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ORIGINAL RESEARCH

Dietary Lipid During Late-Pregnancy and Early-Lactation to Manipulate Metabolic and Inflammatory Gene Network Expression in Dairy Cattle Liver with a Focus on PPARs

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Abstract: Polyunsaturated (PUFA) long-chain fatty acids (LCFAs) are more potent in eliciting molecular and tissue functional changes in monogastrics than saturated LCFA. From −21 through 10 days relative to parturition dairy cows were fed no supplemental LCFA (control), saturated LCFA (SFAT; mainly 16:0 and 18:0), or fish oil (FISH; high-PUFA). Twenty-seven genes were measured via quantitative RT-PCR in liver tissue on day −14 and day 10. Expression of nuclear receptor co-activators (*CARM1, MED1*), LCFA metabolism (*ACSL1, SCD, ACOX1*), and inflammation (*IL6, TBK1, IKBKE*) genes was lower with SFAT than control on day −14. Expression of *SCD*, however, was markedly lower with FISH than control or SFAT on both −14 and 10 days. FISH led to further decreases in expression on day 10 of LCFA metabolism (*CD36, PLIN2, ACSL1, ACOX1*), intracellular energy (*UCP2, STK11, PRKAA1*), de novo cholesterol synthesis (*SREBF2*), inflammation (*IL6, TBK1, IKBKE*), and nuclear receptor signaling genes (*PPARD, MED1, NRIP1*). No change in expression was observed for *PPARA* and *RXRA*. The increase of *DGAT2*, *PLIN2*, *ACSL1*, and *ACOX1* on day 10 versus −14 in cows fed SFAT suggested upregulation of both beta-oxidation and lipid droplet (LD) formation. However, liver triacylglycerol concentration was similar among treatments. The hepatokine *FGF21* and the gluconeogenic genes *PC* and *PCK1* increased markedly on day 10 versus −14 only in controls. At the levels supplemented, the change in the profile of metabolic genes after parturition in cows fed saturated fat suggested a greater capacity for uptake of fatty acids and intracellular handling without excessive storage of LD.

Keywords: dairy cows, fat supplementation, hepatic gene network

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Introduction

The liver plays a pivotal role in whole-body lipid hemostasis and responds rapidly to changes in dietary fat composition in both rodents[1] and ruminants.[2] Previous data with peripartal dairy cattle have underscored the potential for dietary lipid supplementation as a useful nutritional strategy to prepare[3] and facilitate the hepatic metabolism of non-esterified fatty acids (NEFA)[4] released in high amounts from adipose tissue during early lactation.[5] However, there have been contrasting results with respect to type of dietary lipid and the response in blood hydroxybutyrate (BHBA), liver lipid content, dry matter intake (DMI), milk yield, and changes in body weight and body condition score.[2,4,6,7] It is likely that differences across studies are partly due to the specific type (eg, saturated vs. unsaturated) and quantity of lipid supplemented.

In rodent liver, long-chain fatty acids (LCFAs) can bind directly to various nuclear receptors (*PPAR, LXR*) leading to changes in their transcriptional activity, which alters the function of pathways in proportion to changes in the mRNA expression of target genes.[8,9] Pathways associated with LCFA oxidation, inflammation, and ketogenesis were recently evaluated in bovine cells leading to the recognition that saturated LCFA, eg, 16:0 and 18:0, and the fish oil-enriched 20:5n-3 were the most-potent at upregulating genes associated with PPARα activity.[10,11] The response to saturated LCFA likely is a ruminant evolutionary adaptation of PPAR to metabolize the saturated LCFA which are found in large amounts in the circulation due to extensive ruminal hydrogenation.[10,11]

There is in vivo and in vitro evidence suggesting that the PPARα gene network might be responsive to dietary lipids and could be used to monitor the functional changes that might occur in the liver during the transition period.[4,10,12,13] From that perspective, it is important to evaluate if those responses also are observed during the transition period in cows receiving supplemental lipid enriched in one or more of the main dietary LCFA. The objective of the current study was to evaluate the expression of several genes associated with the PPARα transcriptional network in liver tissue harvested from cows fed supplemental saturated fat or fish oil during the transition period.

Methods

Experimental design

The present study involved the same subset of multiparous Holstein cows used by Schmitt et al.[14] Briefly, a completely random subset of 5 multiparous cows (2nd and 3rd lactation) that were fed no supplemental LCFA (control, n = 12 cows total) or supplemental LCFA from either Energy Booster (SFAT; Milk Specialties Co., Dundee, IL, USA; n = 15) or fish oil (FISH; Omega Proteins, Houston, TX, USA; n = 15) were used for hepatic phospholipid (PL) and triacylglycerol (TAG) LCFA analysis and gene expression profiling. The cows from the control, FISH, and SFAT diets were fed the respective diets from −25 (±4), −24 (±3), and −26 (±7) d, respectively, until 10 days post partum.

Biopsies

The liver tissue was harvested via percutaneous biopsy.[15] The average gap between the start of feeding and the first biopsy was 11 ± 3 days. Prepartal liver biopsies were harvested at −14 ± 4, −13 ± 3, and −16 ± 6 days in cows fed the control, FISH, and SFAT diets, respectively. Postpartal biopsies were harvested at 7 ± 4 days in all the groups. Biopsied tissue (1 to 2 g) was weighed and stored in liquid N_2 prior to RNA extraction. A portion of liver tissue collected (0.8 to 1.0 g) was used for analysis of PL and TAG fatty acid concentrations as described by Ballou et al.[15]

RNA extraction and real-time quantitative PCR (qPCR)

Complete details of the procedure for RNA extraction can be found in Schmitt et al.[14] Briefly, approximately 0.2–0.3 g of liver tissue was weighted and immediately placed in ice-cold TRIzol reagent for homogenization. Genomic DNA was removed from RNA with RNase-free DNase, using RNeasy Mini Kit columns. RNA concentration was measured with NanoDrop ND-1000 spectrophotometer, while the RNA quality was assessed using the Agilent Bioanalyzer system (Agilent 2100 Bioanalyzer, Agilent Technologies, Santa Clara, CA, USA). The average RNA integrity number (RIN) value for liver samples was 8 ± 0.4. Protocols for primer design, testing, selection of internal control genes (ICG) for normalization were as previously described.[14] Briefly, genes

selected as suitable ICG based on geNorm analysis included *EDC4, SHPRH, EIF3K, UXT, ACTB,* and *MRPL39*. The geometric mean of these genes was used to normalize gene expression data in the present study.

Genes selected for transcript profiling in this study are associated with fatty acid uptake and transport (*FABP1, CD36*), esterification, desaturation and lipid droplet (LD) formation (*DGAT2, SCD, PLIN2, PLIN4*), fatty acid oxidation (*ACSL1, ACOX1, CPT1A, FGF21*), gluconeogenesis (*PC, PCK1*), and intracellular energy (*UCP2, PRKAA1, STK11*). Of particular importance was the study of transcription regulators (*PPARA, RXRA, PPARD, SREBF2*), nuclear receptor co-activators (*CARM1, MED1*), nuclear receptor co-repressors (*NCOR2, NRIP1*), and inflammation related (*IL6, TBK1, IKBKE*) and apoptosis/signaling related genes (*CIDEB, STK1*). Primer pairs for target genes and ICG and sequencing results of primer products not shown in Tables 1 and 2 were reported previously.[14,16]

Fatty acid analysis

Details of these procedures have already been published by Ballou et al.[15] Briefly, a 100 mg liver sample was used to separate both PL and TAG via thin-layer chromatography using hexane-diethyl etheracetic acid (90:30:1, vol/vol/vol) as the elution phase. Methyl esters of fatty acids (FA) were prepared by incubation with 2 M potassium hydroxide in methanol for 15 min at room temperature. The ester mixture was separated using a Hewlett Packard 5890 gas chromatograph (Hewlett Packard, Avondale, PA, USA) equipped with a flame-ionization detector and a Supelco 2560 100-m capillary column (Supelco, Bellefonte, PA, USA).[15]

Statistical analysis

After normalization with the geometric mean of the ICG, the qPCR data from all treatments (prepartum and postpartum) were log-2 transformed prior to statistical analysis.[14] A repeated measures model was fitted to gene expression, FA, NEFA, and DMI data using Proc MIXED in SAS. The model consisted of time, treatment, and time × treatment interaction as fixed effects, as well as cow as the random effect. An autoregressive covariate structure was used. All means were compared using the PDIFF statement of SAS.

Results

Dry matter intake and blood NEFA

The DMI was significantly ($P < 0.05$) affected by the interaction of diet and day. Supplementing FISH led to lower peripartal DMI compared to SFAT and control cows; the maximum decrease was observed close to parturition. A postpartal increase in DMI was observed in all the groups but cows supplemented with FISH still had lower DMI than the other two groups by the end of the supplementation period (Fig. 1A). The plasma NEFA concentration was not affected significantly by diet ($P < 0.05$). Irrespective of lipid type, NEFA increased at calving and remained elevated for the subsequent 10 days (Fig. 1B). Cows supplemented with FISH had comparatively lower postpartal NEFA relative to controls or SFAT, which agreed with the lower DMI during that time-frame.

Hepatic fatty acid composition

Phospholipids

Mean concentration of palmitic acid (16:0) increased ($P < 0.05$) after parturition in all groups including control. The hepatic PL fatty acid content of stearic acid

Table 1. GenBank accession number, gene symbol, hybridization position, sequence and amplicon size of primers.

