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Effect of the *GDF9* gene on the weight of lambs at birth

Lyubov Getmantseva[1*], Nekruz Bakoev[1], Nadezhda Shirokova[2], Maria Kolosova[2], Siroj Bakoev[1], Anatoly Kolosov[2], Aleksander Usatov[3], Varvara Shevtsova[3], Yuri Kolosov[2]

[1]*L. K. Ernst Federal Science Center for Animal Husbandry, Russia*
[2]*Don State Agrarian University, Persianovsky, Rostov region, Russia*
[3]*Southern Federal University, Rostov-on-Don, Russia*
**Corresponding author:* ilonaluba@mail.ru

Abstract

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The role of the *GDF9* gene in the process of folliculogenesis, oocyte normal maturation and the development of the embryo promoted an investigation of its polymorphism and testing it as a genetic marker of farm animals' reproductive indices. The purpose of this paper was to study the influence of the SNP effect of the *GDF9* gene in point G1 (G260A) on the weight of lambs at birth in Volgograd sheep. The objects of research were 117 sheep of the Volgograd breed, Russia. The results of the first and second lambing were taken into account to determine the weight of lambs at birth. The analysis was carried out by PCR-RFLP method. A mixed linear model was used to quantify the effect of different genotypes of the *GDF9* gene on the weight of lambs at birth. A set of factors included in the model was determined by using one-factorial (ANOVA) and multifactorial (MANOVA) variance analysis**.** The multifactorial variance analysis allowed establishing a high statistical significance of the SNP effect of the *GDF9* gene on weight of lambs at birth. As a result, it was found that weight of lamb at birth in ewes of the AG genotype was 0.156 kg larger than in ewes of the GG genotype. Further studies aimed at studying the *GDF9* gene in sheep of different breeds will allow revealing peculiar influence of different allelic variants on the productive qualities of sheep.

Keywords: SNP effect; GDF9; sheep; weight of lamb at birth; mixed linear model

Introduction

Today in the world there are about 1200 sheep breeds varying in economic features (Kaczor, 2017). At lambing sheep usually bring one or two lambs. However, there are more productive breeds such as Inter alia Cambridge, Thoka, Javanese, Belclare, Lacaune, Woodland, Booroola, Aragonesa, Romney (Inverdale and Hanna), Garole (Bengal), Belle-ile, small-tailed Han, Hu and Kendrapada, whose litter size ranges from three to six lambs (Davis et al., 1982; Juengel et al., 2013).

Studying fertility genes in various breeds is of great importance for increasing the profitability of the sheep industry. Research in this area will provide a better understanding of fertility and infertility process in mammals and thereby to prevent genetic disorders associated with reproduction. Genetically determined differences in the number of maturing and ovulating follicles in sheep have been investigated since 1982, when an attempt was made to explain the genetic foundations of nest multiple lambing in different sheep breeds (Davis et al., 1982). It has been shown that sheep fertility depending on breed may be determined either by a polygenic or a major segregating gene named the Fec gene.

The folliculogenesis and oogenesis process depends on the relationship between the oocyte and the surrounding somatic cells (Huang and Wells, 2010). In the folliculogenesis pro-

cess the oocytes secrete the factors necessary for the growth of surrounding follicular cells and regulate differentiation, proliferation, apoptosis and luteinization of granulosa and cumulus cells. One of these factors is the differential growth factor 9 (GDF9), produced by the oocyte throughout the time of the primary follicle formation to ovulation. GDF9 plays an important role in the development of folliculogenesis in mammals due to its importance for a normal maturation of the oocyte and further development of the embryo, while in the absence of this factor embryos stop developing until they reach the blastocyst stage (Sudiman et al., 2014). In sheep the sequence of the *GDF9* gene (Gene ID: 100217402) comprises 2500 bp and contains two exons and one intron. The gene protein product is represented by 456 amino acids. The first exon comprises 397 bp encoding 134 amino acids, the second exon comprises 968 bp encoding 322 amino acids. The role of the *GDF9* gene in the process of folliculogenesis, oocyte normal maturation and the development of the embryo promoted an investigation of its polymorphism and testing it as a genetic marker of farm animals' reproductive indices. Bodensteiner et al. (1999) were the first to establish the *GDF9* gene expression in sheep oocytes. In studies of Hanrahan et al. (2004) there were 8 polymorphic points of the *GDF9* gene (G1-G8) represented. Three mutations from eight do not lead to a change in the amino acid sequence (G2, G3 and G5). Five remaining nucleotide substitutions (G1, G4, G6, G7 and G8) result in amino acid substitutions (Hanrahan et al., 2004). As a result of studies made by Barzegari et al. (2010) it was found that the presence of G1 mutation was also confirmed in Iranian Moghani and Ghezel breeds. Sheep with heterozygous genotype were more prolific, and double litters were more frequent (53.8%), while in homozygote sheep the proportion of double litters was small (6.3%). Previous studies of the Volgograd and Salsk sheep showed the presence of the *GDF9* gene polymorphism at the G1 (G260A) point and its association with litter size. It was found that the largest number of lambs was observed in ewes of AG genotype (Gorlov et al., 2018).

