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Milk yield and genomewide expression profiling in the mammary gland of beef primiparous cows in response to the dietary management during the pre- and postweaning periods[1]

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ABSTRACT: Accelerated growth programs during prepubertal periods have been promoted to advance the first calving of beef heifers. The objectives of the present study were to evaluate nutrition-induced changes on first lactation milk yield and composition and on gene expression of the mammary gland in Parda de Montaña primiparous cows. Female calves (n = 16) were involved in a 2 × 2 factorial experiment. In the preweaning period (PRE-W; 0–6 mo), female calves were either fed a creep feed supplement (Creep) or fed only their dam's milk (Control). In the postweaning period (POST-W; 6–15 mo), heifers received either a high-energy diet (91.7 MJ/d) or a moderate-energy diet (79.3 MJ/d). All the heifers were managed together from breeding (15 mo) to the end of their first lactation (32 mo). Animal performance; milk production and quantity during the first lactation; plasma glucose, IGF-I, and leptin concentrations; and RNA samples from the mammary gland at the end of the first lactation of the primiparous cows (32 mo) were analyzed. The BW and ADG of the primiparous cow during its first lactation were not different among treatments; however, creep feeding during PRE-W reduced milk production ($P < 0.01$), milk CP, crude fat, lactose, nonfat solids, and casein content throughout lactation and increased somatic cell count in the third ($P < 0.05$) and fourth month of lactation ($P < 0.10$). The energy level during the POST-W had no effect on milk production and quality. Gene expression in the mammary gland was affected by the diet in the PRE-W and POST-W, with the PRE-W diet having the greatest impact. During the PRE-W, creep feeding resulted in upregulation of genes related to immune response and chemokine activity, suggesting that these animals might be in a compromised immune status. Therefore, this strategy would not be recommendable; meanwhile, increasing the energy level in the diet during the POST-W would be recommendable, because it had no deleterious effects on milk yield and composition.

Key words: cattle, creep feeding, dietary treatment, mammary gland, microarray, milk production

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INTRODUCTION

Raising heifers on high energy planes of nutrition during the prepubertal period has been proposed to lower the age at first calving, reducing the "unproductive" period of the animals and thus reducing the environmental impact. However, high energy planes of nutrition during the prepubertal period have been shown to have a negative effect on milk yield

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(Radcliff et al., 2000) and may compromise mammary development and decrease the potential of subsequent milk yield and the health and immune status of the animal (Drackley and Bartlett, 2001; Lohakare et al., 2012). The effects might be different depending on the prepubertal period in which the growth is promoted. In beef heifers, creep feeding had no effect or reduced milk production with changes in the mammary gland composition whereas a moderate or medium energy level during the postweaning period had an effect on the mammary gland composition and milk production in one year but not another year (Buskirk et al., 1996). In addition, the feeding level causing a reduction in milk yield potential is different between breeds (Sejrsen et al., 2000).

Although nutritional science has embraced the tools of genomics, few attempts at large-scale or global evaluation (studying the genomewide expression profile using microarray platforms or next generation sequencing [**NGS**] as RNA sequencing [**RNA-seq**]) of nutritional gene regulation in the mammary gland have been performed in cattle (Finucane et al., 2008; Bionaz et al., 2012; Cui et al., 2014). These analyses offer a great opportunity to understand nutritional gene regulation in the mammary gland. An understanding of impacts of nutrition during the first months of calves' life is crucial to design feeding strategies that optimize lifetime productivity. Therefore, the objectives of the present study were to evaluate early nutrition-induced changes in first-lactation milk yield and composition and in gene expression of the mammary gland in Parda de Montaña primiparous cows.

MATERIAL AND METHODS

All experimental procedures including care of animals and anesthesia were performed in accordance with the guidelines of the European Union regulations for the use and care of animals in research (Directive 2010/63/EU; European Union, 2010) .and approved by the Animal Ethics Committee of the Centro de Investigación y Tecnología agroalimentaria de Aragón (protocol 2010/07).

Animal, Diets, and Sample Collection

The present study was conducted at the Centro de Investigación y Tecnología Agroalimentaria de Aragón – La Garcipollera Research Station in the mountain area of the central Pyrenees (northeast Spain; 42°37′ N, 0°30′ W). Animals were selected to be as unrelated as possible based on pedigree records. Sixteen Parda de Montaña (a breed selected from old Brown Swiss for beef production) female calves were born in the autumn (October 2010) from 16 single-calving cows sired by 5 unrelated bulls. The female calves were randomly but equally assigned at birth to 1 of 4 management strategies, in a 2 × 2 factorial experiment, with 2 feeding treatments in the preweaning period (**PRE-W**) and 2 feeding treatments in the postweaning period (**POST-W**). In the PRE-W (0–6 mo of age), all female calves suckled their dams twice daily for 30 min. Half of the calves (n = 8) were fed a creep feed supplement (**Creep**; composed, on an as-fed basis, of 30% corn, 16.5% soybean flour, 15.5% barley, 15% extruded cereal, 15% wheat bran, 3% milk byproducts, 2% beet pulp, 1.3% palm oil, 1% calcium carbonate, 0.2% vitamin–mineral premix, and 0.5% sodium chloride; 894 g DM/kg, 166 g CP/kg DM, and 214 g NDF/kg DM) whereas the other half were fed only their dam's milk (**Control**). In the POST-W (6–15 mo), the heifers received alfalfa hay (851 g DM/kg, 92 g CP/kg DM, and 462 g NDF/kg DM) plus concentrates (900 g DM/kg, 147 g CP/kg DM, and 252 g NDF/kg DM) in different proportions to have a different energy intake. Half of the heifers of each PRE-W treatment were assigned to each POST-W treatment and had either a high-energy diet (**High**; 91.7 MJ/d, achieved with an average daily intake of 6.8 kg hay and 3.4 kg concentrate) or a moderate-energy diet (**Moderate**; 79.3 MJ/d, achieved with an average daily intake of 8.4 kg hay and 1.3 kg concentrate). Thereafter, all the heifers were identically managed throughout the rest of the study. At 15 mo, heifers were synchronized and bred by AI. Until calving (27 ± 0.2 mo), the heifers continuously grazed on mountain meadows (848 g DM/kg, 145 g CP/kg DM, and 293 g NDF/kg DM), at a stocking rate of 4 heifers/ha. In the last month of gestation, the heifers were housed and fed daily 9 kg per heifer of meadow hay (922 g DM/kg, 82 g CP/kg DM, and 632 g NDF/kg DM). During their first lactation (4 mo), each primiparous cow received daily 10 kg of a total mixed ration (56% forages and 44% grains, byproducts, vitamin, and mineral supplements; 861 g/kg DM, 13.0 MJ ME/kg DM, 85 g CP/kg DM, and 499 g NDF/kg DM), calculated to meet its maintenance, growth, and milk production requirements for energy and protein. The cows were group fed but they were tied up for a maximum of 2 h so that each one could eat its own portion without disturbance of other animals in the same pen and leave no refusals. The calves had access to only their dam's milk until weaning on Day 129 (±5) of lactation. The evolution of BW and ADG of the heifers until calving is detailed in Supplementary Fig. S1 (see the online version of the article at http://journalofanimalscience.org).

