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# Effects of polymorphisms in *DGAT1* and *LEP* genes on milk traits in Holstein primiparous cows

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**Abstract**

The genes encoding the diacylglycerol O-acyltransferase (*DGAT1*) and leptin (*LEP*) became a functional candidate genes for lactation traits in cows. Several studies associated single nucleotide polymorphisms (SNP) in these genes with fat and protein content in milk, fat and protein yield, milk yield and some reproductive traits. Three reported SNPs in these genes were investigated in our study (*DGAT1-K232A*, *LEP-R25C*, *LEP-A80V*). One hundred and sixty-three primiparous dairy cows from one farm were genotyped. The milk yield, fat and protein yield, fat and protein content and the age at first calving were recorded. The frequencies of alleles and genotypes were assessed. The effect of genotypes on milk traits and age at first calving were studied using linear models. The assumption of the different allele frequencies resulting from selection aimed at milk yield was proved in *DGAT1* and *LEP-A80V*. The significant effect ($P<0.01$) of *DGAT1* polymorphism was estimated only for the fat content. No significant effect of individual *LEP* polymorphism on milk trait or age at first calving was estimated. No significant effect of combination of *LEP* polymorphisms was estimated for those traits. The effect of *DGAT1* on the age at first calving was indicated.

*Key words*: dairy cattle, milk production traits, polymorphism, leptin, diacylglycerol acyltransferase

## Introduction

The identification of candidate genes associated with various qualitative and quantitative trait loci for meat and milk production is a tool to promote more efficient selection criteria for breeding purposes in livestock species. *DGAT1* became a functional candidate gene for lactation traits after studies indicated that mice lacking both copies of *DGAT1* are completely devoid of milk secretion (Smith et al., 2000). It was shown that the QTL variation is most likely caused by a nonconservative base substitution in the *DGAT1* gene changing lysine to alanine (*K232A*) in the *DGAT* enzyme. In particular, the allele encoding the lysine 232 variant proved to be more efficient with regard to milk fat synthesis (Grisart et al., 2002; Winter et al., 2002). Similarly, Gautier et al. (2007) reported the *K* allele is associated with an increase in fat content, fat yield and protein content and a decrease in protein and milk yields. On the other hand the allele encoding the alanine at this position is associated with lowered milk fat content (Winter et al., 2002). The leptin gene (*LEP*) has been studied intensively during the last years, especially in cattle, and it has been considered to be a candidate gene for meat and milk production (as reviewed by Van der Lende et al., 2005). A polymorphism in intron 2 of the *LEP* gene was found to affect milk yield

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(Buchanan et al., 2003; Kaminski et al., 2006; Liefers et al., 2002), growth rate (Nkrumah et al., 2005), and body fat percentage (Buchanan et al., 2002; Nkrumah et al., 2004). C to *T* transition in exon 2 of leptin that encodes an Arg25Cys (*R25*C) substitution (position four of the secreted peptide) is associated with body fat deposition in beef cattle (Buchanan et al., 2002). The *T* allele is associated with increased fat deposition and higher leptin mRNA levels in adipose tissue. Statistical analysis indicated that the *A80V* polymorphism has an effect on milk and protein yield. Animals with the *TT* genotype had approximately two-fold higher (P=0.006) estimated breeding values for milk and protein yields (Madeja et al., 2004). The aim of this study was to verify the associations of the *K232A* polymorphism in *DGAT1* gene and *R25*C, and *A80V* polymorphisms in leptin gene with milk traits in Holstein primiparous cows.

## Materials and methods

*Animals and phenotypes*

The herd of purebred Holstein primiparous cows from one farm was used for this study. The performance data were collected during one season in order to minimize this effect. The dataset consisted of 163 primiparous dairy cows originating from 49 sires. Cows were reared in free group stable with individual boxes and milking was performed twice a day. The overall milk yield during 305 days of lactation, fat yield, fat content, protein yield, protein content and age at first calving were recorded. The basic statistics of the studied traits for the 163 animals is shown in Table 1.

