Dietary marine algae and its influence on tissue gene network expression during milk fat depression in dairy ewes

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Abbreviations

MA, marine algae; LCFA, long chain fatty acids; MFD, milk fat depression; SO, sunflower oil; SOMA, sunflower oil marine algae; TAG, triglycerides; GH, Growth Hormone; CLA, conjugated linoleic acid.

ABSTRACT

Supplementation of a linoleic acid-rich diet with marine algae reduces milk fat content while impacting milk fatty acid profile in dairy sheep. Unlike other ruminant species, in ovine there are limited data on the molecular mechanisms that may regulate adipose, liver, and mammary responses to dietary marine lipids. This study was conducted to investigate changes in mRNA expression and relative mRNA abundance of key enzymes involved in lipid metabolism in mammary, subcutaneous adipose and liver tissue in response to long-term milk fat depression induced by marine algae. Eleven Assaf ewes were randomly assigned to 2 experimental diets consisting of a TMR based on alfalfa hay and concentrate (40:60) supplemented with 25 g of sunflower oil/kg DM plus 0 (SO; control diet) or 8 g of marine algae/kg of DM (SOMA diet) for 54 d. Quantitative reverse transcription-PCR was used to study expression of target genes in tissues harvested at slaughter at the end of the feeding period (54 d). There was no effect of SOMA on mammary and adipose tissue expression of genes encoding proteins required for fatty acid uptake and activation (*ACSS2*, *LPL*), intracellular fatty acid transport (*FABP3*, *FABP4*), de novo fatty acid synthesis (*ACACA*, *FASN*), esterification (*DGAT1*, *DGAT2*, *LPIN1*), desaturation (*SCD*), elongation (*ELOVL6*), transcriptional regulation (*INSIG1*, *MED1*, *PPARG*, *RXRA*, *SCAP*, *SREBF1*, *THRSP*) and lipid droplet formation (*ADFP*, *BTN1A1*, *XDH*). Abundance of *PPARG* (0.04%) and *INSIG1* (2.22%) in mammary tissue was markedly greater than that of *SREBF1* (0.002%), suggesting that they may play a more important role in milk fat synthesis regulation. Addition of marine algae did not affect the expression of β -oxidation- and lipoprotein-related genes (*ACOX1*, *APOB*, *CPT1A*, *PPARA*, *RXRA*) in hepatic tissue. However, feeding SOMA up-regulated hepatic *HMGCS2*, which controls ketogenesis. Concentration of plasma β -hydroxybutyrate, NEFA, glucose, triacylglycerol, growth hormone, insulin-like growth factor 1,

insulin, and leptin was not different between groups at d 54. Taken together with the milk fat responses and previous data from bovine fed similar diets, results suggest that transcriptional control mechanisms regulating fat synthesis in mammary secretory tissue were likely established during earlier stages of the feeding period.

Keywords: marine lipids, gene expression, lipid metabolism, sheep

1. INTRODUCTION

Inclusion of marine lipids in the diet of dairy sheep represents an effective nutritional strategy for altering milk fat composition (Papadopoulos et al., 2002; Reynolds et al., 2006; Capper et al., 2007). However, dietary marine algae (MA) have recently been associated with milk fat depression (MFD) in dairy ewes (Toral et al., 2010a; Bichi et al., 2013). Milk fat synthesis is known to involve the coordinated expression of several transcription regulators and their target genes (Bionaz and Loores, 2008). The role of altered lipogenic gene expression has been examined in lactating dairy cows fed a MFD diet (Piperova et al., 2000; Ahnadi et al., 2002; Harvatine and Bauman, 2006), and the transcriptomic adaptations outlined not only in mammary (Invernizzi et al., 2010) but also in adipose tissue (Thering et al., 2009).

Some long-chain fatty acids (LCFA) including 16:0, 18:0, *trans*-10 18:1, and *trans*-10, *cis*-12 18:2 alter bovine mammary cell lipogenic gene networks and in turn lipid droplet synthesis (Kadegowda et al., 2009). In particular, the *trans*-10 LCFA intermediates arising from ruminal metabolism of dietary unsaturated FA (Bauman et al., 2011) are potent inhibitors of lipogenesis via their negative effect on transcriptional control mechanisms regulating target genes (Kadegowda et al., 2009).

Data on the nutritional regulation of expression of lipogenic enzymes in small ruminants are limited (Agazzi et al., 2010; Shingfield et al., 2013; Hussein et al., 2013). Previous studies with goats evaluated lipogenic gene expression in adipose and mammary tissue in response to dietary vegetable and fish oil (Bernard et al., 2009a,b; Li et al., 2012; Toral et al., 2013) but direct comparisons between the sheep and the goat have identified species-specific differences in mRNA levels of mammary lipogenic genes (Tsiplakou et al., 2009). Furthermore, the goat appears to be less responsive to MFD than the ewe (Bernard et al., 2012; Shingfield et al., 2013).

In lactating ewes, exogenous *trans*-10, *cis*-12 18:2 induced MFD (Lock et al., 2006; Sinclair et al., 2010; Oliveira et al., 2012; Hussein et al., 2013), but this isomer does not seem to play a relevant role in diet-induced MFD (Toral et al., 2010a,b; Bichi et al., 2013). To our knowledge, there is only limited information about the molecular adaptations in ovine tissues in response to diets that induce MFD (Hussein et al., 2013).

The objective of this study was to use adipose, liver, and mammary tissue from sheep fed a linoleic acid-rich TMR supplemented with MA that resulted in MFD (Bichi et al., 2013) to evaluate mRNA expression of transcription regulators and target genes to better understand the role of transcriptional mechanisms in the long-term nutritional regulation of diet-induced MFD.