Measurements and Blood Sampling

The BW of the primiparous cows and their calves was recorded once a week throughout the first lactation, and their ADG was calculated using linear regression of BW against time. The cows were milked monthly during 4 mo of lactation using the oxytocin and machine milking technique (Le Du et al., 1979) to determine milk quantity and composition. Milk fat and protein content were analyzed using an infrared scan (MilkoScan 4000TM; Foss Electric Ltd., Hillerød, Denmark). Somatic cell count (**SCC**) was determined using the flow cytometry method with an automated somatic cell counter (Fossomatic 5000; Foss Electric Ltd.). At the end of the first lactation, the primiparous cows were bled from the coccygeal vein at 0800 h, before feeding. Blood samples were collected into tubes that contained EDTA (Vacuette España S.A., Madrid, Spain) to determine plasma glucose concentrations and into heparinized tubes (Vacuette España S.A.) to determine IGF-I and leptin concentrations. Immediately after collection, blood samples were centrifuged at 1,500 × g for 20 min at 4°C, and the plasma was harvested and stored at −20°C until analyses.

Plasma Glucose, IGF-I, and Leptin Concentration Analyses

Plasma concentrations of glucose (glucose oxidase/peroxidase method) were determined with an automatic analyzer (GernonStar; RAL/TRANSASIA, Dabhel, India). The reagents for the glucose analyses were provided by the manufacturer of the analyzer (RAL Técnica para el Laboratorio, S.A., Barcelona, Spain). The intra- and interassay CV were 1.5 and 0.9%, respectively. The sensitivity was 0.056 mmol/L. Circulating IGF-I concentrations were quantified with a solid-phase EIA assay (Immulite; Siemens Medical Solutions Diagnostics Limited, Llanberis, Gwynedd, UK). The mean intra- and interassay CV were 3.1 and 12.0%, respectively. The sensitivity of the technique was 20 ng/mL. Plasma leptin concentrations were determined using RIA with a multispecies commercial kit (Multispecies Leptin Ria kit; Linco Research, Inc., St. Charles, MO). The mean intra- and interassay CV were 3.5 and 6.9%, respectively. The sensitivity was 1.30 ng/mL.

Ribonucleic Acid Isolation, Assessment of RNA Integrity, and Microarray Hybridization and Data Processing

Mammary tissue samples were taken by biopsy at the end of first lactation (32 mo), after calves suckled in the morning (0800 h), using the technique described by Dervishi et al. (2012) modified for cattle. Cows were anesthetized with an intravenous injection of 50 mg xylazine (Xilagesic 20%; Calier, Barcelona, Spain) followed by a subcutaneous local injection of 2.5 mL lidocaine chlorhydrate (Xilocaina Ovejero 2%; Laboratorios Ovejero, León, Spain). The central part of the right rear quarter of the udder was shaved and cleaned with a 10% povidone–iodine solution (Lainco, S.A., Barcelona, Spain). An incision of approximately 1 cm was made approximately 2 cm above the nipple on the biopsy site with a scalpel through the skin and gland capsule. A human Tru-Cut biopsy needle (CareFusion France 309 SAS, Le Pont de Claix, France) with a 20-mm sample notch was used to harvest a sample of approximately 15 mg tissue, collected at a depth of 3 to 5 cm. Afterward, the mammary gland surface was sprayed with Aludemin (Chemical Ibérica, Salamanca, Spain) to prevent infection. Immediately after biopsy, the mammary tissue sample was stored in RNAlater solution (QIAGEN, Madrid, Spain) and then frozen and stored at −80°C. Total RNA was extracted using RNeasy Tissue mini kits (QIAGEN) following the manufacturer's protocol. Prior to microarray analysis, RNA integrity and quality were assessed using a RNA 6000 Nano LabChip on the Agilent 2100 Bioanalyzer (Agilent Technologies Hewlett-Packard, Waldbronn, Germany) and quantified using a nanophotometric spectrophotometer (Implen, Madrid, Spain). All RNA integrity number values were above 8.

Ribonucleic acid samples were analyzed using a Bovine Gene 1.1 ST Array Strip (Affymetrix UK Ltd., High Wycombe, UK). Microarray hybridization and scanning were performed at the Functional Genomics Core facility (Institute for Research in Biomedicine, Barcelona, Spain) following the recommendations of the manufacturer. Scanned images (DAT files) were transformed into intensities (CEL files) using Affymetrix GeneChip Operating Software (Affymetrix UK Ltd. ,High Wycombe, UK). Overall array intensity was normalized between arrays to correct for systematic bias in data and remove the impact of nonbiological influences on biological data. The imported data were analyzed at the gene level, with exons summarized to genes, using the mean expression of all the exons of a gene. The robust multiarray average algorithm using quantile normalization, median polish probe summarization, and $\log_2$ probe transformation was used for normalization. The data sets have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (**GEO**) repository (https://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE78173 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?Acc=GSE78173; accessed 22 Feb 2016).

Validation of Microarray Data by Quantitative PCR

To quantify mature microRNA (**miRNA**) expression, 1 μg of total RNA isolated of each sample was polyadenylated and reverse transcribed using the NCode miRNA first-strand synthesis kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For miRNA, the resulting cDNA was subjected to real-time quantitative real-time quantitative PCR using the NCode universal reverse primer (Invitrogen) in conjunction with a sequence-specific forward primer for each miRNA (forward primers for NCode miRNA detection are the exact sequence of the mature miRNA). For coding genes, 1 μg of RNA of each sample was treated with DNase (Invitrogen), and single-stranded cDNA was synthesized using the SuperScriptIII Reverse Transcriptase kit (Invitrogen), following the manufacturer's recommendations. Specific exon primers covering different exons for each gene were generated and confirmed for specificity using the basic local alignment search tool (BLAST; National Center for Biotechnology Information, U.S. National Library of Medicine,Bethesda, MD; https://blast.ncbi.nlm.nih.gov/Blast.cgi; accessed 10 Sept 2016). Quantitative reverse-transcriptase PCR was performed using SYBR Green Master Mix SYBR Premix Ex Taq II (Tli RNase H Plus; Takara, Sumalsa, Zaragoza, Spain) and a Bio-Rad CFX96 Touch Real-Time PCR detection system (Bio-Rad, Sumalsa, Zaragoza, Spain) in triplicate for all samples. The standard curve method was used to assess the performance of the quantitative PCR (**qPCR**) through 4-fold serial dilution of cDNA pooled from the mammary gland. Two samples were replicated in all plates to remove technical variation from this source of variability. Sequences of primers, amplicon lengths, the annealing temperatures, and primer concentrations for genes (*PTRG*, *ANKRD26*, *SLC25A3*, *CXCL11*, *PYCR1*, *KHDRBS*, *RPS9*, and *RPL32*) and miRNA (*Mir296*, *Mir1940*, *Mir143*, *Mir197*, *Mir200B*, *mir191*, and *Let7F*) are described in Supplementary Table S1 (see the online version of the article at http://journalofanimalscience.org). Reference genes (*RPS9* and *RPL32*) and miRNA (*mir191* and *Let7F*) were chosen because in previous studies, they have been shown to be the most stable (Bionaz and Loor, 2007; Li et al., 2014; Kinoshita et al., 2016).