*DNA extraction and genotyping*

The DNA from hair roots was isolated by the Maxwell 16 Magnetic Particle Processor and Maxwell purification kit (Promega, USA) following the manufacturer´s instructions. Genomic DNA was genotyped by PCR-RFLP assays for the locus responsible for the *DGAT1* K232A substitution, and two bovine leptin (*LEP*) *R25*C and *A80V* substitutions. Briefly, PCR reactions for *DGAT1* genotyping were performed according to Bauer et al., 2011.

For *LEP* polymorphisms, PCR reactions were performed in a total volume of 25 $\mu$L using 10-50 ng genomic DNA as template, 1X Colorless GoTaq Flexi Buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.8 U GoTaq DNA polymerase (Promega, USA) and 0.4 $\mu$M of each primer for *R25*C polymorphism (Buchanan et al., 2002) or *A80V* polymorphism (Haegeman et al., 2000). The PCR profile for *R25*C included an initial denaturation step at 95 °C for 5 min, 35 cycles of 94 °C (20 s), 52 °C (30 s), 72 °C (30 s) and a final extension step of 10 min at 72 °C and for *A80V* included an initial denaturation step at 95 °C for 5 min, 35 cycles of 94 °C (20 s), 50 °C (30 s), 72 °C (30 s) and a final extension step at 72 °C for 10 min.

For genotyping the *LEP R25*C polymorphism the restriction endonuclease *Kpn2 I* (Fermentas, Germany) was used to digest a 94 bp PCR product at 55 °C overnight. For genotyping the *LEP A80V* polymorphism the restriction *HphI* (Fermentas, Germany) was used to digest a 330 bp PCR product at 37 °C overnight. PCR products were analyzed by the automated microchip electrophoresis system MCE®-202 MultiNA (Shimadzu, Japan) with

Table 1. The mean, standard deviation, minimum, maximum and coefficient of variation of the studied traits

| Trait | Mean | S.D. | Min. | Max. | CV |
|---|---|---|---|---|---|
| Yield (kg) | | | | | |
| Milk | 11243 | 1950 | 5858 | 18611 | 0.17 |
| Milk fat | 393.5 | 72.2 | 248.9 | 763.1 | 0.18 |
| Milk protein | 346.1 | 57.0 | 216.7 | 571.4 | 0.16 |
| Milk composition (%) | | | | | |
| Fat | 3.53 | 0.47 | 2.40 | 5.41 | 0.13 |
| Protein | 3.09 | 0.20 | 2.70 | 3.70 | 0.06 |
| Age (days) | 779 | 67 | 585 | 978 | 0.09 |

Age= age at first calving
CV=coefficient of variation (expressed as ratio of standard deviation and mean value of variable)

a DNA-500 kit according to the manufacturer´s protocol. A SYBR Gold fluorescent dye for DNA staining (Invitrogen, USA) and a 25 bp DNA Ladder (Invitrogen, USA) was used to determine the size of the PCR products.

*Statistical analysis*

The genotype and allele frequencies for each polymorphism were calculated. In order to decide whether the study of both *LEP* polymorphisms is worth fitting the model (single or combination) linkage disequilibrium between two *LEP* loci was determined using the haplotype frequencies (calculated using PHASE v 2.1; Stephens et al., 2001; Stephens et al., 2005) and calculated as a difference between observed and expected frequency of one haplotype with two loci:

$$D(a,b) = P(a,b) - P(a)P(b)$$

where a and b are specific alleles within the loci A and B. The D coefficient was then normalized according to Lewontin (1964).

The effect of genotype on milk traits and age at first calving was determined using the linear models (GLM procedure, SAS Institute, 2002-2009) with fixed effects of genotype and sire:

$$Y_{ijk}=\mu + G_i + S_j + e_{ijk}$$

where $Y_{ijk}$ is a phenotypic observation (milk traits and age at first calving) of the animal, $\mu$ is the overall mean, $G_i$ is the fixed effect of single genotype (*DGAT1-K232A*, *LEP-R25*C, *LEP-A80V*), $S_j$ is effect of sire j (due to selection sire effect was considered as fixed) and $e_{ijk}$ is the random residual effect. As the experiment was conducted within the one herd during the one season only in primiparous cows, these effects were eliminated. The least square means were estimated for genotype groups. Differences were tested by Scheffe`s test. The effect of the *LEP* polymorphism combination was evaluated by a linear model with two polymorphisms, their interaction and the fixed effect of sire:

$$Y_{ijkl}=\mu + G_i + H_j + G_i{*}H_j + S_k + e_{ijkl}$$

where $Y_{ijkl}$ is a phenotypic observation (milk traits and age at first calving) of the animal, $\mu$ is the overall mean, $G_i$ and $H_j$ is the fixed effect of a single genotype (*LEP-R25*C and *LEP-A80V*), $G_i{*}H_j$ is the fixed effect of a genotype combination of *LEP-R25*C and *LEP-A80V* polymorphisms, $S_k$ is the effect of a sire k (due to selection sire effect was considered as fixed) and $e_{ijkl}$ is the random residual effect.

The substitution effects of favourable alleles were calculated. The similar linear model was used with the replacement of genotype effect by the linear regression on the number of desired allele:

$$Y_{ijk} = \mu + b.x_i + S_j + e_{ijk}$$

where $Y_{ijk}$ is a phenotypic observation (milk traits and age at first calving) of the animal, $\mu$ is the overall mean, $x_i$ is the number of desired alleles (0,1,2) and b is the regression coefficient representing the allele substitution effect, $S_j$ is effect of a sire j and $e_{ijk}$ is a random residual effect.

## Results and discussion

*Allele frequencies*

In present study the animals were reared on the one farm, where the emphasis was given on milk production in the last few years. The selection process aimed at the improving the milk production may lead to changes in allele frequencies resulting in increase in allele frequency associated with higher levels of milk yield. The frequencies of the *DGAT1* genotypes could result from this fact, as several authors presented that the *K* allele (coding lysine) is associated with lower milk yield (Banos et al., 2008; Kaupe et al., 2007; Näslund et al., 2008). In our study (Table 2) the *DGAT1 A* allele had much higher frequency (0.88) than the *K* allele (coding lysine) associated with higher fat content. Näslund et al. (2008) also presented low frequencies of the *K* allele ranging from 0.01 to 0.18 in Swedish Holstein and Swedish Red populations. On the other hand Dokso et al. (2015) reported higher frequencies of *K* allele in population of Holstein (0.78), Simmental (0.62) and Brown Swiss cows (0.65).

The allele frequencies of *R25*C leptin polymorphism were almost the same (0.52 (C) vs 0.48 (*T*)) and were similar to frequencies reported by Buchanan et al. (2003) in Holstein population, Szyda et al. (2007) in Polish Black-and-White population and Clempson et al. (2011) in Holstein-Friesian population. Although authors Buchanan et al. (2003) and Banos et al. (2008) reported association of the *T* allele with increased milk yield, this fact was not manifested in allele frequencies in this herd, where the selection for the milk yield was applied for several years.

Regarding the polymorphism *A80V* in leptin gene, the C allele was more frequent (69 %). This is in agreement with Kulig (2005) who reported higher frequency of this variant in Polish Black-and-White population and showed that the CC genotype of *A80V* leptin polymorphism is associated with higher milk yield. The smaller difference between the allele frequencies in the Polish Black-and-White population was presented by Szyda et al. (2007).

The combination of leptin polymorphisms (*R25C/A80V*) was investigated. All combinations were present except for *CT/TT*, and the most frequent combinations were *CT/CT* and *CT/CC*.

*The effect of DGAT1 polymorphism*

Due to low number of the *KK* homozygotes (4 animals) only the *AA* homozygotes and heterozygotes are compared and discussed. There was a statistically significant ($P<0.05$) difference between the *AA* homozygotes and heterozygotes in fat content (3.52 vs 3.82 %) and non-significant difference between those genotypes regarding the protein content (3.11 vs. 3.17 %). The *K* allele was associated with higher fat and protein content. The *DGAT1* allele substitution effect (represented as regression coefficient) was determined by regression of number of *K* allele copies. According to Table 3, changing one copy of the *A* allele by the *K* allele leads to significant ($P<0.01$) increase of fat content by 0.31 %, non significant increase of fat yield, non significant decrease of the milk yield, protein yield and decrease of age at first calving by 19 days. The association of the *K* allele with higher fat and protein content was presented by several authors (Kadlecová et al., 2014; Signorelli et al., 2009; Thaller et al., 2003). These findings are in agreement with general knowledge of negative correlation of milk yield and content of fat and protein also tested in our study. The trend of decreasing age at first calving may be explained by higher level of fatness, which is one of criterions for entering the mating. Concerning the reproduction, Oikonomou et al. (2008) presented results showing the *A* allele of this polymorphism to be associated with more inseminations required per conception, reduced conception rate during lactation and increased incidence of reproductive problems.