2. MATERIALS AND METHODS

2.1. Animals, Experimental Diets and Management

All experimental procedures were performed in accordance with the Spanish Royal Decree 1201/2005 for the protection of animals used for experimental and other scientific purposes. Details of the experimental procedures have been described previously (Bichi et al., 2013). Briefly, eleven fat-tailed Assaf ewes (82.4 ± 3.26 kg BW) in mid-lactation (84 ± 2.3 DIM at the beginning of the experiment) were randomly assigned to two experimental diets consisting of a TMR based on alfalfa hay and a concentrate (40:60) supplemented with 25 g of sunflower oil (Carrefour S.A., Madrid, Spain)/kg of DM plus 0 (SO; control diet, n=5) or 8 g of marine algae (DHA Gold Animal Feed Ingredient, Martek Biosciences Corp., Columbia, MD)/kg of DM (SOMA diet, n=6). Ingredients, chemical composition and FA profile of the experimental diets have been reported previously (Bichi et al., 2013). Fresh diets were offered daily ad libitum at 0900 and 1900 h and clean water was always available. Ewes were milked twice daily at

approximately 0830 and 1830 h in a 1 × 10 stall milking parlor (DeLaval, Madrid, Spain) throughout the 54 d experiment.

2.2 Measurements, Sampling Procedures and Chemical Analysis

Blood samples from the jugular vein were individually collected in 10-mL Vacutainer tubes (BD Vacutainer, Plymouth, UK) containing lithium heparin, before the morning milking on d 54. Plasma obtained after centrifugation (3,000 rpm, 10 min, 4°C) was stored at -30°C. Concentrations of glucose, triacylglycerol (TAG), BHBA, and NEFA were analyzed at the Veterinary Diagnostics Laboratory, College of Veterinary Medicine, University of Illinois. Concentration of insulin was analyzed using a commercial kit from Mercodia (Cat#10-1201-01). Leptin, growth hormone (GH), and IGF-1 were measured via Radioimmunoassay (RIA).

At the end of the experiment, the ewes were slaughtered humanely via intravenous injection of an euthanasia solution (T-61, Intervet, Salamanca, Spain). Then, mammary, subcutaneous adipose from the tail-head region, and liver tissue samples were collected from each animal under sterile conditions. Tissue samples were immediately frozen in liquid nitrogen and stored at -80 °C until RNA extraction.

2.3. RNA Extraction, Quantitative Reverse Transcription-PCR, Normalization, and Percentage Relative mRNA abundance

Complete details of these procedures can be found in the Supplemental material. Briefly, the total RNA of mammary, adipose and liver tissue was extracted using ice-cold Trizol (Invitrogen Corp., Carlsbad, CA) as described by Loores et al. (2005). Genomic DNA was removed using RNeasy Mini Kit columns (Qiagen, Hilden, Germany). The purity and concentration of the RNA

from each sample were measured using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Quantitative reverse transcription-PCR was carried out as described by Graugnard et al. (2010). The final data were normalized using the geometric mean of 4 internal control genes (*UXT*, *EIF3K*, *TUBB*, and *YWHAZ*). The relative % mRNA abundance for each gene was calculated using the median ΔC_t values, corrected for the efficiency of PCR amplification (Bionaz and Loor, 2008).

2.4. Statistical Analysis

Data were analyzed using the MIXED procedure of SAS (Version 9.2; SAS Institute Inc., Cary, NC). The statistical model included the fixed effect of experimental treatment and the random effect of animal, nested within the diet to contrast the effect of the algae supplementation. Before statistical analysis, data of gene expression were log-transformed ($\log_2$ scale). The UNIVARIATE procedure of SAS was used to identify and remove outliers from the data set. Significant differences were declared at $P < 0.05$ and tendencies at $P < 0.10$.

3. RESULTS

3.1. Plasma Metabolite Concentrations

No differences between diets were observed for the plasma concentration of glucose, BHBA, NEFA, TAG, GH, IGF-1, insulin, and leptin concentration ($P > 0.05$; Table 1).

3.2. mRNA Expression in Mammary, Adipose and Liver Tissue

In mammary and adipose tissue, feeding MA had no effect ($P > 0.05$; Table 2) on the expression of mRNA encoding for genes associated with LCFA uptake (*LPL*), intracellular acetate activation (*ACSS2*), intracellular LCFA transport (*FABP3*), desaturation (*SCD*), elongation (*ELOVL6*), esterification (*DGAT1*, *DGAT2*, *LPIN*), and de novo FA synthesis (*ACACA*, *FASN*). Furthermore, MA did not affect ($P > 0.05$) the expression of transcription regulators (*INSIG1*, *MED1*, *PPARG*, *RXRA*, *SCAP*, *SREBF1*, *THRSP*). Expression of milk fat globule membrane proteins (*BTN1A1*, *XDH*) in mammary tissue did not differ ($P > 0.05$) due to MA.

In the liver, there were no differences ($P > 0.05$) between treatments in mRNA encoding genes associated with lipoprotein metabolism (*APOB*), LCFA oxidation (*ACOX1*, *CPT1A*) and regulation of transcription (*PPARA*, *RXRA*); whereas mRNA expression of *HMGCS2*, a gene associated with ketogenesis, was greater ($P < 0.05$) in response to SOMA (Table 2).

3.3 Percentage Relative mRNA Abundance Among Measured Genes

Analysis of percentage relative mRNA is presented in Figure 1. Results revealed that in mammary gland tissue the most-abundant genes, accounting for >20% of total measured mRNAs, were *BTN1A1* (27%), *SCD* (24%), *LPL* (24%), and *XDH* (21%). The abundance of *PPARG* and *INSIG1* was 0.04% and 2.2% compared with *SREBF1* and *THRSP* which averaged 0.002% and 0.001%. All other genes had relative abundance <1.0%.

In the adipose tissue the highest relative % mRNA abundance was observed for *FABP4* (47%) followed by *SCD* (31%) and *LPL* (13%). The abundance of *PPARG* was ~1% and that of *THRSP* and *SREBF1* (the least-abundant gene) 1.4% and 0.003%. Expression of *INSIG1* averaged 0.45%

and, except for *ACACA* (1.8%) and *ELOVL6* (2.5%), most other lipogenic and esterification enzymes had relative abundance <1.0%.

In the liver the highest relative % mRNA abundance was observed for *APOB* (85%). The abundance of *PPARA* was markedly lower (0.25%) compared with *RXRA* (6.3%) and the ketogenic enzyme *HMGCS2* (7.3%). The lowest abundance was observed for *CPT1A1* (0.13%).

