Journal of Animal Science, 2025, **103**, skaf320
https://doi.org/10.1093/jas/skaf320
Advance access publication 18 September 2025
Lactation and Mammary Gland Biology

Increased growth rate post-weaning affects mammary gene expression of two-year-old ewes during their second pregnancy and lactation

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Abstract

This study examined the long-term effects of increased growth rates between three and seven months of age on gene expression in the mammary gland of nondairy ewes during their second lactation. A total of 19 twin-bearing, two-year-old ewes that either had an increased growth rate between three and seven months of age (heavy; n = 9; 153 ± 2.2 g/d and 47.9 ± 0.38 kg at breeding) or did not have an increased growth rate (control; n = 10; 127 ± 1.9 g/d and 44.9 ± 0.49 kg at breeding) were selected. Mammary gland biopsies were collected at day 128 of pregnancy and day 30 of lactation to examine the expression of 37 genes involved in mammary cell development and milk fat metabolism using NanoString nCounter technology. Ewes in the heavy group tended to exhibit lower expression ($P < 0.05$) of acyl-CoA synthetase short-chain family member 1 (*ACSS1*), a gene critical for mitochondrial acetyl-CoA synthesis, energy production, and milk fat synthesis, and a trend toward ($P = 0.11$) lower expression of signal transducer and activator of transcription 5A (*STAT5A*), a regulator of mammary epithelial cell differentiation and survival. These lower expressions suggest potential carry-over effects of increased growth rate between three and seven months of age. However, no phenotypic differences were observed in lamb growth or live weight, and no differences were detected in the expression of downstream target genes or modulators of these pathways, suggesting limited functional impact on mammary gland development and lamb performance outcomes. Further investigations, including a functional assessment of lactation and use of comprehensive transcriptomic analyses, would be needed to understand the effects of increased growth rates between three and seven months of age on ewe mammary function and milk composition.

Lay Summary

This study examined how faster growth between three and seven months of age affects mammary glands in nondairy ewes during their second lactation. A total of 19 twin-bearing ewes, including 9 heavy ewes with a faster growth rate between three and seven months of age and 10 control ewes without a faster growth rate were selected. Mammary gland samples were collected during pregnancy and early lactation to examine the expression of 37 genes involved in mammary gland development and lipid metabolism. Ewes in the heavy group tended to show lower expression of acyl-CoA synthetase short-chain family member 1 (*ACSS1*), a gene for energy production and milk fat synthesis, and a trend toward lower expression of signal transducer and activator of transcription 5A (*STAT5A*), a regulator of mammary cell development. These results suggest potential carry-over effects of faster growth rate. However, no differences were observed in lamb growth, live weight, or in the expression of genes regulated by *ACSS1* or *STAT5A*, indicating limited impact on mammary gland development and lamb performance. Additional research on milk production and detailed gene analysis is needed to better understand how faster growth after weaning might influence mammary gland development and subsequent milk quality in nondairy ewes.

Key words: acyl-CoA synthetase short-chain family member 1, ewe lamb, mammary gland development, mammary lipid metabolism

Abbreviations: ABCG2, ATP binding cassette subfamily G member 2; ACACA, acetyl-CoA carboxylase alpha; ***ACC1***, acetyl-CoA carboxylase 1; ACSL1, acyl-CoA synthetase long-chain family member 1; ACSS1, acyl-CoA synthetase short-chain family member 1; ACSS2, acyl-CoA synthetase short-chain family member 2; ADF, acid detergent fiber; ATP, adenosine triphosphate; BAX, BCL2 associated X, apoptosis regulator; BCL2, BCL2 apoptosis regulator; BCLX, BCL2 like 1; BCS, body condition score; BDH1, β-hydroxybutyrate dehydrogenase; CCND1, cyclin D1; CISH, cytokine inducible SH2 containing CIS 1 suppressor of cytokine signaling (SOCS1); CP, crude protein; DEPC, diethylpyrocarbonate; DM, dry matter; DNA, deoxyribonucleic acid; ELF5, E74 like ETS transcription factor 5; ENSOARG13395 (CEBPB), no official name (CCAAT enhancer binding protein beta); ERBB4, Erb-b2 receptor tyrosine kinase 4; FASN, fatty acid synthetase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GHR, growth hormone receptors; GLM, generalized linear model; IGF1, insulin-like growth factor 1; IGF1R, insulin-like growth factor 1 receptor; IGFBP-1, insulin-like growth factor binding protein 1; IGFBP3, insulin-like growth factor binding protein 3; IGFBP5, insulin-like growth factor-binding protein 5; JAK2, janus kinase 2; L30, day 30 of lactation; LALBA, lactalbumin alpha; LEP, leptin; LEPR, leptin receptor; LTF, lactotransferrin; ME, metabolizable energy; MEC, mammary epithelial cell; mRNA, messenger ribonucleic acid; NDF, neutral detergent fiber; P128, day 128 of pregnancy; PR, progesterone receptors; PRL, prolactin; PRLR, prolactin receptor; PPARA, peroxisome proliferator-activated receptor-alpha; PPARD, peroxisome proliferator-activated receptor–delta; PPARG, peroxisome proliferator-activated receptor–gamma; RCC, reporter code count; RNA, ribonucleic acid; RQS, RNA quality score; SLC16A1/MCT1, monocarboxylic

Received January 28, 2025. Accepted September 10, 2025

acid transporter 1; SLC25A4, solute carrier family 25, mitochondrial carrier; adenine nucleotide translocator, member 4; SLC25A5, solute carrier family 25, mitochondrial carrier; adenine nucleotide translocator, member 5; SOCS2, suppressor of cytokine signaling 2; SOCS3, suppressor of cytokine signaling 3; SREBP-1, sterol regulatory element-binding transcription protein 1; STAT5A, signal transducer and activator of transcription 5A; TCA, tricarboxylic acid cycle; TGFB1, transforming growth factor beta 1; TGFBR1, transforming growth factor beta receptor 1; TGFBR2, transforming growth factor beta receptor 2;

