EXPRESSION OF SELECTED LIPOGENIC GENES AND FATTY ACID TRANSPORTERS CHANGES ACROSS STAGES OF LACTATION IN DAIRY EWES †

[EXPRESIÓN DE GENES LIPOGÉNICOS SELECCIONADOS Y TRANSPORTADORES DE ÁCIDOS GRASOS CAMBIAN ATRAVÉS DE LAS ETAPAS DE LACTANCIA EN OVEJAS LECHERAS]

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SUMMARY

Background. During lactation the mammary fat synthesis is increased compared to adipose tissue. **Objective.** This study evaluated the promoter specific gene expression of acetyl-CoA carboxylase alpha (*ACACA*) transcripts from promoters II and III, fatty acid synthase (*FASN*), sterol regulatory element binding transcription factor 1 (*SREBF1*) in mammary gland and adipose tissue and, peroxisome proliferator-activated receptor gamma (*PPARG*), fatty acid transporters acyl-CoA synthetase long chain family member 1 (*ACSL1*), solute carrier family 27 member 6 (*SLC27A6*), fatty acid binding proteins 3 and 4 (*FABP3* and *FABP4*), CD36 molecule (*CD36*) and lipoprotein lipase (*LPL*) in the mammary gland. **Methodology.** Eight Lacaune lactating ewes at 15, 70, and 120 days in milk (DIM) were used. **Results.** There was no effect of tissue, lactation stage, or tissue by lactation stage interaction for PIII *ACACA* transcripts. Mammary PII transcripts changed according to stage of lactation with higher expression at 15 than 120 DIM. In adipose tissue at 120 DIM, expression of PII *ACACA* transcripts were higher than 15 and 70 DIM. Expression of *FASN* in adipose tissue was higher at 70 and 120 DIM when compared to mammary tissue at 120 DIM. The *SREBF1* expression at 70 was higher than 15 DIM in mammary tissue and higher than 70 DIM in adipose tissue. In the mammary gland, the gene expression of *LPL*, *CD36* and *FABP3* was decreased from 15 to 120 DIM. **Implications.** Collectively, our results highlight the fact that in early lactation (15 to 70 DIM) milk fat synthesis is prioritized over adipose tissue in dairy sheep. **Conclusion.** This prioritization is managed through an orchestrated increase in mammary gene expression of *ACACA* transcripts, *FASN*, *SREBF1*, *LPL*, *CD36*, and *FABP3*.
Key words: Fatty acid synthesis; lactation cycle; milk fat synthesis.

RESUMEN

Antecedentes. Durante la lactancia hay la síntesis de grasa en la glándula mamaria es mayor en comparación con el tejido adiposo. **Objetivo.** Este estudio evaluó la expresión génica específica de la acetil-CoA carboxilasa alfa (*ACACA*), transcripciones de los promotores II y III, sintasa de ácidos grasos (*FASN*), factor de transcripción de unión al elemento regulador de esteroles 1 (*SREBF1*) en tejidos mamarios y adiposos y, el receptor gamma activado del proliferador de peroxisoma (*PPARG*), transportadores de ácidos grasos acil-CoA sintetasa de cadena larga 1 (*ACSL1*), portador de soluto compañia familia 27 miembro 6 (*SLC27A6*), proteínas de unión a ácidos grasos 3 y 4 (*FABP3* y *FABP4*), molécula CD36 (*CD36*) y lipoproteína lipasa (*LPL*) en la glándula mamaria. **Metodología.** Ocho ovejas de la raza Lacaune a los 15, 70 y 120 días en lactación (DEL). **Resultados.** No hubo efecto del tejido, la etapa de lactancia o la interacción tejido y etapa de lactancia para las transcripciones de PIII *ACACA*. Las transcripciones de PII mamaria cambiaron de acuerdo a la etapa de lactancia con una mayor expresión a los 15 que 120 DIM, la expresión de la transcripcion PII *ACACA* fue superior a 15 y 70 DIM. La expresión de *FASN* en el tejido adiposo fue mayor a 70 y 120 DIM en comparación con el tejido mamario a 120 DIM. La expresión de *SREBF1* a 70 fue superior a 15 DIM en tejido mamario y superior a 70 DIM en tejido adiposo. En la glándula mamaria, la expresión génica de *LPL*, *CD36* y *FABP3* se redujo de 15 a 120 DIM. **Implicaciones.** Colectivamente, nuestros resultados resaltan el hecho de que en la lactancia temprana (15 a 70 DIM) la síntesis de grasa de la leche tiene prioridad sobre el tejido adiposo en ovejas lecheras. Conclusión. Esta priorización se gestiona a través de un aumento orquestado en la expresión génica mamaria de las transcripciones de *ACACA*, *FASN*, *SREBF1*, *LPL*, *CD36* y *FABP3*.
Palabras clave: Síntesis de ácidos grasos; ciclo de lactancia; síntesis de grasa láctea.