Accession #	Gene	Primers[a]	Primers (5′-3′)[b]	bp[c]
XM_002695200.1	*FGF21*	F.223 R.328	CAGAGCCCCGAAAGTCTCTTGAAAGTGCAGCGATCCGTACAG	106
NM_001034036.1	*PPARA*	F.729 R.830	CATAACGCGATTCGTTTTGGACGCGGTTTCGGAATCTTCT	102
NM_001083636.1	*PPARD*	F.460 R.559	TGTGGCAGCCTCAATATGGAGACGGAAGAAGCCCTTGCA	100
NM_001035289.2	*ACOX1*	F.180 R.279	ACCCAGACTTCCAGCATGAGATTCCTCATCTTCTGCACCATGA	100
NM_173980.2	*PLIN2*	F.1607 R.1706	TTTATGGCCTCATGCTTTTGCCTCAGAGCAGACCCCAATTCA	100
FJ415874.1	*CPT1A*	F.141 R.240	TCGCGATGGACTTGCTGTATACGGTCCAGTTTGCGTCTGTA	100
BC111622	*FABP1*	F.183 R.283	GTTCATCATCACCGCTGGCTCCACTGCCTTGATCTTCTCCC	101

Notes: [a]Primer direction (F—forward; R—reverse) and hybridization position on the sequence; [b]exon-exon junctions are underlined; [c]amplicon size in base pairs (bp).

Table 2. Sequencing results of PCR primer products.

Gene	Sequence
FGF21	CGAGATCTGAAGCAAATTGAGGCAGAAATCCTTACGTGTGAGCATGACCTAGAAGATTCCGAAACCGCGA
PPARA	CGAGATCTGAAGCAAATTGAGGCAGAAATCCTTACGTGTGAGCATGACCTAGAAGATTCCGAAACCGCGA
PPARD	GCATGGGGACGGCGTCGGGCTCACTACGGCGTTCACGCTTGTGAGGGATGCAAGGGCTTCTTCCGTCC ACAAA
ACOX1	ATCCTCGTATCCGCGTTCAGGGTGCGTTTAAGAAGAGTGCCATCATGGTGCAGAAGATGAGGAAATCCCC
PLIN2	ACGTGCGTCGTCGTTCGTATAAAACACCTTCATGTAGGCTGTTGTATGAATTGGGGTCCGCTCTGAGAC
CPT1A	GGACTATGAAGGTAAACCAGGCCCGGGACGCCCTTCGTACAGGCCTCTCGCTCCAGCTGGCTCATTACA AGGGACCA
FABP1	GAGGGAGGAGTGTGAGATGGAGTTCATGACTGGGAGAGAAGATCAAGGCAGTGGA

Note: Best hits using BLASTN (http://www.ncbi.nlm.nih.gov) are shown.

Table 3. Percentage of long-chain fatty acids in liver phospholipids during the peripartal period in cows fed (n = 5/treatment) control, fish oil (FISH), or saturated lipid (SFAT).

Fatty acid†	Treatment	Day				SEM†	*P* value		
		–21	–10	1	11		Diet	Time	D × T‡
16:0	CON	9.47	11.28	12.86	12.90	0.96	0.34	<0.01	0.39
	SFAT	10.58	10.98	12.55	12.12				
	FISH	12.55	10.32	13.25	13.33				
18:0	CON	30.58^{α}	$30.07^{a,\alpha}$	$27.34^{a,b,\beta}$	27.01^{γ}	1.11	0.62	<0.01	<0.01
	SFAT	31.08^{α}	$31.01^{a,\alpha}$	$26.80^{a,\beta}$	27.65^{β}				
	FISH	30.03^{α}	$25.59^{b,\beta}$	$29.68^{b,\alpha,\gamma}$	$28.44^{\alpha,\gamma}$				
18:1cis9	CON	0.57	0.82	0.90	0.78	0.11	0.21	<0.01	0.73
	SFAT	0.75	0.81	0.88	0.88				
	FISH	0.43	0.70	0.83	0.84				
18:1trans11	CON	0.76^{a}	0.91^{a}	0.87^{a}	$0.79^{a,b}$	0.14	<0.01	<0.01	<0.01
	SFAT	0.74^{a}	0.56^{a}	0.86^{a}	0.54^{a}				
	FISH	$0.37^{b,\alpha}$	$4.95^{b,\beta}$	$1.50^{b,\gamma}$	$0.97^{b,\delta}$				
18:2c9t11	CON	0.16	0.20	0.27	0.22	0.02	<0.01	<0.01	0.23
	SFAT	0.14	0.13	0.28	0.16				
	FISH	0.07	0.13	0.21	0.17				
18:2n-6	CON	9.64^{α}	$10.35^{a,\alpha}$	$13.16^{a,\beta}$	13.15^{β}	0.88	<0.01	<0.01	0.03
	SFAT	9.62^{α}	$9.87^{a,\alpha}$	$13.25^{a,\beta}$	13.45^{β}				
	FISH	10.29^{α}	$7.09^{b,\beta}$	$9.46^{b,\alpha}$	13.16^{γ}				
18:3n-3	CON	$0.93^{b,\alpha}$	0.95^{α}	$1.37^{a,b,\beta}$	1.24^{α}	0.12	0.49	<0.01	0.03
	SFAT	$1.12^{a,b,\alpha}$	0.89^{α}	$1.64^{a,\beta}$	1.17^{α}				
	FISH	$1.26^{a,\alpha}$	0.79^{β}	$1.14^{b,\alpha}$	1.28^{α}				
20:4n-6	CON	$11.76^{\alpha,\beta}$	10.60^{α}	$10.92^{b,\alpha}$	$12.29^{a,\beta,\gamma}$	0.53	<0.01	<0.01	0.02
	SFAT	11.29^{α}	$10.45^{\alpha,\beta}$	$9.87^{a,b,\beta}$	$11.42^{a,\alpha}$				
	FISH	11.24^{α}	$10.34^{\alpha,\beta}$	$9.01^{a,\beta,\gamma}$	$8.82^{b,\gamma}$				
20:5n-3	CON	1.43	1.43^{a}	1.44^{a}	1.69^{a}	0.21	<0.01	<0.01	<0.01
	SFAT	1.59^{α}	$1.51^{a,\alpha}$	$1.52^{a,\alpha}$	$2.21^{a,\beta}$				
	FISH	1.30^{α}	$4.03^{b,\beta}$	$4.33^{b,\beta}$	$3.94^{b,\beta}$				
22:5n-3	CON	0.71	0.68	0.43	0.36	0.07	0.01	<0.01	0.10
	SFAT	0.64	0.65	0.41	0.28				
	FISH	0.70	0.93	0.65	0.27				
22:6n-3	CON	0.94	0.92^{a}	0.92^{a}	0.89^{a}	0.29	<0.01	<0.01	<0.01
	SFAT	0.97	0.84^{a}	0.83^{a}	0.76^{a}				
	FISH	0.90^{α}	$7.72^{b,\beta}$	$7.97^{b,\beta}$	$6.58^{b,\gamma}$				

Notes: †Standard error of the mean; ‡diet × time interaction. $^{a\text{–}c}$Difference ($P < 0.05$) between diets on the same day. $^{\alpha,\beta,\gamma,\delta}$Significant interactions ($P < 0.05$) within a diet and between days.

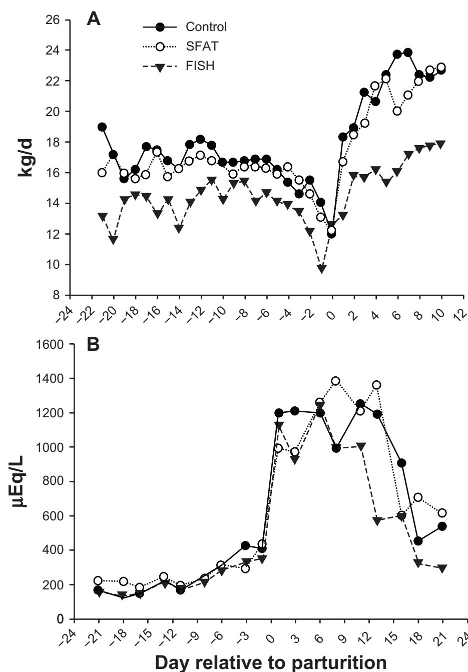

Figure 1. Dry matter intake (**A**) and blood NEFA concentration (**B**) during the peripartal period in cows fed (n = 5/treatment) control, fish oil (FISH), or saturated lipid (SFAT). There was a significant ($P < 0.05$) Diet × Time effect for dry matter intake; whereas, for NEFA only Time was significant ($P < 0.05$).

(18:0) decreased (diet × day $P < 0.05$) after parturition in control and SFAT but increased with FISH and remained elevated by day 11 (Table 3). Supplementing FISH increased (diet × day $P < 0.05$) the proportion of trans-18:1 isomers, with a maximal concentration on day −10 followed by a gradual decrease by day 11 (Tables 1 and S1). Concomitantly, FISH led to lower (diet × day $P < 0.05$) prepartal linoleic acid (18:2n-6) content at −10 days followed by a steady increase after parturition. By day 11, there were no differences in 18:2n-6 among diets. Overall concentration of 18:1trans11 was greatest ($P < 0.05$) in FISH compared with control or SFAT from −10 through 11 days. In contrast, conjugated linoleic acid (CLA) concentration was lower (diet $P < 0.05$) in lipid-supplemented cows than control cows. However, CLA concentration peaked in all the groups (day $P < 0.05$) at parturition (1 day) followed by a gradual decrease by day 11. The concentrations of other 18:1trans isomers were little affected by supplemental lipid (Table S1).

SFAT and FISH-supplemented cows had greater (diet × day $P < 0.05$) α-linolenic acid (18:3n-3) content at −21 days compared with controls, while a lower concentration was observed at −10 days in the same groups (Table 3). As compared with SFAT and control, the FISH-supplemented cows had a lower (diet × day $P < 0.05$) proportion of γ-linolenic acid (18:3n-6) at −10 days but a gradual increase was observed at 1 and 11 days (Table S1). As expected, the concentration of eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) was greatest with FISH compared with control or SFAT from −10 days through 11 days. However, arachidonic acid (20:4n-6) decreased markedly with FISH and SFAT after parturition as compared with control (Table 3).