Introduction

Increased growth rates between weaning and puberty can have detrimental effects on mammary gland development and subsequent milk production in ewes (Johnsson and Hart, 1985; Umberger et al., 1985; Villeneuve et al., 2010a). During development, the mammary gland undergoes a significant phase of ductal elongation and parenchymal development, influencing future alveoli development and ewe milk production (Johnsson and Hart, 1985; Berryhill et al., 2017; Geiger, 2019). This allometric phase was previously reported to occur between two and five months of age in ewes (Anderson, 1975); however, studies in heifers indicate that this prepubertal phase can begin prior to birth (Geiger and Hovey, 2023). The response of the mammary gland to nutrition is greater during this phase compared with the post-pubertal period (Geiger and Hovey, 2023). A high plane of nutrition with an increased post-weaning growth rate has been reported to result in an earlier attainment of puberty in ewes, interrupting the allometric phase (Meyer et al., 2006; Geiger and Hovey, 2023). This affects parenchymal development and increases fat accumulation in the mammary fat pad (Johnsson and Hart, 1985; Hovey et al., 1999; Hue-Beauvais et al., 2021). Consequently, milk production was reduced for up to two lactations from ewes with increased postweaning growth rates compared with ewes with slower growth rates (Villeneuve et al., 2010b), indicating there are carry-over effects. A series of studies using ultrasound scanning, however, reported that an increased growth rate between weaning and ewe breeding did not subsequently affect the internal structures of the mammary gland in nondairy ewes up to the weaning of their second litter (Haslin et al., 2021a,b). The presence of carry-over effects from increased growth rate prior to puberty on the mammary gland function in ewes, therefore, remains unclear.

Nutrition has been reported to influence gene expression in the mammary gland of ruminants (Conte et al., 2021; Hue-Beauvais et al., 2021). In dairy heifers, an elevated plane of nutrition preweaning was associated with an upregulation of pathways associated with cell growth in the mammary parenchyma (Vailati-Riboni et al., 2018), epithelial-mesenchymal transition, and lipid accumulation in the mammary gland (Hare et al., 2019). However, pathways associated with molecule transport and lipid metabolism were downregulated in dairy heifers on a high-protein, low-fat diet (Piantoni et al., 2012). A recent study reported that a high-quality diet (high energy and high protein) in nondairy ewes preweaning (30 to 120 d of age) increased the expression of growth hormone receptors (***GHRs***), progesterone receptors (***PRs***), and insulin-like growth factor binding protein-1 (***IGFBP-1***) compared to ewes on a lower-quality diet (Mirzaei-Alamouti et al., 2023). In the same study, the quality of the diet postweaning (121 to 210 d of age) did not affect *PR, IGFBP-1*, or sterol regulatory element-binding transcription protein-1 (***SREBP-1***) in ewes (Mirzaei-Alamouti et al., 2023). A high-quality diet postweaning, however, downregulated estrogen receptor and GHR (Mirzaei-Alamouti et al., 2023), which have a role in ductal expansion into the mammary fat pad. There is little information regarding the effect of a higher plane of nutrition between weaning and puberty on gene expression of the mammary gland in ewes and its potential long-term effects.

The potential carry-over effects on the ewe's mammary gland have been investigated using either productive measures (e.g. milk production or lamb growth; Villeneuve et al., 2010b; Haslin et al., 2021b) or ultrasound scanning techniques (Haslin et al., 2021b). Both examination techniques, however, did not provide information on mammary cell activity and metabolism. The objective of this study, therefore, was to investigate potential carry-over effects of increased growth rates in nondairy ewes between weaning and first breeding at seven months of age on the expression of genes involved in mammary development, cell survival, and lipid metabolism when they were 2-year-old ewes in their second lactation. It was hypothesized that ewes that experienced an increased growth rate would present differences in the expression of genes related to mammary development, cell survival, and lipid metabolism.

Materials and Methods

Our experimental and all animal handling procedures were approved by the Massey University Animal Ethics Committee (MUAEC 17/16). The experiment was conducted at Massey University Keeble Farm (latitude: 40°24′03″S; longitude: 175°35′51″E), 5 km south of Palmerston North, New Zealand.

Experimental design

Background

The management and feeding treatment of the ewes were previously described in detail by Haslin et al. (2022). Briefly, 270 twin-born Romney ewes born in 2017 were allocated to one of the two treatments at weaning (approximately 86 d of age) using a stratified randomly sampling procedure to ensure that the average weaning weight of the treatments did not differ (28.6 kg ± 0.16). The 'heavy' group (n = 135) was preferentially fed until their first breeding (10/05/2018; approximately 213 d of age), achieving a growth rate of 153 ± 2.2 g/d (average ± SEM) and an average live weight of 47.9 ± 0.38 kg (average ± SEM). The 'control' group (n = 135) had a growth rate of 127 ± 1.9 g/d and an average live weight of 44.9 ± 0.49 kg at breeding. The live weight difference was achieved by differing herbage allowances on either lucerne (*Medicago sativa* L.) or a ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.) sward and cereal-based concentrate feed allowances. Prior to breeding, both treatments were offered a cereal-concentrate feed (CP 10.5%, NDF 17.6%, ADF 7.1%, ME 12.8 MJ/kg DM): up to 200 g/d for 43 d for the control group and 200 g/d for 68 d and 300 g/d for 51 d for the heavy group. From the ewe's breeding onward, both treatments were bred and managed together and grazed on perennial ryegrass and white clover pastures under commercial New Zealand grazing conditions.

