Research Article

Association of Growth Differentiation Factor 9 (GDF9) Gene with Birth Type, *In vitro* Maturation and Selected Physiological Traits in Awassi Sheep

Hadi Awad Hassooni[1], Safaa Sabbar Atiyah[2*], Alaa S. J. Mohamed[1]

[1]University of Al-Muthanna, Faculty of Agriculture, Department of Animal Production, Al-Muthanna, Iraq; [2]Al-Furat Al-Awsat Technical University, Technical Institute of Kufa, Department of Animal Production Techniques, Kufa, Iraq.

Abstract | One of the most economically important traits in sheep is the type of birth (single or twin). The formation of ovarian follicles and the ovulation rate are influenced by the Growth Differentiation Factor 9 (GDF9) gene. This study was conducted to investigate the relationship between the GDF9 gene polymorphism and birth type (single vs. twin), as well as its association with *in vitro* maturation (IVM) of oocytes with and without cumulus cells across atretic, immature, and mature stages. The study also examined some physiological traits of Awassi sheep and assessed the potential for early genetic selection based on these traits. Sequencing analysis of a 634 base pair (bp) segment of the GDF9 gene revealed a single nucleotide polymorphism (SNP), where guanine (G) was replaced with adenine (A) at position 205. Significant differences ($P \leq 0.05$) were observed in the percentage of immature oocytes lacking cumulus-oocyte complex (COC) cells among animals with different genotypes (GG, GA, and AA) at this SNP site. Specifically, animals with the GA genotype associated with twin births had a higher percentage of immature oocytes without COC cells compared to those with the GA genotype linked to single births. However, no significant differences ($P \geq 0.05$) were found in the percentages of atretic and immature oocytes without COC cells among the three genotypes (GG, GA, AA). Additionally, no significant differences ($P \geq 0.05$) were observed in the *in vitro* maturation rates of oocytes with cumulus cells across atretic, immature, and mature stages among the different GDF9 genotypes. Hormonal analysis showed no significant differences ($P \geq 0.05$) in LH and FSH levels among animals carrying the AA, GA, and GG genotypes. The study concluded that GDF9 and litter size in Awassi ewes can be utilized as biological indicators of ovulation or to increase fertility.

Keywords | *In vitro* maturation, GDF9 gene, Oocytes, Litter size, Awassi sheep

Received | December 17, 2024; **Accepted** | May 26, 2025; **Published** | July 03, 2025
***Correspondence** | Safaa Sabbar Atiyah, Al-Furat Al-Awsat Technical University, Technical Institute of Kufa, Department of Animal Production Techniques, Kufa, Iraq; **Email:** safaa.sabbar.iku@atu.edu.iq
Citation | Hassooni HA, Atiyah SS, Mohamed ASJ (2025). Association of growth differentiation factor 9 (GDF9) gene with birth type, *In vitro* maturation and selected physiological traits in awassi sheep. J. Anim. Health Prod. 13(3): 617-623.
DOI | https://dx.doi.org/10.17582/journal.jahp/2025/13.3.617.623
ISSN (Online) | 2308-2801

INTRODUCTION

Awassi sheep are the most widespread breed in Iraq and represent more than half of the local sheep breeds. Selecting genetic variety and key genes for genetic improvement is made possible by incredible developments in statistics and molecular biology. Selecting individuals with the finest phenotypic appearance is the basis of traditional

genetic improvement of farm animals. In the area of genetic manipulation, it has also attained good commercial viability. Since economic features are influenced by the dominance of several genetic sites, sometimes referred to as quantitative trait sites, scientific advancements have also contributed to a better knowledge of how the genome functions, allowing for the creation of more precise selection programs with less effort and expense. by choosing these linked sites (Al-Dabbagh, 2019).