*The effect of LEP polymorphisms*

Due to location of two polymorphisms in the gene (exon2 and exon3, respectively) we tested their linkage. Absolute linkage disequilibrium value was -0.073 and normalized value 0.48. These values suggest that although two loci are not in linkage equilibrium (random combination of alleles) some information may be gained by using both of two polymorphisms.

The effect of the *R25C* leptin genotype was not statistically significant in any of the models. There were trends shown in fat content and protein content, where least-square means were higher in *TT* homozygotes in comparison with CC homozygotes and heterozygotes. The pattern of fat yield and protein yield followed the pattern of milk yield, where least-square means of heterozygotes were lower than those of CC and *TT* homozygotes. According to Table 3 changing one copy of C allele by *T* allele leads to increase of fat content by 0.07 % ($P=0.08$) and protein content by 0.04 %. Substitution effect of *T* allele also shows that the one copy of this allele variant increases the fat yield by 5.5 kg, protein yield by 3.3 kg and decreases milk yield by 43.8 kg

Table 2. The genotype and allele frequencies of *DGAT1* and *LEP* polymorphisms, observed ($H_o$) and expected ($H_e$) heterozygosity, *Chi* and *p* values

| Polymorphism | Genotype frequencies | | | Allele frequencies | | *Ho* | *He* | *Chi* | *p* value |
|---|---|---|---|---|---|---|---|---|---|
| *DGAT1* [*K232A*] | *AA* | *KA* | *KK* | *A* | *K* | | | | |
| | 0.78 | 0.20 | 0.02 | 0.88 | 0.12 | 0.20 | 0.21 | 1.302 | $p>0.05$ |
| *LEP* [*R25C*] | CC | *CT* | *TT* | C | *T* | | | | |
| | 0.28 | 0.48 | 0.24 | 0.52 | 0.48 | 0.48 | 0.50 | 0.274 | $p>0.05$ |
| *LEP* [*A80V*] | CC | *CT* | *TT* | C | *T* | | | | |
| | 0.48 | 0.43 | 0.09 | 0.69 | 0.31 | 0.43 | 0.43 | 0.012 | $p>0.05$ |

Table 3. The DGAT1, LEP R25C, LEP A80V genotype effects and allele substitution effects on milk traits and age at first calving

| Trait | LS means (*DGAT1*) | | | Substitution effect of *K* allele | LS means (*LEP R25C*) | | | Substitution effect of *T* allele | LS means (*LEP A80V*) | | | Substitution effect of *T* allele |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | AA | KA | KK | | CC | CT | TT | | CC | CT | TT | |
| Yield (kg) | | | | | | | | | | | | |
| Milk | 11135 | 10832 | 10837 | -259 | 11387 | 10781 | 11284 | -43.8 | 11355 | 10762 | 10947 | -341 |
| Milk fat | 392.0 | 411.5 | 446.3 | 21.6 | 405.9 | 387.4 | 416.4 | 5.5 | 407.5 | 387.3 | 418.3 | -3.7 |
| Milk protein | 346.4 | 342.8 | 342.7 | -3.1 | 349.7 | 338.7 | 355.9 | 3.3 | 354.3 | 335.6 | 351.5 | -7.5 |
| Composition (%) | | | | | | | | | | | | |
| Fat | 3.54a | 3.83b | 4.23a,b | 0.31++ | 3.60 | 3.61 | 3.73 | 0.07 | 3.63 | 3.60 | 3.90 | 0.07 |
| Protein | 3.12 | 3.17 | 3.19 | 0.05 | 3.09 | 3.15 | 3.17 | 0.04 | 3.13 | 3.12 | 3.23 | 0.03 |
| Age (days) | 791.7 | 766.8 | 786.5 | -18.7 | 789.3 | 784.7 | 783.6 | -2.9 | 787.5 | 787.7 | 755.4 | -10.3 |