Present study

In 2019, a subset of two-year-old ewes from each treatment were selected at pregnancy diagnosis (93 d of pregnancy; heavy n = 9, 65.7 ± 1.5 kg and control n = 10, 70.1 ± 1.6 kg; least square-mean ± SEM) from two-year-old ewes that had weaned at least one lamb as a one-year-old ewe, were successfully mated during the first 17 d of breeding, and were identified as twin-bearing ewes at pregnancy diagnosis with transabdominal ultrasound. Live weight change of these ewes between the first breeding at seven months of age and pregnancy diagnosis at two years of age is shown in Figure 1. Prelambing, ewes were allocated to lambing paddocks, with ewes from the heavy and control groups present in all lambing paddocks. During the lambing period, ewes were checked daily to identify and ear-tag newborn lambs. Mammary biopsies were performed on day 128 of pregnancy (P128) and day 30 of lactation (L30). Two-year-old ewes whose entire litter died at birth or during lactation were not biopsied and were excluded from the experiment (control n = 1). During the lambing period, five twin-born lambs died, resulting in five ewes (control n = 4 and heavy n = 1) rearing a single lamb and 13 ewes rearing twin lambs (control n = 5 and heavy n = 8).

Animal measurements and sample collection

Live weight and body condition score (**BCS**; Jeffries 1969) of ewes were recorded at P128 and L30. Within 18 h of birth, the sex of lambs, their birth rank, and date of birth were recorded. Live weights of lambs were recorded within 18 h of birth and again in early lactation (L30).

Mammary tissue collection and processing

A Bard Magnum biopsy gun with a 12 G needle (Bard Biopsy Systems, AZ, USA) was used to collect mammary gland tissue of the ewes at P128 and L30. Mammary gland tissues were collected from one udder half per biopsy; these were alternated to sample both glands. The biopsy method was based on the procedure described by Paten et al. (2015). Approximately 2–3 mL of local anesthetic (Lopaine, 2% lignocaine HCl, Ethical Agents, Manukau, NZ) was administered to the biopsy site prior to the procedure. An analgesic (1 mL/30 kg, Ketoprofen 10%, Ethical Agents, Manukau, NZ) and an antibiotic (0.2 mL/10 kg, Engemycin [100 mg oxytetracycline/mL], MSD Animal Health, Buckinghamshire, UK) were also administered to alleviate pain and reduce the risk of infection. The tissues were collected in the caudal section and midline of one udder half where the udder protrudes to ensure collecting the ductal and lobulo-alevolar tissues of the parenchyma. Biopsied tissues were snap-frozen in liquid nitrogen, then placed in a cryo-vial and stored at −80 °C until later use for ribonucleic acid (**RNA**) extraction and gene expression analyses.

mRNA methods

mRNA extraction procedure

Cryo-vials containing mammary biopsy tissue were removed from −80 °C and stored on dry ice. Entire frozen biopsy samples were ground in 1 mL of Trizol reagent (Invitrogen, CA, USA) with a disposable, sterile pestle until partially homogenized. The solution was left for 5 min at room temperature to allow complete dissociation of nucleoprotein complexes, after which 200 µL of chloroform was added and mixed by vortex for 15 s. The solution was left to incubate at room temperature for 3 min. The samples were then centrifuged at 12,000 *g* for 20 min at 4 °C, to separate the solution into aqueous (RNA-containing) and organic phases. A minimum of 400 µL of the aqueous layer was removed and added to 500 µL of 2-propanol. The solution was then centrifuged at 12,000 *g* for 10 min at 4 °C. The RNA precipitated and formed a pellet on the bottom of the microcentrifuge tube. The flow-through was discarded. The RNA pellet was then washed with 1 mL of 70% ethanol, and the solution was centrifuged at 7,500 *g* for 5 min at 4 °C. This step was repeated, and the supernatant was discarded. The messenger RNA (**mRNA**) pellet was dried for 5 to 10 min at room temperature, and 25 µL of diethylpyrocarbonate (**DEPC**)-treated water was added to the RNA to resuspend it. The mRNA was stored at −80 °C.

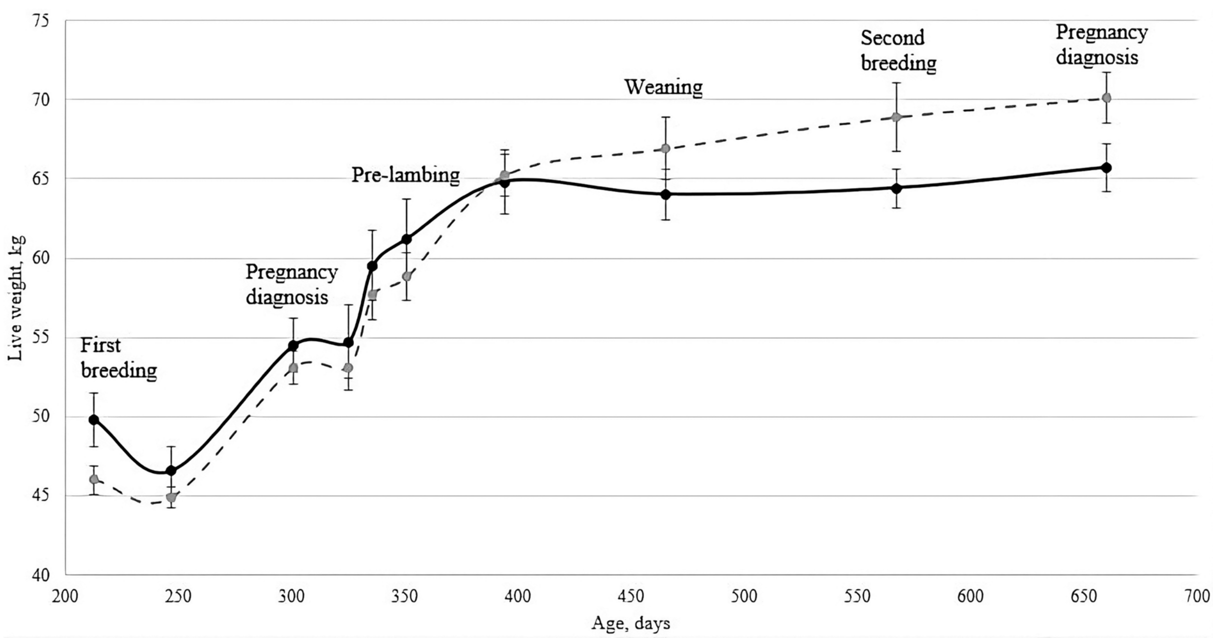

Figure 1. Live weight (±SEM) of the subsets of ewes from the control (n = 10; dotted line) and heavy (n = 10; solid line) treatment groups between their first breeding at seven months of age (213 d of age) and pregnancy diagnosis at two years of age (660 d of age).