Statistical Analysis

Performance of the Cow and Calf and Milk Yield and Composition. Statistical analyses were performed using the SAS statistical package version 9.3 (SAS Inst. Inc., Cary, NC). Models fitting different residual variance–covariance structures were tested to model the experimental error and the one with the lower Akaike information criterion and Bayesian information criterion was chosen. The mixed model or cow BW included PRE-W and POST-W feeding treatments, months of lactation, and their interactions as fixed effects, and the cow was included as a random effect. For milk production and composition, PRE-W and POST-W feeding treatments, milking day, and their interaction were included as fixed effects, the BW as a covariate, and the cow as a random effect. The equation of the model was the following:

$$y_{ijkl} = \mu + \tau_i + t_k + (\tau \times t)_{ik} + \rho_l + (\rho \times t)_{lk} + b(\mathrm{BW})_{ij} + a_{ij} + \varepsilon_{ijk},$$

in which y_{ijkl} is observation *ijk*, μ is the overall mean, τ_i = the effect of PRE-W treatment *i*, t_k is the effect of the month of lactation, $(\tau \times t)_{ik}$ is the effect of the interaction between PRE-W treatment *i* and the month of lactation, ρ_l is the effect of POST-W treatment *l*, $(\rho \times t)_{lk}$ is the effect of the interaction between POST-W treatment *l* and the month of lactation, *b* is the regression coefficient of BW on *y*, BW_{ij} = BW of the animal, a_{ij} is the random animal effect with mean 0 and variance σ^2_a, and ε_{ijk} is the random residual with mean 0 and variance $\sigma^2_i\sigma^2_j\rho^{|i-j|}$.

The cow's ADG and plasma glucose and hormone concentrations at the end of the first lactation and the calf's parameters were analyzed using PROC GLM, including PRE-W and POST-W feeding treatments and their interaction as fixed effects. Pairwise comparisons were tested and corrected using Bonferroni correction to take into account multiple tests. Treatment differences were considered significant when $P < 0.05$, and a trend was declared when $0.05 < P < 0.10$.

Differentially Expressed Genes in the Mammary Gland. Gene expression normalized data were analyzed using Babelomics (http://babelomics.bioinfo.cipf.es/; accessed 15 Jan 2016) and MetaboAnalyst software (http://www.metaboanalyst.ca/, accessed 15 Jan 2016; Xia et al., 2009), following the workflow described by González-Calvo et al. (2017). Briefly, genes were considered differentially expressed between treatments when the statistically significant value of the Limma test was $P < 0.01$. In addition, a significance analysis of microarray (**SAM**) was used to identify and reconfirm differentially expressed genes and to detect false positive significant genes from Limma test. We have chosen the value of $\Delta = 0.5$, which resulted in a false discovery rate (**FDR**) < 0.005.

To cluster the samples based on their genes expression profile, principal components analysis (**PCA**), partial least squares discriminate analysis (**PLS-DA**), and variable importance of projection (**VIP**) analysis

were performed. We also studied patterns of changes in gene expression, and finally, cluster analysis was performed and the results are shown in a heat map. In addition, hierarchical clustering analysis was performed using MetaboAnalyst. For distance measurements, we used the Euclidian and Ward algorithm for clustering. The results are shown as a heat map.

Functional Annotation Analyses. Functional annotation analysis such as pathways and processes of major biological significance and importance were performed using the Database for Annotation, Visualization and Integrated Discovery (**DAVID**) version 6.8 (Huang et al., 2009). An enrichment score of 1.3 was used as a threshold for cluster significance. g:Profiler (Reimand et al., 2016) was also used to perform statistical enrichment analysis. In addition, all the significant genes were used to identify and visualize metabolic pathways in the Kyoto Encyclopedia of Genes and Genomes (**KEGG**; http://www.genome.jp/kegg/tool/map_pathway2.html; ; accessed 16 Jan 2016).

Gene Expression Validation by Quantitative PCR. MicroRNA and mRNA levels were measured and analyzed using their PCR signal (**Cq**). Because amplification efficiencies of all genes were different from 2, Cq data were transformed using the equation proposed by Steibel et al. (2009) to rescale Cq values.

Determination of gene expression stability of all genes and miRNA included in this study were calculated using NormFinder (Andersen et al., 2004) to select the best reference gene.

Statistic methodology to analyze differences in the expression rate was performed following the method proposed by Steibel et al. (2009). The mixed model fitted was

$$y_{rigkm} = \mathrm{TG}_{gi} + \mathrm{P}_k + \mathrm{A}_m + e_{rigkm},$$

in which y_{rigkm} is the Cq value (transformed data taking into account for Efficiency < 2) of the *g*th gene from the *r*th well in the *k*th plate collected from the *m*th animal subjected to the *i*th treatment (Control and Creep), TG_{gi} is the fixed interaction among the *i*th treatment and the *g*th gene, P_k is the fixed effect of the *k*th plate, A_m is the random effect of the *m*th animal from which samples were collected ($\mathrm{A}_m \sim (0, \sigma^2_{\mathrm{A}})$), and e_{rigkm} is the random residual. Gene specific residual variance (heterogeneous residual) was fitted to the gene by treatment effect ($e_{rigkm} \sim N(0, \sigma^2_{egi})$).

To test differences in the genes of interest (diffGOI) in the expression rate of the target genes between treatments in terms of fold change (**FC**), the approach suggested in Steibel et al. (2009) was used. The significance of the diffGOI estimates was determined with the *t*-statistic. To validate the microarray results, the Pearson correlation coefficient was calculated between FC values estimated in microarray analysis and qPCR expression measures for the 6 and 5 genes and miRNA.

RESULTS AND DISCUSSION

The main results are presented separately for preweaning and postweaning management because the interaction between the preweaning and postweaning management was nonsignificant, except for glucose concentration in plasma. Therefore, only the results for metabolites are presented, considering the interaction of both effects.