One of the most crucial economic characteristics is litter size, which has a direct bearing on the economic benefits from contemporary sheep husbandry. In order to improve litter size using marker-assisted breeding, it can be helpful to investigate the main genes influencing litter size. The GDF9 gene, which encodes a member of the transforming growth factor-β (TGF-β) family (Al-Mutar and Younis, 2020), has important functions in ewe reproduction (Vage *et al*., 2013). The gene is located on chromosome 5 of sheep (Tang *et al*., 2018) and has two exons separated by an intron of 1126 bases. GDF9 is mostly expressed in oocytes and is crucial for sheep follicle growth and ovulation. (Mullen and Hanrahan, 2014). In previous studies, several mutations in the gene were identified in Belclare sheep in Europe (Eghbalsaied *et al*., 2012), Afshari sheep (G2, G3, and G4) (Abdoli *et al*., 2018), fat tailed sheep (G1, G2, G3, and G4) (Saleh *et al*., 2020), and FecG H in Rahmani sheep in Africa (Bravo *et al*., 2016). It was reported that c.978A>G, c.994G>A, FecG H, and c.1040T>C (Phe 347 Ser) were associated with birth size in Creole Araucana, Rahmani, and Mongolian sheep, respectively (Tong *et al*., 2020). The present study aims to clarify the association between GDF9 gene polymorphisms and type of parturition, as well as their relationship with *in vitro* maturation of Awassi sheep oocytes.

MATERIALS AND METHODS

The experimental design of the current study was in in line with the international animal ethical protocols. Blood samples were collected from ewes immediately before slaughter. Blood samples were collected from the jugular vein using sterile 10 ml medical syringes after thoroughly cleaning the puncture site. A total of 8 ml of blood was collected from each animal. The samples were then divided into two portions. The first portion, comprising 3 ml, was placed into tubes containing an anticoagulant and stored in a refrigerator until DNA extraction was carried out. The second portion, consisting of 5 ml, was transferred into gel tubes without anticoagulant to allow clotting and facilitate serum separation. These tubes were centrifuged at 3000 rpm for 10 minutes. The resulting serum was carefully extracted using sterile syringes, transferred to new sterile test tubes, and stored at −15 °C for subsequent hormone analysis.

Collection of Oocytes

Oocytes were extracted from the ovaries by aspiration, which involved removing the follicular fluid from the large and medium-sized follicles on the ovary's surface using an 18 mm medical syringe, Since the diameter of the needle influences the flow of sheep oocytes during aspiration and a medical syringe filled with 0.5 ml of RBMI-1640 culture medium and 20 IU/ml of heparin, an anticoagulant, to stop the oocytes from adhering to one another. Following extraction, the oocytes were placed in a Petri plate, and a manually adapted Pasteur pipette was used to collect the oocytes under a dissecting microscope. The Petri dish was used to wash the oocytes three times with culture medium to eliminate the remaining cell components affixed to the oocytes, and the oocytes with a atretic and irregular shape, which were characterized by shrinkage and a wide gap between them and the Zp region, or damage to this region were removed.

Oocyte Classification

After isolating the oocytes in a Petri dish and washing them three times in RBMI-1640 culture medium, the oocytes were classified based on their external appearance according to the method of Nogueira *et al*. (2009), in terms of whether they contained layers of germinal aggregate cells or not and whether they were mature or immature, as mature, atretic, and abnormally shaped ones were excluded.

In vitro Maturation of Oocytes

After washing the oocytes three times with RBMI-1640 culture medium supplemented with some hormones (5 IU/mL ECG, 10 IU/mL hCG, and 1µg/ml Estrogen) for the purpose of using them as a medium for oocytes *in vitro* maturation, The oocytes were moved to a four-well petri plate, which was separated into five halves. and 6-4 oocytes were placed equally in each dish containing The usual culture medium, RBMI-1640, was coated with paraffin oil and kept in a CO2% incubator for 24 hours at 38.5°C and 95% relative humidity. To differentiate between mature and immature oocytes, the oocytes were subsequently examined under a microscope.