mRNA quality and quantification

To assess the purity of each sample, the ratio of the absorbance of an RNA sample at 260 nm and at 280 nm (A_{260}/A_{280}) was measured with the NanoDrop Spectrophotometer (Thermo Scientific, MA, USA). An A_{260}/A_{280} ratio of 2.0 was considered as an indicator of pure RNA, and a minimum of 1.9 was required to use the NanoString technology. The RNA quality score (**RQS**) was also measured with a fragment analysis. Two of the samples collected in lactation (control n = 1 and heavy n = 1) had very low quantities of RNA per microliter and were not included in the gene expression analysis.

NanoString gene expression analysis

The analysis of mRNA expression was performed using the nCounter Analysis System (NanoString Technologies Inc., Seattle, WA). The NanoString nCounter system was selected for its sensitivity and reliability in quantifying gene expression from small mammary gland biopsies. This platform provides a direct digital readout of mRNA levels without amplification, minimizing technical variability (Veldman-Jones et al., 2015). Rigorous quality control ensured reliable gene expression analysis across treatment groups and time points, even with low RNA yield. NanoString's precision and adaptability make it ideal for studies with limited samples or challenging experimental conditions.

The mRNA samples prepared from tissues were analyzed using a ProbeSet consisting of probes specific for 86 genes from *Ovis Aries* (Oar_v3.1 [GCA_000298735.1]). This study specifically targeted 37 genes that were selected from the literature based on their function in mammary cell activity (i.e. proliferation, differentiation, survival, and apoptosis; Hennighausen and Robinson, 2005; Gallardo et al., 2019; Petridis and Fthenakis, 2019) and in mammary lipid metabolism (Bionaz and Loor, 2008; Mu et al., 2021; 2022). To run complete plates, additional sheep samples and genes were included. Additionally, nine reference genes were included based on published recommendations (Paten et al., 2014; Szczęsna et al., 2015; Gallardo et al., 2019). A complete list of genes and probe sequences is provided in the supplementary file "SAESheep_C3183 Summary.xlsx." Furthermore, mRNA was hybridized with the ProbeSets according to the manufacturer's instructions (nCounter Elements XT Reagents User Manual; NanoString MAN-10086-01 June 2018). Briefly, samples were hybridized by adding 8 μL of MasterMix and 7 μL of mRNA to each tube of a 12-tube strip immediately before placing the strip at 67 °C for 22 h. After hybridization, samples were transferred to the nCounter Prep Station, which automatically removed excess probe and aligned and immobilized the probe-target complexes in the nCounter cartridge. Sample cartridges were placed in the nCounter Digital Analyzer, which counted and tabulated color codes on the surface of the cartridge for each target molecule. Data were retrieved from the Analyzer as raw data (Reporter Code Count [**RCC**]) files.

For analysis, RCC files were imported into nSolver Analysis Software v4.0 (https://www.nanostring.com/products/analysis-software/nsolver) and underwent the software's sample quality control routine set to the following criteria: 1) imaging: fields of view registration <75%; 2) binding density outside the 0.05 to 2.25 range; 3) positive control linearity: positive control R2 value <0.95; and 4) positive control limit of detection: 0.5 fM positive control ≤2 SDs above the mean of the negative controls. All samples used for statistical analysis passed the quality control routine. Background subtraction was performed by subtracting the geometric mean of eight internal negative controls from each sample. Positive control normalization was performed using the geometric mean of six internal positive controls to compute the normalization factor. The normalization factor of all samples was inside the 0.15 to 15 range.

Reference gene normalization was performed using the geometric mean of counts for the nine reference genes included in the ProbeSet. The average of these geometric means across all lanes was used as the reference against which each lane is normalized. A normalization factor was then calculated for each of the lanes based on the geometric mean of counts for the reference genes in each lane relative to the average geometric mean of counts for the reference genes across all lanes. This normalization factor was then used to adjust the counts for each gene target and control in the associated lane. The normalization factor of all samples was inside the 0.1 to 30 range.

Statistical analysis

All statistical analyses were conducted using SAS v9.4 (SAS Institute Inc., Cary, NC, USA). All possible models were examined with statistical significance of terms and interactions thereof accepted at $P < 0.05$, and tendencies were considered at $P < 0.10$. Live weights and live weight change of ewes between P128 and L30 and lambs at each time point (P128 and L30; birth and L30, respectively) were analyzed using the general linear model (**GLM** procedure) with treatment (control vs. heavy) fitted as a fixed effect. Ewe BCS for each time point (P128 vs. L30) and BCS change between P128 and L30 were analyzed with generalized linear models using Poisson distributions and natural log transformations and fitted treatment as a fixed effect. Normalized gene counts were analyzed with linear mixed models (Proc MIXED procedure) with treatment, time point, rearing rank to weaning (single vs. twins), and the two-way interaction between treatment and time point fitted as fixed effects, and ewe fitted as a random effect. The three-way interaction between treatment, time point, and rearing rank was tested within the model and was removed from the models, as it was not significant ($P > 0.05$).

Results

Live weight and BCS of ewes at P128 did not differ between treatments ($P > 0.05$), but ewes in the control group tended to be heavier ($P < 0.10$) than ewes in the heavy group at L30 (Table 1). Ewe live weight and BCS change between P128 and L30 did not differ between treatments ($P > 0.05$; Table 1). Live weight of lambs and growth between birth and L30 did not differ between treatments ($P > 0.05$; Table 1).