Triacylglycerol

Concentration of 16:0 was greatest ($P < 0.05$) overall in cows fed SFAT, but 18:0 was not affected by diet (Table 4). A postpartal increase (day $P < 0.05$) in 16:0 and a decrease (day $P < 0.05$) in stearic acid was observed (Table 4). Similar to the PL fatty acid fraction, FISH-supplemented cows had a tendency (diet × day $P = 0.06$) for a postpartal increase in TAG concentration of trans-vaccenic acid (18:1trans11) as compared with control and SFAT (Table 4). Overall, however, concentration of 18:1trans11 was greatest ($P < 0.05$) for cows fed FISH than SFAT or control. Similar to PL, the CLA concentration of hepatic TAG also increased soon after calving and remained elevated compared with prepartal concentrations. A postpartal increase (day $P < 0.05$) was observed in the concentration of 18:2n-6 regardless of diet. The concentrations of other 18:1trans isomers were little affected by supplemental lipid (Table S1).

Compared with the PL fraction, supplementing lipid was not effective (diet $P > 0.05$) in altering the 18:3n-3 and 20:4n-6 concentration of the hepatic TAG fraction. However, a postpartal increase (day $P < 0.05$) in 18:3n-3 and a concomitant decrease in 20:4n-6 concentration was observed regardless of diet (Table 4). Similar to PL, there was an increase (diet × day $P < 0.05$) in the proportion of all n-3 fatty acids in the TAG fraction of FISH-supplemented cows; 20:5n-3, 22:5n-3, and 22:6n-3 had a steady increase in concentration by day 1 followed by still greater concentrations relative to the prepartum levels. Overall, the postpartal concentration of 20:5n-3, 22:5n-3, and 22:6n-3 was markedly lower in SFAT and control than FISH (Table 4). A postpartal increase

Table 4. Percentage of long-chain fatty acids in liver triacylglycerol during the peripartal period in cows fed (n = 5/treatment) control, fish oil (FISH), or saturated lipid (SFAT).

Fatty acid	Treatment	Day				SEM†	*P* value		
		–21	–10	1	11		Diet	Time	D × T‡
16:0	CON	32.21	33.23	38.76	39.37	1.80	0.03	<0.01	0.87
	SFAT	35.10	36.70	39.14	40.42				
	FISH	33.05	33.03	35.46	37.10				
18:0	CON	20.59	18.00	6.87	7.56	1.93	0.18	<0.01	0.82
	SFAT	18.11	18.87	9.37	6.27				
	FISH	16.86	15.56	6.59	5.77				
18:1cis9	CON	14.28	13.82	20.90	20.72	2.63	0.92	<0.01	0.21
	SFAT	14.35	12.81	21.16	19.57				
	FISH	20.04	14.00	14.92	21.69				
18:1trans11	CON	0.85	0.99	1.07	0.72	0.24	0.04	0.01	0.06
	SFAT	0.84	0.66	1.22	0.86				
	FISH	0.41	1.47	1.76	1.43				
18:2c9t11	CON	0.07	0.12	0.26	0.26	0.04	0.85	<0.01	0.33
	SFAT	0.12	0.08	0.26	0.20				
	FISH	0.11	0.04	0.29	0.28				
18:2n-6	CON	4.48	4.88	6.45	7.75	0.60	0.14	<0.01	0.80
	SFAT	4.29	4.36	5.52	6.42				
	FISH	3.68	4.01	6.22	7.31				
18:3n-3	CON	0.50	0.73	1.36	1.34	0.20	0.33	<0.01	0.19
	SFAT	0.89	0.46	1.54	1.16				
	FISH	0.62	0.54	1.74	1.74				
20:4n-6	CON	2.24	2.77	0.60	0.81	0.59	0.90	<0.01	0.44
	SFAT	2.92	2.42	0.45	0.51				
	FISH	1.81	3.93	0.66	0.57				
20:5n-3	CON	0.06$^{\alpha}$	0.24$^{\beta}$	0.07$^{a,\alpha}$	0.11$^{a,\alpha,\beta}$	0.06	<0.01	0.23	0.04
	SFAT	0.15	0.11	0.06^{a}	0.10^{a}				
	FISH	0.09$^{\alpha}$	0.25$^{\alpha,\beta}$	0.36$^{b,\beta,\gamma}$	0.29$^{b,\beta,\gamma}$				
22:5n-3	CON	0.62$^{\alpha}$	1.30$^{b,\beta}$	0.62$^{a,\alpha}$	0.61$^{a,\alpha}$	0.20	<0.01	0.02	<0.01
	SFAT	0.52	0.69^{a}	0.61^{a}	0.56^{a}				
	FISH	0.47$^{\alpha}$	0.84$^{a,b,\alpha}$	1.61$^{b,\beta}$	1.57$^{b,\beta}$				
22:6n-3	CON	0.04^{b}	0.27^{a,b}	0.04^{a}	0.06^{a}	0.10	<0.01	<0.01	<0.01
	SFAT	0.35$^{a,\alpha}$	0.04$^{a,\beta}$	0.06$^{a,\beta}$	0.04$^{a,\beta}$				
	FISH	0.09$^{a,b,\alpha}$	0.46$^{b,\beta}$	1.26$^{b,\gamma}$	1.01$^{b,\gamma}$				

Notes: †Standard error of the mean; ‡diet × time interaction. $^{a–c}$Difference ($P < 0.05$) between diets on the same day. $^{\alpha,\beta,\gamma,\delta}$Significant interactions ($P < 0.05$) within a diet and between days.

(diet × day $P < 0.05$) was observed in the proportion of 18:3n-6, namely due to the response in the control group. The overall increase, however, was of a lower magnitude in FISH as compared with SFAT (Table S2).

Hepatic gene expression

Fatty acid transport and LD formation

The expression of genes involved in fatty acid uptake/transport, storage, and oxidation changed in a different fashion with lipid supplementation as compared to the control cows. The expression of the LCFA-uptake protein *CD36* in the prepartal period was lower (diet × day $P < 0.05$) with SFAT than FISH and control, and was markedly down regulated after parturition in response to FISH. In contrast, an increase (diet × day $P < 0.05$) in *FABP1* expression postpartum was observed in cows fed SFAT and the controls, at which point expression was greater in those cows compared with FISH (Table 5).

Feeding SFAT led to lower (diet × day $P < 0.05$) *DGAT2* prepartum when compared to control or FISH, however that diet resulted in an increase in expression of *DGAT2* postpartum, at which point expression of *DGAT2* was similar between control and FISH. Quite remarkably, the expression of *SCD* was lowest (diet

Table 5. Relative expression (treatment means, log-2 scale) of genes involved in fatty acid uptake, esterification, desaturation, lipid droplet formation, fatty acid oxidation, gluconeogenesis and cellular energy during the peripartal period in cows fed (n = 5/treatment) control, fish oil (FISH), or saturated lipid (SFAT).

Gene	Prepartum			Postpartum			SEM†	*P* value		
	Control	FISH	SFAT	Control	FISH	SFAT		Diet	Time	D × T‡
Fatty acid uptake/transport										
CD36	–0.02[a]	–0.59[a,b,*]	–1.48[b]	0.12[a]	–1.91[b,*]	–1.07[a,b]	0.43	0.06	0.18	<0.01
FABP1	1.34*	1.39	0.99*	1.99[a,*]	0.99[b]	1.50[a,b,*]	0.24	0.20	0.13	<0.01
Esterification, desaturation, lipid droplet formation										
DGAT2	1.49[a]	0.96[a,b]	0.33[b,*]	2.09[a]	1.00[b]	1.52[a,b,*]	0.34	0.13	<0.01	<0.05
SCD	–0.43[a,*]	–4.11[c]	–2.02[b]	–1.69[a,*]	–4.21[b]	–1.72[a]	0.38	<0.01	0.22	0.09
PLIN2	2.59	1.97*	1.42*	3.21[a]	0.97[b,*]	2.29[a,*]	0.41	<0.05	0.43	<0.01
PLIN4	–2.57	–2.38	–2.71	–1.90	–3.38	–3.12	0.51	0.45	0.33	0.09
Fatty acid oxidation										
ACSL1	3.95[a,*]	2.88[a,b,*]	2.72[b,*]	4.76[a,*]	2.12[c,*]	3.40[b,*]	0.41	<0.01	0.21	0.02
ACOX1	2.71[a]	2.24[a,*]	1.22[b,*]	2.24[a,b]	1.36[b,*]	2.48[a,*]	0.33	0.10	0.9	<0.01
CPT1A	3.08	2.92	2.20	3.54	1.76	2.20	0.71	0.20	0.54	0.14
FGF21	–0.51*	–0.06	1.07	5.55[a,*]	2.10[b]	1.08[b]	1.09	0.33	<0.01	<0.01
Gluconeogenesis										
PC	3.57*	3.25	2.83	5.73[a,*]	2.95[b]	3.54[b]	0.53	0.06	<0.01	<0.01
PCK1	3.76*	3.72	3.49	5.13[a,*]	3.10[b]	3.21[b]	0.51	0.17	0.56	0.04
Intracellular energy										
UCP2	2.98	2.76*	1.60	1.96	0.76*	1.62	0.67	0.33	0.04	0.04
PRKAA1	3.80	3.59*	2.90	4.19[a,b]	2.36[b,*]	2.90[a,b]	0.50	0.22	0.16	0.01
STK11	4.09	3.78*	2.96	3.57	2.39*	2.81	0.44	0.21	<0.01	0.04

Notes: †Standard error of the mean; ‡diet × time interaction. [a–c]Difference (D × T, $P < 0.05$) between diets at prepartum or postpartum times. *Difference (D × T, $P < 0.05$) prepartum vs. postpartum within diet.

$P < 0.05$) for SFAT and FISH than controls during the prepartal period. Its expression, however, decreased markedly after parturition in control cows but not in lipid-supplemented cows. The LD-associated proteins *PLIN2* (formerly known as adipophilin) and *PLIN4* (formerly known as S3-12) did not differ in expression among groups in the prepartal period. However, there was a decrease in expression of *PLIN2* in cows fed FISH while an increase was observed in cows fed SFAT (diet × day $P < 0.05$). The overall result was that *PLIN2* expression postpartum was similar between SFAT and controls and lower with FISH.