Statistical Analysis

The data of the current study were analyzed using statistical analysis system (SAS Institute Inc.2018) and analysis of variance (ANOVA). For the analysis, a significance level of $p<0.05$ was used. The Duncan (1955) multinomial test was used to compare the significant differences between the means using the least squares means approach. Mathematical model of the relationship of genetic conformations of the GDF9 gene to the studied traitswas:

$$Yil=\mu + Gi + eil$$

RESULTS AND DISCUSSION

The growth differentiation factor 9 (GDF9) gene is an important gene that affects reproductive traits and is also called FecG (Margawati et al., 2023). The growth differentiation factor gene is a member of the large family of transforming growth factors (TGF-β) (Al-Mutar and Younis, 2020; Elshareef et al., 2023; Hassooni *et al.*, 2024). GDF9 is mainly expressed in oocytes and plays an important role in follicle development and ovulation in sheep. It also has important functions in ewe reproduction, including its favorable effect on ovulation rate and reproduction (Tang *et al.*, 2018; Munoz et al., 2021). Several *in vitro* studies were conducted to determine the effect of the genotypes of the GDF-9 gene on the growth and vitality of ovarian follicles. Mery et al. (2007) took 2348 follicles at different stages of growth from the ovaries of five ewes and cultured them in the laboratory. They found that the speed of development of these follicles was linked to the gene expression of the GDF-9 gene and its receptors.

Electrophoresis was performed using 1% agarose gel as described in the Materials and Methods section. Each sample consisted of 5 µL of DNA mixed with 2 µL of loading dye, and was loaded into the wells of a 1% agarose gel prepared with Diamond™ Nucleic Acid Dye (Promega, USA). The dye was diluted in TE buffer at a ratio of 1:100 (v/v) using a micropipette and applied to the gel. After setting the appropriate voltage, DNA migration was visualized using a UV gel documentation system, and images were captured using a dedicated gel imaging camera (Figure 1).

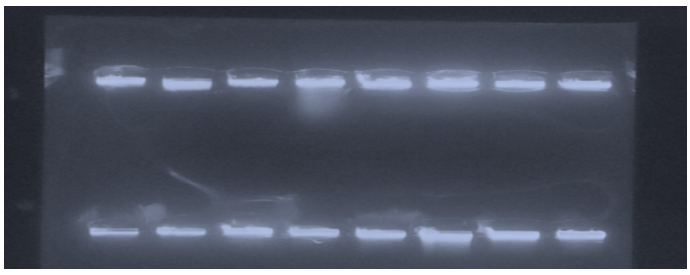

Figure 1: Electrophoresis of the extracted DNA, using a 1% agarose gel at a voltage of 70 V 85 mA for 20 minutes.

Relationship Between Genetic Polymorphisms in the Studied Segment of the GDF9 Gene and the Percentages of Cumulus Cells in Awassi Sheep Oocytes

Animals carrying the three genetic variants (GG, GA, and AA) resulting from the mutation at position 205 of the examined segment of the GDF9 gene did not show significant differences (P > 0.05), as presented in Table 1. This finding applies to *in vitro* maturation outcomes of mature, immature, and atretic oocytes in the presence of cumulus cells, taking into account the type of birth (single or twin).

Table 1: The relationship between the genetic polymorphisms of GDF9 gene (G<A) and in vitro maturation rates of Awassi sheep oocytes with cumulus cells (mean ± standard error).

Genetic polymorphisms	Type of birth	Presence of cumulus cells		
GG	Single	Atretic oocytes	Immature oocytes	Mature oocytes
	Twins	9.63±2.10	14.21±2.41	76.14±4.28
GA	Single	13.20±4.08	13.48±3.83	73.29±7.67
	Twins	14.16±2.26	20.76±3.77	65.06±5.72
AA	Single	18.87±8.06	20.25±5.23	60.87±11.5
	Twins	10.68±2.52	18.01±5.42	71.31±7.41
Significance level		P>0.05	P>0.05	P>0.05

In cases of single births, the percentages of atretic oocytes were 9.63% for GG, 14.16% for GA, and 10.68% for AA genotypes. The corresponding values for immature oocytes were 14.21%, 20.76%, and 18.01%, while mature oocytes showed percentages of 76.14%, 65.06%, and 71.31% for GG, GA, and AA, respectively. For twin births, atretic oocyte percentages were 13.20% (GG), 18.87% (GA), and 16.27% (AA). Immature oocyte percentages were 13.48%, 20.25%, and 18.77%, and mature oocytes were 73.29%, 60.87%, and 64.94% for GG, GA, and AA genotypes, respectively.

