than CON, and had reached expression values observed on day –14. No interaction of $D \times T$ was observed for *IGFALS* and *STAT5A*. However, for *IGFALS* there was an overall treatment effect ($P < 0.05$) because of the greater expression in cows fed OVE.

TAG and lipoprotein metabolism, and nuclear receptor co-regulators. Results of these target genes and the pertinent discussion are reported in the supplementary file.

Discussion

Metabolic adaptations. Previous studies with cows fed to meet or exceed (100 or 150% of NE_L) prepartal energy

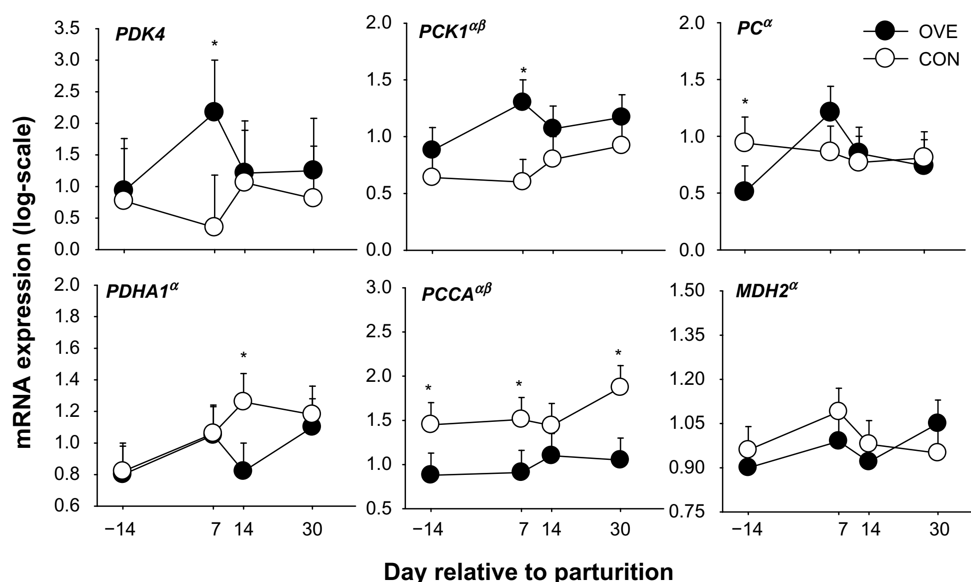

Figure 6. mRNA expression of carbohydrate metabolism genes in liver of cows (n = 6/diet) fed a control diet (CON; 1.34 Mcal/kg of DM) or a moderate energy diet (overfed, OVE; 1.62 Mcal/kg of DM) during entire dry period.
Notes: *D × T ($P < 0.05$). $^{\alpha}$Day $P < 0.05$ and $^{\beta}$Treatment $P < 0.05$.

requirements[19,20] reported similar results to those observed with OVE in terms of EBAL, NEFA, BHBA, insulin, liver TAG composition, and milk production. It has already been observed that overfeeding energy prepartum resulted in EBAL of 160% of requirements at week 3 prepartum followed by a marked decrease to less than 72% during the first week postpartum.[20] Studies from Dann et al.,[19,21] Loor et al.,[22] Janovick et al.,[23] and Ji et al.[24] provided evidence that overconsumption of energy during the dry period results in chronic hyperinsulinemia prepartum and worse adaptations to lactation, including lower postpartum DMI, milk production, and a more severe NEB. Although postpartum DMI and milk production did not differ between groups, the marked NEB in the OVE group likely rendered those cows more susceptible to disease including a more pronounced inflammatory status and compromised liver activity.[25,26]

Serum NEFA is an useful indicator of EBAL status, and the liver is the most important site for removing the excess bloodstream NEFA and its oxidation by mitochondrial β-oxidation to produce energy.[1] LCFA can also be oxidized via β-oxidation in hepatic peroxisomes, thus providing an alternative pathway to catabolize the excess uptake of NEFA around parturition.[27] Both sites of β-oxidation, mitochondria and peroxisome, might have contributed to the lower hepatic accumulation of TAG in CON cows, which were fed to meet and not greatly exceed energy requirements during the dry period. With this in mind, and based on its well-established function in non-ruminants, the activity of hepatic PPARα gene networks during the transition from pregnancy into lactation would represent an important feature of the high-genetic merit dairy cow.