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INTRODUCTION

Studies about the processes of lactation biology in the mammary gland of ruminants advanced significantly during the twentieth century (Baumgard et al., 2017). These, initially, defined and quantified the main aspects of lipid metabolism in the mammary gland, including milk fatty acid (FA) synthesis through *de novo* synthesis and preformed FA from bloodstream (Bauman and Davis, 1974).

During lactation, there is a coordination between milk fat synthesis by the mammary gland and the adipose tissue. The fatty acids (FA) in both tissues originate either from *de novo* synthesis (≤C16 carbons) by the action of acetyl-CoA carboxylase alpha (*ACACA* - EC 6.4.1.2) and fatty acid synthase (*FASN* - EC 2.3.1.85) or uptake from the bloodstream (preformed FA originating from the diet ≥C16 carbons) (Palmquist, 2006). The tissue where lipid synthesis will occur is determined by several factors such as the nutritional status, milk production and lactation stage.

The coordination of the enzymes responsible for lipid synthesis and mobilization is highly transcriptionally regulated (Barber et al. 1997; Travers and Barber, 2001). We recently demonstrated the effects of *trans*-10, *cis*-12 conjugated linoleic acid (CLA) on the gene expression of *ACACA* isoforms, *FASN*, *SREBF1* and leptin at each stage of lactation (Ticiani et al., 2016), where we showed that *ACACA* alpha transcripts are down-regulated in the mammary gland and up-regulated in adipose tissue of lactating ewes under *trans*-10, *cis*-12 CLA-induced milk fat depression through a promoter and tissue-specific manner. However, the expression behavior of both lipogenic genes and FA transporters in both tissues in the course of a normal lactation is still unknown. Understanding the mechanisms of regulation of mammary lipogenesis in ruminants is central to making changes in fat content and milk composition, as well as making strategic changes in order to improve animal health status.

The central hypothesis of our study was that higher gene expression of transcriptionally regulated lipogenic genes and FA transporters occurs in mammary gland compared to adipose tissue at early stages of lactation. To test this, we evaluated in mammary gland and adipose tissue, or only in mammary gland, the expression of selected transcriptionally regulated genes.

MATERIAL AND METHODS

cDNA samples, primers and quantitative real time PCR (RT-qPCR)

It is important to mention that we used samples from the "control" treatment from another published study (Ticiani et al., 2016), and although part of the samples came from the same animals, the research question and objectives of the current study are different and independent of those of Ticiani et al. (2016). The genes that were evaluated in both studies are ACACA, FASN and SREBF1 in mammary and adipose tissues. All others genes were studied only in this manuscript. Additionally, we would like to point out that Ticiani et al. (2016) compared the effects of CLA vs. no-CLA (Control) in each stage throughout lactation (always as follow: CLA vs. Control at 15; CLA vs. Control at 70 and CLA vs. Control at 120 DIM) and did not compare the lactation cicle separately.

