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Effect of prolactin, β-lactoglobulin, and κ-casein genotype on milk yield in East Friesian sheep

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ABSTRACT

The effect of prolactin (*PRL*), β-lactoglobulin (*β-LG*), and κ-casein (*CSN3*) on milk yield was estimated in an East Friesian dairy sheep population from Old Chatham Sheepherding Company, New York. Genotypes were determined by PCR amplification followed by digestion with *Hae*III and *Rsa*I for *PRL* and *β-LG*, respectively, and by PCR amplification for *CSN3*. Monthly milking records and pedigree information were used to evaluate the effect of each polymorphism on milk yield. Results indicated that *PRL* genotype had a significant effect on milk yield. Ewes carrying one *A* allele produced 110.6 g more milk per day than ewes with no *A* alleles. There was no statistical difference between ewes with only one *A* allele and ewes with 2 *A* alleles. No association among polymorphisms at the *β-LG* and *CSN3* loci and milk yield was found. The results presented in this study indicate that the *PRL* gene is a potential marker that could be used in selection programs for improving milk yield in dairy sheep.
Key words: candidate gene, dairy sheep, milk production

INTRODUCTION

Dairy sheep production is a relatively new but growing industry in North America. Sheep-milk cheeses have become very popular among US consumers in recent years. Imports of sheep-milk cheeses by the United States increased from 14.8 million kg in 1984 to 33.1 million kg in 2006 (FAO, 2006), a 123.6% increase in 20 yr. Although there is great economic opportunity for this new industry, sheep breeds in North America have not been selected for milk production, and therefore, the milk yield is well below that achieved by many European breeds.

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Experience with dairy cattle shows that milk yield, a sex-limited trait expressed at maturity with moderate heritability, can be improved through a classical selection program essentially based on selection of bull progeny tested across many herds, followed by intensive use of selected bulls as parents of the next generation. For the dairy sheep industry in the United States, several limitations make the traditional dairy-cattle selection program for increasing milk yield less effective. Lack of widespread use of AI and lack of a standardized milk recording system integrated across flocks are 2 obvious limitations. Given these limitations, the need for development of DNA markers and other genomic tools to increase milk yield is critical for the future of the US dairy sheep industry.

In dairy cattle, several genes have been investigated for their relationships with milk yield (for a review, see Ogorevc et al., 2009). If their associations with milk yield could be proven in dairy sheep, they could provide tools needed for more efficient breeding programs by providing additional information to identify genetically superior individuals. In addition, these individuals could be identified early in life, leading to shorter generation intervals and increased rates of genetic progress.

In this study, we tested the association between genetic polymorphism of several genes and a quantitative trait (daily milk yield) in a dairy sheep population with a pedigree structure. A straightforward method to measure the association is to evaluate the contrasts between the means of genotype classes for the trait of interest using a fixed linear model. Although this method is simple and fast, in a population with a pedigree structure, it ignores the covariance among the individuals due to genetic relationships among them (and possible common environmental factors) and may lead to high levels of false-positive associations (Aulchenko et al., 2007).

The measured genotype approach (**MG**; Hopper and Mathews, 1982; Boerwinkle et al., 1986; George and Elston, 1987) represents a powerful methodology for association analysis (Lange et al., 2002; Havill and Dyer, 2005) in such a population. In this approach, the genetic polymorphism under study is included as

a fixed effect in a mixed model that also includes a random animal effect that accounts for genetic relationships among members of the pedigree population.

In a simulation study, Aulchenko et al. (2007) compared type 1 error, power, and operational characteristics of the MG approach with other methods utilizing within-family variation such as the quantitative-trait transmission disequilibrium test and the family-based association test. For low to moderately heritable (30%) traits in populations with a pedigree structure, the power of the MG approach was much greater than that of transmission disequilibrium test–based approaches and had a type I error that was closest to the prespecified α. Therefore, we would expect that the false-positive error rate was also closest to the prespecified α. The MG approach, as described by Aulchenko et al. (2007), was used in this study.

The present study investigated the association between polymorphisms in prolactin (***PRL***), β-lactoglobulin (***β-LG***), and κ-casein (***CSN3***) genes and daily milk yield. The study was conducted in a population of East Friesian dairy sheep.