PPAR and liver fatty acid metabolism. Activation of the PPARα network in non-ruminants is important during periods of under-nutrition in terms of coordinating lipid metabolism including cellular uptake, activation, and oxidation of LCFA.[8] It has been hypothesized that lipolysis of adipose tissue around calving would provide NEFA to activate PPARα and RXRα signaling, hence up-regulate the expression of its target genes.[15] Although there have been inconsistent reports regarding changes in hepatic *PPARA* expression around calving,[4] other factors such as the degree of inflammation could play a role in controlling *PPAR* expression.[28] For instance, we recently observed that an inflammatory challenge postpartum did not alter hepatic *PPARA,* but up-regulated *PPARD.*[18] Palin and Petit[29] and Carriquiry et al.[7] also reported no effect on hepatic expression of *PPARA* postpartum when cows were fed more than 100% of their energy requirements prepartum, even when the source of energy came from dietary LCFA supplementation.

Despite the lack of interaction for *PPARA* expression, the up-regulation of *RXRA* at 14 days postpartum along with several target genes was indicative of greater activity of this NR, without change in its mRNA expression. The pattern of NEFA and BHBA concentrations in the OVE group agreed with the expression profiles of lipid metabolism genes such as *CPT1A*, *ACADVL*, *ACOX1*, *APOA5*, *CROT*, and *HMGCS2* that were highly expressed in the OVE cows even during the prepartum period. All these are well-established PPARα targets in non-ruminants, and there is some published evidence supporting a similar role in ruminants.[15]

The coordinated response in the blood and gene expression data suggests that influx of NEFA into the liver might