Animal BW and ADG of the Primiparous Cow and its Calf during Lactation

The performance of the primiparous cows during first lactation was not affected by the feeding during the PRE-W or POST-W (Table 1). Similar results were reported by Hixon et al. (1982), Martin et al. (1981), and Buskirk et al. (1996). The calf performance was not affected by either the PRE-W or POST-W feeding treatment of its dam (Table 1). Our results are in agreement with Hixon et al. (1982), who reported that creep feeding during the PRE-W of the dam does not affect calf's BW at birth or at 120 d of age. Similarly, Freetly and Cundiff (1998) reported that the level of nutrition of the heifer between weaning and breeding did not affect the calf's performance. However, other studies found that calf performance was impaired in dams that had previously been creep fed during lactation (Martin et al., 1981) or fed high-energy diets in the pubertal phase (Buskirk et al., 1996)

Plasma Glucose, IGF-I, and Leptin Concentrations

Plasma glucose concentration at the end of the first lactation of the primiparous cows was affected by the interaction of PRE-W and POST-W treatments ($P = 0.045$; Table 2). During the PRE-W, creep feeding decreased glucose concentration, while High POST-W treatment increased glucose concentration by 15% ($P < 0.05$). During the PRE-W, creep feeding decreased glucose concentration ($P < 0.001$), while High POST-W treatment increased glucose concentration by 15% ($P < 0.001$). This differs from results from Reis et al. (2015), who found only a transient effect on heifer plasmatic glucose during the time when different feeding treatments were applied and not in later phases, whereas in the current study, a longer-lasting metabolic imprinting would have been promot-

ed. Concentration of IGF-I at the end of the first lactation was not affected by the feeding management in the PRE-W or POST-W (Table 2), which agrees with our previous observations in growing animals, where IGF-I did not differ among treatments in the long term (Blanco et al., 2008). Cows' leptin concentration at the end of lactation was affected by the feeding management during the POST-W; cows on the High energy level had 36% greater leptin concentration than their cohorts ($P < 0.05$). However, despite in vivo studies suggesting that leptin can mediate the effects of a high plane of nutrition on mammary cell proliferation in prepubertal heifers (Silva et al., 2008) and on the adaptive immune responses (Bruno et al., 2005; Matarese et al., 2005), no differences were found in this regard between cows from the High and Moderate POST-W treatments (compare below).

Milk Production and Composition

Milk traits during the first lactation were not affected by the feeding management during the POST-W ($P > 0.05$; Fig. 1), as described by other authors (Marston et al., 1995; Freetly and Cundiff, 1998). During the PRE-W creep treatment reduced the daily yield of milk, CP, casein, lactose, and nonfat solids ($P < 0.05$) throughout lactation (Fig. 1). It also tended to decrease the daily production of fat on the first and third month of lactation ($P = 0.06$) and decreased it by 24% in the second month of lactation ($P = 0.04$). Hixon et al. (1982) reported that creep feeding during the PRE-W did not affect milk composition but decreased milk yield, especially at the fourth month of lactation (by 28% when compared with non-creep-fed cows). In addition, Sexten et al. (2004) also reported that creep feed during the PRE-W had a negative impact on milk yield. The fact that this different milk yield did not result in different calf growth rates during lactation could be due to the young age of calves at weaning. Maybe, in a longer lactation, the lower milk supply from Creep dams would have resulted in the current differences in weaning weight reaching significance. Most of the studies on the effects of growth rates in early life on mammary development and future milk production have been conducted in dairy heifers and have sometimes found contradictory results, as reported in the review of Lohakare et al. (2012). In beef heifers, Prichard et al. (1989) found that creep feeding beef females during lactation did not affect udder weight, percentage of lipid, or total lipid in the udder, although they observed adipocyte hypertrophy in their udder fat depots. However, they did not assess to what extent future milk production potential could be affected. Other authors indicate that increased prepubertal ADG can retard mammary development by reducing the amount of parenchyma (Meyer et al., 2006) and result in a fatty udder syndrome that decreases the future milk yield potential (Radcliff et al., 2000).

Zanton and Heinrichs (2005) reported that milk yield responses of dairy heifers were quadratically associated with increasing prepubertal ADG and recommended an optimal growth rate of 800 g/d from 150 to 320 kg BW for maximum first lactation milk and protein yields. In the current study, ADG of creep-fed animals during the PRE-W treatment exceeded this recommended value (Supplementary Fig. S1; see the online version of the article at http://journalofanimalscience.org) and resulted in subsequent decreased milk yield, whereas both POST-W treatments provided ADG around this value and had similar milk yields.

In addition, creep feeding during the PRE-W increased SCC on the third month of lactation ($P < 0.049$) and tended to increase it on the fourth month of lactation ($P = 0.05$; Fig. 1). The International Dairy Federation (Schaerbeek, Belgium) has determined that a SCC > 200,000 cells/mL of milk (which would be a logarithmic somatic cell count value of 2.3) suggests the presence of mastitis and considered 100,000 cells/mL as a healthy threshold (Hillerton, 1999).

Differentially Expressed Genes by Microarray Analysis in the Mammary Gland

In the present study, we have identified 307 genes differentially expressed between feeding managements during the PRE-W with a FDR < 0.005 in the mammary gland (Supplementary Fig. S2 ; see the online version of the article at http://journalofanimalscience.org). More specifically, in the Creep group, 81 genes were upregulated and 223 genes were downregulated compared with Control cohorts (all the genes ranked by FC are shown in Supplementary Table S2 [see the online version of the article at http://journalofanimalscience.org]). The top 50 genes identified with SAM are shown in Table 3. However, only 7 genes differentially expressed between feeding managements during the POST-W feeding with a FDR < 0.005 in the mammary gland (*MIR1291*, *CXCL3*, *LOC616819*, *RASD1*, *LBP*, *NFKBIA*, and *CEBPD*) were identified. These results are in accordance with the effect observed on milk production and quality of the primiparous cows, where no effects of POST-W treatments were found. Because no effects of POST-W treatments were observed on milk production and quality and the low impact on gene expression, the subsequent analyses were performed considering only the PRE-W feeding treatment.