MATERIALS AND METHODS

Population and Phenotypic Data

All animal experiments were approved by the Oklahoma State University Institutional Animal Care and Use Committee. Blood samples were collected from all East Friesian ewes in milking during year 2005 from the Old Chatham Sheepherding Company (**OCSC**), New York. The OCSC began in 1994 and is today one of the largest dairy sheep farms in the Unites States with purebred East Friesian and crossbred Lacaune milking ewes. Ewes are milked twice daily, and individual milk yields are recorded once monthly in the OCSC database for the entire lactation period. All records collected in the OCSC flock from January 1, 1997, to July 31, 2007, were made available for this study. Milking records; pedigree information; and *PRL*, *β-LG*, and *CSN3* gene genotypes were available on 566, 519, and 475 ewes, respectively, and were used to measure and test the association between these polymorphisms and milk yield in this population.

Genotyping

Blood (10 mL) was collected into a heparinized tube (143 USP) from the jugular vein of each sheep. Genomic DNA was extracted from whole blood, using a commercial kit (Qiagen, Valencia, CA). A 2.5-kbp fragment spanning intron 2 of the ovine *PRL* gene was amplified, as described by Vincent and Rothschild (1997), and subsequently digested with *Hae*III restriction enzyme (Invitrogen, Carlsbad, CA). Allele *A* contained 3 restriction sites for *Hae*III and resulted in 4 fragments of 1,400, 530, 360, and 150 bp, whereas the presence of an additional restriction site in the *B* allele resulted in 5 fragments of 1,400, 530, 360, 150, and 20 bp. For the *β-LG* gene, a 120-bp fragment including parts of intron 1 and exon 2 was amplified, as described by Feligini et al. (1998), and subsequently digested with *Rsa*I restriction enzyme (Invitrogen). Allele *A* contained 2 restriction sites for *Rsa*I and resulted in 3 fragments of 66, 37, and 17 bp, whereas the absence of one restriction site in the *B* allele resulted in only 2 fragments of 103 and 17 bp. Genotyping of the SNP described by Feligini et al. (2005) in the sheep *CSN3* was performed with a common forward primer and 2 reverse primers (SNP-C and SNP-T) that differed in the 3′ end base and by the presence of a 12-bp poly-G tail in SNP-C. The presence of the poly-G tail allowed the discrimination of the 2 alleles: allele *T* was 85 bp long and allele *C* was 97 bp long.

Statistical Analysis

Gene and genotypic frequencies were calculated using the ALLELE procedure of SAS (SAS Institute Inc., Cary, NC). The data used to estimate the association of each gene polymorphism with milk production consisted of monthly test-day production records for the genotyped ewes. The following mixed model was used to describe the data:

$$Y_{ijknm} = YRMO_i + GENE_j + A_k + DL_{nm(k)} + E_{ijknm},$$

where Y_{ijknm} is the amount of milk in a test-day sample from an individual ewe, $YRMO_i$ is the fixed effect due to ewes tested in the same year and month, $GENE_j$ is the fixed effect of the gene polymorphism evaluated, A_k is the random effect of ewes, $DL_{nm(k)}$ is the random effect of test day and lactation nested within ewe and assumed to follow a first-order autoregressive process, and E_{ijknm} are the random residuals.

In matrix notation, the model can be represented as

$$\mathbf{y} = \mathbf{X\beta} + \mathbf{Za} + \mathbf{Qd} + \mathbf{e},$$

where $\mathbf{X}$ = incidence matrix that relates the records in y to the fixed effects; $\boldsymbol{\beta}$ = unknown vector of fixed effects; $\mathbf{a}$ and $\mathbf{d}$ = vectors representing random effects of ewes and of test day and lactation nested within ewe, associated with records in $\mathbf{y}$ by design matrices $\mathbf{Z}$ and $\mathbf{Q}$, respectively; $\mathbf{e}$ = vector of residuals. With