4. DISCUSSION

As pointed out in the companion study (Bichi et al., 2013), it was evident that the addition of MA to a linoleic acid-rich diet in dairy sheep not only led to a lower milk fat yield and content, but also to an important change in milk FA profile, with a strong decrease in the concentration of 18:0 and *cis*-9 18:1 and an increase in *trans*-10 18:1, as well as *cis*-9, *trans*-11 18:2 and n-3 PUFA (Table 1). Therefore, the objective of the present study was to investigate if the MFD induced by MA and the related changes in milk FA profile are associated with a coordinated down-regulation in mRNA expression of genes with key functions in the overall process of milk fat synthesis as recently observed in CLA-induced MFD (Hussein et al., 2013).

4.1. Mammary Lipid Metabolism

The changes in milk FA profile and the MFD due to SOMA were not associated with statistical differences in mRNA expression of mammary genes at the end of the 54 d feeding period. This contrasts recent findings by Hussein et al. (2013) during CLA-induced MFD in ewes where the remarkable increase in milk *trans*-10, *cis*-12 18:2 concentration was correlated with the coordinated downregulation in transcript abundance of lipogenic enzymes involved in mammary lipid synthesis. Even though a slight but significant increase of *trans*-10, *cis*-12 18:2

concentration also was evident in the present study (Table 1), our previous study partly ruled out an involvement of this CLA in causing and maintaining MFD due to feeding SOMA because of its low content and the lack of correlation with milk fat content (Bichi et al., 2013). The lack of difference in mRNA expression of mammary genes in our study is consistent with data from cows fed fish oil and experiencing MFD demonstrated that changes in the mammary transcriptome, as well as in milk FA profile, occur relatively quickly after initiation of treatments (Invernizzi et al., 2010) such that once milk fat concentration was set and remained nearly unchanged, there were no differences in gene expression despite marked MFD.

The absence of differences in mammary mRNA expression of genes involved in the de novo synthesis (*ACACA*, *FASN*), as well as uptake, transport and trafficking of FA in the cells (*LPL*, *FABP3*) or coding for the major proteins of the milk fat globule membrane (*BTN1A1*, *XDH*), was similar to previous observations in goats fed plant oils (Ollier et al., 2009; Bernard et al., 2012). In relative terms our data revealed that sheep mammary tissue expresses similar amounts of *BTN1A1* and *XDH*, while in cows *XDH* is more abundant (Bionaz and Loor, 2008). Conversely, marine lipid in cows has sometimes been associated with a down-regulation of mammary *LPL* (Ahnadi et al., 2002; Harvatine and Bauman, 2006). The marked abundance of *LPL* relative to both *ACACA* and *FASN* in our study contrasts with data from cows in which both *LPL* and *FASN* were among the most-abundant genes during lactation (Bionaz and Loor, 2008). Such differences, however, might be attributable to the fact that we supplemented oil and therefore more *LPL* was probably necessary to handle an increase in circulating chylomicrons and very low density lipoproteins (Thering et al., 2009).

In accordance with the present study, Bernard et al. (2010) reported no changes in *SCD* expression in goats despite a putative decreased supply of 18:0 from the rumen. Desaturation of

18:0 in *cis*-9 18:1 by mammary Δ 9-desaturase is considered the predominant mechanism that maintains and regulates milk fluidity (Shingfield et al., 2010). The response of *SCD* in cows fed a blend of soybean and fish oil for 4-d was non-significant in the study of Harvatine and Bauman (2006); whereas, Invernizzi et al. (2010) reported a gradual up-regulation of *SCD* over time as a consequence of the reduction in 18:0 supply from the rumen induced by dietary fish oil feeding for 3 wk. The analysis of percentage relative mRNA abundance among the genes measured in the present study revealed that, despite the lack of difference in expression between treatments, *SCD* was one of the most abundant genes involved in mammary lipid metabolism (Fig. 1). Its relative abundance was similar to that observed in bovine mammary tissue during lactation (Bionaz and Loores, 2008). Thus, just as in cows fed fish oil (Invernizzi et al., 2011), it appears that when the diet is supplemented with marine lipids, milk fat synthesis in sheep also is dependent on other factors such as endogenous synthesis of oleic acid from rumen-derived 18:0.

A recent in vitro study with bovine kidney cells demonstrated that dietary 16:0, 20:5 and 22:6 n-3 LCFA, whose content was significantly greater in SOMA than SO (Bichi et al., 2013), are able to modulate lipid metabolism by increasing the expression of some key transcription factors or their co-activators (Bionaz et al., 2012). The fact that MA did not affect transcription regulators of milk fat synthesis in the present study partly differs from data in the bovine during MFD (Invernizzi et al., 2010; Harvatine and Bauman, 2006; Bauman et al., 2011). Species-specific differences among ruminants in the response of milk fat secretion and composition cannot be discarded to explain these results. This point is particularly relevant because our analyses revealed that expression of *PPARG* and *INSIG1* is markedly greater relative to *SREBF1*, which is opposite to cows (Bionaz and Loores, 2008). This suggests a different adaptive response to the reduction in milk fat synthesis between lactating dairy cows and ewes. Even though the

present study analyzed only one time point, our results underscore the importance of *PPARG* in the control of milk fat synthesis in sheep.

To further clarify mechanisms, an investigation of the temporal adaptation of the transcriptome in sheep mammary tissue is needed. Particularly to better understand the role of molecular mechanisms in this species as it relates to the supply of dietary and/or ruminally-derived long-chain FA.

4.2. Adaptations of Peripheral Tissues to Changes in Milk FA Secretion

Despite the high percentage of mRNA abundance of some key lipid-related genes such as *FABP4*, *SCD*, and *LPL* (see Fig. 1), on d 54 there was no difference between treatments on mRNA expression of genes encoding for lipid metabolism-related networks in subcutaneous adipose and liver tissue. A similar lack of response was observed in the expression pattern of *LPL*, *ACACA*, *FASN*, of *SCD* in adipose tissue of goats receiving a diet rich in n-3 PUFA (i.e., linseed oil) for 21 d (Bernard et al., 2009a,b). These results contrast the observed up-regulation at 21 d of feeding of several lipid-related genes (particularly, *LPL* and *SCD*) in adipose tissue of cows with MFD in response to supplemental fish oil (Thering et al., 2009), suggesting that the energy spared from reduced milk fat synthesis might be partitioned towards non-mammary tissues, specifically adipose (Harvatine et al., 2009). However, based on mammary and adipose data from studies evaluating long-term nutritional regulation of milk fat synthesis during MFD (Thering et al., 2009; Invernizzi et al., 2010) it is not possible to conclude that in the ovine MA did not affect the lipogenic network in subcutaneous adipose tissue. It is likely that control mechanisms regulating fat synthesis were established before the time of sampling in the present study. Support for this is the fact that in the parent study involving all animals (Bichi et al., 2013)

there was a clear temporal adaptation in the profile of FA in milk, as shown previously in cows (Shingfield et al., 2006).