In this study, we used six cDNA samples from the six animals of control treatment in Ticiani et al. (2016) plus the samples from two animals not used before, also receiving none treatment to run the RT-qPCR analysis. Briefly, samples were obtained at 15, 70 and 120 days in milk (DIM) from the same eight Lacaune ewes and all procedures for mammary and adipose tissues biopsies, total RNA extraction, complementary cDNA, and Real-Time PCR analysis (RT-qPCR) were described in detail by Sandri et al. (2017). The lipogenic genes evaluated were: acetyl-CoA carboxylase alpha (*ACACA*), transcripts only from promoters II and III because those from promoter I are not expressed in mammary gland (Molenaar et al., 2003), fatty acid synthase (*FASN*) and the transcription factor sterol regulatory element binding factor 1 (*SREBF1*), in the mammary gland and adipose tissue. Peroxisome proliferator-activated receptor gamma (*PPARG*), FA transporters [acyl-CoA synthetase long chain family member 1 (*ACSL1*), solute carrier family 27 member 6 (*SLC27A6*), fatty acid binding proteins 3 and 4 (*FABP3* and *FABP4*), and CD36 molecule (*CD36*)] and triglyceride uptake lipoprotein lipase (*LPL*)] were evaluated in mammary tissue only because we had a power outage that cause the lost of RNA.

Primers were obtained from published studies or designed using Primer Express software (PE Applied Biosystems, v. 3.0) using gene sequences publically available in GenBank [National Center for Biotechnology Information (NCBI); http://www.ncbi.nlm.nih.gov/genbank/]. Briefly, the primers used were: a) *ACACA*, *FASN*, *SREBF1*, actin-beta (*ACTB*) and ribossomal protein S18 (*RPS18*) from Ticiani et al. (2016); b) *LPL*, *CD36*, *FABP3* and *PPARG* from Hussein et al. (2013) and; c) designed for this study: *SLC27A6* (F: GCCCATGTCTTCCTGAACCA, R: TGCCCAAACGCTGATACCTT (Predicted - XM_004008652.3), *ACSL1* F: AAATAGCATCCGGACCCAGC, R: CGATGAACTTGTCTGGGGCT (Predicted - XM_015104562.1), and *FABP4* F: ATGGCCAAACCCACTGTGAT, R: GGCCCAATTTGAAGGACATCT (NM_001114667.1). All primers were tested

according the melting curve and amplification efficiency. All samples were run with a seven-point standard curve with a "pool" of cDNA for each tissue with serial dilution (100, 50, 25, 12.5, 6.25, 3.125 and 1.5625%). Subsequently, a regression equation was generated by plotting the cycle threshold values from RT-qPCR against the log from each value from the standard curve. The slope of the equation described the efficiency of the reaction. All reactions had a determination coefficient higher than 0.95 and efficiency between 90 and 115% (Supplemental Table 1).

Statistical analysis

Data were analyzed using MIXED procedure of SAS (2017). For *ACACA*, *FASN*, and *SREBF1* the model included the fixed effect of tissue (mammary or adipose), the repeated measure stage of lactation within animal, and their interaction. Data for FA transporters, *PPARG*, and *LPL* were analyzed only in mammary gland with the MIXED procedure with stage of lactation as fixed effect and animal as random. Also, as described by Vandesompele et al. (2002), the geometric mean of the housekeeping genes ribosomal protein S18 (*RPS18*) and actin-beta (*ACTB*) was used as a covariate. Data were log2 transformed when necessary and the back transformed data is reported. The variance-(co) variance matrix was chosen according the lowest Akaike information. Data points with Studentized residuals outside of ± 2.5 were considered outliers and excluded from analysis (only two data point were excluded for *FASN* in adipose tissue at 15 and 70 DIM, respectively). The PDIFF option in LSMEANS statement adjusted for Tukey Test was used to assess differences among stages of lactation. Significance was declared at $P < 0.05$.

RESULTS

In the current study, there was no effect of tissue, lactation stage, or tissue and lactation stage interaction for PIII *ACACA* transcripts ($P > 0.05$). Mammary PII transcripts changed according to stage of lactation ($P = 0.0001$) with higher expression at 15 (~ 3.2 fold) than 120 DIM. Also, the PII transcripts were less expressed at 15, 70, and 120 in mammary gland compared to adipose tissue at 120 DIM (Table 1). In adipose tissue at 120 DIM, PII *ACACA* transcripts were ~11 fold higher expression than 15 and 70 DIM (Table 1).