Table 1. Effect of feeding management during the preweaning period (PRE-W) and the postweaning period (POST-W) on the performance of the primiparous cows and their calf's during the first lactation

	PRE-W		POST-W			*P*-value[6]	
Performance	Creep[1]	Control[2]	High[3]	Moderate[4]	RootMSE[5]	PRE-W	POST-W
Cow's BW, kg					154[7]		
Parturition	476	500	508	468		1.00	1.00
End of lactation	474	481	494	460		1.00	1.00
Cow's ADG, kg/d	−0.012	−0.08	−0.063	−0.03	0.133	0.36	0.60
Lactation length, d	132	124	128	128	19	0.48	0.99
Calf's BW, kg							
Birth	38.8	39.3	39.9	38.1	3.6	0.83	0.35
Weaning	119	124	125	118	15	0.62	0.35
Calf's ADG, kg/d	0.680	0.714	0.688	0.706	0.120	0.66	0.78

[1]Creep = calves fed a creep feed supplement.
[2]Control = calves fed only their dam's milk.
[3]High = high-energy diet (91.7 MJ/d).
[4]Moderate = moderate-energy diet (79.3 MJ/d).
[5]RootMSE = root mean square error.
[6]After Bonferroni correction.
[7]Residual.

Principal components analysis of 304 genes showed that the first 2 principal components explain 56.3% of the observed variance of the sample set (Fig. 2A). The PCA scores plot showed the differences corresponding to the primiparous cows fed differently during the PRE-W. Moreover, PLS-DA (Fig. 2B) identified genes that were most important for the separation observed in the scores plots. Genes such as *TCEB2* and *NFU1* had the highest VIP score followed by *RNA18S*, *LOC789005*, and *CBX8* (Fig. 2C). Analysis of patterns in gene expression changes revealed that *MIR1940* and *ANKRD26* were positively correlated in the Creep treatment (Fig. 2D). On the other hand, they were negatively correlated with *PSMG3*, *MRPL23*, *MRPS18C*, and other genes. These genes play an important role in view of the PCA and PLS-DA cluster analysis.

In the current study, 15 miRNA were differentially expressed. Some key milk-related miRNA were identified using a miRNA microarray assay of mammary gland tissue from dairy cows (Wang et al., 2014). In this sense, Hou et al. (2012) and Li et al. (2015) found that *mir296* and *mir1940* were upregulated in mastitis-infected mammary glands. In the current study, these miRNA were upregulated in the Creep group and showed a significant enriched cluster related to immune response. *Mir181D* was downregulated in the Creep group, as in another study in which mammary tissue infected with the Gram-positive bacteria *Streptococcus uberis* was compared with normal tissue and a lower expression of this miRNA was found in infected tissues (Naeem et al., 2012). In addition, miR-143 has also been associated with bovine intramuscular fat proliferation

Table 2. Effect of the feeding management during the preweaning period (PRE-W) and the postweaning period (POST-W) on glucose, IGF-I, and leptin concentrations of the primiparous cows at the end of its first lactation

	PRE-W		POST-W		Creep[1]		Control[2]			*P*-value		
Metabolite	Creep	Control	High[3]	Moderate[4]	High	Moderate	High	Moderate	Root-MSE[5]	PRE-W	POST-W	PRE-W × POST-W
Glucose, mol/L	3.67[b]	4.14[a]	4.24[a]	3.58[b]	3.89[b]	3.46[b]	4.59[a]	3.70[b]	0.19	0.0006	0.0001	0.045
IGF-I, ng/mL	69.9	57.8	73.1	54.6	80.52	59.2	65.7	49.92	29.9	0.45	0.25	0.86
Leptin, ng/mL	2.62	2.80	3.30[a]	2.12[b]	3.53	1.71	3.08	2.52	0.99	0.74	0.04	0.24

[a,b]Means within a row with different superscripts significantly differ ($P < 0.05$) after Bonferroni correction.
[1]Creep = calves fed a creep feed supplement.
[2]Control = calves fed only their dam's milk.
[3]High = high-energy diet (91.7 MJ/d).
[4]Moderate = moderate-energy diet (79.3 MJ/d).
[5]RootMSE = root mean square error.