The relative abundance of mammary *FABP3* (0.52% of total measured genes) and adipose *FABP4* (47% of total measured genes), along with low *DGAT1* (0.005%) in mammary and higher *DGAT2* (0.05%) in adipose, are suggestive of active utilization of LCFA in adipose likely for esterification into triacylglycerol (Graugnard et al., 2009). However, this hypothesis is contrasted by the absence of differences in concentrations of NEFA, insulin and glucose, which are known to be involved in the regulation of adipose lipogenesis (Vernon et al., 1980). Similar to our study, no effect on plasma concentration of metabolites, including glucose, NEFA, BHBA, or metabolic hormones, such as leptin, insulin and IGF-1, were observed during short- (Baumgard et al., 2000; 2002) and longer-term MFD (Thering et al., 2009; Oliveira et al., 2012).

The lack of change in liver mRNA expression of genes encoding proteins associated with cholesterol and lipoprotein metabolism (*APOB*), FA oxidation (*ACOX1*, *CPT1A*) and with regulation of oxidation and ketogenesis (*PPARA*, *RXRA*) is in agreement with recent findings in dairy cows with MFD induced by supplemental CLA (Schlegel et al., 2012). There was evidence, indeed, that 14 wk of CLA supplementation persistently decreased the milk fat content without apparently influencing hepatic lipid metabolism and plasma concentration of TAG (Schlegel et al., 2012).

Regression analysis revealed a weak negative correlation between *HMGCS2* mRNA expression and plasma BHBA ($r = -0.49$, $P < 0.01$) or NEFA ($r = -0.50$, $P < 0.01$) concentrations. The lack of a strict interdependence between *HMGCS2*, a key enzyme controlling hepatic ketogenesis, and BHBA agrees with the study of van Dorland et al. (2009), in which the increase in BHBA in cows after parturition was not associated with any increase in *HMGCS2* expression.

However, the up-regulation of this gene was in line with the numerically, albeit not statistically significant, greater *PPARA* and *RXRA* suggesting a mechanistic response in liver to handle greater flux of LCFA as a result of MFD. Recently, an in vitro study provided support for several of the major LCFA found in SOMA as being *PPARA* agonists (Bionaz et al., 2012) and the present results seem to corroborate the suggestion that, once activated, *PPARA* is able to up-regulate target genes associated with ketogenesis (Lor et al., 2005). Consistent also with previous researches in dairy goats (Bernard et al., 2009a,b), the present results would indicate that hepatic lipid metabolism had a low relevance in nutritional mechanisms altering milk FA composition.

5. CONCLUSIONS

Altogether the present results highlight several differences among ruminant species in the mechanisms regulating the response to an MFD diet not only at the mammary level but also in adipose depots and liver. Transcriptional control mechanisms regulating mammary fat synthesis during long-term MA-induced MFD in sheep likely were established during earlier stages of the feeding period. Further studies are necessary to clarify the role of the transcriptome on the regulation of the onset of MFD in dairy ewes.

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Table 1. Milk and fat yield, fat content, and fatty acid (FA) profile in dairy ewes after 54 days on the experimental diets¹

Item	Treatment		SEM	P-value ²
	SO	SOMA		
Milk yield, g/d	1,274	1,608	204.6	0.26
Fat yield, g/d	80.9	75.1	13.19	0.75
Fat content, g/100 g of raw milk	6.57	4.80	0.323	0.003
FA, g/100g of total FA				
4:0	3.60	3.50	0.146	0.65
6:0	2.65	2.15	0.240	0.16
8:0	2.46	1.85	0.278	0.14
10:0	7.01	5.44	0.837	0.20
12:0	3.83	3.48	0.342	0.46
14:0	9.89	11.16	0.490	0.07
<i>cis</i> -9 14:1	0.25	0.28	0.042	0.55
15:0	0.72	0.73	0.026	0.72
16:0	20.88	23.36	0.860	0.06
<i>cis</i> -9 16:1	0.76	0.87	0.093	0.41
17:0	0.44	0.42	0.028	0.64
18:0	8.26	2.04	0.553	<0.001
<i>cis</i> -9 18:1 ³	16.80	10.33	1.115	0.002
Other <i>cis</i> -18:1	1.27	1.21	0.120	0.70
<i>trans</i> -10 18:1	0.77	6.91	1.465	0.01
<i>trans</i> -11 18:1	5.89	8.85	1.182	0.10
Other <i>trans</i> -18:1	2.49	3.04	0.200	0.07
<i>cis</i> -9, <i>cis</i> -12 18:2 ⁴	2.57	2.01	0.213	0.08
<i>cis</i> -9, <i>trans</i> -11 18:2 ⁵	2.93	3.52	0.670	0.53
<i>trans</i> -9, <i>cis</i> -11 18:2	0.14	0.26	0.038	0.04
<i>trans</i> -10, <i>cis</i> -12 18:2	0.04	0.07	0.006	0.01
<i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15 18:3	0.41	0.31	0.041	0.10
<i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17 20:5	0.03	0.06	0.006	<0.001
<i>cis</i> -4, <i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19 22:6	0.06	0.49	0.033	<0.001
Other FA	5.85	7.64	0.231	<0.001

¹Refers to TMR containing 25 g of sunflower oil/kg of DM and supplemented with 0 (SO; control diet) or 8 g of marine algae (SOMA diet)/kg of DM.

²Probability of significant differences between diets.

³Coelutes with *trans*-13+14 18:1.

⁴Coelutes with 9,15 18:2.

⁵Coelutes with *trans*-7 *cis*-9 18:2 + *cis*-14 20:1.