Fatty acid oxidation

The expression of *ACSL1* and *ACOX1* in the prepartal period was greater in cows fed FISH than SFAT. After parturition, however, cows fed FISH had lower expression and cows fed SFAT had greater expression of both genes resulting in an overall interaction effect (diet × day $P < 0.05$). The control cows also had an increase in *ACSL1* expression after parturition, actually resulting in greater overall expression than SFAT and FISH. Whereas expression of the PPARα target *CPT1A* did not differ due to treatment or day, the expression of *FGF21* which is another PPARα target increased to the greatest extent (diet × day $P < 0.05$) postpartum in the controls. Thus, in the postpartal period *FGF21* expression was greatest in controls relative to FISH and SFAT.

Genes involved in gluconeogenesis and intracellular energy sensing

There was no interaction (diet × day $P > 0.05$) in the prepartal period for the expression of PC and *PCK1*; however, expression of both genes increased (diet × day $P < 0.05$) markedly postpartum in the control cows, at which point cows fed FISH and SFAT had lower overall expression largely due to a lack of change in expression from the prepartal period. In a similar fashion, there was no significant prepartal interaction effect on the expression of *UCP2*, *PRKAA1*, and *STK11*, which are involved in intracellular sensing of ATP and signaling via the AMPK pathway. However, cows fed FISH had

a marked decrease (diet × day $P < 0.05$) in expression of these three genes in the postpartal period leading to a lower overall *PRKAA1* expression postpartum.

Transcription regulators and nuclear co-activators and repressors

No obvious pre- and postpartal differences were observed in the expression of *PPARA*, while *RXRA* had an overall downregulation after parturition (time $P = 0.06$), due primarily to feeding control and FISH (Table 6). Feeding SFAT resulted in lower (diet × day $P < 0.05$) *SREBF2* prepartum compared with controls, but unlike FISH it did not elicit a decrease in expression in the postpartum period. Thus, overall *SREBF2* expression postpartum was similar in controls and SFAT and lower in FISH. Similar to *SREBF2*, feeding FISH led to lower (diet × day $P < 0.05$) expression of *PPARD* between the pre and postpartal period at which point cows fed both FISH and SFAT had lower (diet × day $P < 0.05$) expression than controls.

Feeding FISH and SFAT vs. control led to lower expression in the prepartal period of the nuclear receptor co-regulators *CARM1, NCOR2, MED1*, and *RIP1* (diet × day $P < 0.05$). Expression of all these genes decreased (diet × day $P < 0.05$) in the postpartum period exclusively in cows fed FISH such that control cows still had greater overall expression followed by cows fed SFAT.

Inflammation and apoptosis

The expression of *IL6, TBK1*, and *IKBKE* in the prepartal period was lowest (diet × day $P < 0.05$) in cows fed SFAT in comparison to the control or FISH cows (Table 7). Interestingly, cows fed FISH experienced a decrease (diet × day $P < 0.05$) in postpartal expression of these genes while expression in SFAT-fed or control cows remained unchanged. Except for *TBK1* and *IKBKE*, expression of *IL6* was lower (diet × day $P < 0.05$) overall postpartum in cows fed SFAT and FISH. Cows fed FISH also had a decrease (diet × day $P < 0.05$) in *CIDEB* expression postpartum at which point expression was lower compared with controls and SFAT.

Discussion

Hepatic PL and TAG fatty acid composition

Changes observed in the hepatic fatty acid profiles of PL and TAG were likely driven by a combination of (1) alterations in LCFA composition of adipose tissue induced by feeding, (2) mobilization of LCFA postpartum in response to lipolytic signals, and (3) biohydrogenation of PUFA in the rumen leading to increases in trans fatty acids. The contribution of adipose tissue LCFA to the PL and TAG fraction of lipids in dairy cattle liver has been demonstrated previously.[2,17] Schmitt et al[14] using the same cows from this study, reported that all groups had an increase in liver TAG after parturition, which is a typical

Table 6. Relative expression (treatment means, log-2 scale) of genes encoding transcription regulators, nuclear receptor co-activators and co-repressors during the peripartal period in cows fed (n = 5/treatment) control, fish oil (FISH), or saturated lipid (SFAT).

Gene	Prepartum			Postpartum			SEM†	*P* value		
	Control	FISH	SFAT	Control	FISH	SFAT		Diet	Time	D × T‡
Transcription regulators										
PPARA	0.41	0.63	0.26	0.38	0.49	0.14	0.23	0.54	0.31	0.88
RXRA	3.17	2.85	2.31	2.60	1.36	2.24	0.57	0.30	0.06	0.77
PPARD	3.18	2.86*	1.93	3.72[a]	1.19[b,*]	2.22[b]	0.45	0.04	0.18	<0.01
SREBF2	4.43[a]	3.74[a,b,*]	3.18[b]	3.72[a]	2.59[b,*]	3.74[a]	0.36	0.01	0.66	0.02
Nuclear receptor co-activators										
CARM1	4.20[a]	3.48[b,*]	2.84[b]	4.04[a]	2.10[b,*]	3.24[a,b]	0.37	0.03	0.08	<0.01
MED1	4.30[a]	3.57[b,*]	2.89[b]	4.21[a]	2.15[c,*]	3.37[b]	0.38	0.02	0.12	<0.01
Nuclear receptor co-repressors										
NCOR2	3.67[a]	3.00[a,b,*]	2.35[b]	3.69[a]	1.91[b,*]	2.73[b]	0.33	0.01	0.30	0.04
NRIP1	3.60[a]	2.78[b,*]	2.40[b]	3.48[a]	1.64[b,*]	2.72[b]	0.36	0.03	0.14	0.03

Notes: †Standard error of the mean; ‡diet × time interaction. [a–c]Difference (D × T, $P < 0.05$) between diets at the prepartum or postpartum times. *Difference (D × T, $P < 0.05$) prepartum vs. postpartum within diet.

Table 7. Relative expression (treatment means, log-2 scale) of genes involved in inflammation and cell growth during the peripartal period in cows fed (n = 5/treatment) control, fish oil (FISH), or saturated lipid (SFAT).

Gene	Prepartum			Postpartum			SEM[†]	*P* value		
	Control	FISH	SFAT	Control	FISH	SFAT		Diet	Time	D × T[‡]
Inflammation related genes										
IL6	3.04[a]	2.11[a,*]	1.06[b]	2.32[a]	0.90[b,*]	1.48[b]	0.35	0.01	0.03	0.02
TBK1	4.37[a]	3.68[a,*]	2.78[b]	4.04[a]	1.98[b,*]	3.44[a]	0.38	0.03	0.05	<0.01
IKBKE	3.58[a]	3.28[a,*]	2.17[b]	3.34[a]	1.39[b,*]	2.76[a]	0.37	0.05	0.03	<0.01
Apoptosis										
CIDEB	5.38	4.40*	4.18	5.63[a]	3.52[b,*]	4.82[a]	0.40	0.02	0.99	0.04

Notes: [†]Standard error of the mean; [‡]diet × time interaction. [a–c]Difference (D × T, $P < 0.05$) between diets at the prepartum or postpartum times. *Difference (D × T, $P < 0.05$) prepartum vs. postpartum within diet.

response observed due to the increase in circulating NEFA (ie, from mobilization of adipose tissue) (Fig. 1B). Although not measured in these cows, it is likely that the pool of LCFA stored in adipose tissue TAG and also in liver PL during the prepartal period in particular, contributed to the observed changes in gene expression profiles. This idea is supported by the fact that both FISH and SFAT upregulated several genes associated with lipid metabolism and the PPAR pathway namely in the prepartal period.[14]

Dietary fat supplementation often produces a pronounced effect on hepatic PL composition.[2] In the present study, the postpartal decrease of 18:0 in the concentration and the increase in 18:1trans isomers (particularly trans11) with FISH was consistent with greater biohydrogenation of dietary PUFA.[15] The postpartal increase in 16:0 and 18:2n-6 regardless of diet likely was associated with increased adipose tissue lipolysis and subsequent transport and storage in the liver.[17] The observed decrease in the PL concentration of γ linolenic acid (18:3n-6) might have been due to allosteric inhibition or transcriptional regulation of the elongases as well as the Δ5- and Δ6-desaturases or to competition between n-6 and n-3 substrates for incorporation into PL.[18]

The observed increase in the hepatic PL and TAG content of α linolenic acid (18:3n-3) likely accounted for the increase in content of 20:5n-3, 22:5n-3, and 22:6n-3 at calving[2]; α linoleic acid can be desaturated and elongated to 20:5n-3 and 22:6n-3. The decrease in dihomo-γ-linoleic acid (20:3n-6) and arachidonic acid (20:4n-6) with FISH and SFAT after parturition as compared with the control (Table 3) might have been due to a low baseline concentration of linoleic acid (18:2n-6) in hepatic PL[15] as linoleic acid is desaturated and elongated to 20:3n-6 and subsequently to 20:4n-6.[2] Another possibility is that greater supply of 18:2 resulted in competition and displacement of other long chain PUFAs for esterification into PL.[2] Similarly, increased tissue content may inhibit elongation and desaturation of 18:2 and 18:3 as observed in calves.[19] The lower level of γ linolenic acid in FISH could have affected the concentration of arachidonic acid as γ linolenic acid is the intermediate in the conversion of linoleic acid to arachidonic acid.[20]

Intracellular metabolism and LCFAs in bovine

The metabolism of specific LCFAs has been studied previously in vitro using hepatocytes isolated from neonatal calves.[21,22] Despite the inherent difficulties in comparing in vitro and in vivo studies, in the context of our study, data generated with incubations of 16:0 alone or in combination with 18:0, 18:1, 20:5n-3, or 22:6n-3 are of interest. For instance, greater total oxidation (CO_2 and acid-soluble products) of ^{14}C-16:0 were observed with incubations of 1 mM 16:0 plus 1 mM 20:5n-3 and 18:1 when compared with incubation of 1 mM 16:0 alone.[21] In contrast, the incubation containing 20:5n-3 did not increase use of ^{14}C-palmitic acid for cellular TAG synthesis, while 18:1 did. Palmitate plus 22:6n-3 did not affect total oxidation of ^{14}C-palmitic acid or its esterification to TAG.[21] Results from that initial study clearly underscored the differential utilization of LCFA for esterification or fatty acid oxidation in liver cells. Although there were no data on mRNA or protein expression of lipid metabolism enzymes, it would not be unreasonable to suspect (based on data from model organisms) that changes in expression would have been partly responsible for the changes observed. For instance, 18:1

either from exogenous sources or synthesized from 18:0 via SCD, is central for cellular TAG synthesis and LD formation,[23] and is probably an essential step in very-low-density lipoprotein (VLDL) synthesis in the liver.