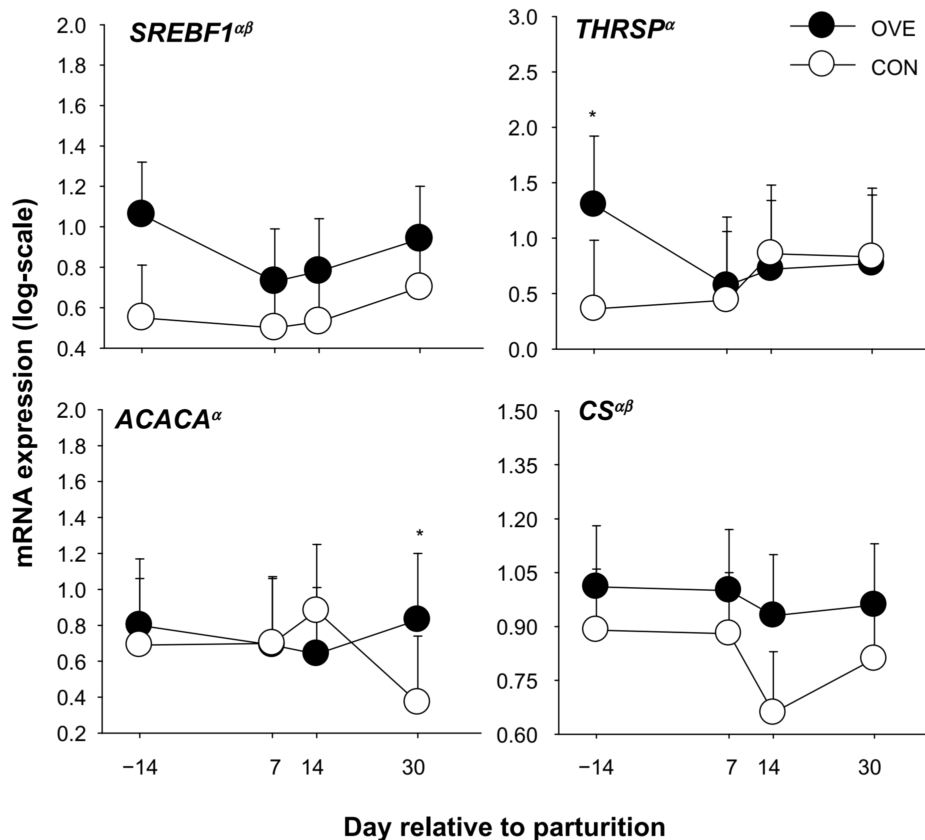

Figure 7. mRNA expression of lipogenic transcription regulators and enzymes in liver of cows (n = 6/diet) fed a control diet (CON; 1.34 Mcal/kg of DM) or a moderate energy diet (overfed, OVE; 1.62 Mcal/kg of DM) during entire dry period.
Notes: *D × T ($P < 0.05$). $^{\alpha}$Day $P < 0.05$ and $^{\beta}$Treatment $P < 0.05$.

have activated the PPARα signaling pathway. For instance, the oxidation of NEFA to produce energy and also ketone bodies would have been enhanced by the greater influx of NEFA and down-stream activation of the expression of *HMGCS2*.[30] Both CPT1 A and ACADVL are key enzymes regulating hepatic fatty acid β-oxidation pathways in mitochondria; ACOX1 regulates β-oxidation in peroxisomes; and HMGCS2 is considered the rate-limiting enzyme of hepatic ketogenesis.[31] Once in the peroxisome, acyl-CoAs are initially oxidized and after conversion to acylcarnitines, by carnitine octanoyltransferase (CROT) or peroxisomal CRAT, these intermediates are exported from the peroxisome by a still unknown mechanism for further oxidation to acetyl-CoA in mitochondria.[32]

The gene *MLYCD* also is activated by PPARα in non-ruminants and encodes a protein that acts to increase the rate of fatty acid oxidation by catalyzing the decarboxylation of cytosolic malonyl-CoA.[33] A recent study, using a mouse model of non-alcoholic fatty liver disease, demonstrated that the increase in expression of *MLYCD* decreased the concentration of hepatic malonyl-CoA and increased CPT1 A activity, favoring the fatty acid oxidation and decreasing steatosis.[34]

The other NR measures that could be involved in the regulation of hepatic metabolism are PPARD and RORA. Signaling via PPARD could be associated with the liver adaptations to inflammation[18] rather than control hepatic energy availability and metabolism. It was demonstrated that adeno-PPARD infection of murine liver led to lower tissue damage and a reduction in MAPK8 (a major intracellular pro-inflammatory molecule) stress signaling.[35] Thus, this NR can potentially play a similar anti-inflammatory role in bovine as in mouse. The fact that overfeeding energy down-regulated the postpartal expression of *PPARD* seems to suggest those cows might have been more susceptible to inflammation signals.

In non-ruminants, the regulatory role of RORA over aspects of hepatic lipid metabolism involves several lipogenic genes such as *SREBF1*, *LXR*, and *LPIN1*, and recently links have been identified with the hepatic expression and secretion of *FGF21*.[36] In an in vitro study using HepG2 cells, there was a positive relationship between the overexpression of RORα and the secretion of FGF21. In contrast, the suppression of RORα led to a decrease in *FGF21* expression and secretion.[37] RORα also exerts a control function of the circadian rhythms, eliciting a positive effect on the activity of BMAL1.[38] Activation of RORA can enhance the release of glucose from liver, via gluconeogenesis, through the activation of the enzyme glucose-6-phosphatase.[39] Overfeeding energy to cows in the present study induced *RORA* expression, as