Together with litter size the weight of lambs at birth is an important breeding trait. In this regard the purpose of this paper was to study the influence of the SNP effect of the *GDF9* gene in point G1 (G260A) on the weight of lambs at birth in Volgograd sheep.

Materials and Methods

Experiment material

The objects of research were 117 sheeps of the Volgograd breed, Russia. The results of the first and second lambing were taken into account to determine the weight of lambs at birth.

PCR-RFLP analysis

Genomic DNA was isolated from small pieces (1–10 mm^2) of ear tissue using a kit Diatom DNA Prep100 according to the manufacturer's instructions (Isogene Lab Ltd, Russia). The analysis was carried out by PCR-RFLP method (polymerase chain reaction-restriction fragment length polymorphism). Special oligonucleotide primers proposed by Hanrahan et al. (2004) were used to amplify the *GDF9* (G1) gene: 5'-GAAGACTGGTATGGGGAAATG -3' and 5'-CCAATCTGCTCCTACACACT -3'. PCR conditions: initial denaturation - 2 min at 94°C; denaturation 94˚C – 30 s, annealing 63°C – 40 s, elongation 72˚C - 30 s (35 cycles), final elongation at 72˚C 4 min. The *GDF9* gene fragment restriction with 462 bp length was performed using BstHH1 endonuclease. The restriction fragments were separated in a 2% agarose gel with adding ethidium bromide.

Statistical analysis

A mixed linear model was used to quantify the effect of different genotypes of the *GDF9* gene on the weight of lambs at birth (Getmantseva et al., 2017). A set of factors included in the model was determined by using one-factorial (ANOVA) and multifactorial (MANOVA) variance analysis**.** The following factors were considered as potential factors of the model:

a) Fixed effects: the sex of the lamb (male, female), litter size (single litter, twins litter), lambing number (first, second), the *GDF9* gene genotype (AG, GG). All factors are classified as discrete with a fixed set of levels.

b) Random effects: the additive genotype of the mother.

Calculation of the mixed linear model parameters was performed using the R language lme package in the R-studio.

Results

The results of molecular genetic studies showed that the polymorphism of the *GDF9* at the G1 (G260A) point in the population under study is represented by two alleles A and G with frequencies 0.04 and 0.96 respectively and two genotypes AG and GG with frequencies 8.55 and 91.45% respectively. The homozygous AA genotype in the sample was absent.

As a result of studying the Volgograd sheep it was found that an average weight of the AG genotype ewes' lambs at the first lambing was 3.62 kg. At the second lambing the weight of lambs in single litters averaged 3.32 kg, and in twins litters - 2.71 kg (Table 1).

Lambs of GG genotype ewes had the weight of 3.48 kg at first lambing. At the second lambing in single litters the weight of lambs was 3.38 kg, and in twins litters - 2.53 kg

Table 1. Weight of lambs at birth in ewes with AG genotype of *GDF9* gene

Genotype of *GDF9* gene in ewes	Weight of lambs at birth					
	Single-litter			Twins-litter		
	Female	Male	All	Female	Male	All
First lambing						
AG	3.54 ± 0.07	3.73 ± 0.09	3.62 ± 0.06	-	-	-
GG	3.37 ± 0.03	3.59 ± 0.03	3.48 ± 0.03	-	-	-
Second lambing						
AG	3.20 ± 0.15	3.50 ± 0.20	3.32 ± 0.13	2.56 ± 0.20	2.86 6 ± 0.17	2.71 ± 0.14
GG	3.24 ± 0.07	3.48 ± 0.04	3.38 ± 0.04	2.35 ± 0.07	2.67 ± 0.06	2.53 ± 0.05

(Table 1).The results obtained showed that the AG genotype of the *GDF9* gene is associated with a better weight of lambs at birth. To evaluate the reliability of the findings a mixed linear model was used.