A second study confirmed the response of 18:1 only in terms of enhancing the use of 16:0 for esterification, but did not confirm the effect of 20:5n-3 on ketone body formation from 16:0.[22] Furthermore, there was an increase in cellular TAG with incubations of 22:6n-3. However, contrary to the first study incubation of 1 mM 16:0 plus 1 mM 18:0 resulted in greater BHBA concentration in culture media as a result of greater oxidation rate of ^{14}C-16:0.[22] Although we did not detect statistical differences in blood BHBA due to lipid supplementation,[14] the likely fluctuations in BHBA metabolism during lactation, when compared with in vitro systems, would make it difficult to use this marker to discern physiological effects in vivo.

Additional analyses from this second study included rate of gluconeogenesis and concentration of cellular glycogen. Linolenic acid plus 16:0 resulted in the highest rates of gluconeogenesis from ^{14}C-propionic acid and greatest amounts of intracellular glycogen, along with reduced TAG production.[22] An interesting finding was that 22:6n-3 plus 16:0 incubation, or either plus 20:5n-3, increased cellular TAG content and incorporation of ^{14}C-palmitic acid into cellular TAG. In addition, 22:6n-3 plus 16:0 decreased metabolism of ^{14}C-propionic acid to glucose or to cellular glycogen in the medium.[22]

The in vitro work with hepatocytes has, by necessity, been short-term compared with the longer-term feeding studies. Furthermore, the above studies used supra-physiological concentrations of each LCFA. Their expected concentration in the circulation of the cow after parturition is unlikely to reach the 1 mM level. In fact, unless the cow is under a ketotic situation, the peak total NEFA concentration after parturition is rarely greater than 1.5 mM.[24] Although the resulting data from these in vitro studies have helped expand our knowledge of the metabolic effects of specific LCFA, the observed results are challenging to place in the in vivo context. Thus, gene expression data obtained over a longer time-frame from cows in field conditions may provide a more physiologically-relevant view of the likely mechanistic effects of enriching diets with specific LCFA.

Hepatic gene expression

Fatty acid transport and metabolism

The expression of *FABP1* in dairy cows has been previously characterized as increasing between parturition and 14 days postpartum, a response which was proposed to denote downstream activation of PPARα via NEFA metabolism.[5] However, in the present study, even with the increase in NEFA postpartum (Fig. 1B), the *FABP1* expression remained stable in cows fed SFAT and FISH while it was upregulated in controls. That response suggests that irrespective of the type of fat supplemented, this intra-cellular transporter could be modulated without the influence of the rising postpartum NEFA. On the other hand, the pronounced down-regulation of *CD36* after parturition in cows fed FISH (Table 5) suggested a possible biological role of PUFA in the control of the intracellular flux of LCFA. Such a response would be contradictory with non-ruminant data (ie, upregulation of *CD36* with PUFA) indicating an intra-species difference in the response of liver cells to supplementation with PUFA, as already proposed in bovine kidney cells.[10]

The contrasting response between liver tissue in the present study and bovine kidney cells[10] in the same species is not surprising and obviously is likely related with the different types of cells studied in addition to the endocrine and metabolic environment. The complexity of the LCFA pool that hepatocytes are exposed to also has shown to influence the degree of PPAR gene network activation in rodent liver.[25–27] As stated before, feeding FISH led to a large difference in the concentration of 18:1trans11, 18:1trans12, 22:6n-3, and 20:5n-3, all of which could potentially play a role in the activation of the PPARα gene network at least judging from in vitro data.[28]

Esterification, desaturation, and LD formation

The enzyme *SCD* is responsible for biosynthesis of monounsaturated fatty acids 18:1 and 16:1 from 16:0 and 18:0, which are substrates for de novo synthesis of PL, cholesterol esters, and TAG.[29,30] Our results with *SCD* agree with reports from other animal species where both omega-3 and omega-6 PUFA decreased *SCD* expression partly by decreasing mRNA stability.[30–34] On the other hand, the *SCD* down-regulation with SFAT is contrary to in vitro reports with bovine kidney cells[10] in which *SCD* was upregulated by exogenous 16:0 and 18:0, the main LCFA in the blood of

ruminants. However, the downregulation of *CD36* could partly explain the lower *SCD* if in fact there was less 18:0 and 16:0 taken up from blood, leading to reduced substrate availability.

In rodents, *SCD* and *DGAT2* are located adjacently in the outer endoplasmic reticulum membrane.[35] It has been proposed that *SCD* indirectly influences the activity of *DGAT2* by supplying substrates (16:1 and 18:1) for synthesis of TAG.[29] Our data revealed a similar pattern of expression response for *SCD* and *DGAT2* across diets, namely postpartum. The *DGAT2* enzyme plays a key role in the cytosolic accumulation of TAG,[36–38] which could in the post-absorptive state provide LCFA for lipoprotein synthesis. The lower expression of this gene postpartum with the FISH group than with the control and the lack of difference between FISH and SFAT could be taken as an indication of decreased use of LCFA for TAG synthesis. Such response would have channeled LCFA towards PL and/or cholesterol ester synthesis, VLDL synthesis, or oxidation.[23]

The lower *SCD* expression also could have been associated with the response observed for *PLIN2*, another enzyme linked with liver TAG accumulation and size of LDs.[39] Studies in vitro with different non-ruminant cell lines[40,41] demonstrated that *ADFP* is upregulated by PUFA, which was obviously not induced by FISH. The increase of *PLIN2* expression postpartum we observed in the SFAT group agrees with previous data from rodent hepatocytes[40] and underscores the fact that the increase in NEFA alone is not the sole mechanism for activating transcription of this gene. Hepatic TAG accumulation typically follows the rise in NEFA postpartum.[14,24] As an integral LD protein, *PLIN2* may serve more critical roles in managing the turnover of neutral lipid stores to facilitate the coordinated release of LCFA into lipoproteins in response to changes in metabolic state.[23]

Unlike *PLIN2*, *PLIN4* is an exchangeable cytosolic LD protein that facilitates rapid protein association with the immature LD.[5,42] Generally in the liver, the *PLIN4* is associated with smaller and peripheral LD and is stimulated by high concentrations of LCFA while *PLIN2* is associated with bigger and more mature LD.[43] The observed changes postpartum for *PLIN2* in cows fed SFAT (upregulation) and FISH (downregulation) seem to suggest a difference in potency of LCFA, or alternatively, as in the case of the FISH group, a protective mechanism of the cell to buffer from excessive LCFA influx.

Fatty acid oxidation

In the liver, *ACSL1* is central for the synthesis of LCFA-acyl-CoA, which can then be channeled towards β-oxidation.[44] The regulation of expression of this enzyme by PPARα activation in rodents is well-established,[44–46] and recent work demonstrated a similar response in bovines.[10,47] Previous work with peripartal dairy cows fed diets without supplemental lipids revealed that *ACSL1* increases expression between −14 to 1 day postpartum after which it remains unchanged through 14 days. This response could help in the metabolism of incoming NEFA, thus channeling them towards β-oxidation.[5,48] We observed a similar response in the control cows and those fed SFAT. Thus, the down regulation of *ACSL1* in cows fed with FISH, along with *CD36* in particular, would agree with the concept of different LCFA eliciting opposite effects on transcription of *ASCL1* and *CD36*. It could be envisioned that such a response would prevent excessive influx of LCFA but also serve to control their use for esterification or oxidation.

The lack of difference for *ACOX1* expression postpartum between control and SFAT suggested that, on the one hand, enhanced dietary LCFA flux into liver did not alter peroxisomal oxidation (ie, at a greater availability of LCFA, the liver from SFAT-fed cows might have been capable of oxidizing a greater fraction) and on the other hand, that not enough dietary LCFA reached the liver to cause an effect. The latter case is likely because of the mammary gland's preference for taking up LCFA from the circulation, and thus in a way buffering other tissues.[15] The effect of fish oil on *ACOX1* expression has been evaluated in several rodent studies which demonstrated that PUFA are potent activators.[25,49–51] Mechanistically, such a response makes sense as it is the first and rate-limiting enzyme of the peroxisomal fatty acid β-oxidation pathway,[52] which is important in peripartal liver lipid metabolism.[53] The prepartal response with FISH vs. SFAT appeared to be in line with rodent data. However, the postpartal decrease in *ACOX1* with FISH coupled with the increase with SFAT suggested that saturated LCFAs were more

potent in activating *ACOX1*, which agrees with in vitro data.[10] These results highlight that in peripartal cows, feeding FISH (at least at the levels of this study) might not be effective in enhancing LCFA oxidative capacity.

In our analysis of adipose tissue gene expression from these cows, we found some evidence that lipid supplementation could influence the production of adipokines by adipose tissue, hence, influence indirectly the hepatic capacity of β-oxidation.[14] It has been proposed in rodents that adiponectin binding to its hepatic receptor (*ADIPOR2*) leads to activation of both AMPK and PPAR signaling pathways and, consequently, *ACOX1* activation.[5,54] We observed that FISH led to lower postpartal expression of *ADIPOR2* and *ADIPOQ* in the adipose, and lower *ADIPOR2* in liver tissue.[14] In contrast, *ADIPOR2* expression in liver and *ADIPOQ* in adipose tissue were the same in the control and SFAT. Such responses could be mechanistically related with the lower postpartal *ACOX1* and a potential overall reduction in β-oxidation when dietary FISH supplementation.