Table 2. Concentration of plasma metabolites in dairy ewes after 54 days on the experimental diets^a

Item	Treatment		SEM	<i>P</i> -value ^b
	SO	SOMA		
BHBA, mmol/L	0.59	0.49	0.061	0.23
Glucose, mg/dL	49.7	58.8	4.07	0.13
NEFA, mmol/L	0.16	0.12	0.018	0.19
Triacylglycerol, mg/dL	34.5	29.4	4.15	0.38
Growth hormone, ng/mL	1.20	0.87	0.305	0.43
IGF-1, ng/mL	219.7	195.9	14.62	0.26
Insulin, µg/L	0.59	0.76	0.172	0.49
Leptin, ng/mL	17.9	16.6	2.062	0.66
NEFA:Insulin	0.32	0.21	0.067	0.24
Glucose:Insulin	113.2	82.6	16.49	0.20
IGF-1:GH	242.9	260.1	89.72	0.89

^aRefers to TMR containing 25 g of sunflower oil/kg of DM and supplemented with 0 (SO; control diet) or 8 g of marine algae (SOMA diet)/kg of DM.

^bProbability of significant differences between diets.

Table 3. Gene expression (Log₂-transformed) in mammary, adipose and liver tissue in dairy ewes after 54 days on the experimental diets^a

	Treatment		SEM	P-value ^b
	SO	SOMA		
Mammary				
<i>ACACA</i>	-1.03	-1.26	0.210	0.43
<i>ACSS2</i>	1.75	1.36	0.325	0.38
<i>BTN1A1</i>	1.51	1.20	0.342	0.49
<i>DGAT1</i>	0.27	0.11	0.317	0.71
<i>ELOVL6</i>	-5.13	-5.20	0.389	0.89
<i>FABP3</i>	1.40	1.05	0.620	0.67
<i>FASN</i>	-1.28	-1.66	0.470	0.54
<i>INSIG1</i>	0.77	0.28	0.325	0.27
<i>LPIN1</i>	0.77	0.59	0.464	0.78
<i>LPL</i>	1.08	1.06	0.237	0.96
<i>MED1</i>	0.03	-0.06	0.077	0.39
<i>PPARG</i>	-1.34	-1.79	0.350	0.35
<i>RXRA</i>	-0.24	-0.31	0.052	0.35
<i>SCAP</i>	0.55	0.50	0.102	0.72
<i>SCD</i>	0.82	0.54	0.241	0.41
<i>SREBF1</i>	-0.03	-0.33	0.323	0.50
<i>THRSP</i>	-10.59	-10.50	0.693	0.93
<i>XDH</i>	1.41	0.97	0.245	0.21
Adipose				
<i>ACACA</i>	0.61	1.13	0.411	0.37
<i>ACSS2</i>	-0.35	-0.48	0.229	0.69
<i>ADFP</i>	-4.26	-4.05	0.307	0.63
<i>DGAT2</i>	-0.72	-0.19	0.675	0.58
<i>ELOVL6</i>	0.44	1.16	0.525	0.33
<i>FABP4</i>	0.93	0.98	0.194	0.86
<i>FASN</i>	-2.79	-2.88	0.717	0.93
<i>INSIG1</i>	-1.89	-1.88	0.419	0.99
<i>LPIN1</i>	0.12	0.19	0.240	0.83
<i>LPL</i>	-0.26	-0.02	0.302	0.56
<i>MED1</i>	-0.18	-0.34	0.112	0.31
<i>PPARG</i>	2.48	2.90	0.232	0.22
<i>RXRA</i>	0.96	0.58	0.165	0.12
<i>SCAP</i>	0.55	0.64	0.235	0.80
<i>SCD</i>	-0.00	0.81	0.570	0.32
<i>SREBF1</i>	0.71	0.63	0.373	0.87
<i>THRSP</i>	0.30	0.66	0.976	0.79

Liver

<i>ACOX1</i>	-1.37	-1.12	0.207	0.40
<i>APOB</i>	-0.58	-0.36	0.195	0.42
<i>CPT1A</i>	-0.83	-0.75	0.110	0.58
<i>HMGCS2</i>	-0.96	-0.26	0.204	0.03
<i>PPARA</i>	-0.60	-0.36	0.118	0.17
<i>RXRA</i>	0.03	0.17	0.148	0.51

^aRefers to TMR containing 25 g of sunflower oil/kg of DM and supplemented with 0 (SO; control diet) or 8 g of marine algae (SOMA diet)/kg of DM.

^bProbability of significant differences between diets.

SUPPLEMENTAL MATERIAL

RNA extraction

RNA samples were extracted using established protocols (Loor et al., 2005). Briefly, tissue was weighed (~0.3 g for mammary gland, ~1 g for adipose tissue, ~0.1 g for liver) and placed straightway inside a 15 mL centrifuge tube (Cat. No. 430052, Corning Inc.®) with 1 µL of Linear Acrylamide (Ambion® Cat. No. 9520, Austin, TX) as a co-precipitant, and 5 mL ice-cold Trizol reagent (Invitrogen Corp., Carlsbad, CA). Tissue was then homogenized. Genomic DNA was removed from RNA with DNase using RNeasy Mini Kit columns (Qiagen, Hilden, Germany). RNA concentration was measured using a Nano-Drop ND-1000 spectrophotometer (Nano-Drop Technologies,). The purity of RNA (A_{260}/A_{280}) for all samples was above 1.81. Also, RNA quality was evaluated using the Agilent Bioanalyzer system (Agilent Technologies,). The average RIN number of the samples used was >8.0.

qPCR Analysis

For qPCR analysis, cDNA was synthesized using 100 ng RNA, 1 µL dT18 (Operon Biotechnologies, Huntsville, AL), 1 µL 10 mmol/L dNTP mix (Invitrogen Corp., CA), 1 µL random primer p(dN)₆ (Roche Cat. No 11034731001, Indianapolis, IN), and 7 µL DNase/RNase free water. The mixture was incubated at 65 °C for 5 min and kept on ice for 3 min. A total of 9 µL of master mix composed of 4.5 µL 5X First-Strand Buffer, 1 µL 0.1 M DTT, 0.25 µL (50 U) of SuperScript™ III RT (Invitrogen Corp., Carlsbad, CA), 0.25 µL of RNase Inhibitor (10 U, Promega, Madison, WI) and 3 µL DNase/RNase free water was added. The reaction was performed in an Eppendorf Mastercycler® Gradient (Eppendorf, Hauppauge, NY) using the following temperature program: 25 °C for 5 min, 50 °C for 60 min and 70 °C for 15 min. cDNA was then diluted 1:4 (v:v) with DNase/RNase free water.