The expression of *FASN* was reduced 5.8 fold in mammary tissue at 120 compared to 15 DIM ($P = 0.03$). Expression of *FASN* in adipose tissue was higher at 70 and 120 DIM when compared to mammary tissue at 120 DIM.

The *SREBF1* expression was 16 fold higher at 70 than 15 DIM ($P = 0.03$) in mammary tissue, and 9 fold higher in adipose tissue than in the mammary tissue at 70 DIM (Table 1). On the other hand, *PPARG*, another transcription factor regulating *ACACA* and *FASN* that is well studied in adipose tissue, showed no change in mammary gland (Table 2).

The expression of *LPL* and *CD36* was up-regulated by more than 2 fold at 15 compared with 120 DIM. The *SLC27A6*, a membrane protein transporter was consistently unaltered throughout lactation and no changes in *ACSL1* gene expression during lactation was also observed. *FABP3* was up-regulated at 15 DIM suggesting a main role for this isoform compared with *FABP4* (Table 2).

DISCUSSION

Barber and Travers (2001) described that the *ACACA* alpha gene is transcribed from multiple tissue specific promoters (P) and Molenaar et al. (2003) showed the existence of differences between species (e.g the expression from promoter I "PI" is low or absent in the ovine mammary gland but present in the lactating bovine mammary gland).

Changes in expression of different *ACACA* transcripts could be expected in adipose and mammary tissues as a part of metabolic changes during lactation corroborating the concept of homeorhesis described more than 30 years ago by Bauman and Currie (1980). Supporting that, Travers and Barber (2001) showed in sheep an increased gene expression of *ACACA* in mammary gland with a corresponding repressed expression in adipose tissue at the same time. Our results showed that *ACACA* from promoter PIII is transcribed in a similar way for both tissues throughout the lactation cycle being important for fat synthesis in both tissues during lactation. However, our results pointed out for an important role of PII transcripts in mammary gland at early lactation and an essential role in adipose tissue at the end of the lactation cycle.

The regulation of *de novo* fat synthesis between mammary and adipose tissues occurs by regulation of the mRNA levels in lipogenic enzymes, according to the physiological prioritization of milk fat synthesis during the lactation cycle (Barber et al., 1997). As proposed by Molenaar et al. (2003), *ACACA* transcripts from PI are not essential for milk fat synthesis in sheep. Indeed, in the current experiment PI transcripts were not detected in lactating mammary tissue.

In the current experiment, the abundance of the *ACACA* PII transcript and *FASN* was higher in adipose tissue in late lactation, highlighting the increase of lipogenesis in the adipose tissue at the end of lactation, in agreement with the concept of homeorhesis described by Bauman and Currie (1980).

Table 1. Gene expression[1] in mammary and adipose tissue of dairy ewes at three stages of lactation.

	ACACA PII					
	Tissue[2]		SEM[3]	P value		
Stage[4]	MG	AT		Tissue	Stage	Tissue*Stage
15	101.3^{Ab}	60.9^{Bb}	1.18	0.0001	0.003	0.0001
70	45.7^{ABb}	64.9^{Bb}	1.17			
120	31.8^{Bb}	701.8^{Aa}	1.20			
	ACACA PIII					
	Tissue		SEM	P value		
Stage	MG	AT		Tissue	Stage	Tissue*Stage
15	165.6	96.3	29.3	0.25	0.66	0.10
70	43.3	147.4	26.6			
120	72.8	153.1	31.7			
	FASN					
	Tissue		SEM	P value		
Stage	MG	AT		Tissue	Stage	Tissue*Stage
15	142.6^{A}	66.7^{BC}	1.45	0.02	0.89	0.0003
70	80.8^{AB}	127.9^{Ba}	1.40			
120	24.4^{Bb}	298.7^{Aa}	1.39			
	SREBF1					
	Tissue		SEM	P value		
Stage	MG	AT		Tissue	Stage	Tissue*Stage
15	24.0^{B}	124.8	1.86	0.89	0.25	0.002
70	401.9^{Aa}	44.4^{b}	1.57			
120	103.0^{AB}	146.5	1.61			

[1]Arbitrary units of mRNA abundance; [2] MG = mammary gland and AT = adipose tissue; [3] Standard error mean; [4] DIM = days in milk; Means within a column with different capital letters and in the row with different small superscripts are significantly different.