Although these values showed some variation, they were not statistically significant, suggesting that the GDF9 polymorphism at site 205 may not have a notable effect on oocyte maturation outcomes when cumulus cells are present. These findings differ from those reported by Abdelgadir *et al.* (2021) in Sudanese sheep, possibly due to breed-specific genetic differences affecting oocyte development and maturation. This highlights the importance of considering genetic background in reproductive biotechnology studies.

Association Between Polymorphisms in the Studied Segment of the GDF9 Gene and the Absence of Cumulus Cells in Awassi Sheep Oocytes

The results presented in Table 2 showed significant differences (P ≤ 0.05) among genetic variants in the *in vitro* maturation of immature oocytes from Awassi sheep in the absence of cumulus cells. However, no significant differences were observed in mature and atretic oocytes, regardless of whether the birth type was single or twin. The GA genotype showed superior performance in twin births for immature oocytes.

In the case of single births, the percentages for atretic oocytes were 21.37% (GG), 27.53% (GA), and 25.86% (AA); for immature oocytes, 34.55% (GG), 25.08% (GA),

and 38.91% (AA); and for mature oocytes, 39.63% (GG), 47.38% (GA), and 46.68% (AA).

Table 2: The relationship between the genetic polymorphisms of GDF9 gene (G<A) and in vitro maturation rates of Awassi sheep oocytes without cumulus cells (mean ± standard error).

Genetic polymorphisms	Type of birth	Absence of cumulus cells		
GG	Single	Atretic oocytes	Immature oocytes	Mature oocytes
	Twins	9.63±2.10	14.21±2.41	76.14±4.28
GA	Single	13.20±4.08	13.48±3.83	73.29±7.67
	Twins	14.16±2.26	20.76±3.77	65.06±5.72
AA	Single	18.87±8.06	20.25±5.23	60.87±11.5
	Twins	10.68±2.52	18.01±5.42	71.31±7.41
Significance level		P>0.05	P≤0.05	P>0.05

For twin births, the percentages for atretic oocytes were 23.85% (GG), 21.85% (GA), and 21.82% (AA); for immature oocytes, 29.42% (GG), 45.01% (GA), and 38.91% (AA); and for mature oocytes, 46.71% (GG), 33.75% (GA), and 39.26% (AA).

The observed variations in these results may be attributed to differences in genetic composition, the number of animals used, and the average age of the sheep, as well as environmental conditions across experimental settings.

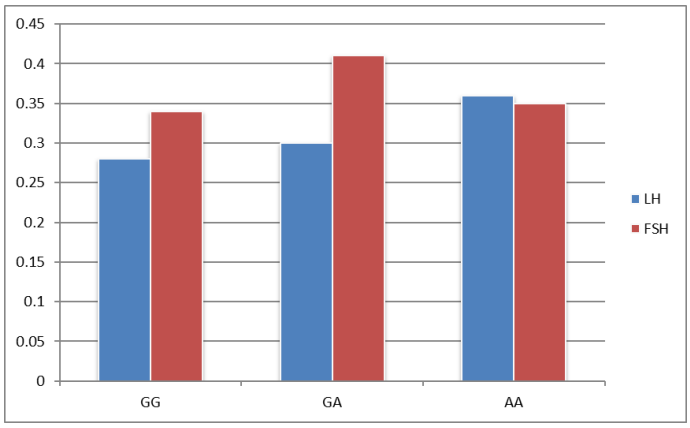

Figure 2: The relationship between the genetic polymorphisms of the studied trait of the GDF9 gene and the concentrations of LH and FSH hormones in twin-birth sheep.