Figures 1-3 show the distribution of the weight of lambs at birth depending on the levels of fixed factors. The influence significance of the factors before incorporating them into the mixed model was determined by means of the variance analysis. The

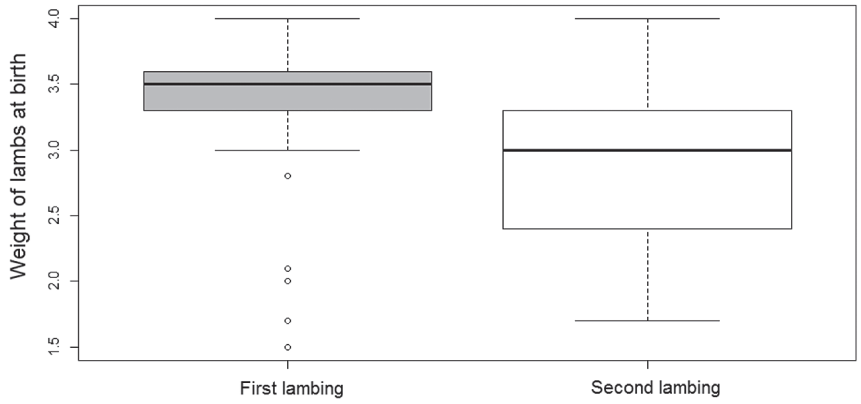

Fig. 1. Distribution of weight of lambs at birth depending on lambing number

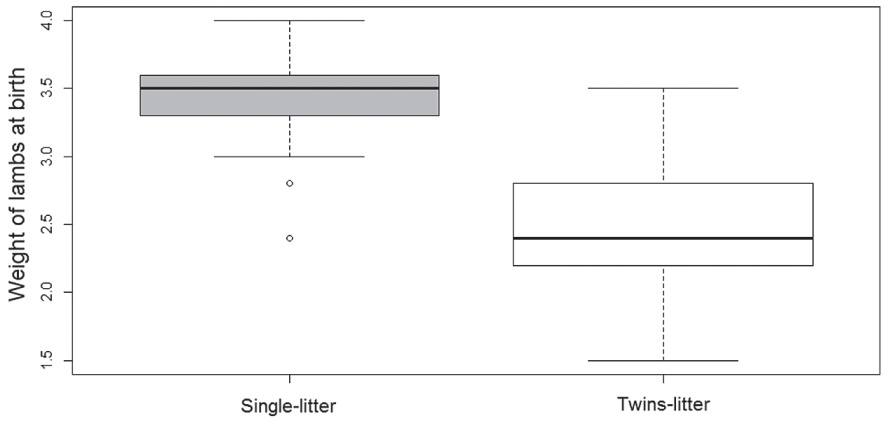

Fig. 2. Distribution of f weight of lambs at birth depending on the litter size (Single-litter/Twins-litter)

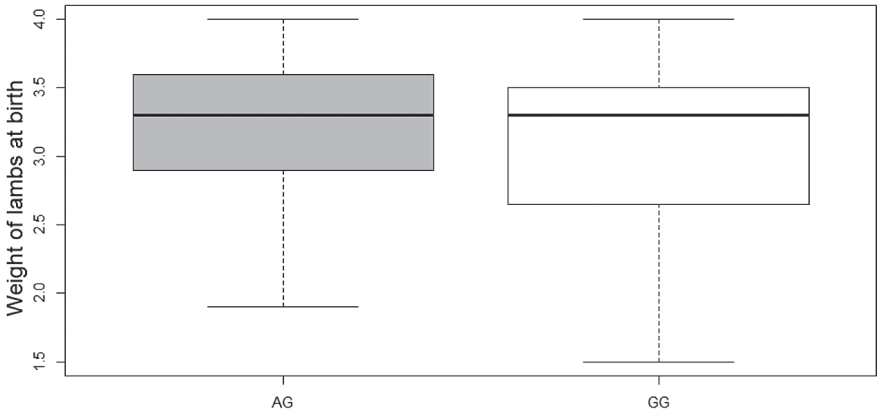

Fig. 3. Distribution of weight of lambs at birth depending on the genotype of ewes by the *GDF9* gene