Quantitative PCR (qPCR) was performed using 4 μ L diluted cDNA (dilution 1:4) combined with 6 μ L of a mixture composed of 5 μ L 1x SYBR Green master mix (Applied Biosystems, Foster City, CA), 0.4 μ L each of 10 μ M forward and reverse primers, and 0.2 μ L DNase/RNase free water in a MicroAmp™ Optical 384-Well Reaction Plate (Applied Biosystems, Foster City, CA). Each sample was run in triplicate and a 7 point relative standard curve plus the non-template control (NTC) were used (User Bulletin #2, Applied Biosystems, Foster City, CA). The reactions were performed in an ABI Prism 7900 HT SDS instrument (Applied Biosystems, Foster City, CA) using the following conditions: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C (denaturation) and 1 min at 60 °C (annealing + extension). The presence of a single PCR product was verified by the dissociation protocol using incremental temperatures to 95 °C for 15 s plus 65 °C for 15 s. Data were calculated with the 7900 HT Sequence Detection Systems Software (version 2.2.1, Applied Biosystems, Foster City, CA).

Selection of internal control genes

The final data were normalized using the geometric mean of 4 selected ICG (Table S1): ubiquitously expressed transcript (*UXT*), eukaryotic translation initiation factor 3, subunit K (*EIF3K*), which were identified as suitable internal controls in bovine adipose and mammary tissues (Thering et al., 2009; Invernizzi et al., 2010), tyrosine 3-monooxygenase (*YWHAZ*), and tubulin beta, subunit 2A (*TUBB*), which were identified as suitable internal controls in ovine tissues (Zang et al., 2011).

Primer design and testing

Primers were designed using Primer Express 3.0 with minimum amplicon size of 100 bp and limited 3' G+C (Applied Biosystems, CA). When possible, primers were designed to fall across exon–exon junctions. Primers were aligned against publicly available databases using

BLASTN at NCBI and UCSC's Sheep (*Ovis aries*) Genome Browser Gateway (Table S3). Prior to qPCR, primers were tested in a 20 μ L PCR reaction using the same protocol described for qPCR except for the final dissociation protocol. For primer testing we used a pool of cDNA samples (mixture from 3 different ovine tissues) to ensure identification of desired genes. Five μ L of the PCR product were run in a 2% agarose gel stained with ethidium bromide (2 μ L). The remaining 15 μ L were cleaned using QIAquick® PCR Purification Kit (QIAGEN) and sequenced at the Core DNA Sequencing Facility of the Roy J. Carver Biotechnology Center at the University of Illinois, Urbana-Champaign. Only those primers that did not present primer-dimer, a single band at the expected size in the gel, and had the right amplification product (verified by sequencing) were used for qPCR. The accuracy of a primer pair also was evaluated by the presence of a unique peak during the dissociation step at the end of qPCR. Sequencing results for all genes are reported in Table S2 and in previous publication (Loor et al., 2007; Bionaz and Loor, 2008a,b; Kadegowda et al., 2009; Schmitt et al., 2011; Naeem et al., 2012; Ji et al., 2012).

Genes selected for transcript profiling in the present study were grouped as follows: FA uptake from blood, lipoprotein lipase (*LPL*); intracellular FA trafficking, fatty acid binding protein 3, muscle and heart (*FABP3*) and 4, adipocyte (*FABP4*); intracellular activation of VFA, acyl-CoA synthetase short-chain family member 2 (*ACSS2*); de novo FA synthesis, acetyl-coenzyme A carboxylase α (*ACACA*), fatty acid synthase (*FASN*); desaturation, stearoyl-CoA desaturase (*SCD*); triacylglycerol synthesis, diacylglycerol acyltransferase 1 and 2 (*DGAT1*, *DGAT2*), lipin 1 (*LPIN1*); long chain FA elongation, ELOVL family member 6 (*ELOVL6*); lipid droplet formation, adipose differentiation related protein (*ADFP*), butyrophilin, subfamily 1, member A1 (*BTN1A1*), xanthine dehydrogenase (*XDH*); transcription regulation, insulin induced

gene 1 (*INSIG1*), mediator of RNA polymerase II transcription subunit 1 (*MED1*), SREBP cleavage activating protein (*SCAP*), sterol regulatory element-binding transcription factor 1 (*SREBF1*), thyroid hormone responsive SPOT14 (*THRSP*), peroxisome proliferator activated receptor gamma (*PPARG*); cholesterol transport and lipoprotein synthesis, apolipoprotein B (*APOB*); ketogenesis, 3-hydroxy-3-methylglutaryl-CoA synthase 2 (*HMGCS2*); long-chain fatty acid oxidation, acyl-CoA oxidase 1, palmitoyl (*ACOX1*), carnitine palmitoyl-transferase 1A (*CPT1A*); transcriptional regulation of fatty acid oxidation, retinoid X receptor, alpha (*RXRRA*), peroxisome proliferator-activated receptor alpha (*PPARA*).

Relative mRNA abundance among transcripts

Efficiency of PCR amplification for each gene was calculated using the standard curve method ($E = 10^{-1/\log \text{ curve slope}}$). Relative mRNA abundance among measured genes was calculated as previously reported (Bionaz and Loores, 2007), using the inverse of PCR efficiency raised to ΔC_t (gene abundance = $1/E^{\Delta C_t}$, where $\Delta C_t = C_t \text{ sample} - \text{geometric mean } C_t \text{ of 4 internal control genes}$). Overall mRNA abundance for each gene among all samples measured was calculated using the median ΔC_t . Use of this technique for estimating relative mRNA abundance among genes was necessary because relative mRNA quantification was performed using a standard curve (made from a mixture of RNA from different ovine tissues, which precluded a direct comparison among genes). Together, use of C_t values corrected for the efficiency of amplification plus internal control genes as baseline overcome this limitation.