this model, the data vector $\mathbf{y}$ has expected value $E(\mathbf{y}) = \mathbf{X}\boldsymbol{\beta}$ and (co)variance matrix $\mathbf{V} = \text{Var}(\mathbf{y}) = \mathbf{ZGZ}' + \mathbf{QJQ}' + \mathbf{R}$, where $\mathbf{G} = \mathbf{A}\sigma^2_a$, $\mathbf{A}$ is the relationship matrix among animals, and σ^2_a is the additive genetic variance; $\mathbf{J} = \Sigma\sigma^2_{dl}$, where Σ is the autoregressive correlation matrix between test-day measurements for the same ewe and lactation n time periods apart with correlation ρ^n, and σ^2_{dl} is test-day and lactation variance; and $\mathbf{R} = \mathbf{I}\sigma^2_e$, where $\mathbf{I}$ is an identity matrix and σ^2_e is residual variance.

The analysis was performed using REML and the MIXED procedure of SAS (SAS Institute Inc., Cary, NC). The procedure works in 2 steps, first the variance matrix $\hat{\mathbf{V}}$ with its components $\hat{\mathbf{G}}$, $\hat{\mathbf{J}}$, and $\hat{\mathbf{R}}$ are estimated, and $\hat{\mathbf{V}}$ is subsequently used to estimate the fixed effects as

$$\hat{\boldsymbol{\beta}} = (\mathbf{X}'\hat{\mathbf{V}}^{-1}\mathbf{X})^{-1}\mathbf{X}'\hat{\mathbf{V}}^{-1}\mathbf{Y}.$$

The variance matrix of $\hat{\boldsymbol{\beta}}$ is

$$\text{Var}(\hat{\boldsymbol{\beta}}) = (\mathbf{X}'\hat{\mathbf{V}}^{-1}\mathbf{X})^{-1}.$$

The statistical inference about linear combinations of fixed effects, including contrasts described by vector $\mathbf{L}$, is based on linear combinations of the form $\mathbf{L}'\hat{\boldsymbol{\beta}}$ with variance

$$\text{Var}(\mathbf{L}'\hat{\boldsymbol{\beta}}) = \mathbf{L}'(\mathbf{X}'\hat{\mathbf{V}}^{-1}\mathbf{X})^{-1}\mathbf{L}.$$

This method might underestimate the true variance of $\hat{\boldsymbol{\beta}}$ because no account is made for uncertainty in estimating $\hat{\mathbf{V}}$. The Kenward–Roger option in SAS (Kenward and Roger, 1997) was used to account for this potential bias. The hypothesis testing of $\mathbf{L}'\hat{\boldsymbol{\beta}}$ (an estimable linear combination of fixed effects) was performed using the statistics

$$\{(\mathbf{L}'\hat{\boldsymbol{\beta}})'[\text{Var}(\mathbf{L}'\hat{\boldsymbol{\beta}})](\mathbf{L}'\hat{\boldsymbol{\beta}})\}/[\text{rank}(\mathbf{L})],$$

which has an approximate F-distribution with numerator degrees of freedom equal to the rank($\mathbf{L}$) and denominator degrees of freedom equal to the degrees of freedom required to estimate variance of $\mathbf{L}'\hat{\boldsymbol{\beta}}$.

The Satterthwaite option was used to ensure that the correct degrees of freedom were used for every test of interest. This option controls the computation of degrees of freedom for the t- or F-statistics used in the test of the fixed effects in the model, linear combinations of fixed effects, least squares means, and contrasts. The Tukey–Kramer multiple comparison adjustment for the P-values and confidence limits for the differences of least squares means was also used to ensure that the false-positive error rate was not inflated.

To ensure the validity of our inference regarding the genetic polymorphism fixed effect, the χ^2 statistic associated with the likelihood ratio test was also used. This statistic compares 2 models, the full model and the reduced model without the gene fixed effect, and the difference in -2 times the log-likelihoods between the 2 models is compared against χ^2 distribution with 2 degrees of freedom. To perform this test, the maximum likelihood method was used to fit the full and restricted models because the penalty term associated with the REML method depends on the fixed-effect specification.

Table 1. Number of observations and levels of fixed effects in the analyses of the effect of genetic polymorphisms of the 3 genes[1] on test-day milk yield

Item	*β-LG*	*PRL*	*CSN3*
Ewes	566	519	475
Test-day records	5,127	4,653	4,600
Year-month levels	98	93	98
Lactation-DIM levels	78	78	78

[1]*β-LG* = β-lactoglobulin; *PRL* = prolactin; *CSN3* = κ-casein.