Recently, it was reported that FISH inhibits de novo lipogenesis and β-oxidation, and decreases insulin resistance in non-ruminants. The improvement in insulin sensitivity is mediated by down regulating the PPARG network, *ChREBP,* and *SREBF1*. Furthermore, FISH also increases adiponectin (powerful insulin-sensitizing agent) production in adipose tissue.[55] The decrease in DMI (Fig. 1A) along with the lower milk yield,[14] and the observed down regulation of *SCD* and *DGAT2* in the present study and in adipose tissue (*SCD, DGAT2 ChREBP,* and *SREBF1*) in the study of Schmitt et al,[14] suggests that FISH may actually have had a negative impact on genes involved in LCFA oxidation. Additional research seems warranted to elucidate the underlying mechanisms and their physiological relevance.

Peroxisomal oxidation results in the increased production of shorter-chain fatty acyl-CoA that generally are channeled to be completely oxidized in mitochondria.[5] Although *CPT1A* has a key role in this process, the lack of change in expression in our study seems to confirm previous data, providing evidence that this enzyme is not strongly controlled at the transcription level even during periods of severe negative energy balance.[56] Rodent *CPT1A* is markedly upregulated in response to undernutrition and fasting, thus underscoring additional differences between species in the control of hepatic LCFA oxidation. Despite the apparent lack of *CPT1A* activation (ie, it is a PPARα target in rodents) the upregulation of *FGF21* (another PPARα target) postpartum provided evidence of transcriptional adaptations that could have been driven via PPARα.

In rodents, the PPARα protein is required for the normal activation of hepatic LCFA oxidation, TAG clearance, and ketogenesis.[57] Despite obvious biological variation in its expression across treatments, the pattern of *FGF21* expression that was observed in this study seemed to confirm that this protein is as important in coordinating hepatic adaptations to undernutrition in ruminants as in rodents. However, the lack of postpartal increase in cows fed SFAT or FISH could indicate that excess LCFA influx into liver actually might feedback-inhibit the transcriptional activation of *FGF21* and more potently when feeding PUFA (eg, FISH) than saturated LCFA. From a mechanistic standpoint, the attenuation of *FGF21* did not seem to curtail LCFA oxidation in cows fed SFAT (ie, *ACOX1* expression increased) and blood BHBA was similar among treatments.[14] Overall, the present and previous data[4] suggest that *FGF21* in cows is associated with negative energetic balance.

Genes involved in gluconeogenesis and intracellular energy sensing

The predominant glucose precursors in dairy cows are propionate and lactate, although the contribution of alanine and glycerol becomes quantitatively more important during conditions of propionate deficit (ie, the early postpartal period).[58,59] Partitioning of lactate and alanine towards gluconeogenesis is under the control of *PC*, the expression of which increases sharply after parturition,[13,60] feed restriction,[61] and experimentally-induced glucose deficit[62] to facilitate flux of alanine and lactate toward oxaloacetate rather than acetyl-CoA.

The upregulation of *PC* at calving is linked with increased concentrations of NEFA in plasma. This is not surprising as several metabolic reactions are regulated by LCFA at the level of the activation of genes that encode key regulatory enzymes,[63,64] including gluconeogenesis and fatty acid metabolism. The observed increase in linoleic acid concentration in PL after calving in controls (and comparatively lower

in FISH) seems to support in vitro data, demonstrating that linoleic acid could serve to activate *PC* and enhance the capacity for fatty acid oxidation and glucose synthesis.[64] Furthermore, there is evidence that greater supply of linoleic acid can enhance the rate of gluconeogenesis in bovine hepatocytes.[22] Cellular-membrane bound linoleic acid also can serve as a ligand for NR that regulate gene expression. For example, in hepatocyte cultures, this LCFA increased the *PCK1* mRNA level in a dose-dependent manner.[65]

The exogenous LCFA can enhance the rates of hepatic mitochondrial oxidation by uncoupling oxidation from ATP production.[5] The uncoupling proteins (UCPs) are key players in mitochondrial oxidation, the activity of which can be induced by LCFA. Monounsaturated and PUFA appear more effective than saturated LCFA in activating the liver specific isoform *UCP2*.[66] *UCP2* is a demonstrated non-ruminant PPARα target in vivo and has been proposed to play a role in lipid metabolism, insulin resistance, glucose utilization, regulation of reactive oxygen species, and macrophage-mediated immunity.[67,68]

Armstrong and Towle[66] reported induced expression of hepatic *UCP2* with high concentrations of LCFA. To some extent, that effect lends support to the observed lower postpartal *UCP2* expression in cows fed FISH vs. SFAT as they had numerically-lower blood NEFA (Fig. 1B). Insulin[69] and aspirin[66] significantly reduced the expression of *UCP2* mRNA in rodent liver. Because the high NEFA concentration postpartum seems to directly impair the ability of the pancreas to secrete insulin in postpartal dairy cows,[70] it is unlikely that in the present study insulin had a mechanistic role on the expression of *UCP2*. In contrast, the fact that aspirin reduced *UCP2* in rodent liver despite high concentrations of LCFA could indicate that in our study the prostaglandin pathway might have been physiologically relevant. This idea is supported by the lower hepatic PL concentration of 20:4n-6 (the immediate precursor of prostaglandins) in cows fed FISH when compared with those fed SFAT.

Nuclear receptor co-activators and co-repressors

The co-activator-associated arginine methyltransferase I (CARM1) is a critical component of glucose metabolism in rodent hepatocytes.[71] There it physically interacts with cAMP-responsive element binding factor CREB before both being recruited to the PCK1 and glucose-6-phosphatase promoters in a cAMP-dependent manner particularly during periods of dietary glucose short-falls (eg, undernutrition, starvation, negative energy balance).[72] CARM1 regulates gene expression by multiple mechanisms including methylation of histones and co-activation of steroid receptor transcription.[73] The observed postpartal decrease of *CARM1* with a FISH supplemented diet agrees with the lower expression of *PCK1* (Table 5). In addition, it is possible that differences in intracellular cAMP concentration also might have played a role in both *CARM1* and *PCK1* upregulation. We speculate that because of the lower rate of milk production and DMI in response to FISH, the intracellular levels of cAMP (driven by the increase in glucagon after parturition)[74] would have been lower in those cows. The lower DMI due to feeding FISH was clearly a long-term response which preceded parturition. Thus, cows had likely adapted by the time of parturition in a way that the lower rate of milk production was driven by a level of DMI which was appropriate to meet the energy demands of the mammary gland.

Mediator 1 (MED1) is required for high-fat diet-induced hepatic steatosis via PPARγ, and loss of MED1 protects rodents against fatty liver.[73] A biologically-similar role for *PPARG* in ruminant liver is unlikely because this isoform is substantially lower in abundance than *PPARA* or *PPARD* (data not shown). In fact, in this study the expression of *PPARD* and not *PPARA* was affected by the onset of lactation and by the type of lipid fed. Our data seem to suggest a mechanistic link between *PPARD*, *CARM1*, and *MED1*, particularly postpartum when the responses due to diet for the three genes were the same. This suggestion is supported by the observed responses in the expression of the co-repressors *NCOR2* and *NRIP1*.

Nuclear receptor co-repressor 2 (*NCOR2*), in tandem with specific NR and different DNA binding transcription factors, represses the transcription of target genes.[75] In non-ruminants, *NRIP1* seems to play dual roles in metabolic tissues but the precise mechanisms driving its co-activator role remain elusive.[76] What seems evident from previous rodent studies is that *NRIP1* is required for expression of genes associated with energy metabolism (eg, *UCP1*, *CPT1A*) partly

under control of β-adrenergic stimulation and PPARα and PPARδ.[77]

Transcription regulators of lipid metabolism

In non-ruminants, sterol regulatory element binding proteins *SREBF1* and *SREBF2* act as a central hub to control the transcription of genes required for cholesterol, fatty acid, TAG, and PL.[78,79] In the liver, *SREBF1* preferentially regulates genes involved in fatty acid synthesis while *SREBF2* regulates genes associated with cholesterol biosynthesis.[80,81] The activation of *SREBF2* in the liver is mediated by *SREBF* cleavage binding protein (SCAP). CIDEB controls cholesterol biosynthesis by regulating the levels of SCAP at the transcriptional level.[80] In the present study, the postpartal decrease in *SREBF2* with FISH (Table 6) could have been a consequence of lower *CIDEB* expression (Table 7), which may have decreased abundance of SCAP and, thus, reduced the transport and activation of *SREBF2*. From a physiological perspective, such decrease in *SREBF2* expression could have resulted in lower endogenous synthesis of cholesterol, which is an important process in liver as a part of the lipoprotein synthesis pathway.

Inflammation and apoptosis

Dietary LCFA can impact immunity through the production of cytokines and molecules involved in the regulation of immune responses.[82] Omega-3 and omega-6 PUFAs are important immunomodulators.[83] The transcription factors interferon regulatory factor 3 (IRF3) and NFkB are the central points of an integrated network of genes involved in the innate immune response, whereas inhibitors of kappa light polypeptide gene enhancer in B-cells, kinase epsilon (*IKBKE*) and TANK-binding kinase (*TBK1*), play a pivotal role in coordinating the activation of both those genes.[84] The decrease in expression of *IL6, TBK1*, and *IKBKE* postpartum in cows fed FISH could have been associated with lower concentration of 20:4n-6 in PL. This fatty acid is the major substrate for prostaglandins and a key link between PUFA and inflammation.[82,85]

The observed postpartal increase in 20:5n-3 and 22:6n-3 in PL of cows fed FISH likely inhibited 20:4n-6 metabolism directly, via substrate competition, or indirectly, by altering the expression of inflammatory genes through effects on transcription factor activation.[85] Feeding plant or fish oil rich in omega-3 PUFA generally reduces inflammatory reactions and production of interleukin (IL)-1, IL-6, MMP-3 (STR1), and tumor necrosis factor.[86,87] Such type of effects might be less pronounced in dairy cattle because of the substantial degree of biohydrogenation of PUFA in the rumen and also because of the substantial uptake of LCFA by the mammary gland during lactation. However, the data provides strong evidence that the enrichment of omega-3 PUFA in liver PL due to feeding FISH likely was important in lowering the expression of inflammatory genes. A possible mechanism for such an effect could have encompassed PPARδ, which was recently shown to be activated in dairy cattle liver during inflammation.[88]

The cell death-inducing DFFA-like effector b (CIDEB) protein has a high level of expression in non-ruminant liver, and its deficiency affects energy expenditure, plasma TAG levels,[89] and also alters the expression of genes involved in various metabolic and signaling networks.[80] The *CIDEB* protein is localized to the LD, endoplasmic reticulum, and Golgi apparatus and facilitates VLDL lipidation and maturation in the liver by interacting with ApoB.[80] In the present study, the decrease in *CIDEB* expression with FISH may have led to reduced expression of *SREBF2*, de novo cholesterol biosynthesis, and potentially an augmentation of the cellular inflammatory status.