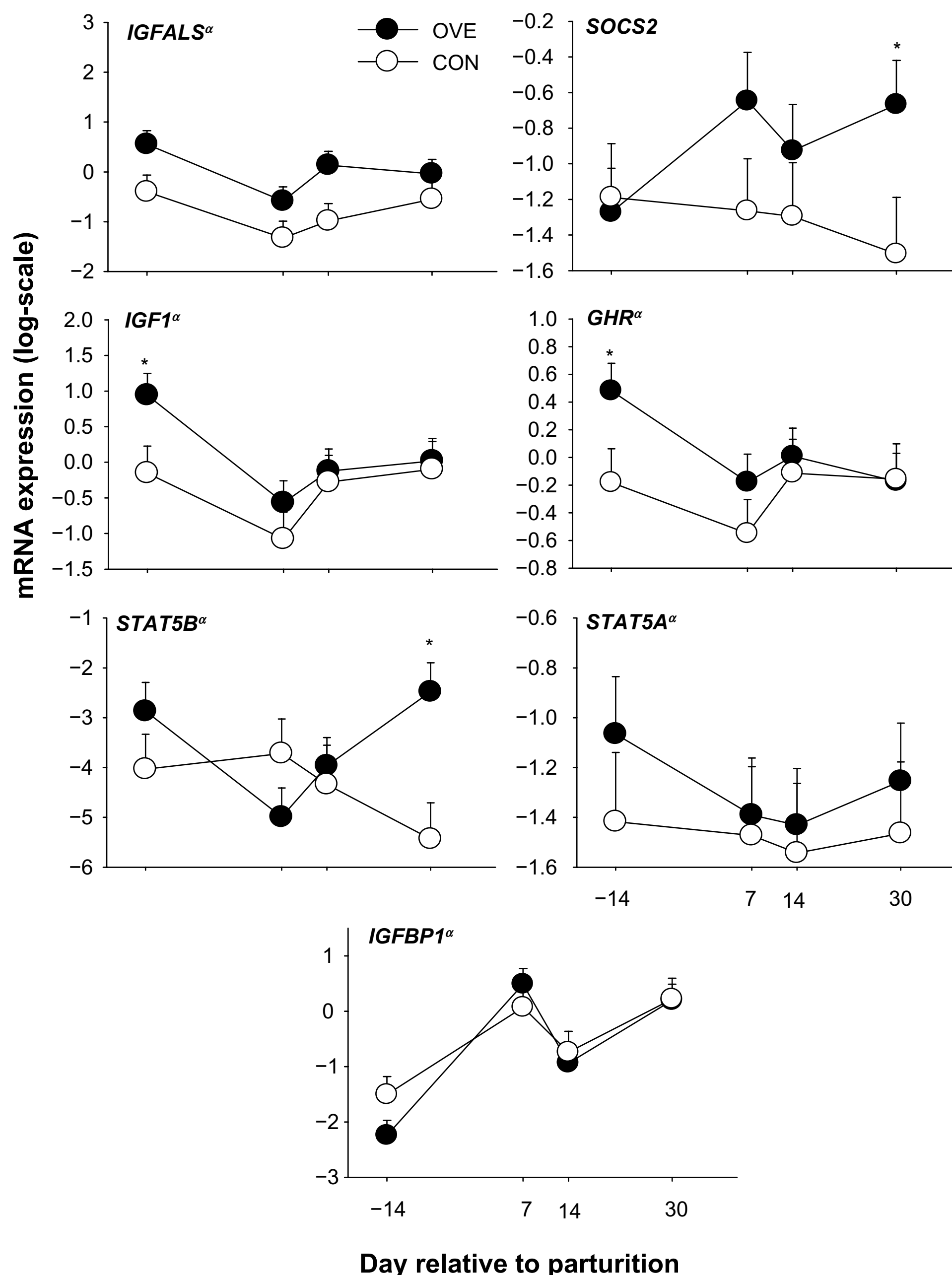

Figure 8. mRNA expression of genes associated with GH signaling in liver of cows (n = 6/diet) fed a control diet (CON; 1.34 Mcal/kg of DM) or a moderate energy diet (overfed, OVE; 1.62 Mcal/kg of DM) during entire dry period.
Notes: *D × T ($P < 0.05$). $^{\alpha}$Day $P < 0.05$.

well several oxidative target genes, indicating high activity of the oxidative pathway in these animals.