RESULTS AND DISCUSSION

Milk Yield

Year-month fixed effects consisted of all year-months with at least 2 ewes with milking records, for a total of 98, 93, and 98 year-month levels for *PRL*, *β-LG*, and *CSN3* analysis, respectively. Days in milk by lactation effects consisted of 10-d intervals between 10 and 260 d for lactation 1, 2, and >2, for a total of 78 levels. The size of the data set and the number of levels for each fixed and random effect in the model for each of the 3 genes are shown in Table 1.

Type III tests for fixed effects are presented in Table 2. The year-month effect was, as expected, significant. The trend in average production by month was cyclical, with a lesser yield in winter and spring months and a greater yield in summer and fall months. Seasonal breeding was a major factor responsible for seasonal variation in yield per ewe.

The lactation curves were of expected shape. The curves showed lesser total yield and flatter curves for first lactation and greater total yield, greater peak yield, and lesser persistency for subsequent lactations.

Table 2. Type III tests for year-month and gene polymorphism fixed effects[1]

		SATdf					
Gene[2]	Fixed effect	NM	DN	F	$P > F$	LRT	$P > \chi^2_2$
PRL	Year-month	92	4,205	12.73	<0.0001		
	Polymorphism	2	594.9	4.55	0.01	9.2	0.01
β-LG	Year-month	97	4,634	13.35	<0.0001		
	Polymorphism	2	697.1	0.63	0.53	1.16	0.55
CSN3	Year-month	97	4,178	11.99	<0.0001		
	Polymorphism	2	547.6	0.32	0.72	0.7	0.70

[1]Separate analyses for each gene (*PRL*, *β-LG*, *CSN3*) showing numerator (NM) and denominator (DN) degrees of freedom calculated with the Satterthwaite option (SATdf), F-statistic (F), probability > F ($P > F$), likelihood ratio test statistic (LRT), and probability > χ^2_2 ($P > \chi^2_2$).
[2]*β-LG* = β-lactoglobulin; *PRL* = prolactin; *CSN3* = κ-casein.

Gene and Genotypic Frequencies

Gene frequencies were 0.13 and 0.87 for the *PRL Hae*III polymorphism (alleles *A* and *B*, respectively), 0.69 and 0.31 for the *β-LG Rsa*I polymorphism (alleles *A* and *B*, respectively), and 0.51 and 0.49 for the *SCN3 Rsa*I polymorphism (alleles *C* and *T*, respectively). Genotypic frequencies for all 3 genes are presented in Table 3.

The gene frequencies for the *PRL Hae*III locus reported in this study in the East Friesian breed differs from the frequency reported by Ramos et al. (2009) in the Serra da Estrela and Merino breeds, in which the *A* allele occurred more frequently than the *B* allele. Although breed differences could explain these discrepancies, another possible explanation is the presence of founder effects in the respective populations. Our gene and genotypic frequencies at the *β-LG Rsa*I locus were similar to the frequencies reported in Pag and Valle del Belice sheep (Cubric-Curik et al., 2002) and in East Friesian sheep from Saxony, Germany (Wessels et al., 2004), in which the *B* allele was found to be predominant. Our gene and genotypic frequencies at the *SCN3 Rsa*I locus were similar to the frequencies reported in Prameka (Serbia) and Sarda breeds (Feligini et al., 2005).

Table 3. Genotypic frequencies for *PRL Hae*III, *β-LG Rsa*I, and *SCN3 Rsa*I polymorphisms,[1] LSM, and SE of LSM for test-day milk yield (g of milk/d)

Gene[2]	Genotype	Frequency	LSM	SE
PRL	*AA*	0.02	1,180.76	142.66
	AB	0.24	1,215.90	51.84
	BB	0.74	1,105.29	45.50
β-LG	*AA*	0.44	1,159.91	50.23
	AB	0.51	1,125.00	50.09
	BB	0.05	1,133.12	80.30
CSN3	*CC*	0.13	1,147.18	64.8
	CT	0.76	1,158.91	49.06
	TT	0.11	1,115.32	68.44

[1]Total number of ewes was 566, 519, and 475 for *β-LG*, *PRL*, and *CSN3*, respectively.
[2]*β-LG* = β-lactoglobulin; *PRL* = prolactin; *CSN3* = κ-casein.