Conclusions

The present study revealed the type of dietary fatty acid which affects the hepatic fatty acid profile of PL and TAG. At the levels supplemented, the change in the profile of metabolic genes after parturition in cows fed saturated fat suggested a greater capacity for uptake of fatty acids and intracellular handling without excessive storage of LD. The lack of difference in liver TAG concentration between lipid-supplemented groups and the downregulation of metabolic genes after parturition in cows fed fish oil suggested that it might not be effective in enhancing oxidative capacity of LCFA. Such response contrasts the effect of very-long chain PUFA in monogastric species. The results highlighted that both saturated and very-long chain PUFA seemed equally effective at helping decrease inflammatory gene expression but FISH had a more potent effect after parturition. Based on the combined data from this study additional studies to better delineate the

effective doses of saturated and very-long chain PUFA to feed around parturition seem warranted.

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Author Contributions

Conceived and designed the experiment: MAB, EJD. Conceived and/or performed the analyses: HA, ES, JJL. Wrote the manuscript: HA, ES, MNC, JJL. Agree with manuscript results and conclusions: HA, ES, MAB, MNC, EJD, JJL. Jointly developed the structure and arguments for the paper: HA, ES, JJL. Made critical revisions and approved final version: HA, ES, MAB, MNC, EJD, JJL. All authors reviewed and approved of the final manuscript.

Competing Interests

Author(s) disclose no potential conflicts of interest.

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.

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Supplementary Tables

Table S1. Concentration of fatty acids in hepatic phospholipids during the peripartal period.

Fatty acid	Diet†	Day				SEM‡	*P* value		
		–21	**–10**	**1**	**11**		**Diet**	**Time**	**D × T¶**
14:0	CON	0.25	0.35	0.20	0.20	0.09	0.71	<0.01	0.22
	SFAT	0.53	0.31	0.18	0.16				
	FISH	0.52	0.19	0.20	0.17				
14:1trans	CON	<0.01	0.02	<0.01	<0.01	0.01	0.60	0.17	0.82
	SFAT	<0.01	<0.01	<0.01	<0.01				
	FISH	<0.01	0.01	<0.01	<0.01				
14:1cis	CON	0	<0.01	<0.01	<0.01	<0.01	0.43	0.61	0.19
	SFAT	0	0	<0.01	<0.01				
	FISH	<0.01	<0.01	<0.01	<0.01				
15:0	CON	0.34	0.34	0.22	0.22	0.04	0.76	<0.01	0.45
	SFAT	0.39	0.32	0.19	0.18				
	FISH	0.43	0.25	0.19	0.18				
15:1trans	CON	<0.01	0.04	<0.01	<0.01	0.01	0.44	0.15	0.78
	SFAT	<0.01	<0.01	<0.01	<0.01				
	FISH	<0.01	0.02	<0.01	<0.01				
16:1trans	CON	0.29	0.28	0.18	0.22	0.03	0.03	<0.01	0.09
	SFAT	0.27	0.24	0.18	0.16				
	FISH	0.28	0.39	0.22	0.17				
16:1cis	CON	0.62	0.66	0.70	0.65	0.06	<0.01	0.52	0.85
	SFAT	0.68	0.67	0.73	0.66				
	FISH	0.63	0.55	0.59	0.53				
17:0	CON	1.35	1.27	0.83	0.82	0.10	0.95	<0.01	0.37
	SFAT	1.39	1.30	0.75	0.81				
	FISH	1.12	1.43	0.84	0.80				
17:1trans	CON	0.09	0.12	0.04	0.05	0.02	0.01	<0.01	0.67
	SFAT	0.11	0.09	0.06	0.04				
	FISH	0.04	0.09	0.01	<0.01				
18:1trans5	CON	<0.01	0	<0.01	<0.01	0.03	0.10	0.56	0.55
	SFAT	<0.01	<0.01	<0.01	<0.01				
	FISH	0.10	0.06	0.01	<0.01				
18:1trans7	CON	<0.01	<0.01^{a}	<0.01	<0.01	<0.01	0.21	0.09	<0.01
	SFAT	0.01	<0.01^{a}	<0.01	0.01				
	FISH	<0.01$^{\alpha}$	0.03$^{b,\beta}$	<0.01$^{\alpha}$	<0.01$^{\alpha}$				
18:1trans8	CON	0.08$^{b,\alpha}$	<0.01$^{c,\beta}$	<0.01$^{\beta}$	<0.01$^{b,c,\beta}$	0.03	0.78	0.25	<0.01
	SFAT	<0.01$^{a,\alpha}$	<0.01$^{a,b,\alpha}$	<0.01$^{\alpha}$	0.11$^{a,\beta}$				
	FISH	<0.01$^{a,\alpha}$	0.07$^{b,\beta}$	<0.01$^{\alpha}$	<0.01$^{b,\alpha}$				
18:1trans9	CON	0.09	0.11	0.09	0.08	0.05	0.84	0.16	0.39
	SFAT	0.09	0.10	0.16	0.03				
	FISH	0.09	0.17	0.03	0.02				
18:1trans10	CON	0.17	<0.01	<0.01	0.02	0.07	0.91	0.59	0.12
	SFAT	<0.01	<0.01	<0.01	<0.01				
	FISH	<0.01	0.16	<0.01	0.04				
18:1trans12	CON	0.39$^{\alpha,\beta}$	0.17$^{a,\alpha}$	0.53$^{a,b,\alpha,\beta}$	0.63$^{\beta}$	0.15	<0.01	0.01	<0.01
	SFAT	0.35	0.38^{a}	0.50^{a}	0.49				
	FISH	0.18$^{\alpha}$	1.24$^{b,\beta}$	0.90$^{b,\beta,\gamma}$	0.71$^{\gamma}$				
18:1t13-14	CON	<0.01	<0.01	<0.01	2.86	0.93	0.39	0.41	0.45
	SFAT	<0.01	0	0	<0.01				
	FISH	<0.01	<0.01	<0.01	<0.01				

(*Continued*)

Table S1. (*Continued*)

Fatty acid	Diet[†]	Day				SEM[‡]	*P* value		
		–21	**–10**	**1**	**11**		**Diet**	**Time**	**D × T**[¶]
18:1cis11	CON	0.57	0.82	0.90	0.78	0.11	0.21	<0.01	0.73
	SFAT	0.75	0.81	0.88	0.88				
	FISH	0.43	0.70	0.83	0.84				
18:1cis12	CON	0.29	0.19	0.26	0.24	0.07	0.74	0.99	0.22
	SFAT	0.23	0.22	0.26	0.13				
	FISH	0.14	0.29	0.17	0.30				
18:1cis13	CON	0.05	0.01	0.04	0.04	0.02	0.57	0.63	0.42
	SFAT	<0.01	0.01	0.04	0.02				
	FISH	0	0.06	0.03	0.03				
18:1cis16	CON	0.04	0.10	0.14	0.13	0.03	0.38	0.01	0.11
	SFAT	0.09	0.09	0.13	0.06				
	FISH	0.05	0.16	0.12	0.16				
20:0	CON	0.08	0.10	0.06	0.06	0.03	0.02	<0.01	0.11
	SFAT	0.08	0.07	0.05	0.04				
	FISH	0.21	0.12	0.07	0.05				
18:3n-6	CON	0.40	0.42^{a}	0.39^{a}	0.38^{a}	0.08	<0.01	0.34	0.04
	SFAT	$0.34^{\alpha,\beta}$	$0.48^{a,\alpha,\beta}$	$0.33^{a,\alpha}$	$0.53^{a,\beta}$				
	FISH	0.39^{α}	$0.08^{b,\beta}$	$0.11^{b,\beta}$	$0.15^{b,\beta}$				
18:2alltrans	CON	0.13	0.14	0.07	0.06	0.02	<0.01	<0.01	0.38
	SFAT	0.14	0.16	0.08	0.10				
	FISH	0.08	0.03	0.01	0.01				
20:3n-3	CON	6.55	6.75	4.53	4.27	0.59	<0.01	<0.01	0.14
	SFAT	5.92	6.86	4.00	4.38				
	FISH	5.54	3.26	2.14	2.38				
22:1	CON	0.01	0.01	0.01	0.02	0.10	0.39	0.46	0.48
	SFAT	<0.01	0.01	0.01	0.01				
	FISH	0.30	0.03	<0.01	<0.01				
22:2n-6	CON	0.01	0.01	<0.01	<0.01	0.12	0.07	0.59	0.75
	SFAT	<0.01	<0.01	<0.01	<0.01				
	FISH	0.35	0.14	0.07	0.04				
22:3n-3	CON	<0.01	0.01	0	0.11	0.07	0.92	0.72	0.25
	SFAT	0.02	0.03	0.09	0.01				
	FISH	0.18	<0.01	<0.01	<0.01				
22:4n-6	CON	2.71	2.58	1.60	1.27	0.22	<0.01	<0.01	0.08
	SFAT	2.36	2.45	1.59	1.10				
	FISH	2.07	0.93	0.36	0.26				
Unknown	CON	5.33	3.89	2.04	2.60	0.80	0.92	<0.01	0.10
	SFAT	3.93	3.81	1.70	2.74				
	FISH	3.30	6.00	1.71	2.26				

Notes: $^{a–c}$Difference ($P < 0.05$) between different diet at same day. $^{\alpha,\beta,\gamma,\delta}$Denote significant interactions ($P < 0.05$) within a same diet at different days. [†]CON = control diet containing no supplemental lipid: SFAT = CON supplemented with Energy Booster: FISH = CON supplemented with fish oil; [‡]standard error of the mean; [¶]diet by time interaction.