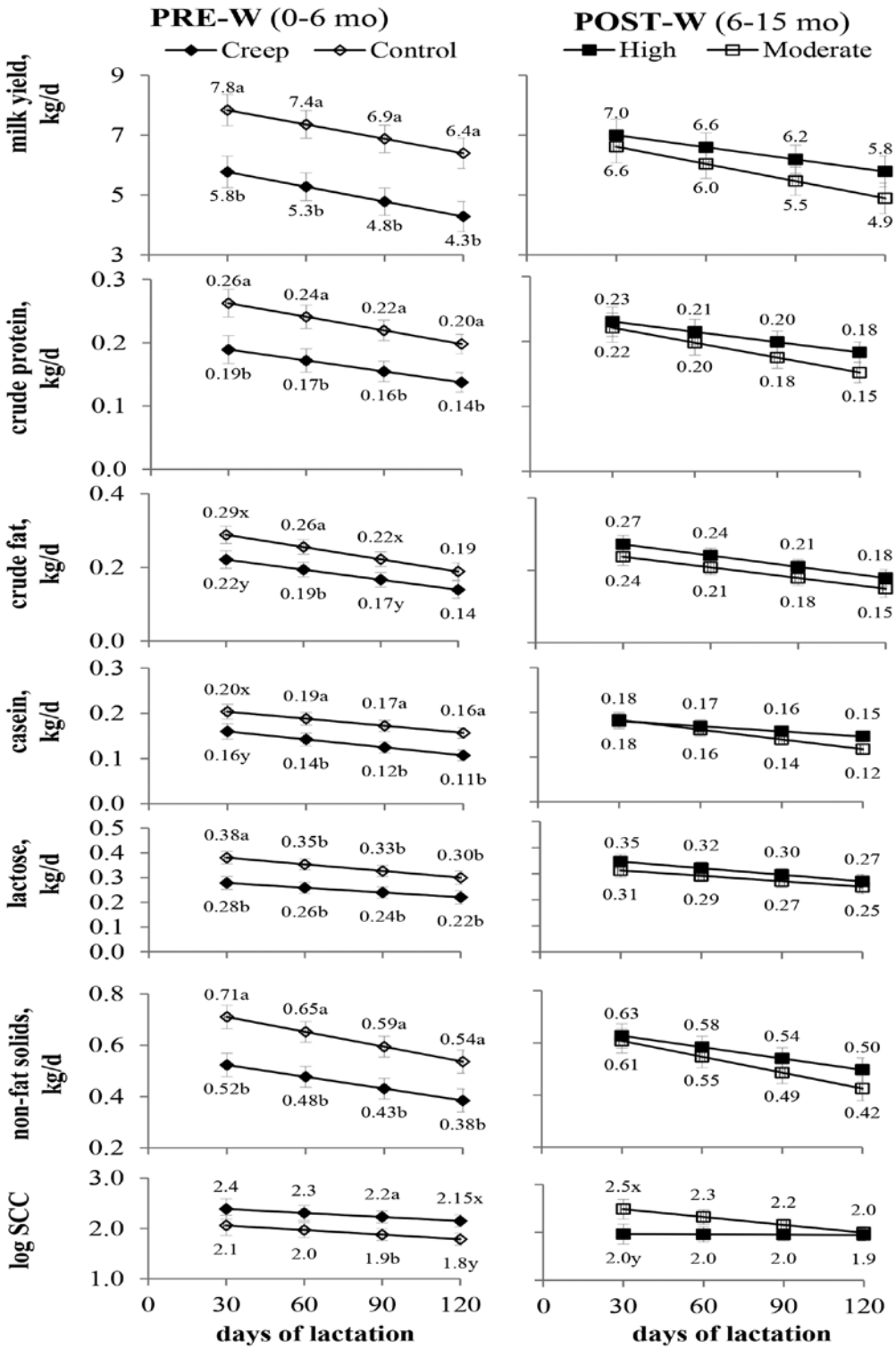

Figure 1. Effect of feeding treatments established during the preweaning period (PRE-W) and the postweaning period (POST-W) on milk production and quality during the first lactation. [a,b]Different letters indicate a significant difference with at least $P < 0.05$. Creep = calves fed a creep feed supplement; Control = calves fed only their dam's milk; High = high-energy diet (91.7 MJ/d); Moderate = moderate-energy diet (79.3 MJ/d); SCC = somatic cell count.

and differentiation (Li et al., 2011) and high back fat deposition in crossbred beef cattle, suggesting that this miRNA may promote bovine adipogenesis that may accelerate adipose tissue development. Klöting et al. (2009) found that mir197 and other miRNA play a role in the link between adipose tissue dysfunction and the development of obesity-associated disorders including type 2 diabetes, showing depot-specific expression patterns. In this sense, expression of mir197 in adipose tissue correlates with fasting plasma glucose and was also positively correlated in the current study. In the current study, mir143 is overexpressed in the Creep

treatment, with low levels of glucose in plasma, which may be an indicator of fatty mammary glands.

Because of the widespread expression of Ankrd26 in most organs and tissues, it is possible that the Ankrd26 protein may act locally within each cell to control organ size, and it is the level of Ankrd26 protein that regulates this process producing more fat cells, more liver cells, etc. Moreover, *ANKRD26* RNA is present in many normal tissues. In our study, *mir1940* and *ANKRD26* were positively correlated. In this sense, Bera et al. (2008) produced a mutant mouse and reported that the homozygous *Ankrd26* mutant mice have extreme obesity and an increase in organ and body size.

The results of hierarchical clustering analysis for the mammary gland are presented in Fig. 3A and 3B. The expression profile of these genes was able to cluster and correctly classify the samples within their corresponding group. The heat map shows the presence of 2 different clusters containing different genes. The responses of each variable to the different diets during the PRE-W are indicated with changes in the color intensity on the heat map. The Control and Creep groups showed different gene expression pattern. The first cluster of genes is upregulated in the Creep group compared with the Control group, whereas the second cluster is downregulated in the Creep group.

Functional Clustering Annotation

The results of DAVID functional annotation clustering of 307 significant genes are shown in Supplementary Table S3 (see the online version of the article at http://journalofanimalscience.org). These analyses revealed that only 2 significant enriched clusters were found upregulated in the Creep group, which included the rhodopsin-like G protein-coupled receptors (*OR2A2*, *C3AR1*, *OR7A17*, *OR7G1*, *GPR75*, *GPR39*, *TACR1*, *CXCL11*, *CCL6*, and *OR1F1*) and immune response (*TMEM173*, *VAMP7*, *TFE3*, *LILRA6*, *CX3CL1*, *CXCL11*, and *CCL16*).

Interestingly enough, in the Creep group, the cluster of genes related to immune response and chemokine activity was overexpressed. Inflammation and infections are associated with changes in the defense system of the mammary gland and cause a reduction in milk yield and quality. The number of somatic cells in milk is correlated with intramammary infection, and cattle breeders have used SCC in genetic selection for reducing mastitis. There is a strong transcriptional regulation of metabolism (particularly lipid and glucose), immune response, epigenetics, protein synthesis machinery, angiogenesis, and transport, among others, into the biological adaptation of the bovine mammary gland during the end of pregnancy and during the whole lactation (Bionaz et al., 2012). These authors found that the mammary gland appeared to have placed substantial effort in preparing the immune system based on KEGG pathways analysis. The data in the current experiment confirmed the lack of clinical mastitis in the mammary tissue used; however, the possibility that the animals might be at greater risk to develop subclinical mastitis cannot be excluded. It could be speculated that, based on a change in mammary gland architecture, creep feeding heifers during PRE-W favors the development of cell types related to immune response. In the Control group, which had higher protein and casein content in the milk, the most enriched clusters are related to "gene expression," "ribonucleoprotein," and "ribosome," all related to protein biosynthesis mechanisms, protein translation including ribosomal proteins, and protein folding. These results were confirmed with g:Profiler software, which identified an additional KEGG pathway: RNA degradation. Interestingly enough, the results of KEGG analysis showed that the ribosome, spliceosome, and RNA degradation pathways were downregulated in Creep animals (Supplementary Fig. S3, S4, and S5; see the online version of the article at http://journalofanimalscience.org), suggesting that a Creep animal put greater effort into immune response rather than into milk protein biosynthesis. The proline biosynthetic process was also activated in Control animals, which corresponds with greater production of casein and protein than in Creep animals. Milk proteins are synthesized in the mammary gland, but 60% of the AA used to build the proteins are obtained from the cow's diet. The casein family of protein consists of several types of caseins (α-s1, α-s2, β, and 6), and caseins have high proline content (van Meijl et al., 2010). Furthermore, results of recent findings suggest that proline may play a role in regulating the mechanistic target of rapamycin (**mTOR**) activation pathway (van Meijl et al., 2010) and, together with arginine, glutamine, and leucine, activate the mTOR and regulators of polyamine production, to enhance protein synthesis in cells and tissues. In this sense, Bionaz and Loor (2007) found increased expression of the mTOR pathway in the regulation of milk protein synthesis during lactation.