Table S1. Description of 4 internal control genes

Symbol	Entrez Gene Name
<i>EIF3K</i>	Eukaryotic translation initiation factor 3, subunit K
<i>TUBB</i>	Tubulin beta, subunit 2A
<i>UXT</i>	Ubiquitously expressed transcript
<i>YWHAZ</i>	Tyrosine 3-monooxygenase

Table S2. Sequencing results of PCR products from primers of genes designed for this experiment. Best hits using BLASTN (<http://www.ncbi.nlm.nih.gov>) are shown

Gene	Sequence
<i>ACACA</i>	GGGGCCCTGTCAACGGTGACGTCGGATAGCATCTCCAACCTTCCTTCACTCCTTAGAGAGGGGGGGTCAAGTCCTC TCTGCTCATACTTCTAA
<i>ACSS2</i>	CGCATCAGTCTCGGGTGCCTCACAGGGACAGACAACAAGGGTCCCACATGGGGGAATCTGGATGTTTCTCTTGAG CAGGAGATGGGAC
<i>ADFP</i>	GCTCCTCTCTCCGTGATTGGACTGTGCCGGGAGATGGTGGCATAATGGCCAACCAGAAGATTCACGATGGCTCAA
<i>DGAT1</i>	ACCGGTTTGTTCGGATTGGGACGGCTTCCTCCTCCCTCCCCACTTTCATCTTAGAGACTGGCTGCATATCCGGGT CAGTGTGGTTGTAA
<i>FABP3</i>	CCCTTCCTCTCGTGAGTCCTGTGCCTAAAATAACCTTGCTCCTGAATGGACCCAAAAACCTAGAGGAATGGGAAT GAGAA
<i>FABP4</i>	AGAGACTTCACACGTGGGATGGAATCACCCCATAAAGAGAAAACCTTGTTGGATGATAAGCCTGGTGCTGGAATGT ACCCAGCAGCACCTTCATCTAAGGTTTAA
<i>LPL</i>	GACTTCGACGTCTCGTTCTCTTATTGACTCTCTGTTGAATGAAGAAAATCCAAGGTAAGGGCCTACCGGG
<i>PPARG</i>	CAGATCTCGGGCTTGTTGACCCTTGACTGGAATGACCAAGGTAACCTCTCCTAAAATACGGGCGTGACGAGATAA
<i>SCD</i>	GGGAGAAAGGCAAGGCCAGCCTTGAGGTATGTTTGGAGAAACATCATCCTCATGGGTCTGTTACACTTGGGAA
<i>SREBF1</i>	CACCAACGTCCGGCCCGTCAGACCCTGGTGAAGTGGCGGGGCCATCCCTGGCCACTGTGTCCACTGGTAGTGGAC ACCTGTACAAGCTGCCAA
<i>TUBB</i>	CAGAGCGTCCGAACAGGACACGGCCGGCCTACCTTCGTGGGAATGGGATCCCCAACAACGTCAAGGACAGCTGG TTTGGCGACATCCAA
<i>XDH</i>	AGGTAGATGACTCTTCCGGGCTAGATGCCCTGCCACCCCGGAGGAAGATCCGCAATGCCTGTGTGGACAAGGTTT CAA
<i>YWHAZ</i>	GTTGTACTCTCCTTTTTGCAAGACGGAAGGGTGTCTTGAGAAAACAGCAGATGTGGCTTCGAGAATTACAGAGAG AAA

Table S3. Sequencing results of genes using BLASTN from NCBI against nucleotide collection (nr / nt) with total score

Gene	Best hits	Score
<i>ACACA</i>	<i>Ovis aries</i> acetyl-CoA carboxylase alpha (ACACA), mRNA >emb X80045.1 <i>O. aries</i> mRNA for acetyl-CoA carboxylase	105
<i>ACSS2</i>	<i>Ovis aries</i> acetyl-CoA synthetase 2 (ACAS2) mRNA, partial cds	93.3
<i>ADFP</i>	<i>Ovis aries</i> perilipin 2 (PLIN2), mRNA >gb EF660332.1 <i>Ovis aries</i> adipose differentiation-related protein (ADFP) mRNA, complete cds	53.6
<i>DGAT1</i>	<i>Ovis aries</i> diacylglycerol acyltransferase 1 (DGAT1) gene, complete cds	114
<i>FABP3</i>	<i>Ovis aries</i> heart fatty acid binding protein (H-FABP) gene, exons 2 through 4 and partial cds	62.6
<i>FABP4</i>	<i>Ovis aries</i> fatty acid binding protein 4, adipocyte (FABP4), mRNA >gb EU301804.1 <i>Ovis aries</i> adipocyte fatty acid-binding protein 4 (FABP4) mRNA, complete cds	73.4
<i>LPL</i>	<i>Ovis aries</i> lipoprotein lipase (LPL), mRNA	77.0
<i>PPARG</i>	<i>Ovis aries</i> proliferator-activated receptor gamma mRNA, partial cds	84.2
<i>SCD</i>	<i>Ovis aries</i> stearyl-CoA desaturase (SCD) gene, complete cds	93.3
<i>SREBF1</i>	<i>Ovis aries</i> sterol regulatory element-binding transcription factor 1 gene, exons 6, 7 and partial cds	95.1
<i>TUBB</i>	<i>Ovis aries</i> beta-tubulin mRNA, partial cds	59.0
<i>XDH</i>	<i>Ovis aries</i> xanthine dehydrogenase (XDH) mRNA, partial cds	87.8
<i>YWHAZ</i>	<i>Ovis aries</i> tyrosine 3-monooxygenase (YWHAZ) mRNA, partial cds	46.4

Table S4. Gene ID, GenBank accession number, hybridization position, sequence and amplicon size of primers for *Ovis aries* used to analyze gene expression by qPCR

