Turkish Journal of Veterinary & Animal Sciences

Volume 49 | Number 4 Article 2

8-14-2025

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ŞEYMA KÖKKAYA

FADİME DALDABAN

DAVUT BAYRAM

MEHMET ULAŞ ÇINAR

KORHAN ARSLAN

See next page for additional authors

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KÖKKAYA, Ş, DALDABAN, F, BAYRAM, D, ÇINAR, M, ARSLAN, K, & AKYÜZ, B (2025). An investigation of the association of GDF9-c.260G>A polymorphism with lamb live weight in the Akkaraman breed. *Turkish Journal of Veterinary & Animal Sciences 49* (4): 144-150. https://doi.org/10.55730/1300-0128.4383



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Authors
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This research article is available in Turkish Journal of Veterinary & Animal Sciences: https://journals.tubitak.gov.tr/veterinary/vol49/iss4/2

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Turkish Journal of Veterinary and Animal Sciences

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Research Article

Turk J Vet Anim Sci
(2025) 49: 144-150
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doi:10.55730/1300-0128.4383

An investigation of the association of *GDF9*-c.260G>A polymorphism with lamb live weight in the Akkaraman breed

Şeyma KÖKKAYA[1,*], Fadime DALDABAN[1], Davut BAYRAM[2], Mehmet Ulaş ÇINAR[3], Korhan ARSLAN[1], Bilal AKYÜZ[1]

[1]Department of Genetics, Faculty of Veterinary Medicine, Erciyes University, Kayseri, Turkiye
[2]Department of Animal Science, Faculty of Veterinary Medicine, Erciyes University, Kayseri, Turkiye
[3]Department of Animal Science, Faculty of Agriculture, Erciyes University, Kayseri, Turkiye

Received: 17.05.2024 • **Accepted/Published Online:** 03.05.2025 • **Final Version:** 15.08.2025

Abstract: In this study, the relationship between the Growth Differentiation Factor-9 (*GDF9*) gene polymorphism and birth weight, as well as weights on days 30, 60, 90, was investigated in Akkaraman lambs, one of the indigenous sheep breeds of Türkiye. For this purpose, a total of 314 lambs were genotyped by PCR-RFLP method for the single nucleotide polymorphism (SNP) c.260G>A in the *GDF9* gene. While GG and GA genotypes were observed in the studied breed, the AA genotype was not observed. The results showed that Akkaraman lambs were in Hardy–Weinberg (H–W) equilibrium for the *GDF9*-c.260G>A polymorphism ($p < 0.05$). In this study, a significant association was observed between *GDF9*-HhaI polymorphism and 30th day weight and 60th day weight in Akkaraman lambs. When the animals were analyzed according to birth type, it was observed that this factor was associated with birth weight, as well as weights on days 30, 60, and 90. It is suggested that the c.260G>A coded SNP of the *GDF9* gene has potential use in increasing lamb live weight from birth to weaning.

Key words: Lamb, growth differentiation factor-9 gene, live weight, Akkaraman

1. Introduction

Daily dietary protein intake levels are important for growth, development and, biological functions in humans [1]. Animal-derived foodstuffs are the primary source of protein for humans [2]. As recommended by the World Health Organization (WHO), the daily protein intake of a healthy individual should be 0.8 g per kg live weight, and 42% of the daily protein intake should be of animal origin. However, meeting the demand for animal protein is becoming increasingly difficult due to the rapidly growing world population [3]. Red meat is one of the most popular animal protein sources [4]. In Türkiye, all red meat comes from cattle and small ruminants [5].

The climate and geographical conditions of Türkiye, as well as the species distribution of the country, make sheep meat a good alternative to bovine meat. Being rich in digestible essential amino acids, sheep meat is described as a good protein source and is also known to be rich in micronutrients (iron, zinc, selenium, and vitamins B3, B6 and particularly B12) [6]. Sheep are highly valuable livestock as they require less feed and water than cattle, readily adapt to less favorable climatic and environmental conditions and are fit for extensive breeding [7].

In Türkiye, sheep are mostly bred for meat production [8]. Thus, the country ranks among the global leaders in sheep meat production [9]. According to the Food and Agriculture Organization (FAO), the sheep population of Türkiye is approximately 46 million (FAO, 2021). Nearly half (45%) of Türkiye's sheep population consists of the Akkaraman breed, which is disease-resistant, high-yielding, fat-tailed, and well-adapted to diverse climates and poor pasture conditions [10,11]. As the global population increases, meat consumption is also increasing. Therefore, improving sheep breeding programs and increasing productivity is of great importance [12].