Of the 37 genes included in this study, 14 presented low expression levels with a gene count lower than 150 copies at each time point (Table 2). The level of expression of ATP binding cassette subfamily G member 2 (***ABCG2***), BCL2 like 1 (***BCLX***), and peroxisome proliferator-activated receptor-alpha (***PPARA***) showed an interaction between treatment, time point, and rearing rank ($P < 0.05$, meaning presented in Supplementary Appendix A). *ABCG2* was overexpressed ($P < 0.05$) in heavy ewes rearing a single lamb and control ewes rearing twins at L30 compared to heavy ewes rearing twins at L30. Heavy

Table 1. Effect of treatment (control vs. heavy) on body condition score (BCS) and live weight of two-year-old ewes in late pregnancy (P128) and early lactation (L30), ewe live weight and BCS change between late pregnancy and early lactation, and live weight of their lambs at birth and early lactation and lamb growth rate between birth and early lactation (LSM ± SEM)

Parameters	Time points	*n*	Control	*n*	Heavy	*P*-values
Ewe live weight (kg)	P128	10	79.0 ± 1.70	9	76.4 ± 1.84	0.317
	L30	9	70.2 ± 1.14	9	66.0 ± 1.89	0.074
Ewe live weight change (kg)	P128 to L30	9	8.3 ± 1.45	9	10.4 ± 1.47	0.328
Ewe BCS	P128	10	3.10 ± 0.56	9	3.17 ± 0.59	0.935
	L30	9	2.61 ± 0.54	9	2.44 ± 0.52	0.824
Ewe BCS change	P128 to L30	9	0.56 ± 0.27	9	0.72 ± 0.28	0.682
Lamb live weight (kg)	Birth	19	7.07 ± 0.24	18	6.99 ± 0.18	0.795
	L30	13	13.0 ± 0.57	17	12.7 ± 0.61	0.700
Lamb growth rate (g/d)	Birth to L30	13	241 ± 15.0	17	223 ± 28.2	0.623

ewes at L30 had greater expression ($P < 0.05$) than ewes at P128, where the treatments did not differ ($P > 0.05$) from each other (Appendix A). *BCLX* was overexpressed ($P < 0.05$) in heavy ewes rearing a single lamb and control ewes rearing twins at L30 compared to control ewes rearing a single lamb and heavy ewes rearing twins at L30 and ewes at P128 (Appendix A). *PPARA* was overexpressed ($P < 0.05$) in control ewes rearing a single lamb at L30 compared to heavy ewes rearing twins at L30, which in turn was overexpressed ($P < 0.05$) compared to all ewe groups at P128.

The normalized count of genes and the differences between rearing rank, treatment, time point, and their two-way interactions are presented in Table 2. Erb-b2 receptor tyrosine kinase 4 (***ERBB4***) had a greater level of expression in control ewes at L30 than heavy ewes at L30 and control ewes at P128 ($P < 0.05$) but did not differ from heavy ewes at P128 ($P > 0.05$). There was a tendency for an interaction between treatment and time point ($P < 0.10$) in the expression of acyl-CoA synthetase short-chain family member 1 (***ACSS1***) and *PPARA*. The expression of *ACSS1* was greater ($P < 0.05$) in control ewes at L30 than in heavy ewes at L30 and was greater ($P < 0.05$) than that of control and heavy ewes at P128. The expression of *PPARA* was greater ($P < 0.05$) at L30 for control and heavy ewes, which did not differ ($P > 0.05$), than at P128 for control and heavy, which did not differ ($P > 0.05$).

Heavy ewes tended to have a lower ($P < 0.05$) level of expression of *ACSS1* than control ewes, irrespective of time point. Although the expression of signal transducer and activator of transcription 5A (***STAT5A***) did not differ between treatments ($P = 0.117$; Table 2), a trend toward lower expression in the heavy group was observed. Rearing rank showed a tendency ($P < 0.10$) for a difference in the expression of BCL2 associated X, apoptosis regulator ***(BAX)***, and solute carrier family 25, mitochondrial carrier (***SLC25A5***). Both genes tended to be lower for ewes that reared a single lamb to weaning than ewes that reared twins, irrespective of treatment and time point. *BAX*, BCL2 apoptosis regulator (***BCL2***), glyceraldehyde 3-phosphate dehydrogenase (***GAPDH***), insulin-like growth factor 1 (**IGF1**), insulin-like growth factor binding protein 3 (***IGFBP3***), leptin receptor (***LEPR***), prolactin receptor (***PRLR***), *SLC25A5*, and transforming growth factor beta 1 (***TGFB1***) were overexpressed at P128 compared to L30 ($P < 0.05$). ABCG2, acyl-CoA synthetase long-chain family member 1 (***ACSL1***), *ACSS1*, acyl-CoA synthetase short-chain family member 2 (***ACSS2***), β-hydroxybutyrate dehydrogenase (***BDH1***), cyclin D1 (***CCND1***), cytokine inducible SH2 containing CIS 1 suppressor of cytokine signaling SOCS1 (***CISH***), E74 like ETS transcription factor 5 (***ELF5***), no official name (CCAAT enhancer binding protein beta) ***(ENSOARG13395 (CEBP))***, Janus kinase 2 (***JAK2***), lactalbumin alpha (***LALBA***), leptin (***LEP***), *PPARA*, peroxisome proliferator activated receptor delta (***PPARD***), prolactin (***PRL***), monocarboxylic acid transporter 1 (***SLC16A1/MCT1***), and solute carrier family 25, mitochondrial carriers; adenine nucleotide translocator, member 4 (***SLC25A4***), suppressor of cytokine signaling 2 (***SOCS2***), suppressor of cytokine signaling 3 (***SOCS3***), transforming growth factor beta receptor 1 (***TGFBR1***), transforming growth factor beta receptor 2 (***TGFBR2***) were overexpressed at L30 compared to P128 ($P < 0.05$). However, ERBB4, insulin-like growth factor-binding protein 5 (***IGFBP5***), insulin-like growth factor 1 receptor (***IGF1R***), lactotransferrin (***LTF***), peroxisome proliferator activated receptor gamma (***PPARG***), and *STAT5A* did not differ between time points ($P > 0.05$).