Relationship Between Genetic Variants of the Studied GDF9 Gene Segment and LH and FSH Hormone Concentrations in Single- and Twin-Born Awassi Sheep

The hormonal analysis presented in Figure 2 revealed no significant effect (P ≥ 0.05) of the genetic variants (GG, GA, and AA) resulting from the mutation at position 205 of the GDF9 gene segment on the concentrations of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in twin-birth Awassi sheep. The observed hormone levels (in ng/ml) for LH were 0.36 (GG), 0.30 (AA), and 0.28 (GA), while FSH concentrations were 0.41 (GG), 0.34 (AA), and 0.36 (GA), respectively. A slight decrease in hormone levels may have resulted from animal stress during blood collection, as previously reported by Kohanski and Redmond (2017).

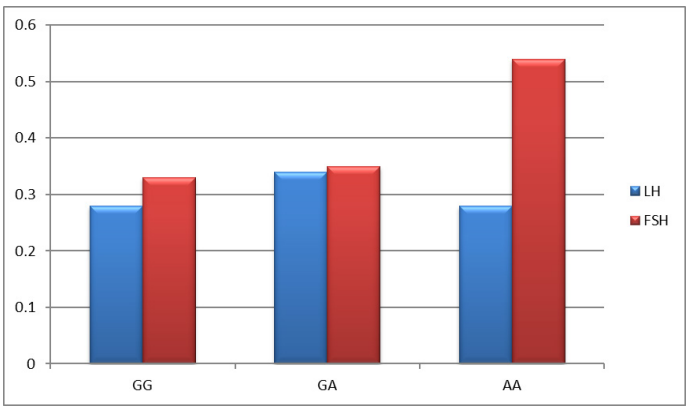

Figure 3: The relationship between the genetic polymorphisms of the studied segment of the GDF9 gene and the concentrations of LH and FSH hormones in single-born sheep.

Similarly, Figure 3 shows no significant differences (P ≥ 0.05) in LH and FSH concentrations across genotypes in single-born Awassi sheep. LH levels were 0.28, 0.34, and 0.28 ng/ml for the GG, GA, and AA genotypes, respectively. FSH levels were recorded at 0.33, 0.35, and 0.54 ng/ml for the same genotypes. These results are not consistent with the findings of Jumaa and Kassim (2022), who reported that hormonal treatments significantly affected reproductive organ development and sex hormone concentrations. The discrepancies may be due to differences in the physiological, morphological, and structural characteristics of the animals used, particularly in ovarian structure and hormone production. Additionally, environmental factors and management conditions may have influenced the hormonal activity and reproductive performance in the studied sheep (Ahmed and Atiyah, 2022).

Reproductive traits in ewes are strongly influenced by the GDF9 gene, a member of the TGF-β (Transforming Growth Factor Beta) superfamily. GDF9 is predominantly expressed in oocytes and plays a critical role in follicular development and ovulation (Tang *et al.*, 2018). It has been identified as one of the key genes contributing to sheep prolificacy (Vage *et al.*, 2013; Mullen and Hanrahan, 2014; Souza *et al.*, 2014), by enhancing oocyte developmental competence and regulating ovarian function (Stocker *et al.*, 2020).

Moreover, GDF9 interacts with other key factors in the TGF-β signaling pathway, including BMP15 and BMPR1B. These molecules work together to control oocyte maturation, follicular atresia, and granulosa cell differentiation (Liu *et al*., 2019). GDF9 and BMP15 are secreted by oocytes and bind to BMPR1B receptors on granulosa cells, thus potentially increasing litter size and ovulation rate. The combined action of GDF9 and BMP15 also promotes the expression of anti-Müllerian hormone (AMH), which is critical for ovarian function and follicular recruitment (Roy et al., 2018). Previous studies have shown that sheep with concurrent mutations in FecB and BMP15 had significantly higher litter sizes than those with only one mutation (Chu et al., 2007). Similarly, Hanrahan *et al*. (2004) demonstrated that sheep carrying both GDF9 and BMP15 mutations ovulate more frequently than those with only one mutation. However, the interaction between BMPR1B and GDF9 remains poorly understood and warrants further investigation.