In addition to the well-established role of PPARα in hepatic lipid metabolism, at least in rodents, we sought to evaluate other genes encoding proteins that play an important role in the overall process of ATP generation during fatty acid oxidation. For instance, *ETFB* and *ETFDH* are components of the electron transport chain and accept electrons for several mitochondrial matrix flavoprotein dehydrogenases.[40] It has been demonstrated in vitro with rat primary hepatocytes that their expression is modulated by PPARα, because of the existence of PPRE, suggesting the involvement of this NR in the regulation of some components of the respiratory chain.[41]

Carbohydrate and lipid metabolism. The transition from gestation to lactation involves important changes in carbohydrate and lipid metabolism because of the requirements for milk production.[42] The main gluconeogenic substrate in ruminants is propionate but alanine also is quantitatively important after parturition when feed intake is lower. In non-ruminants, the gluconeogenic pathway is tightly regulated at the transcriptional and post-transcriptional levels by the concentrations of hormones such as glucagon, insulin, and GH.[43] The mitochondrial enzyme *PC* and the cytosolic

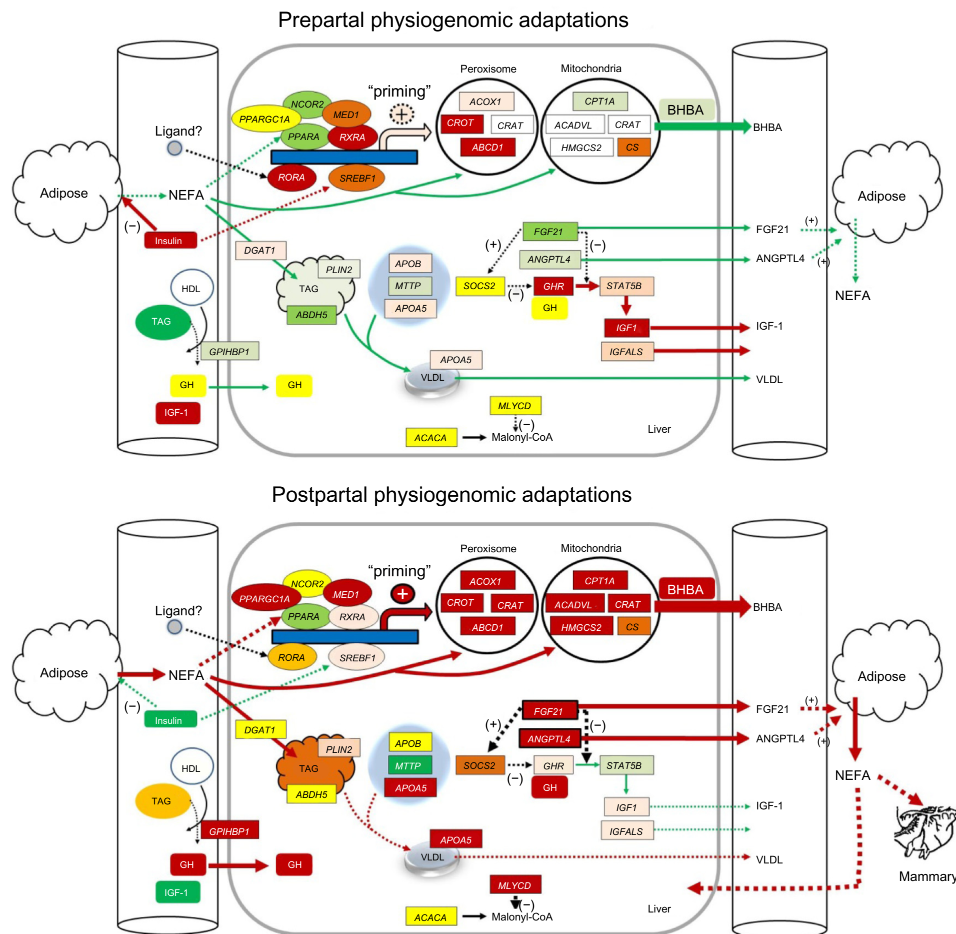

Figure 9. Integrative model of the physiogenomic adaptations in adipose and liver tissue induced by energy overfeeding during the last 45 days of pregnancy. PPAR co-regulator expression prepartum suggests the existence of a "priming" effect such that after parturition the marked increase in blood non-esterified fatty acids (NEFA) represent a signal for induction of fatty acid oxidation, synthesis of hepatokines, and reduction of lipogenic intermediates that could inhibit oxidation (e.g. malonyl-CoA). The greater insulin concentration induced by overfeeding energy prepartum likely accounts for the up-regulation of the transcription regulator *SREBF1*. The postpartal uncoupling of the GH/IGF-1 axis appears to be partly a response of the marked increase in local FGF21 synthesis and activation of *SOCS2* signalling to inhibit GH action and synthesis of IGF-1.

Notes: The different shades of color for genes and metabolites are indicative of the relative changes induced by overfeeding energy compared with the control. Yellow to red denote modest increase/up-regulation to marked increase/up-regulation; gray to dark green denote no change to marked decrease/down-regulation. Positive or negative signs denote activation or inhibition. Dotted lines denote a likely effector function on a particular gene or pathway.

enzyme *PCK1* are thought to be key for the control of gluconeogenesis,[43] but other enzymes that participate in the activation and metabolism of propionate, eg, *PCCA* and *MUT*, or the control of pyruvate oxidation (eg, *PDK4* and *PDHA1*) likely are important in the overall process.