Discussion

The expression of specific genes in the mammary gland of two-year-old ewes during their second lactation was affected by treatment and time point. During lactation (L30), *ACSS1* tended to be underexpressed in the mammary gland of ewes in the heavy group compared to the control group. Although ewes were managed under similar grazing conditions and were not feed-restricted during lambing and lactation, with pasture covers averaging 2209 ± 167 kg DM/ha during pregnancy and 1592 ± 63 kg DM/ha during lactation (Haslin et al., 2022b). However, individual intake was not measured, and the heavy group tended to be lighter in lactation than the control group. While live weight and BCS change did not differ between treatments, the lower live weight of heavy ewes in lactation may reflect a lower nutrient intake or energy status. These factors could contribute to the observed trend in *ACSS1* expression, as mitochondrial acetyl-CoA synthesis from acetate is linked to energy demand and availability during lactation.

It is reported that *ACSS1* is expressed in mitochondria and encodes an enzyme that synthesizes acetyl-CoA from acetate (Sivanand et al., 2018). Mitochondrial acetyl-CoA subsequently condenses with oxaloacetate to form citrate (Sivanand et al., 2018; Arnold and Finley, 2023). Citrate can either be oxidized in the tricarboxylic acid (**TCA**) cycle to generate a significant quantity of adenosine triphosphate (**ATP**) through oxidative phosphorylation or be exported to the cytosol for

Table 2. Effect of treatment (control vs. heavy), time point (late pregnancy [P128] vs. lactation [L30]) and rearing rank to weaning (rr; single vs. twin lambs, noting *P*-values shown but values not shown) and the two-way interaction between treatment and time point (inter)[1] on the normalized count of genes (LSM ± SEM) in the mammary gland of two-year-old ewes

Gene	Late pregnancy (P128)		Lactation (L30)		P-values$_{treatment}$	P-values$_{time}$	P-values$_{rr}$	P-values$_{inter}$[1]
	Control	Heavy	Control	Heavy				
ABCG2	739.6 ± 393	716.9 ± 454	5222 ± 435	4337 ± 473	0.329	<0.001	0.887	0.321
ACSL1	252.2 ± 119	222.5 ± 138	1833 ± 132	1546 ± 144	0.264	<0.001	0.611	0.328
ACSS1	219.3 ± 65.3[c]	186.3 ± 75.5[c]	1221 ± 72.4[a]	946.6 ± 78.8[b]	0.058	<0.001	0.294	0.105
ACSS2	1351 ± 152	1182 ± 175	1807 ± 168	1610 ± 183	0.309	0.016	0.678	0.933
BAX	139.2 ± 9.93	127.4 ± 11.5	93.1 ± 11.0	92.8 ± 12.0	0.599	0.002	0.076	0.596
BDH1	78.3 ± 108	258.3 ± 127	875.6 ± 118	847.1 ± 132	0.591	<0.001	0.978	0.304
BCL2	28.5 ± 2.05	27.7 ± 2.37	13.1 ± 2.27	15.6 ± 2.47	0.713	<0.001	0.282	0.458
BCLX	122.1 ± 8.81	130.3 ± 10.2	171.5 ± 9.77	150.4 ± 10.6	0.533	0.002	0.546	0.142
CCND1	2.40 ± 0.87	2.42 ± 1.01	4.38 ± 0.97	6.02 ± 1.06	0.424	0.010	0.574	0.402
CISH (SOCS1)	36.6 ± 9.07	33.5 ± 10.5	100.2 ± 10.1	87.1 ± 10.9	0.445	<0.001	0.736	0.613
ELF5	290.5 ± 44.2	266.0 ± 51.1	462.3 ± 49.0	383.6 ± 53.3	0.325	0.008	0.393	0.574
ENSOARG13395 (CEBPB)	853.0 ± 88.1	714.3 ± 102	1089 ± 97.6	905.2 ± 107	0.145	0.033	0.300	0.805
ERBB4	49.8 ± 6.96[b]	58.4 ± 8.04[ab]	70.9 ± 7.72[a]	49.6 ± 8.40[b]	0.439	0.419	0.927	0.063
GAPDH	761.2 ± 51.3	791.3 ± 60.1	656.5 ± 56.6	668.3 ± 62.5	0.751	0.038	0.742	0.863
IGF1	138.4 ± 15.3	109.5 ± 17.7	26.8 ± 17.0	34.9 ± 18.5	0.562	<0.001	0.746	0.275
IGF1R	1.82 ± 1.71	0.80 ± 1.99	2.05 ± 1.90	5.29 ± 2.07	0.586	0.210	0.435	0.256
IGFBP3	562.4 ± 60.1	536.1 ± 69.5	149.5 ± 66.7	224.1 ± 72.5	0.731	<0.001	0.204	0.445
IGFBP5	380.3 ± 75.1	435.0 ± 86.7	209.0 ± 83.3	303.4 ± 90.6	0.400	0.079	0.729	0.807
JAK2	103.6 ± 9.21	112.4 ± 10.7	161.7 ± 10.2	141.7 ± 11.2	0.617	<0.001	0.735	0.148
LALBA	7177 ± 19352	6033 ± 22358	156450 ± 21463	177420 ± 23339	0.661	<0.001	0.574	0.601
LEP	2.26 ± 0.67	1.67 ± 0.76	3.47 ± 0.74	3.89 ± 0.82	0.926	0.013	0.745	0.420
LEPR	59.5 ± 4.88	53.4 ± 5.64	18.5 ± 5.41	23.1 ± 5.89	0.898	<0.001	0.518	0.327
LTF	134.3 ± 487	101.6 ± 568	37.3 ± 539	1589 ± 592	0.217	0.175	0.758	0.125
PRL	1.61 ± 0.36	1.74 ± 0.42	2.15 ± 0.40	3.36 ± 0.44	0.132	0.013	0.188	0.178
PRLR	1698 ± 135	1508 ± 156	1175 ± 149	1009 ± 163	0.271	0.003	0.695	0.932
PPARA	83.0 ± 9.90[a]	92.6 ± 11.4[a]	180.2 ± 11.0[b]	151.6 ± 11.9[b]	0.419	<0.001	0.883	0.092
PPARD	78.8 ± 20.2	93.3 ± 23.4	123.9 ± 22.4	142.5 ± 24.4	0.492	0.043	0.634	0.924
PPARG	99.8 ± 35.3	149.6 ± 40.8	134.0 ± 39.2	161.4 ± 42.6	0.356	0.551	0.543	0.769
SLC16A1/MCT1	2069 ± 305	2462 ± 353	3672 ± 339	4285 ± 368	0.172	<0.001	0.459	0.742
SLC25A4	224.5 ± 90.9	271.3 ± 105	1207 ± 101	1035 ± 110	0.557	<0.001	0.423	0.278
SLC25A5	937.6 ± 48.6	823.7 ± 56.8	547.4 ± 53.7	492.6 ± 59.2	0.178	<0.001	0.104	0.544
SOCS2	213.7 ± 20.1	177.9 ± 23.3	269.4 ± 22.3	253.8 ± 24.3	0.284	0.009	0.714	0.647
SOCS3	20.6 ± 11.5	17.5 ± 13.3	39.5 ± 12.8	63.7 ± 13.9	0.443	0.018	0.459	0.273
STAT5A	1346 ± 121	1164 ± 140	1403 ± 134	1114 ± 146	0.117	0.980	0.496	0.675
TGFB1	88.2 ± 7.71	82.9 ± 8.93	53.1 ± 8.55	62.1 ± 9.31	0.838	0.004	0.659	0.395
TGFBR1	149.9 ± 19.6	151.2 ± 22.7	314.9 ± 21.7	296.3 ± 23.6	0.709	<0.001	0.655	0.635
TGFBR2	279.8 ± 26.5	238.5 ± 30.6	337.1 ± 29.4	313.9 ± 31.9	0.305	0.034	0.249	0.753