Additionally, the present study observed a significant superiority in *in vitro* maturation (IVM) rates of oocytes containing cumulus-oocyte complexes (COCs) compared to those without cumulus cells. This may be due to the crucial physiological role of the cumulus cell layer, which supports both cytoplasmic and nuclear maturation of the oocyte by maintaining it in the germinal vesicle (GV) stage and facilitating molecular exchange between the oocyte and its microenvironment (Shimada and Terada, 2002).

In contrast, the current findings disagree with Singh et al (2020), possibly due to several factors affecting oocyte quality. These include animal age, season of ovary collection, time elapsed between slaughter and oocyte retrieval, environmental conditions during collection, nutritional status, and the functional state of the ovary at the time of sampling (Amer *et al*., 2008). These variables can significantly impact oocyte competence and maturation success *in vitro*.

CONCLUSIONS AND RECOMMENDATIONS

The current study suggests that the GDF9 gene and litter size in Awassi ewes could serve as biological indicators of ovulation and potentially be used to enhance fertility. Mutations in the GDF9 gene represent important genetic resources for livestock production. These mutations influence growth factor profiles, which, in turn, affect litter size. Further investigation is needed to understand how GDF9 interactions impact follicular growth. Since abnormal expression of GDF9 can lead to infertility, breeders should recognize the importance of this gene as a marker for fertility.

ACKNOWLEDGEMENTS

The administration and staff of the Al-Nahrain Station in Al-Qadisiyah are acknowledged by the authors for their help in completing the study's initial phase. A special thanks goes out to the Animal Production Department and Postgraduate Studies Laboratory at Al-Muthanna University's College of Agriculture, which provided all the resources required to complete the second half of his research.

NOVELTY STATEMENTS

This work aims to provide us a better knowledge of the connection between significant reproductive and physiological features in Awassi sheep and genetic polymorphisms in the growth and differentiation factor 9 (GDF9) gene. Since the majority of earlier studies on the GDF9 gene and how it affects fertility have concentrated on sheep breeds from around the world. Awassi sheep are a significant native breed with distinctive traits in our area, particularly in Iraq and the Middle East. Information that might not be available in other breeds is revealed by specifically examining the GDF9 gene in the Awassi breed. The importance of GDF9 in sheep fertility has found specific correlations between GDF9 genotypes, parturition type (number of litters), in vitro maturation success, and selected physiological traits. Other physiological traits include lamb birth weight, growth rate, and even the length of the breeding season in Awassi sheep. This information is important for developing breeding improvement techniques and artificial insemination. This information not only fills a research gap but also provides a strong scientific foundation for developing targeted genetic improvement strategies to boost fertility and productivity in the Awassi breed.

AUTHOR'S CONTRIBUTIONS

The design of the experiment, in vitro oocyte maturation (IVM) phase, samples collection and molecular biology tools to analyse genetic diversity was contributed by Safaa S. Atiyah and Hadi A. Hassooni. As the field supervisor, Hadi A. Hassooni oversaw the gathering of information from Awassi sheep flocks on physiological characteristics and parturition type. He also oversaw the statistical analysis of the data to connect these characteristics to the genotypes that were found. Alaa S. J. Mohamed was supported both theoretical and practical elements, reviewing scientific literature, interpreting results, and highlighting the study's importance.

Conflict of Interest

Regarding the current work, the authors state that they have no conflicts of interest.

REFRENCES

Abdelgadir AZ, Musa LM, Jawasreh KI, Saleem AO, El-Hag F, Ahmed MKA (2021). G1 point mutation in growth differentiation factor 9 gene affects litter size in Sudanese desert sheep. Vet. World., 14(1): 104. https://doi.org/10.14202/vetworld.2021.104-112

Abdoli R, Ziaeddin Mirhoseini S, Ghavi Hossein-Zadeh N, Zamani P (2018). Screening for causative mutations of major prolificacy genes in Iranian Fat-Tailed sheep. Int. J. Fertil. Steril., 12: 51–55.