Validation of Microarrays Results using Quantitative PCR

Six genes (*PTRG*, *ANKRD26*, *SLC25A3*, *CXCL11*, *PYCR1*, and *KHDRBS*), and 5 miRNA (*Mir296*, *Mir1940*, *Mir143*, *Mir197*, and *Mir200B*) were selected to validate the microarray results using qPCR. The stability values calculated by NormFinder are

Table 3. Top 50 significant features identified using significance analysis of microarray in calves fed a creep feed supplement (Creep) vs. calves fed only their dam's milk (Control; in the preweaning period) contrast in the mammary gland

Symbol	Gene name	*q*-value	FC[1]
LOC781858	*uncharacterized LOC781858*	0	2.93
LOC100301407	*uncharacterized LOC100301407*	0	2.67
LOC784741	*uncharacterized LOC784741*	0	2.49
MIR296	*microRNA mir-296*	0	2.36
CXCL11	*chemokine (C-X-C motif) ligand 11*	0	2.22
VAMP7	*vesicle-associated membrane protein 7*	0	2.17
MIR143	*microRNA mir-143*	0	1.99
LOC788334	*apolipoprotein L, 3-like*	0	1.90
LOC785386	*ribosomal protein L10-like*	0	1.78
SLC25A3	*solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3*	0	1.71
MIR1940	*microRNA mir-1940*	0	1.70
LOC783362	*uncharacterized LOC783362*	0	1.67
MIR200B	*microRNA mir-200b*	0	1.67
LOC100336993	*T-cell receptor beta chain V regionYT35-like*	0	1.67
LOC100847684	*uncharacterized LOC100847684*	0	1.57
LOC789667	*olfactory receptor, family 7, subfamily A, member 17-like*	0	1.51
LOC616977	*interferon beta-2-like*	0	1.49
EFNA5	*ephrin-A5*	0	1.49
ANKRD26.1	*ankyrin repeat domain 18A, mRNA*	0	1.47
LOC781001///RAB21	*uncharacterized LOC781001///RAB21, member RAS oncogene family*	0	1.45
SLC9A6	*solute carrier family 9 (sodium/hydrogen exchanger), member 6*	0	1.42
TACR1	*tachykinin receptor 1*	0	1.42
EEF1A1	*similar to human eukaryotic translation elongation factor 1 alpha 1*	0	1.41
LOC782989	–	0	1.39
MIR2366	*microRNA mir-2366*	0	−2.30
KHDRBS1	*KH domain containing, RNA binding, signal transduction associated 1*	0	−2.23
PTGR1	*prostaglandin reductase 1*	0	−2.11
LOC788523	–	0	−1.84
ACYP1	*acylphosphatase 1, erythrocyte (common) type*	0	−1.83
ULBP3	*NKG2D ligand 3-like, transcript variant X1*	0	−1.77
CTDNEP1	*CTD nuclear envelope phosphatase 1*	0	−1.75
NFU1	*NFU1 iron-sulfur cluster scaffold homolog (S. cerevisiae)*	0	−1.74
MIRLET7G	*microRNA let-7g*	0	−1.71
PRPF8	*PRP8 pre-mRNA processing factor 8 homolog (S. cerevisiae)*	0	−1.68
CDKN2AIPNL	*CDKN2A interacting protein N-terminal like*	0	−1.68
LOC781151	*uncharacterized LOC781151*	0	−1.67
LOC789005	*Ig kappa chain V-III region NG9*	0	−1.66
RNA18S	*18S ribosomal RNA*	0	−1.66
LOC539802	*cGG triplet repeat binding protein 1-like*	0	−1.63
C2H2orf76	*chromosome 2 open reading frame, human C2orf76*	0	−1.62
MIR197	*microRNA mir-197*	0	−1.58
PSMG3	*proteasome (prosome, macropain) assembly chaperone 3*	0	−1.56
C24H18orf21	*chromosome 24 open reading frame, human C18orf21*	0	−1.56
POLR2K	*polymerase (RNA) II (DNA directed) polypeptide K, 7.0kDa*	0	−1.56
MIEN1	*migration and invasion enhancer 1*	0	−1.54
LOC782021	*60S ribosomal protein L21-like*	0	−1.54
MRPS18A	*mitochondrial ribosomal protein S18A*	0	−1.53
MRPS18C	*mitochondrial ribosomal protein S18C*	0	−1.53
SNRNP200	*small nuclear ribonucleoprotein 200kDa (U5)*	0	−1.53
MRPL23	*mitochondrial ribosomal protein L23*	0	−1.53

[1]FC = fold change.