Despite having enabled significant genetic advancements in livestock, conventional breeding methods have achieved only limited success in improving traits that are controlled by additive genes and influenced by environmental factors. To address this limitation, marker-assisted selection using genes that influence trait expression has been suggested as an effective method for estimating the genetic value of animals [13]. Therefore, the adaptation of genetic selection methods to livestock production systems is highly important for maximizing farm profits [14,15].

* Correspondence: seymakcam@gmail.com

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The Growth Differentiation Factor-9 (*GDF9*) gene is a gene that encodes the synthesis of the GDF9 protein, a growth differentiation factor belonging to the Transforming Growth Factor-Beta (TGF-β) superfamily [14,16]. The ovine *GDF9* gene is of a length of 1126-bp and is composed of two exons and one intron [17]. Several reports indicate that the *GDF9* gene is associated with multiple births in sheep [8,12,18,19]. This gene, known for its role in multiple births, may also be linked to increased body weight due to its involvement in muscle development and cell growth [20].

Despite being a member of the TGF-β subfamily, which is associated with growth traits, the ovine *GDF9* gene has been scarcely investigated for its association with live weight gain, according to literature reports. Hence, this study aimed to investigate the c.260G>A SNP, located in the first intron of the *GDF9* gene and responsible for an arginine-to-histidine amino acid substitution, in relation to live weight gain from birth to weaning in the Akkaraman breed. For this reason, the study also examined the relationship between the *GDF9* gene variant, previously linked to live weight, and weight gain until weaning in twin and single Akkaraman lambs. Thus, this candidate gene associated with phenotypic traits will contribute to the advancement of animal breeding.

2. Materials and methods

2.1. Experimental animals

All data used in this study were obtained from a local farm in the Kayseri region. The study was conducted with approval (dated 13.11.2013, approval no: 13/130) from the Local Ethics Committee for Animal Experiments at Erciyes University. Blood samples were collected in accordance with the same ethics committee approval. A total of 314 male Akkaraman lambs were evaluated. The lambs, all male, were born to 2-year-old ewes. Live weights of lambs were measured at birth, 30 days, 60 days, and 90 days. DNA was extracted from jugular vein blood collected in 10 mL EDTA-coated vacutainers. DNA isolation from the whole blood samples was performed using the phenol-chloroform-isoamyl alcohol (25:24:1) method [21].

2.2. Primer synthesis and PCR–RFLP reactions

The final volume of the PCR mixture was 20 µL and contained 2.5 mM $MgCl_2$, 50 µM dNTPs, 0.2 µM primer, 1.25 U Taq DNA polymerase, and 50 ng/µL DNA. After undergoing an initial denaturation at 95 °C for 5 min, the PCR products were amplified by 35 cycles of 45 s at 94 °C, 40 s at 58 °C and 1 min at 72 °C. The last cycle was followed by a final extension at 72 °C for 10 min. After PCR, the 462 bp products were digested with the *Hha*I endonuclease at 37 °C for 16 h (Table 1). Genotypes were determined using 2% agarose gel electrophoresis. Based on the agarose gel results, animals were genotyped as **GG** (254, 156, 52) or **GA** (410, 254,156, 52).

The primers were described by Kasiriyan et al. [22].

2.3. Statistical analysis

The phenotypic data of the lambs were checked for normality of distribution using histograms, q-q graphs and the Shapiro–Wilk test. Qualitative data were assessed with the chi-square test. Since all lambs were raised on the same farm under identical environmental conditions, these factors were excluded from the statistical model. In statistical testing, sex is an important factor affecting weight. However, all lambs in the study were male. A one-way ANOVA test was used to analyze the association between weight phenotypes and genotypes, while a two-way ANOVA was used to evaluate the effects of genotype and birth type (twin vs. single). Statistical analyses were performed using the JMP Pro 17 (SAS Institute Inc., Cary, NC, USA) software, and statistical significance was set at $p \leq 0.05$.