Ahmad A, Atiyah SS (2022). Effect of D-Aspartic acid on the level of some sex hormones and the biochemical parameters of the blood of Shami Bucks. Revis Bionat., 7(4): 36. https://doi.org/10.21931/RB/2022.07.04.36

Al-Dabbagh SF (2019). Study of the relationship between the production of milk and some of its components with the growth of lambs in two breeds of Iraqi sheep. Iraqi J. Vet. Sci., 33(2):8795. https://doi.org/10.33899/ijvs.2019.163172

Al-Mutar H, Younis L (2020). Effect of point mutation in the growth differentiation factor 9 gene of oocytes on the sterility and fertility of Awassi sheep. Arch. Razi Inst., 75: 101–108.

Amer HA, Hegab AO, Zaabal SM (2008). Effects of ovarian morphology on oocyte quantity and quality, granulosa cells, *in vitro* maturation, and steroid hormone production in buffaloes. J. Anim. Reprod., 5: 55-62.

Bravo S, Larama G, Paz E, Inostroza K, Montaldo HH, Sepulveda N (2016). Polymorphism of the GDF9 gene associated with litter size in Araucana creole sheep. Anim. Genet., 47: 390–391. https://doi.org/10.1111/age.12404

Chu MX, Liu ZH, Jiao CL, He YQ, Fang L, Ye SC, Chen GH, Wang JY (2007). Mutations in BMPR-IB and BMP-15 genes are associated with litter size in Small Tailed Han sheep (Ovis aries). J. Anim. Sci., 85: 598–603. https://doi.org/10.2527/jas.2006-324

Duncan DB (1955). Multiple Rang and Multiple F-test. Biometrics.,11: 4-42.https://doi.org/10.2307/3001478

Eghbalsaied S, Ghaedi K, Shahmoradi S, Pirestani A, Amini H, Saiedi T, Nicol L, McNeilly A (2012). Presence of SNPs in GDF9 mRNA of Iranian Afshari Sheep. Int. J. Fertil. Steril., 5: 225–230.

Elshareef KH, Musa HH, Adam SY, Fedailb JS, Cai D, Ahmed AA (2023). Genetic polymorphism of the BMP15 gene in West African sheep (Fulani) breed in Sudan. Applied Veterinary Research, 2(2), pp.2023009-2023009

Hanrahan JP, Gregan SM, Mulsant P, Mullen M, Davis GH, Powell R, Galloway SM (2004). Mutations in the genes for oocyte-derived growth factors GDF9 and BMP15 are associated with both increased ovulation rate and sterility in Cambridge and Belclare sheep (Ovis aries). Biol. Reprod., 70: 900–909. https://doi.org/10.1095/biolreprod.103.023093

Hassooni HA, Atiyah SS, Jassim AS (2024). The relationship between the genetic marker DIK20 and a range of physiological and productive traits in Holstein cattle. J. Anim. Health Prod., 12(2): 189-195. https://doi.org/10.17582/journal.jahp/2024/12.2.189.195

Jumaa AJ, Kassim WY (2022). The Influence of Age, Gender and Weight on Sex Hormone Levels and Histological Development of Reproductive Organs in Arabi Sheep. Al-Mustansiriyah J. Sci., 33(5): 27-32. https://doi.org/10.23851/mjs.v33i5.1309

Kohanski K, Redmond SB (2017). The effects of psychological and physical stressors on the secretion of immunoglobulin A in humans and mice. Bioscience, 88(1): 1-8. https://doi.org/10.1893/BIOS-D-14-00035.1

Liu MN, Zhang K, Xu TM (2019). The role of BMP15 and GDF9 in the pathogenesis of primary ovarian insufficiency. Hum. Fertil., 1–8. https://doi.org/10.1080/14647273.2019.1672107

Margawati ET, Putra WPB, Raadsma HW, Volkandari SD (2023). The Polymorphisms Determination of The FecG/PstI andFecX/HinfI Genes in Indonesian Backcross Sheep (75%Merino ×25% Garut). AIP Conference Proceedings 2606, 040013 (2023); https://doi.org/10.1063/5. 0118413.