Table 2. Gene expression[1] in mammary tissue of dairy ewes at three stages of lactation.

	Stage of lactation (DIM[2])				
	15	70	120	SEM[3]	P value
PPARG	184.6	89.7	168.8	30.5	0.15
LPL	117.9^{a}	68.2^{b}	49.6^{b}	11.5	0.004
CD36	152.4^{a}	98.7^{b}	62.7^{b}	12.2	0.001
FABP3	276.7^{a}	101.6^{b}	47.3^{b}	24.2	0.01
FABP4	183.5	128.8	131.6	25.4	0.27
SLC27A6	122.9	110.2	190.9	46.0	0.50
ACSL1	119.1	95.3	70.7	1.51	0.70

[1]Arbitrary units of mRNA abundance; [2] DIM = days in milk; [3] Standard error mean; Means within a row with different superscripts are significantly different.

Interestingly, *SREBF1* showed a different pattern of gene expression compared with its targeted genes *ACACA* and *FASN*. The higher transcription at the middle of lactation and the fairly gene expression of *PPARG* suggests that both transcription factors are present regulating lipogenic genes during the lactation. Bionaz and Loor (2008a) showed an increased in *PPARG* gene expression throughout lactation in dairy cows. However, our results did not corroborate these findings in sheep suggesting maybe a constitutive level of *PPARG* expression throughout the lactation cycle (Table 2).

Bionaz and Loor (2008a) showed that the gene expression of LPL had a similar pattern to the lactation curve in dairy cows. Our results showed an increased gene expression at the beginning of lactation (15 DIM), which indicate an important role of LPL to hydrolyze VLDL and chylomicron to support mammary fat synthesis at early lactation. In addition, as the FA uptake by mammary cells is mainly protein-mediated, our data suggested a role for *CD36* as a transporter for FA into the mammary epithelial cells during early stage of lactation. Corroborating that, the membrane protein transporter *SLC27A6* was consistently unaltered throughout lactation, which suggest a role for FA uptake during all the lactation cycle.

ACSL1 is mainly an activator of LCFA to acyl-CoA before they can be driven to intracellular metabolic pathways. As expected, our data showed no changes in *ACSL1* gene expression during lactation. This is may be because activation of LCFA is required before triacylglycerol synthesis regardless of stage of lactation.

FABP3 and *FABP4* are the two isoforms found in the mammary gland, being responsible for the diffusion of LCFA into the cells and transporting the FA to organelles for metabolism (Bionaz and Loor, 2008b). *FABP3* was up-regulated at 15 DIM suggesting a main role for this isoform compared with *FABP4* (Table 2). Collectively, our data on expression of genes responsible for fatty acid uptake and transit inside the cells suggest a collaborative work between synthesizing and transporting FA prioritizing milk fat synthesis at early stages of lactation.

From an evolutionary point of view, the coordinated change in lipid synthesis between mammary and adipose tissues can be explained by animal energy needs. At the beginning of lactation, the priority is to feed the offspring, which increases the energy requirements for milk production. As the lactation progresses, the progeny is less maternal-dependent and the female is able to replenish body stores and prepare for a new lactation cycle.

Also, it is important to mention that, care must be taken when interpreting results of gene expression. Although they can give valuable information, they do not always reflect activity of the corresponding enzymes.

CONCLUSION

Collectively our results highlighted the fact that in early and mid lactation (15 to 70 DIM) milk fat synthesis is prioritized over adipose tissue in dairy sheep. This prioritization appeared to be managed through an orchestrated increase, at early stages of lactation, in mammary gene expression of *ACACA* PII transcripts, *FASN*, *SREBF1*, *LPL*, *CD36*, and *FABP3*.

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Conflict of interest. The authors declare that they have no conflict of interest.

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Compliance with ethical standards. The authors declare that Santa Catarina State University Animal Ethical Committee approved all animal management and care procedures.

Data availability. Data are available up on reasonable requesting to the correspondent author.

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