3. Results

For *GDF9*-c.260G>A, the 462 bp DNA fragment was successfully amplified by PCR. Then, all PCR products were subjected to RFLP. After digestion of the 462 bp PCR products with the *Hha*I endonuclease, GG (254 bp, 156 bp, 52 bp) and GA (410 bp, 254 bp, 156 bp 52 bp) genotypes were identified in the study population; however, the AA genotype (410 bp, 52 bp) was not detected (Figure; Table 2). Allele frequencies were calculated as 0.92 for G and 0.08 for A. Genotype frequencies were determined as 0.83 for GG and 0.17 for GA. The observed allele and genotype frequencies are presented in Table 2.

The genotype-phenotype association analysis revealed a significant correlation between *GDF9* gene polymorphism and live weights on days 30 and 60 (Table 3).

Table 1. Primer sequencing and genotyping of *GDF9*-c.260G>A polymorphism.

Gene	Primers (forward-reverse)	AT (°C)	PR	Genotype	RE
GDF9	**F:**GAAGACTGGTATGGGGAAATG **R:**CCAATCTGCTCCTACACACCT	58	462 bp	**GG:** 254, 156, 52 **GA:** 410, 254,156, 52 **AA**: 410, 52	*Hha*I

AT: Annealing temperature; RE: Restriction enzyme; PR; Amplicon length

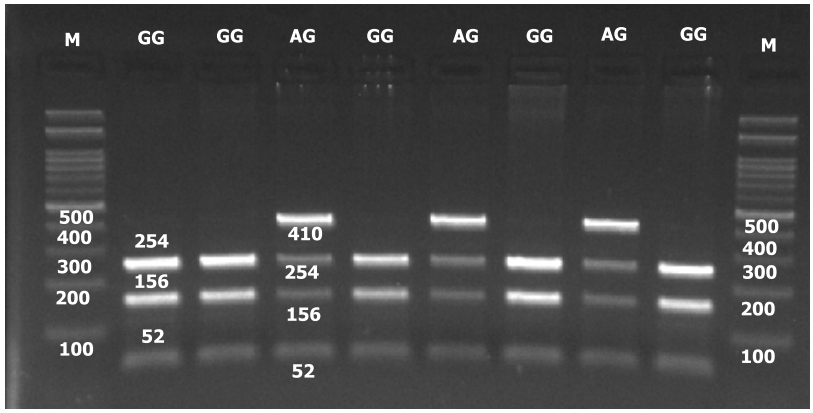


Figure. Gel-electrophoresis of the restriction fragment length polymorphism (RFLP) products of the (c.260G>A) SNP in the *GDF9* gene. Lane M: 100 bp-DNA ladder. GG genotype yielded fragments of 254 bp, 156 bp and 52 bp; GA genotype yielded fragments of 410 bp, 254 bp, 156 bp, and 52 bp.

Table 2. Genotype and allele frequencies and Hardy–Weinberg equilibrium analysis of the *GDF9*- c.260G>A polymorphism in 314 Akkaraman lambs.

Gene	**n**	**Genotype frequency**			**Allele frequency**		**Chi-square (H-W)**
***GDF9* (c.260G>A)**	314	**GG (OG-EG)**	**GA (OG-EG)**	**AA (OG-EG)**	**A**	**G**	$X^2 = 2.45$ p = 0.11
		0.83 (263–265.07)	0.17 (51–46.86)	0 (0–2.07)	0.08	0.92	

n: Number of animals; OG: Observed genotype; EG: Expected genotype; X^2: Chi-square

Table 3. The association of the c.260G>A SNP genotypes with live weight gain in Akkaraman lambs.

Gene	**Genotypes**	**Live weight (LW) measurements, g ($\bar{X} \pm S_{\bar{X}}$)**			
		BW (g)	**LW on day 30 (g)**	**LW on day 60 (g)**	**LW on day 90 (g)**
***GDF9* (c.260G>A)**	**GG**	4600.15±52.89	11447.79±275.71 ^{A}	20621.13±318.34 ^{A}	30624.47±364.91
	GA	4781.57±106.41	13207.16±559.88 ^{B}	22318.72±683.60 ^{B}	31997.11±800.44
p		0.160	**0.009**	**0.031**	0.128

BW: Birth weight; $\bar{X}$: Arithmetic mean; $S_{\bar{X}}$: Standard error

Heterozygous (GA) individuals showed greater live weight gain than homozygous (GG) individuals on days 30 and 60 after birth in Akkaraman lambs carrying the c.260G>A SNP in the *GDF9* gene.