differential evaluation by the single-factor variance analysis showed a high statistical significance of the factors' influence with the exception of the SNP effect of the *GDF9* gene: lamb sex (male lamb / female lamb); litter size (single litter / twins litter); lambing number; mother additive genotype. However, a multivariate variance analysis of all factors in total allowed to establish that in the given model the factor of lambing number does not significantly affect the analyzed feature, while the SNP-effect of the *GDF9* gene in ewes it is authentically associated with the weight of lambs at birth (Table 3). Visual analysis of primary data made it possible to establish the cause of this discrepancy. At the first lambing there were only single litters, while at the second lambing there were 44% twin litters. Obviously, the litter size negatively correlates with the weight lamb at birth. Thus, the evaluation of the effect of lambing number in the weight lamb at birth model without considering the number of lambs would show a decrease in the weight of lambs at birth**.** In fact, the observed decrease in weight of lambs at birth is due to an increase in other important indicators – such as litter size (single litter or twins litter) and weight of litter. Including the factor of litter size in the weight of lambs at birth model allowed establishing the absence of influence of lambing number on this indicator. Taking into account the data in Table 2 and the justifications given, the mixed linear model of the weight of lambs at birth was compiled as follows:

$$WLB \sim Sex + Litter\ size + GDF9 + (1 \mid Mother) \quad (1)$$

where WLB – the weight of lambs at birth; Sex – sex of the lamb (male, female), Litter sizes - (single litter, twins litter), GDF9 - the *GDF9* gene genotype (AG, GG).

In our studies the weight of female lamb from single litter of the mothers with genotype AG was determined as a baseline in the mixed linear model. The results presented in Table 3 showed that the weight of lambs at birth in ewes of the AG genotype was 0.156 kg larger than in ewes of the GG genotype. It has also been established that male-lamb weight is 0.26 kg larger than female-lamb weight on average, and twin lambs on average are 0.98 kg less in weight than their single-born analogues.

Table 2. Assessment of the significance of the differences between the levels of factors affecting the weight of lambs at birth

Factor	P-value (ANOVA)	P-value (MANOVA)
Sex of lamb	0.0004*	0.0000*
Litter size	0.0000*	0.0000*
Lambing number	0.0000*	0.8600
GDF9 gene genotype	0.3940	0.0170*
Additive genotype of the mother	0.0003*	0.0001*

* - statistically significant

Table 3. Weight of lambs at birth with allowance for fixed factors of the mixed linear model

Fixed factors	Level of factor	Weight of lambs at birth	P-value
Sex of lamb	Female	3.4635±0.0770	0.0000*
	Male	3.7201 ± 0.0393	
Litter sizes	Single-litter	3.4635±0.0770	0.0000*
	Twins-litter	2.4815 ± 0.0411	
GDF9 gene genotype	AG	3.4635±0.0770	0.0387*
	GG	3.3075 ± 0.0765	

* - statistically significant

Discussion

Thus, the obtained results showed the presence of a positive SNP-effect of the *GDF9* gene on weight of lambs at birth. However, it should be noted that the homozygous AA genotype was

not established in the population under study and the frequency of the favorable AG genotype was 8.55%. Similar results were obtained by other scientists who investigated polymorphism of the *GDF9* gene in sheep of different breeds. Studies conducted on Salsk sheep also showed the lack of AA genotype and frequencies of genotypes AG and GG were 10 and 90%, respectively (Kolosov et al., 2015). The study of the Iranian Baluchi sheep conducted by Moradband et al. (2011) also showed the lack of the homozygous AA genotype by the *GDF9* gene. Eghbalsaied et al. (2017) studied the polymorphism of the *GDF9* (G1) gene in the Shal, Ghezel, Afshari, Lori-Bakhtyari sheep and found out a high frequency of the GG genotype, which was 85.7%, 73.7%, 85.4% and 88.2% respectively. However, the Shal, Ghezel and Afshari breeds demonstrated the AA genotype with a frequency of 6.1%, 1.7% and 3.6% respectively. It should be noted that the AA genotype was found in these breeds only in ewes, with the AA genotype being absent in rams.

Nicol et al. (2009) suggested that a mutation in the *GDF9* gene increases the rate of ovulation in animals with heterozygous genotype, but in two of the four mutations the development of follicles is disrupted in homozygous ewes leading to infertility. Ovarian insufficiency is caused by blocking follicular growth at the early development stage. The phenotype manifestation may depend on other conjugated alleles or interaction between multiple mutations, which may be the reason that not all breeds manifest this affect (Våge et al., 2013). In our study the low frequency of the desired genotype AG in the Volgograd sheep may be related both to the negative influence of the AA genotype on the fertility of the animals and to the fact that the Volgograd sheep breeding was not targeted at increasing fertility. Further studies aimed at studying the *GDF9* gene in sheep of different breeds will allow revealing peculiar influence of different allelic variants on the productive qualities of sheep.

In conclusion, multifactorial variance analysis allowed establishing a high statistical significance of the SNP effect of the *GDF9* gene on the weight of lambs at birth. As a result, it was found that weight of lambs at birth in ewes of the AG genotype was 0.156 kg larger than in ewes of the GG genotype.

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