A previous study that compared different prepartal dietary energy supplementation levels did not observe differences in the hepatic expression of *PCK1* and *PC* early postpartum, and authors suggested that increasing the supplementation of glucose precursors may decrease the hepatic rates of gluconeogenesis.[44] To the contrary, our data revealed no impairment in the transcriptional response of *PCK1* after parturition in cows fed OVE. The fact that *PDK4* also increased further supported that, despite a likely increase in blood insulin during the prepartal period (eg, Ref. 22), the liver in these cows was "primed" by the greater propionate resulting from overfeeding to respond to the marked NEB. In this context, the lower overall expression of *PCCA* in cows fed OVE seems to suggest that there might be a threshold of intrahepatic propionate above which there is no need to up-regulate mRNA and/or protein expression of this enzyme to increase flux through gluconeogenesis.

In a previous study with peripartal dairy cattle, the expression of *PC* and *SREBF1* did not change after parturition but the prepartal expression of *CS* was higher namely in cows with greater concentration of BHBA.[45] The fact that we observed greater overall expression of *SREBF1*, *THRSP*, and *CS* namely prepartum in cows overfed energy is suggestive that the lipogenic pathway in liver could adapt or respond (as in non-ruminants) to greater dietary carbohydrate and the hyperinsulinemic response (Fig. 2). Furthermore, the fact that expression of some of these genes remained greater postpartum in those cows also is suggestive of a carry-over effect induced

by the chronic energy overfeeding. It is unlikely that such effect would be associated with insulin because its concentration decreased after parturition. Whether the up-regulated lipogenic response is associated with liver TAG accumulation postpartum is unclear, but the greater *MLYCD* postpartum in response to OVE would have reduced malonyl-CoA availability for the fatty acid synthase reaction.

Hepatokines. In non-ruminants, circulating ANGPTL4 is a key regulator of plasma cholesterol and TAG concentrations, and is highly expressed in the liver of early postpartal dairy cattle.[15] The utilization of PPAR agonists (including high-fat diet feeding)[15] results in the up-regulation of *ANGPTL4* and can lead to inhibition of LPL activity in adipose tissue leading to reduced VLDL-TAG utilization and potentially greater lipolysis.[46] The expression of *ANGPTL4* was up-regulated in the liver of ketotic cows,[9] and in cows with severe NEB,[26] hence, it is possible that the up-regulation of *ANGPTL4* has an indirect role in promoting lipolysis during early lactation, potentially to ensure the availability of NEFA to generate energy in the liver or provide LCFA for lactating mammary gland. Obviously, a side-effect of enhanced flux of NEFA into the liver is the overaccumulation of TAG, which could be detrimental. In our study, the greater expression level of *ANGPTL4* postpartum in cows fed OVE provides additional evidence of a more "precarious" condition, particularly in relation to the more severe NEB in these cows. These results agree with those of McCabe et al.,[46] where a greater expression of *ANGPTL4* was observed in dairy cows with severe NEB.