[1] *P*-values of the two-way interaction between treatment (control and heavy) and time point (late pregnancy [P128] and lactation [L30]).
[a,b,c]Within rows, least square means with different superscripts are different ($P < 0.05$).
Control: Ewe lambs grown to 44 kg at ewe lamb breeding; heavy: ewe lambs grown to 48 kg at ewe lamb breeding.

use in de novo milk fatty acid synthesis (Sivanand et al., 2018; Arnold and Finley, 2023). Fujino et al. (2001) reported that bovine ACSS1 synthesized four times more acetyl-CoA for oxidation and ATP production than for lipid synthesis. In dairy cattle, *ACSS1* was upregulated between day 1 and 120 of lactation compared to pregnancy (Bionaz and Loor, 2008), which is consistent with the present results. The demand for energy for milk production increases significantly after parturition and could lead to an overexpression of *ACSS1* during lactation. Furthermore, in lactating mice, *ACSS1* was overexpressed in inflamed mammary glands, reflecting an increased energy requirement to support the activation of the inflammation pathways (Ivanova et al., 2023). In the present study, the two-year-old ewes in the heavy group may have generated less acetyl-CoA from acetate in their mammary mitochondria compared to control ewes. This indicates either a reduced energy production to support lactation through mitochondrial acetate activation or a possible greater incidence of mammary gland inflammations in control ewes.

Although the expression of *STAT5A* did not differ between treatments, there was a trend toward a lower expression in the heavy group. In this regard, *STAT5A*, a key element of the JAK2/STAT5 pathway, plays a key role in mammary epithelial cell (**MEC**) proliferation, differentiation, and survival in pregnancy and lactation (Hennighausen and Robinson, 2005; Tian et al., 2020). Prolactin binds to its receptor PRLR, activating the kinase *JAK2*, which, in turn, phosphorylates *STAT5*. Activated *STAT5* translocates to the nucleus, where it binds to promoter sequences of target genes to increase their expression (Guo et al., 2023; Hannan et al., 2023). The expression of *STAT5A* target genes (*BCLX*, *ELF5*, *LALBA*, and *SOCS3*) or known modulators (*ERBB4*, *PRL*, *PRLR*, *SOCS1*, and *SOCS2*), selected for this study, did not differ between treatments. This suggested that mammary cell development and structure during late pregnancy and lactation were not impacted by increased growth rates between three and seven months of age.

Activated *STAT5A* binds to a promoter of the acetyl-CoA carboxylase alpha gene (*ACACA*), coding for the enzyme acetyl-CoA carboxylase 1 (ACC1) in the bovine mammary gland (Mao et al., 2002). Furthermore, the ACC1 enzyme carboxylates acetyl-CoA to form malonyl-CoA, which is used to synthesize medium-chain fatty acids in de novo fatty acid synthesis (Tian et al., 2022; Wang et al., 2022). The upregulation of *ACACA* by *STAT5A* stimulates the production of fatty acids (Tian et al., 2020), potentially increasing the demand for acetyl-CoA, synthesized from acetate by ACSS1 and ACSS2 enzymes (Bionaz and Loor, 2008). This demand could either indirectly increase citrate export for fatty acid synthesis, consequently increasing acetyl-CoA synthesis by *ACSS1*, or directly increase the synthesis of acetyl-CoA by *ACSS2*. In this study, the expression of *ACSS2* did not differ between treatments, while *ACSS1* expression tended to be lower in the heavy group, potentially indicating a lower demand in de novo fatty acid synthesis leading to fewer medium-chain fatty acids and a reduction of the milk fat content. Further investigations would be required to clarify the potential carry-over effects of increased growth rates between three and seven months of age on fatty acid synthesis in milk in nondairy ewes.