In single lambs, the frequencies of GG and GA genotypes were 0.84 and 0.16, respectively, and the AA genotype was not observed. In twin lambs, the frequencies of GG and GA genotypes were 0.89 and 0.11, respectively; the AA genotype was also not observed (Table 4).

The average birth weights for the GG and GA genotypes, according to birth type, are shown in Table 5. Significant differences in birth weight and weights on days 30, 60, and 90 were observed between twin and singleton lambs when evaluated by birth type. However, when evaluated by allele and by the interaction between birth type and allele, significant differences in live weight were observed (Table 6).

Table 4. Hardy–Weinberg chi-square analysis, observed genotypes, genotype frequencies, and allele frequencies in twin and single lambs.

Birth type	Genotypes	n	Genotype frequency	Allele frequency		Chi-square (H-W)
				G	A	
Single	GG	205	0.84	0.92	0.08	X^2=2.23 p=0.13
	GA	43	0.16			
	AA	0	0			
Twin	GG	58	0.89	0.94	0.06	X^2=0.27 p=0.60
	GA	8	0.11			
	AA	0	0			

n: number of animals; X^2: Chi-square

Table 5. Relationship between *GDF9*-c.260G>A genotypes and live weight in twin and single lambs.

Gene	Birth type	Alleles	Live weight (LW) measurement, g ($\overline{X} \pm S_{\overline{X}}$)			
			BW (g)	30th day LW (g)	60th day LW (g)	90th day LW (g)
GDF-9	**Single**	GG	4723.1220±59.70	11823.29±301	21119.0976±349.73	31237.2098±409.15
		GA	4962.7907±99.10	13590.14±562.04	22726.8837±715.97	32346.2791±852.64
	Twin	GG	4165.5172±94.63	10120.57±631.38	18861.0862±703.74	28458.7759±743.16
		GA	3807.5000±195.24	11148.63±1835.2	20124.8750±1982.8	30120.3750±2265.5

BW: Birth weight; $\overline{X}$: Arithmetic mean; $S_{\overline{X}}$: Standard error

Table 6. Evaluation of the significance of differences between factors affecting lamb birth weight.

	Birth weight	30th day weight	60th day weight	90th day weight
Birth type	**0.000***	**0.022***	**0.020***	**0.037***
Allele	0.720	0.121	0.169	0.248
Birth type / allele	0.071	0.681	0.869	0.818

**: Statistically significant*

4. Discussion and conclusion

Sheep production systems can achieve success and profitability by selecting the breed(s) and type(s) most suited to the prevailing climate and grassland conditions of a given region. In countries like Türkiye, characterized by steppe conditions, low annual rainfall, a large sheep population (reported as 44.5 million heads in 2022 for Türkiye by the Turkish Statistical Institute, TURKSTAT) and a predominantly extensive sheep production pattern, it is not possible to improve the population in its entirety and transit the majority into high-producing breeds through crossbreeding. In fact, crosses such as the Karacabey Merino, Anatolian Merino, Central Anatolian Merino, Sönmez, Tahirova, Ramlıç and Asaf, originating from crossbreeding programs aimed at improving the production yields of the native sheep breeds of Türkiye, have not been well-received by local breeders. Thus, the best option for improving the yield traits of low-producing native sheep breeds is selection. In recent years, the increased use of marker or candidate genes in selection programs has enabled a more rapid genetic advancement. Today, sheep farms in Türkiye almost completely depend on lamb meat production for income, and lamb meat production can be increased either by increasing the

number of slaughter lambs through increased fertility or by increasing the carcass weight per slaughter lamb.

To date, the *GDF9* gene has mostly been studied in relation to twin births and fertility. The *GDF9* gene is also associated with live weight gain. The literature review revealed no study investigating the relationship between the *GDF9* gene and live weight gain until weaning in Türkiye. Live weight gain is an important quantitative trait known to be regulated by multiple genes and influenced by environmental conditions [23]. In this context, the present study aimed to investigate the association between the *GDF9-Hha*I polymorphism and live weight gain from birth to weaning in Akkaraman lambs, the most commonly raised native breed in Türkiye.

Previous studies on the functions of the ovine *GDF9* gene have mostly focused on the investigation of the effects of this gene on fertility [12,18,24–26]. However, unlike previous studies, the present study investigated the relationship between the c.260G>A polymorphism in intron 1 of the *GDF9* gene and live weight gain from birth to weaning in Akkaraman lambs, evaluated by birth type.