Mery L, Lefevre A, Benchaib M, Demirci B, Salle B, Guerin JF, Lornage, J (2007). Follicular growth in vitro: detection of growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) during in vitro culture of ovine cortical slices. Molecular Reproduction and Development: Incorporating Gamete Research, 74(6), pp.767-774.

Mullen MP, Hanrahan JP (2014). Direct evidence on the contribution of a missense mutation in GDF9 to variation in ovulation rate of Finnsheep. PLoS ONE., 9: e95251. https://doi.org/10.1371/journal.pone.0095251

Muñoz C, Vaquera-Huerta H, Gallegos-Sánchez J, Becerril-Pérez CM, Tarango-Arámbula, LA, Bravo-Vinaja Á, Cortez-Romero C (2021). Influence of FecG E mutation on the reproductive variables of Pelibuey ewes in the anestrus period. Tropical Animal Health and Production, 53(2), p.328

Nogueira D, Romero S, Vanhoute L (2009). Oocyte *in vitro* maturation In: Gardner DK, Weissman A, Howles CM and Shoham Z. Editors. Textbook of Assisted Reproductive Technologies. Laboratory and Clinical Perspective. 3rd ed. Informa healthcare., UK:111-151.

Roy S, Gandra D, Seger C, Biswas A, Kushnir VA, Gleicher N, Kumar TR, Sen A (2018). Oocyte-derived factors (GDF9 and BMP15) and FSH regulate AMH expression via modulation of H3K27AC in granulosa cells. Endocrinology, 159: 3433–3445. https://doi.org/10.1210/en.2018-00609

Saleh AA, Hammoud MH, Dabour NA, Hafez EE, Sharaby MA (2020). BMPR-1B, BMP-15 and GDF-9 genes structure and their relationship with litter size in six sheep breeds reared in Egypt. BMC Res. Notes, 13: 215. https://doi.org/10.1186/s13104-020-05047-9

SAS (2018). Statistical Analysis System, User's Guide. Statistical. Version 9.6th ed. SAS. Inst. Inc. Cary. N.C. USA.

Shimada M, Terada T (2002). FSH and LH induce progesterone production and progesterone receptor synthesis in cumulus cells, a requirement for meiotic resumption in porcine oocytes. Mol. Hum. Reprod., 8: 612 618. https://doi.org/10.1093/molehr/8.7.612

Singh AK, Prakash C, Rohit K, Santra A, Das SK (2020). Cattle embryos development through *in vitro* techniques using thyroxine hormone as a culture media supplement. Fertil. Steril., 52(2); 319 -324.

Souza CJ, McNeilly AS, Benavides MV, Melo EO, Moraes JC (2014). Mutation in the protease cleavage site of GDF9

increases ovulation rate and litter size in heterozygous ewes and causes infertility in homozygous ewes. Anim. Genet., 45: 732–739. https://doi.org/10.1111/age.12190

Stocker WA, Walton KL, Richani D, Chan KL, Beilby KH, Finger BJ, Green MP, Gilchrist RB, Harrison CA (2020). A variant of human growth differentiation factor-9 that improves oocyte developmental competence. J. Biol. Chem., 295: 7981–7991. https://doi.org/10.1074/jbc.RA120.013050

Tang J, Hu W, Di R, Liu Q, Wang X, Zhang X, Zhang J, Chu M (2018). Expression analysis of the prolific candidate genes, BMPR1B, BMP15, and GDF9 in Small Tail Han ewes with three fecundity (FecB gene) genotypes. Animals, 8: 166. https://doi.org/10.3390/ani8100166

Tong B, Wang J, Cheng Z, Liu J, Wu, Y, Li Y, Bai C, Zhao S, Yu H, Li G (2020). Novel variants in GDF9 gene affect promoter activity and litter size in Mongolia sheep. Genes, 11: 375. https://doi.org/10.3390/genes11040375

Vage DI, Husdal M, Kent MP, Klemetsdal G, Boman IA (2013). A missense mutation in growth differentiation factor 9 (GDF9) is strongly associated with litter size in sheep. BMC Genet., 14: 1. https://doi.org/10.1186/1471-2156-14-1