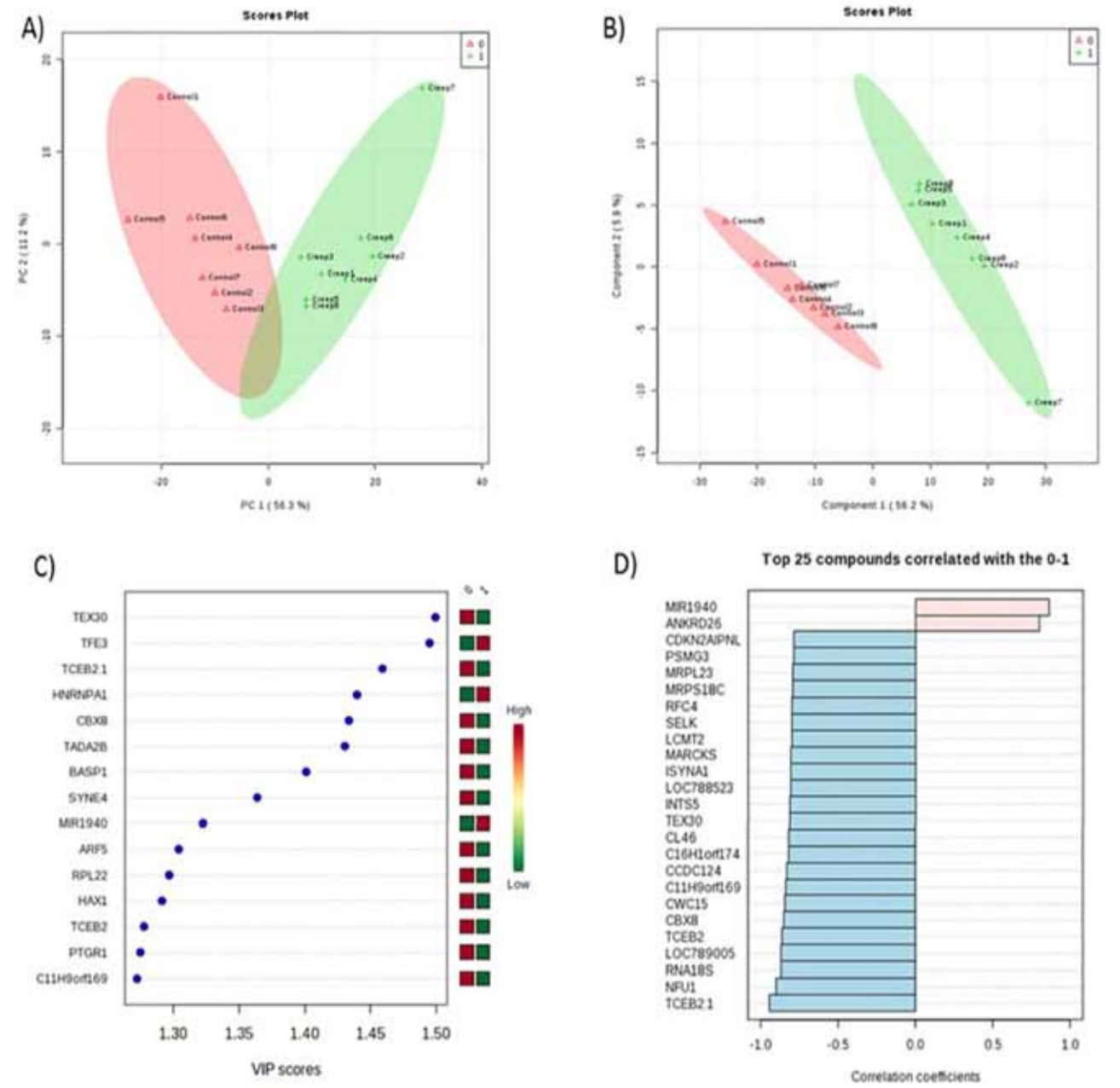

Figure 2. Multivariate analysis based on gene expression profile data in the mammary gland. A) Principal components analysis scores plots discriminating between calves fed only their dam's milk (Control) and calves fed a creep feed supplement (Creep) preweaning period (PRE-W) treatments. B) Partial least squares discriminate analysis (PLS-DA) based on gene expression profile data. C) Important features identified using PLS-DA. The top 15 genes ranked by variable importance of projection (VIP) scores. D) A bar graph showing the top 25 genes correlating with the PRE-W treatment (Control vs. Creep). PC 1 = principal component 1; PC 2 = principal component 2.

presented in Supplementary Fig. S6 (see the online version of the article at http://journalofanimalscience.org). *Mir191* had the best value of stability (M; 0.185), and in the case of coding genes, the most stable gene was *RPL32* gene (M = 0.201). Therefore, *Mir191* and *RPL32* were used to normalize gene expression results. Although the magnitude of the FC obtained using microarray analysis and qPCR was slightly different in some instances, qPCR results showed a trend similar to microarray analysis results, demonstrating the reliability of microarray analysis (Pearson correlation coefficient 0.97, $P = 0.001$ for mRNA and Pearson correlation coefficient 0.95, $P = 0.012$ for miRNA; Supplementary Table S4 [see the online version of the article at http://journalofanimalscience.org]).

Conclusions

During the PRE-W period, creep feeding reduced milk yield and milk fat, protein, casein, and lactose throughout cows' first lactation and increased SCC at the third and fourth month

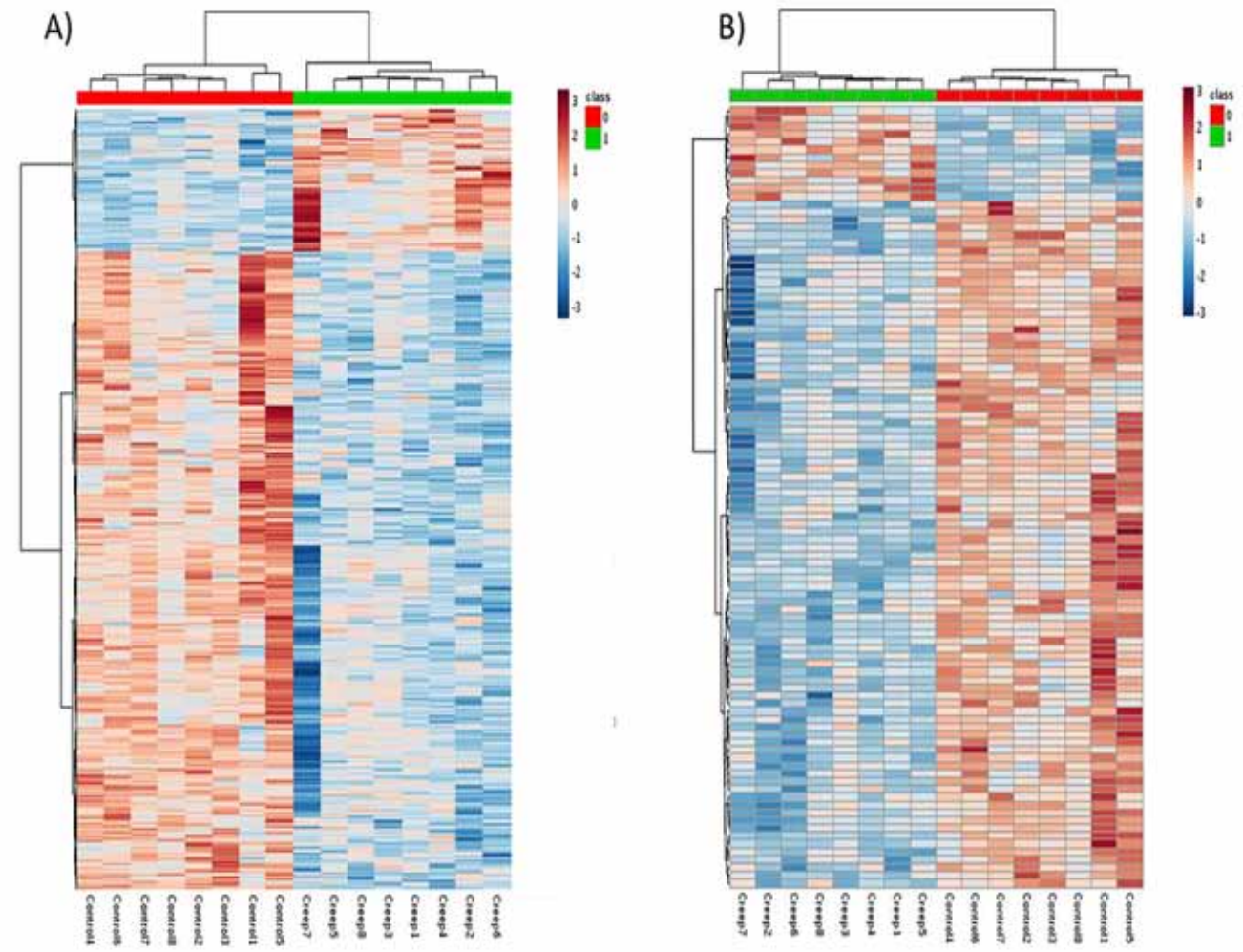

Figure 3. Hierarchical clustering analysis for gene expression in the mammary gland with a) all significant genes and b) the 100 most significant genes. Creep = calves fed a creep feed supplement; Control = calves fed only their dam's milk.

of lactation and affected the gene expression patterns in their mammary glands at the end of the first lactation. Overall, creep feeding during the preweaning period resulted in upregulation of genes related to immune response and chemokine activity and downregulation of ribosome and spliceosome genes. It would be interesting to study whether or not creep feeding in the preweaning period has negative effects on milk yield in subsequent lactations. The postweaning energy level had no impact on milk production and composition; therefore, it could be recommended to accelerate the growth of the beef heifers.

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