In the present study, Akkaraman lambs carrying the c.260G>A polymorphism in the *GDF9* gene were identified as having GG (homozygous wild-type) and GA (heterozygous) genotypes. No individual of the homozygous AA genotype was detected in these breeds. In this study, as in the study by Kırıkçı et al (2023), Akkaraman lambs had a polymorphic structure in the *GDF9* gene [27] Similarly, in a study conducted by Kırıkçı et al. [8] on another native sheep breed of Türkiye, while the AA genotype was reported not to have been detected, the most common genotype was identified as GG (0.79). Moreover, in a study investigating the Volgograd, a cross of Russian origin, only two genotypes, of which GG was the most common (0.92), were reported to have been identified with no indication of the presence of the AA genotype in this particular breed as well [20]. Likewise, the investigation of three native sheep breeds from Egypt showed the absence of the AA genotype and the highest frequency belonging to the GG genotype [28]. Similar results were reported for three different Merino breeds raised in Bulgaria, indicating the AA genotype as absent and the GG genotype as the most common [29]. In a literature report from Greece, the AA genotype was indicated to have been identified in the Chios breed, but not in the Karagouniki, whilst both breeds were characterized by the GG genotype being the most common [30]. Moradband et al. [26] determined the presence of all three genotypes in the local Baluchi sheep, and confirmed the most common genotype as GG (0.72), as was the case in other sheep breeds. The results of both the present study and previous research in various sheep breeds have shown that, in agreement with the report of Moradband et al. [26], for the c.260G>A SNP, the G allele is a wild-type allele. In another study, it was reported that the lamb weight at birth was 0.156 kg higher in sheep with GA genotype than in ewes with GG genotype and there was a statistically significant difference [20].

Amandykova et al. emphasized in their study that the frequency of the AA (mutant) genotype was low and that the low frequency of this genotype could be due to the low frequency of the A allele. As a result, they thought that mutant genotypes could lead to mortality or reproductive failure in the early stages of embryonic development [31]. Since the AA genotype was not observed in any of the 314 lambs examined, this may be attributed to embryonic death or reproductive defects.

Based on the literature review, it appears that the ovine *GDF9* gene has been extensively studied for its association with fertility and multiple births as quantitative traits, and has consistently been shown to be associated with multiple births [8,14,16,17,19,20,25,26,30,32,33]. On the other hand, there are also reports on the association of the *GDF9* gene with various other quantitative traits. In one of these reports by Koyun et al. [34], the *GDF9* gene was indicated to be associated with milk yield and some milk composition traits in the Karakaş, a variety of the Akkaraman breed, and the Norduz. In their research on the Volgograd sheep breed, Getmantseva et al. [20] investigated the association of the c.260G>A SNP of the *GDF9* gene with the birth weight of lambs and determined that lambs of the heterozygous GA genotype had a higher birth weight. Similarly, in the present study, the c.260G>A SNP in the *GDF9* gene was found to be associated with increased live weight gain in Akkaraman lambs on postnatal days 30 and 60, with GA genotype lambs showing higher weights at those time points.

As a quantitative trait affected by genotype and phenotype, growth is a process that starts from birth and continues until the attainment of the adult size. The profitability of animal production depends on livestock showing rapid growth after birth and the timely achievement of the targeted production yields from these rapidly growing animals. The best indicator of growth in farm animals is live weight gain. In sheep production, the income of the farmer depends on the number of lambs that reach the sales age and the live weight of these animals at sale. Therefore, reducing lamb mortality and increasing their live weight at sale would significantly boost farm income. This underscores the need to achieve high live weight gain in lambs until weaning. The findings of both the present study on the Akkaraman breed and Getmantseva et al.'s [20] research on the Volgograd breed suggest that the c.260G>A SNP in the *GDF9* gene may

be beneficial for improving live weight gain in lambs up to weaning. In this context, there is a need for further studies on the association between the *GDF9* gene and the c.260G>A SNP with production traits, using larger sample sizes and other sheep breeds.

In our study, the effect of different allelic variants of the *GDF9* gene on live weight gain in Akkaraman lambs was investigated. The results demonstrated a statistically significant weight gain on days 30 and 60. However, the weight increase on the 90th day was not statistically significant. Furthermore, the difference between twin and single births was not found to have a statistically significant effect in this study. Therefore, it is recommended to increase the number of animals in further studies. Additionally, the relationship between this mutation and live weight in other sheep breeds should be investigated.

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