Table S2. Concentration of fatty acids in hepatic triacylglycerol during the peripartal period.

Fatty acid	Diet†	Day				SEM‡	*P* value		
		–21	–10	1	11		Diet	Time	D × T¶
14:0	CON	3.94	5.34	5.06	3.98	0.53	0.06	0.08	0.62
	SFAT	5.24	5.30	5.17	5.15				
	FISH	4.00	5.39	4.17	4.16				
14:1trans	CON	<0.01^{b}	<0.01	<0.01	<0.01	0.07	0.11	0.08	0.04
	SFAT	0.33$^{a,\alpha}$	<0.01$^{\beta}$	<0.01$^{\beta}$	<0.01$^{\beta}$				
	FISH	<0.01^{b}	<0.01	<0.01	<0.01				
14:1cis	CON	0.06$^{a,\alpha}$	0.14$^{\alpha}$	0.43$^{\beta}$	0.35$^{\beta}$	0.06	0.16	0.01	0.03
	SFAT	0.05$^{a,\alpha}$	0.08$^{\alpha}$	0.34$^{\beta}$	0.34$^{\beta}$				
	FISH	0.32$^{b,\alpha}$	<0.01$^{\beta}$	0.39$^{\alpha}$	0.43$^{\alpha}$				
15:0	CON	1.50	1.58	1.22	1.24	0.24	0.37	0.01	0.72
	SFAT	2.00	1.50	1.11	1.19				
	FISH	1.35	1.53	2.00	1.06				
15:1trans	CON	<0.01^{b}	<0.01	<0.01	<0.01	0.64	0.11	0.08	0.04
	SFAT	0.31$^{a,\alpha}$	<0.01$^{\beta}$	<0.01$^{\beta}$	<0.01$^{\beta}$				
	FISH	<0.01^{b}	<0.01	<0.01	<0.01				
16:1trans	CON	0.65^{a,b}	0.68	0.64^{a}	0.66	0.11	0.02	0.36	0.02
	SFAT	0.90^{a}	0.58	0.74^{a}	0.60				
	FISH	0.58$^{b,\alpha}$	0.85$^{\alpha,\beta}$	1.12$^{b,\beta}$	0.88$^{\beta}$				
16:1cis	CON	1.70	2.01	4.03	3.30	0.42	0.05	<0.01	0.11
	SFAT	1.87	1.74	2.64	3.80				
	FISH	2.44	3.03	3.90	3.41				
17:0	CON	1.87	1.77	0.84	1.04	0.25	0.83	<0.01	0.98
	SFAT	1.73	1.79	0.98	0.84				
	FISH	1.59	1.70	0.91	0.91				
17:1trans	CON	<0.01	<0.01	0.05	0.05	0.02	0.40	<0.01	0.71
	SFAT	0.04	<0.01	0.07	0.09				
	FISH	0.03	<0.01	0.06	0.06				
17:1cis	CON	<0.01	<0.01	0.12	<0.01	0.08	0.58	0.32	0.55
	SFAT	0.07	<0.01	<0.01	0.10				
	FISH	<0.01	<0.01	0.13	0.20				
18:1trans5	CON	<0.01	<0.01	<0.01	<0.01	0.10	0.37	0.63	0.48
	SFAT	0.28	<0.01	0.07	0.01				
	FISH	<0.01	<0.01	<0.01	0.09				
18:1trans7	CON	<0.01	<0.01	<0.01	0.01	0.11	0.40	0.45	0.48
	SFAT	0.32	<0.01	0.01	0.01				
	FISH	<0.01	<0.01	<0.01	<0.01				
18:1trans68	CON	0.04	<0.01	<0.01	0.11	0.34	0.55	<0.01	0.71
	SFAT	<0.01	0.04	0.09	0.11				
	FISH	<0.01	<0.01	0.04	0.06				
18:1trans9	CON	<0.01	0.07	0.12	0.02	0.05	0.67	0.03	0.52
	SFAT	<0.01	0.04	0.12	0.15				
	FISH	0.03	0.04	0.09	0.09				
18:1trans10	CON	<0.01	<0.01	0.06	0.20	0.07	0.61	0.66	0.53
	SFAT	<0.01	0.07	<0.01	<0.01				
	FISH	0.05	0.04	0.04	0.04				
18:1trans12	CON	<0.01	0.04	0.27	0.21	0.12	0.36	<0.01	0.89
	SFAT	0.14	<0.01	0.39	0.28				
	FISH	0.20	<0.01	0.34	0.40				
18:1t13-14	CON	1.21	1.30	0.49	0.35	0.38	0.51	<0.01	0.71
	SFAT	1.68	1.92	0.14	0.43				
	FISH	1.16	1.32	0.56	<0.01				

(*Continued*)

Table S2. (*Continued*)

Fatty acid	Diet†	Day				SEM‡	*P* value		
		–21	–10	1	11		Diet	Time	D × T¶
18:1trans15	CON	<0.01	<0.01	<0.01	0.20	2.07	0.42	0.44	0.47
	SFAT	<0.01	<0.01	<0.01	<0.01				
	FISH	<0.01	<0.01	6.27	<0.01				
18:1cis11	CON	0.53	0.66	1.03	0.64	0.16	0.55	<0.01	0.36
	SFAT	0.63	0.53	1.02	1.13				
	FISH	0.666	0.45	0.97	1.06				
18:1cis12	CON	0.19	0.26	0.49	0.40	0.09	0.02	<0.01	0.70
	SFAT	0.26	0.27	0.49	0.54				
	FISH	0.05	<0.01	0.40	0.44				
18:1cis13	CON	<0.01	0.02	0.12	0.14	0.04	0.17	<0.01	0.98
	SFAT	<0.01	0.03	0.15	0.14				
	FISH	0.05	0.09	0.18	0.15				
18:1trans16	CON	<0.01	0.03	0.08	0.09	0.03	0.70	<0.01	0.61
	SFAT	<0.01	<0.01	0.12	0.09				
	FISH	0.04	<0.01	0.12	0.09				
18:1alltrans	CON	2.07	2.43	2.09	1.92	2.02	0.31	0.36	0.25
	SFAT	3.26	2.70	2.15	1.94				
	FISH	1.91	2.79	9.23	2.21				
18:1allcis	CON	15.00	14.77	22.53	21.90	2.70	0.96	<0.01	0.23
	SFAT	15.25	13.65	22.83	21.38				
	FISH	20.80	14.55	16.47	23.34				
18:3n-6	CON	0.08^{α}	$0.13^{a,\alpha,\beta}$	$0.21^{b,\beta,\gamma}$	$0.35^{a,\gamma}$	0.03	<0.01	<0.01	0.02
	SFAT	0.11^{α}	$0.10^{a,\alpha}$	$0.12^{a,b,\alpha}$	$0.37^{a,\beta}$				
	FISH	$0.08^{\alpha,\beta}$	$0.01^{b,\alpha}$	$0.11^{a,\alpha,\beta}$	$0.13^{b,\beta}$				
18:2alltrans	CON	0.05	0.06	0.09	0.11	0.02	<0.01	<0.01	0.08
	SFAT	0.06	0.07	0.07	0.18				
	FISH	0.03	0.02	0.06	0.04				
20:3n-3	CON	0.93	1.15	0.57	0.51	0.18	0.08	0.01	0.91
	SFAT	0.66	0.89	0.50	0.53				
	FISH	0.66	0.65	0.41	0.34				
22:1	CON	<0.01	<0.01	<0.01	0.01	0.01	0.08	0.33	0.42
	SFAT	<0.01	0.03	<0.01	0.02				
	FISH	<0.01	<0.01	<0.01	<0.01				
22:2n-6	CON	0.36	0.18	<0.01	0.02	0.10	0.14	0.22	0.46
	SFAT	0.01	<0.01	<0.01	0.01				
	FISH	0.08	0.13	0.04	0.02				
22:3n-3	CON	<0.01	$<0.01^{b}$	<0.01	0.26	0.12	0.06	0.10	0.04
	SFAT	$<0.01^{\alpha}$	$0.61^{a,\beta}$	0.05^{α}	0.14^{α}				
	FISH	<0.01	$<0.01^{b}$	<0.01	0.02				
24:1	CON	$<0.01^{b}$	<0.01	<0.01	<0.01	0.01	0.39	0.33	0.04
	SFAT	$0.04^{a,\alpha}$	$<0.01^{\beta}$	$<0.01^{\beta}$	$<0.01^{\beta}$				
	FISH	$<0.01^{b}$	<0.01	0.01	0.02				
22:4n-6	CON	0.51	0.48	0.22	0.19	0.21	0.31	0.01	0.15
	SFAT	0.19	1.12	0.20	0.17				
	FISH	0.33	0.34	0.12	0.13				
22:5n-6	CON	0.14	0.12	0.06	0.04	0.11	0.10	0.17	0.07
	SFAT	0.08	0.49	0.06	0.04				
	FISH	0.08	0.24	0.41	0.28				
Unknown	CON	9.53	6.28	6.44	5.63	1.70	0.08	0.86	0.30
	SFAT	3.72	4.26	5.54	7.09				
	FISH	8.44	8.61	690	6.38				

Notes: [a–c]Difference ($P < 0.05$) between different diet at same day. [α,β,γ,δ]Denote significant interactions ($P < 0.05$) within a same diet at different days. †CON = control diet containing no supplemental lipid: SFAT = CON supplemented with Energy Booster: FISH = CON supplemented with fish oil; ‡standard error of the mean; ¶diet by time interaction.