Genotypic Effects on Milk Production

PRL. The least squares means for milk production grouped according to *PRL Hae*III genotype are presented in Table 3, and the pair-wise comparison of least squares means between different *PRL Hae*III genotypes is presented in Table 4. The *PRL Hae*III polymorphism had a significant effect (Table 2) on milk production ($P < 0.01$). The pair-wise comparison (Table 4) indicated that ewes carrying one *A* allele produced 110.6 g more milk per day than ewes with no *A* alleles (adjusted $P = 0.008$). There was no statistically significant difference in milk yield between *AA* and *AB* ewes or between *AB* ewes relative to the average of *AA* and *BB* ewes.

The likelihood ratio test statistic for testing the *PRL* fixed effect was 9.2 and was statistically significant ($P = 0.01$) compared with χ^2 distribution with 2 degrees of freedom. The results of the likelihood ratio test were essentially identical to the results obtained when using the approximate F-statistics.

Whereas the *β-LG* and *CSN3* genes have been intensely investigated for a potential association with milk production traits, we are aware of only one other study that has investigated the *PRL* effect on milk yield in dairy sheep. Ramos et al. (2009) found a significant effect of the *PRL* genotype on milk yield, fat, and protein content in the Serra da Estrela breed, but no effect was found in the Merino breed. In both breeds, the *A* allele occurred more frequently than the *B* allele, and Serra da Estrela ewes carrying the *AA* genotype had lesser milk yields compared with ewes with *AB* and *BB* genotypes, which is the opposite of the results presented in this study for East Friesian ewes.

Table 4. Pair-wise comparison of LSM (for milk yield, g of milk/d) for *PRL Hae*III, *β-LG Rsa*I, and *SCN3 Rsa*I[1]

Gene[2]	Comparison of genotypes	Constant, g of milk/d	SE	SATdf	*t*-value	$P > \|t\|$	Adj*P*
PRL	*AA* − *AB*	−35.15	139.69	620.7	−0.25	0.80	0.96
	AA − *BB*	75.47	137.23	626.8	0.55	0.58	0.85
	AB − *BB*	110.62	36.92	566.3	3.00	0.002	0.008
	AB − [(*AA* + *BB*)/2]	72.88	75.60	578.4	0.96	0.33	0.45
β-LG	*AA* − *AB*	34.91	31.39	699.6	1.11	0.26	0.51
	AA − *BB*	26.79	71.45	664.4	0.37	0.71	0.92
	AB − *BB*	−8.12	71.38	658.4	−0.11	0.91	0.99
SCN3	*CC* − *CT*	−11.73	48.85	575.5	−0.24	0.81	0.97
	CC − *TT*	31.85	68.61	550.9	0.46	0.64	0.88
	CT − *TT*	43.58	54.94	519.6	0.79	0.43	0.71

[1]Effect estimates, SE, degrees of freedom calculated with Satterthwaite option (SATdf), *t*-value, probability $> |t|$, and probability $> |t|$ adjusted for multiple comparisons (Adj*P*) using the Tukey–Kramer correction.

[2]*β-LG* = β-lactoglobulin; *PRL* = prolactin; *CSN3* = κ-casein.

The *Hae*III polymorphism does not result in a change in amino acid substitution in the PRL polypeptide, and therefore, it is considered a silent mutation. The classical view is that silent mutations do not alter the composition or function of the protein produced by a gene. Recently, it has been shown that silent mutations interfere with several stages of the protein-making process, from DNA transcription to the translation of mRNA into proteins (Chamary et al., 2006). Although the possibility of a direct effect of the polymorphism needs to be explored, the association with milk production is more likely due to linkage with a functional mutation. Different alleles being associated with high milk production in different breeds could, therefore, be the result of different linkage phases between the *Hae*III polymorphism and the functional mutation. In both populations studied, the beneficial allele is present at a low frequency. The East Friesian population in the present study was started in 1993 with a few East Friesian rams, with limited availability of additional East Friesian genetics. Thus, the very low frequency of the beneficial allele could be due to a founder effect.