In rodents it has been demonstrated that hepatic PPARα controls *FGF21* expression during fasting or in neonates during the milk-fed period (ie which provides large amounts of LCFA), and this growth factor induces the synthesis of ketone bodies.[47] As such, FGF21 is central for the provision of alternate sources of energy during extended fasting and starvation.[47] Schoenberg et al.[11] conducted an experiment in dairy cows to measure the blood concentration and the expression of *FGF21* during the transition period, and reported a dramatic increase in plasma FGF21 after parturition. Thus, we confirmed those observations and extended them further to show that overnutrition prepartum likely because of the more severe NEB and greater NEFA results in greater hepatic *FGF21*. We propose that blood FGF21 could be used as a biomarker of "stress" in the postpartal dairy cow, with likely functions on peripheral tissues that help coordinate homeorhetic adaptations.

In rodents, the hepatic expression of *FGF21* is induced by the activation of the complex PPARα-RXR, via NEFA, and through its signaling in the adipose tissue, it can inhibit lipolysis.[14,48] Signaling via FGF21 involves mainly the activation of FGF receptors but only in the presence of the co-receptor KLB.[11] In our study, the increase in *FGF21* expression in cows fed OVE was associated with higher NEFA concentration after parturition but not with the expression of the *FGFRs* or *KLB*. Schoenberg et al.[11] reported that expression of *FGFR* isoforms 1c, 2c, and 4 in liver decreases after calving but *KLB* increased. Blood concentration of FGF21 in the present study also did not correspond closely with the increase in *FGF21* expression. Such differences could be the result, for example, of a delay in post-transcriptional or translational regulatory mechanisms. Furthermore, it appears that in the postpartal cow model, the circulating FGF21 is unable to prevent adipose tissue lipolysis likely because of impairing insulin sensitivity in adipose tissue.[14] Additional studies in this area seem warranted.

GH/IGF-1 axis and metabolism. The increase in GH postpartum is an important aspect of the adaptations of the dairy cow to the onset of lactation. Its anabolic actions are mediated by IGF-1. GH binds to the GHR in liver and activates *IGF1* transcription through a complex series of reactions on the cell surface ie the binding of GH with GHR induces janus kinase 2 (*JAK2*) expression, which in turn phosphorylates members of the *STAT* family. Once phosphorylated STAT proteins translocate to the nucleus where they bind to response elements in the regulatory regions of target genes including *IGF1*.[13]

As the GH/IGF1 axis is involved in many aspects of cell function, its signaling is tightly controlled by several pathways. IGFBP1 protein, as primary function, binds to IGF-1 molecules, hence, helping to modulate its distribution and interaction with IGF-1 receptors in peripheral tissues. During the peripartal period, IGFBP1 could serve as a carrier of IGF-1 to tissues like the mammary gland.[49] Also considered a PPAR target in non-ruminants, an in silico screening approach identified five candidate PPAR response elements located within 10 kb of the transcription start site of the IGFBP1 gene.[50]

The protein IGFALS binds to the complex IGF—IGFBP prolonging the half-life.[51] The protein SOCS2 is another factor that regulates the actions of GH-IGF. It can bind to JAK2–STAT and inhibit the phosphorylation of STAT.[52] The greater *SOCS2* expression suggested that these cows could have a compromised immune response, as this protein is induced in response to pro-inflammatory cytokines and down-regulates the cytokine signaling by inhibiting the JAK/STAT pathway.[53]

Inagaki et al.[13] reported that FGF21 prevents STAT5 signaling and blunts the GH pathway in liver as a means to conserve energy during starvation or under-nutrition ie periods of NEB. The similar pattern of expression of *IGF1, GHR, STAT5A*, and *STAT5B* in cows fed OVE during the peripartal period contrast the marked up-regulation of *FGF21* and of several components of the LCFA oxidation pathway (eg, *CPT1A*, *ACADVL*). Thus, unlike rodents, the data from the present study seem to suggest an inhibitory effect of FGF21 on hepatic GH signaling. The exact mechanisms for such an effect merit further study.