Gene ID	Accession #	Gene	Primers ¹	Primers (5'-3')	bp ²
443186	NM_001009256.1	<i>ACACA</i>	F.2201 R.2319	ACCATGCTGGGAGTTGTCTGT AGAAGTGTATGAGCAGAGAGGACTTG	118
780456	DQ272257.1	<i>ACSS2</i>	F.197 R.303	CGAAGCCATAAAGATCTGTCCAT CCATCTCCTGCTCAAGAGAAACA	106
100125354	NM_001104932.1	<i>ADFP</i>	F.746 R.847	AAGAGGCCAGGAGACCATTTT TGAGCATCGTGAATCTTCTGGTT	101
100126245	EU178818.1	<i>DGAT1</i>	F.2763 R.2877	AGGGATCTGGAAAAGCTTGAATAA ACAACCACACTGACCGGATATG	114
2828237	AY157617.1	<i>FABP3</i>	F.1954 R.2054	AGGGCAAGAACCCCAATTAAA CTCATTCCCATTCTCTAGTTTTTG	100
100137067	NM_001114667.1	<i>FABP4</i>	F.255 R.354	AAACTTAGATGAAGGTGCTCTGGTACA ACATTCCAGCACCAGCTTATCA	99
443408	NM_001009394	<i>LPL</i>	F.942 R.1041	TGGAGATGTGGACCAGCTAGTG CCGGTAGGCCTTACTTGGATT	99
443513	FJ200441.1	<i>PPARG</i>	F.64 R.163	CCGTGCAGGAGATCACAGAGT ATCTCGTGCACGCCGTATTT	99
443185	FJ513370	<i>SCD</i>	F.1100 R.1200	GATGACATCTATGACCCAACTTACCA CCCAAGTGTAACAGACCCATGA	100
100329218	GU206528	<i>SREBF1</i>	F.152 R.267	GGGACAAGGTTTGCTCACATG GGCAGCTTGTGAGTGTCCACTA	115
100303606	AF035420.1	<i>TUBB</i>	F.181 R.280	GAAGGAGGTAGATGAGCAGATGCT GATGTCGCAAACAGCTGTCTTG	99
780499	EF529448.1	<i>XDH</i>	F.631 R.730	GCTCGAGCTCAGCACACAGA TGAACCTTGTCACACAGGCATT	99
780452	AY970970.1	<i>YWHAZ</i>	F. R.	TGTAGGAGCCCGTAGGTCATCT TTCTCTCTGTATTCTCGAGCCATCT	102

¹ Primer direction (F – forward; R – reverse) and hybridization position on the sequence.

² Amplicon size in base pair (bp).

Table S5. Quantitative PCR performance among the genes measured in mammary,,
adipose and liver tissue.

Gene	Median Ct¹	Median ΔCt²	Slope³	(R²)⁴	Efficiency⁶
Mammary gland					
<i>ACACA</i>	23.77	0.63	-3.16	0.996	2.07
<i>ACSS2</i>	23.74	0.60	-2.97	0.990	2.17
<i>BTN1A1</i>	17.69	-5.59	-3.28	0.998	2.02
<i>DGAT1</i>	30.31	7.18	-3.58	0.982	1.90
<i>ELOVL6</i>	27.01	3.73	-3.41	0.988	1.96
<i>FABP3</i>	23.04	0.05	-2.96	0.992	2.18
<i>FASN</i>	23.71	0.52	-2.94	0.979	2.19
<i>INSIG1</i>	20.70	-2.02	-3.29	0.995	2.01
<i>LPIN1</i>	24.15	0.88	-3.14	0.990	2.08
<i>LPL</i>	17.27	-4.96	-3.01	0.991	2.15
<i>MED1</i>	25.03	3.36	-3.59	0.994	1.90
<i>PPARG</i>	25.64	3.30	-2.96	0.983	2.18
<i>RXRA</i>	23.75	1.97	-3.07	0.993	2.12
<i>SCAP</i>	27.27	4.18	-3.03	0.981	2.14
<i>SCD</i>	17.25	-5.10	-3.11	0.993	2.10
<i>SREBF1</i>	28.30	6.51	-2.66	0.981	2.38
<i>THRSP</i>	30.56	7.58	-2.66	0.984	2.38
<i>XDH</i>	18.10	-5.04	-3.17	0.998	2.07
Adipose Tissue					
<i>ACACA</i>	21.01	-1.53	-3.16	0.996	2.07
<i>ACSS2</i>	25.33	2.44	-2.97	0.990	2.17
<i>ADFP</i>	24.99	2.28	-3.12	0.997	2.09
<i>DGAT2</i>	25.95	3.17	-2.94	0.968	2.19
<i>ELOVL6</i>	19.94	-2.25	-3.41	0.988	1.96
<i>FABP4</i>	16.42	-6.30	-3.30	0.997	2.01
<i>FASN</i>	24.44	1.82	-2.94	0.979	2.19
<i>INSIG1</i>	22.92	0.34	-3.29	0.995	2.01
<i>LPIN1</i>	24.05	1.31	-3.14	0.990	2.08
<i>LPL</i>	19.05	-4.10	-3.01	0.991	2.15
<i>MED1</i>	25.48	3.77	-3.59	0.994	1.90
<i>PPARG</i>	22.67	-0.68	-2.96	0.983	2.18
<i>RXRA</i>	22.89	1.14	-3.07	0.993	2.12
<i>SCAP</i>	26.67	4.09	-3.03	0.981	2.14
<i>SCD</i>	17.69	-5.39	-3.11	0.993	2.10
<i>SREBF1</i>	27.77	5.94	-2.66	0.981	2.38
<i>THRSP</i>	21.25	-1.06	-2.66	0.984	2.38

Table S5 (Continued).

Gene	Median Ct¹	Median ΔCt²	Slope³	(R²)⁴	Efficiency⁶
Liver					
<i>ACOX1</i>	27.79	2.95	-3.12	0.988	2.09
<i>APOB</i>	20.03	-4.60	-3.28	0.997	2.02
<i>CPT1A</i>	28.44	3.82	-2.68	0.988	2.36
<i>HMGCS2</i>	23.92	-0.98	-2.92	0.997	2.20
<i>PPARA</i>	28.09	3.19	-2.83	0.989	2.25
<i>RXRA</i>	24.10	-0.89	-3.37	0.991	1.98

¹ The median is calculated considering all ewes.

² The median of Δ Ct is calculated as [Ct gene – geometrical mean of Ct internal controls] for each ewe.

³ Slope of the standard curve.

⁴ R² stands for the coefficient of determination of the standard curve.

⁵ Efficiency is calculated as $[10^{(-1 / \text{Slope})}]$.

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