The region on ovine chromosome 20 in proximity to the *PRL* gene was identified as harboring a putative QTL for fat percentage (Gutierrez-Gil et al., 2009), as well as milk yield, fat yield, and protein yield (Barillet et al., 2005), providing supporting evidence for the importance of this chromosomal region for several milk-related traits. Although our results suggest *PRL* is a very likely positional candidate gene, until fine mapping of this region is performed, we cannot rule out the possibility that other genes in this region might be responsible for the effect we observed or the possibility that more than one QTL exists in this region.

β-LG. There was no significant effect of the *β-LG Rsa*I polymorphism on milk production in the present population. Beta-lactoglobulin is one of the major whey proteins found in milk, and a considerable number of studies have investigated a possible association between the genetic variants of the *β-LG* gene and milk-related traits (Feligini et al., 1998; Cubric-Curik et al., 2002; Nassiry et al., 2007; Dario et al., 2008). The polymorphism investigated in the present study is the most-studied polymorphism; a T→C nucleotide transversion in exon 2 of the *β-LG* gene produces the *Rsa*I RFLP (Feligini et al., 1998) and results in the substitution of the Tyr^{20} with His in the β-LG polypeptide (Kolde and Braunitzer, 1983).

Results from previous studies are conflicting, indicating superiority of either the *A* or *B* allele or no relationship with milk yield. In Valle del Belice ewes, the *AA* genotype was associated with greater milk yield (Giaccone et al., 2000). Another investigation showed that East Friesian ewes with the *AA* genotype had the greatest milk yield in the first lactation, whereas *BB*-genotype ewes had the greatest milk yield in the following lactations (Schmoll et al., 1999). Pietrola et al. (2000) found no significant differences among genotypes in Sarda ewes; however, the authors reported a trend for the *AA* genotype to show greatest values for milk yield and the *AB* genotype to always be the intermediate between the *AA* and *BB* genotypes. Other studies point toward superiority of the *B* allele. Rampilli et al. (1997) presented a trend for Massese ewes with the *BB* genotype to have greater milk production. Similarly, Sardinian ewes with the *BB* genotype had greater milk yields when compared with *AB* and *AA* genotypes (Bolla et al., 1989). In addition, in Serra da Estrela ewes, the *AA* genotype had the least milk yield, with no significant difference in milk yield between *AB* and *BB* genotypes (Ramos et al., 2009). The findings with Serra da Estrela ewes were similar to results for Merino ewes, in which again the *AB* and *BB* genotypes produced greater milk yields (Ramos et al., 2009).

SCN3. The present study did not find a significant effect of the *SCN3 Rsa*I polymorphism on milk pro-

duction. Among the 4 casein genes (α_{S1}-, α_{S2}-, β-, and κ-casein), κ-casein is the least studied with respect to its effect on milk yield and composition. A positive association between genetic variants of *CSN3* and milk traits has been reported in cattle (Ng-Kwai-Hang et al., 1984), and Caravaca et al. (2009) reported an association with total casein and protein content in goats. However, no association between *CSN3* and milk yield has been reported.

CONCLUSIONS

The dairy sheep industry is a new but growing industry in North America. Given the high demand for sheep-milk cheeses currently satisfied by imports, there is an enormous opportunity for growth. For this opportunity to be realized, selection programs that target increased milk production are needed. Marker-assisted selection could play an important role in this development, especially when 2 characteristics of dairy sheep compared with dairy cattle are considered: 1) infrequent use of AI and 2) limited milk production records, which are more costly relative to dairy cattle because of lesser income per ewe. Among the 3 genes studied, only *PRL* showed a strong association with milk production, indicating that the *PRL Hae*III polymorphism could potentially be used as a DNA marker for milk yield in this East Friesian population. The increase in production associated with the *A* allele (110 g of milk/d) has economic relevance because it represents about 7% of the average test-day milk in the study population. However, given the inconsistencies regarding the effect of this polymorphism on milk yield in other dairy sheep populations, its association with milk production requires further validation before it is used in marker-assisted selection.

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