Conclusions

Overall, our results indicated that overfeeding energy prepartum induced relevant changes in the expression of lipid

metabolism- and carbohydrate-related genes, including several PPARα targets (Fig. 9).[29,30] We have proposed an integrative model for changes in hepatic gene expression under the effect of NEFA uptake and activation of PPARα in the liver of dairy cows after parturition (Fig. 9). When NEFA enters into hepatocytes, it can act as a ligand to activate PPARα target genes to mediate LCFA oxidation (peroxisome, mitochondria) and ketogenesis. Activation of *FGF21* could inhibit local GH signaling, and in the circulation, it can reduce adipose tissue insulin sensitivity and contribute to lipolysis. The activation of *ANGPTL4* also contributes to lipolysis by inhibiting lipoprotein lipase activity in adipose (Fig. 9). If the level of NEFA exceeds hepatocyte oxidation capacity, the LCFA can accumulate in liver as TAG, which if severe can cause fatty liver.

The overfeeding of energy prepartum increases the lipid accumulation in adipose tissue before calving at least in part because of the chronic hyperinsulinemia, which up-regulates the adipogenic and lipogenic gene networks.[24] In that context, it was surprising to observe up-regulation of the lipogenic TF *SREBF1* and the enzyme *CS* in liver because classical studies[16] demonstrated that ruminant liver is not a lipogenic tissue such as adipose and lactating mammary gland. Thus, it appears that dairy cattle liver expresses lipogenic enzymes and that under "normal" conditions (eg, feeding of high-forage diets, grazing), the lack of signals such as glucose and insulin is partly responsible for the apparent lack of lipogenic capacity. Whether excess dietary carbohydrate because of long-term overfeeding in late-pregnancy can provide enough signals to the lipogenic pathways and contribute to TAG accumulation remains to be determined. What appears evident, however, is that mechanisms controlling intracellular synthesis of acetyl-CoA and/or malonyl-CoA (eg, MLYCD) also would indirectly determine the lipogenic capacity of the liver (Fig. 9).

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Author Contributions

JJL conceived and designed the experiments. MJK and DEG performed the analyses. MJK, CBJ, and JJL wrote the manuscript. MJK, CBJ, DEG, ES, MNC, FC, and JJL agreed with manuscript results and conclusions. MJK, CBJ, ES, MNC, FC, and JJL jointly developed the structure and arguments for the paper. MJK, CBJ, and JJL made critical revisions and approved the final version. All authors reviewed and approved the final manuscript.

DISCLOSURES AND ETHICS

As a requirement of publication the authors have provided signed confirmation of their compliance with ethical and legal obligations including but not limited to compliance with ICMJE authorship and competing interests guidelines, that the article is neither under consideration for publication nor published elsewhere, of their compliance with legal and ethical guidelines concerning human and animal research participants (if applicable), and that permission has been obtained for reproduction of any copyrighted material. This article was subject to blind, independent, expert peer review. The reviewers reported no competing interests.

Supplementary Material

Supplementary files include information for Validation of the FGF21 ELISA kit for bovine, RNA extraction, quantitative PCR, and primer design.

Supplementary Table 1. Ingredients and chemical composition of experimental diets.

Supplementary Table 2. Sequencing results of PCR products from primers of genes designed for this experiment.

Supplementary Table 3. Gene ID, GeneBank accession number, hybridization position, sequence and amplicon size of primers for Bos taurus used to analyze gene expression by qPCR.

Supplementary Table 4. qPCR performance among the genes measured in liver tissue.

Supplementary Figure 1. Western blot of FGF21 protein from plasma samples of cows (n=6/diet) fed a control diet (CON; 1.34Mcal/kg of DM) or moderate energy diet (overfed, OVE; 1.62 Mcal/kg of DM) during the entire dry period.

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