Of the 37 genes investigated in this study, 32 were differentially expressed between late pregnancy and lactation. However, 10 genes were overexpressed in late pregnancy and were mainly associated with regulation of the cell cycle and survival, including anti- and pro-apoptotic factors (*BAX*, *BCL2*, and *IGFBP5*), stromal cell proliferator and fibroblast activation (*TGFB1*), leptin and prolactin receptors (*LEPR*, *PRLR*), and cell survival factor (*IGF1*). These results contradictprevious studies reporting that *IGF1* was overexpressed in lactation (Gallardo et al., 2019, Abousoliman et al., 2020, Noorgard et al., 2008). In pregnancy, the preparation for lactation is characterized by significant cell proliferation and remodeling to expand the lobulo-alveolar network and secretory cells (Hennighausen and Robinson, 2005; Inman et al., 2015). In contrast, 22 genes were overexpressed in early lactation and were mainly associated with milk fat metabolism (*ACSL1*, *ACSS1*, *ACSS2*, *PPARA*, *PPARD*) and cell remodeling and survival (*ABCG2, BCLX*, *CCND1*, *ELF5*, *SOCS1, SOCS2*, *SOCS3*). These results were consistent with previous studies reporting the overexpression of *ABCG2* (Zhou et al., 2023), *ELF5* (Paten et al., 2015), *LALBA* (Gallardo et al., 2019), *SLC16* and *SLC25* (Patel et al., 2019), *SOCS3* (Szczesna et al., 2020), and genes involved in fatty acid biosynthesis (ASCL1, ACSS2; Paten et al., 2015) in lactation. While the mammary gland undertakes limited cell growth during lactation, activity of MECs increases to synthesize milk protein and fat, along with cell turnover to maintain milk production (Hennighausen and Robinson, 2005; Lérias et al., 2014).

With the exception of *ACSS1*, no other genes were differentially expressed between treatments. This result indicates that the increased growth rate of ewes did not have lasting effects on mammary gene expression or function by the second lactation. These findings are consistent with the lack of phenotypic differences in lamb growth between treatments and previous studies using mammary ultrasound (Haslin et al., 2021a; Haslin et al., 2021b). The magnitude of the ewe growth rate with 150 g/d or less was lower than those achieved in previous studies that reported differences in milk production (Umberger et al., 1985; Villeneuve et al., 2010a). Moreover, the difference of 26 g/d between groups was relatively small, limiting the potential changes in the mammary gene expression. The small sample size and the number of genes analyzed may have limited the ability to detect biologically relevant differences in gene expression. In addition, 38% of the genes had low expression levels, with less than 150 copies (Veldman-Jones et al., 2015). The study of mRNA provides information on the cell activity at the time of sample collection. Hence, the time of biopsy may not have aligned with the expression pattern of the selected genes. Although the sample collection aimed to target mammary parenchyma, which undergoes extensive cell remodeling to support lactation (Lérias et al., 2014), some samples may have included stromal tissue, especially during late pregnancy. Stromal tissue contributes to cell development and lactation preparation (Nagy et al., 2021), but its functions differ from the parenchyma and result in the expression of different genes (Avril-Sassen et al., 2009).

The observed trend in gene expression between the heavy and control groups suggests possible carry-over effects from the increased growth rate during the allometric growth phase, a critical period for tissue development (Johnsson and Hart, 1985; Berryhill et al., 2017; Geiger, 2019). Epigenetic mechanisms, such as noncoding RNA and DNA methylation, are more commonly activated during the developmental stages and can lead to lasting changes in gene expression (Ivanova et al., 2021). Noncoding RNAs are transcribed from genes but do not undergo translation into proteins, instead playing a regulatory role in gene expression (Ivanova et al., 2021). DNA methylation involves the addition of a methyl group to a cytosine nucleotide and alters the expression of associated genes (Ivanova et al., 2021). Although gene expression trends were observed in this study, no corresponding phenotypic evidence was detected in lamb growth rate and live weight. These gene expression differences may represent subtle modifications in mammary tissue that do not translate into differences in milk yield or quality sufficient to impact lamb performance. To understand the long-term effects of increased growth rates between three and seven months of age on ewe mammary function and milk composition, further investigations should include larger sample sizes, functional assessments of lactation, and comprehensive transcriptomic analyses.

In summary, two-year-old ewes that had experienced an increased growth rate between three and seven months of age tended to exhibit lower expression of *ACSS1* during their second lactation than ewes that did not experience an increased growth rate. *ACSS1* is associated with milk lipid metabolism and energy production in mammary epithelial cells, indicating a possible downregulation of both metabolic pathways. This reduction suggests potential carry-over effects of increased growth rate between three and seven months of age. However, no corresponding phenotypic differences were observed in lamb growth or live weight, indicating that these molecular changes may not have translated into performance outcomes. As this study focused on a limited number of genes, possible additional effects could, therefore, be present in the expression of other genes directly impacting milk yield or quality. Further investigations, including a larger sample size, a functional assessment of lactation, and use of comprehensive transcriptomic analyses, would be needed to understand the effects of increased growth rates between three and seven months of age on ewe mammary function and milk composition.

Acknowledgments

The authors wish to acknowledge Sam Peterson, Dean Burnham, Catriona Jenkinson, Geoff Purchas, Emma J. Pettigrew, and Keeble farm staff for their technical assistance and help with data collection, and Neville Haack for his technical assistance in sample and RNA processing. We also wish to acknowledge Beef + Lamb New Zealand for funding this study.

Author Contributions

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Supplementary Data

Supplementary data are available at *Journal of Animal Science* online.

Conflict of interest statement. The authors declare no real or perceived conflicts of interest.

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