Age= age at first calving
++P<0.01
a,b - LS means with different letters differed significantly (P<0.05)

and age at first calving by 3 days. Buchanan et al. (2003) reported that the *T* allele is not significantly associated with fat yield for whole lactation but associated with increased overall protein yield. Our study also shows the decreasing trend of age at first calving when comparing CC and *TT* homozygotes. This can be explained by possible *T* allele association with increased body fat reserves (Buchanan et al., 2002) and selection of heifers entering the mating based on fatness.

No significant effect of *A80V* leptin genotype was observed (Table 3). When comparing the least-square means of fat content and protein content, the higher levels can be found in group of *TT* homozygotes compared to the CC homozygotes. On the other hand, the yield variables (except for fat yield) were higher in the CC homozygotes. The similar results for yield traits were presented by Kulig (2005) in Polish Black-and-White population. Szyda et al. (2007) also reported higher protein yield associated with the C allele. Madeja et al. (2004) pointed out that the substitution of alanine by valine should not affect the protein structure or binding to its receptor, and therefore, this polymorphism should not directly influence production traits, but may be linked to other milk production QTL.

The effect of combination was not significant in any of the models, however, there was a decreasing trend of the age at first calving and increasing trend of the content traits from CC homozygotes to *TT* homozygotes for both polymorphisms showed (results not shown here). However due to low number of *TT/CT* and *TT/TT* genotypes comparison of these genotypes was not reliable. Individual or joint effect of several SNPs in leptin gene was reported in several studies (Banos et al., 2008; Clempson et al., 2011) however no one have studied this combination of polymorphisms. Clempson et al. (2011) reported *R25*C to be in linkage disequilibrium with other SNP located in the leptin promoter region (*UASMS1*), which was not significantly associated with milk production, but associated with some reproductive traits (days to conception, calving interval).

## Conclusion

The results showed that the selection of animals for increased milk production lowered the frequency of alleles associated with the fat and protein content in milk. The analysis of the genotype effect suggested possible association of the *DGAT1* polymorphism with the age at first calving, if the fatness of heifers selected for mating is a criterion. Regarding the *LEP* polymorphisms, the results showed non-significant effect of the combination of two SNPs, however, more animals are needed for proving and enumerating the effect of single genotypes.

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## *Utjecaj polimorfizma DGAT1 i LEP gena na osobine mlijeka holštajnskih prvotelki*

**Sažetak**

Geni koji kodiraju diacilglicerol O-aciltransferazu (DGAT1) i leptin (LEP) su funkcionalni geni za mliječne osobine krava. U nekoliko istraživanja povezani su polimorfizmi na pojedinačnim nukleotidima (SNP) tih gena sa sastavom i koncentracijom masti i proteina u mlijeku, količinom mlijeka, te nekim reproduktivnim svojstvima. U ovom su radu istraživani SNPs u tim genima (DGAT1-K232A, LEP-R25C, LEP-A80V). Ukupno je genotipizirano 163 krava prvotelki s jedne farme. Istraženi su sljedeći parametri: količina mlijeka, koncentracija i sastav masti i proteina, starost (dob) nakon prvog teljenja, frekvencije alela i genotipovi. Utjecaj genotipa na osobine mlijeka i dob kod prvog teljenja analizirani su linearnim modelom. Dokazana je pretpostavka različitih alelnih frekvencija u DGAT1 i LEP-A80V što je posljedica selekcije na količinu mlijeka. Signifikantan utjecaj ($P<0,01$) DGAT1 polimorfizma je utvrđen samo za sastav masti. Nije utvrđen signifikantan utjecaj pojedinačnih niti kombiniranih LEP polimorfizama na osobine mliječnosti, niti dob kod prvog teljenja. Postoje indikacije da DGAT1 gen utječe na dob prvog teljenja.

*Ključne riječi*: mliječne krave, osobine mlijeka, polimorfizam, leptin, diacilglicerol aciltransferaza

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