Iraqi Journal of Biotechnology, 2022, Vol. 21, No. 2, 288-299

Association of Gene Expression of Insulin like Growth Factor-1 (*IGF1*) with some Quantitative Traits for Fallow Deer (*Dama dama*) in Iraq

Israa A Fadhil[1], Basima Q. Alsaadi[2]

[1]Department of Animal Production, College of Agriculture, Al-Qasim Green University
[2] Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, University of Baghdad

Received: 1/6/2022 Accepted: 6/9/2022 Published: December 20, 2022

Abstract: Insulin like growth factor-1 (IGF-1) gene is play a vital role in cell development. The current study was illustrated the impact of IGF1(exon2) gene expression on some quantitative traits in Iraqi Fallow deer. The purity of total RNA samples was ranged from (1.76 to 2.10) in all samples under study. Ct value mean of GAPDH gene is (25.23±0.27), while (17.51 ± 1.65) in target gene. By Sacace qPCR machine, Ct values for GAPDH gene was illustrated and ranged from (22 to 27) and (20 to 27) was appeared for candidate gene. Dissociation curves was taken directly for GAPDH gene (Tm=79.5°C to 81°C) and IGF1 (exon2) gene (Tm=80°C to 81°C) and the peaks were showed single distinct for all samples. The perfect peak appears with the primer temperature about (60ºC) for both genes. The relationship between IGF1 (exon2) gene expression with Fallow deer parameters are showed a significant association ($P \leq 0.05$) was observed for sex (male and female) 1.27±0.11 and 1.006±0.08 respectively. A significant effect ($P \leq 0.05$) were showed among groups of parity with high expression are observed in 3rd parity (1.890 ± 0.12). Among study groups, no difference was observed for IGF1(exon2) gene between either age or antlers production. There is a significant correlation ($P \leq 0.05$) between IGF1 (exon2) gene and body weight of Iraqi Fallow deer (-0.25). Also, the results were mentioned a significant correlation ($P \leq 0.05$) (-0.29) between target gene and body length. The IGF1 (exon2) correlation coefficient observed non-significant effects and negative impacts with heart girth and height at shoulders. The results were observed negative significant of regression ($P \leq 0.05$) for body weight with IGF1(exon2) gene expression were (-0.0344) and the determination coefficient (R^2) was 0.18. A negative significant regression ($P \leq 0.05$) was determined in present study between folding of candidate gene and body length (-0.0296) and R^2 is recorded about (0.27). No significant effects of IGF1 (exon2) folding was observed with heart girth and height at shoulder. The current study was the first study that investigates the gene expression quantity of IGF1 gene in Iraqi Fallow deer.

Keywords: IGF1 (exon2) gene, *Dama dama,* Gene expression.

Corresponding author: (Email: asraar83@agre.uoqasim.edu.iq).

Introduction

Fallow deer (*Dama dama*) are belonging to the sub-family of Cervine and belongs to ruminant mammals (1). The estimated global Fallow deer population is about 5 million, and New Zealand ratio are over one-half of the world's production of deer production (2). Primarily, intensive farming prevails due to Fallow deer importance in Europe meat industry (3). The main products of deer for most countries are meat and antlers as well as musk (4). According to the numerous factors, meat of Fallow deer from farmed-raised is a good source of copper and iron in

the human diet as well as it is rich with total collagen, moisture, protein and mineral (Ca, K, Mn, Mg, Na, P, Fe, Cu), (especially Cu and Fe) and beneficial fatty acid composition make the meat of Fallow deer a valuable component of the human diet and can be recommended as a part of a healthy diet (5).

Molecular genetics are concerned with the identification of genetic variation to determine the phenotypic changing and understanding the biological effects by study of function and structure of gene at molecular level. Therefore, Molecular genetics are important part of the most using applications nowadays, involving test of paternity, genetic diseases diagnostic, reproductive bio techniques, hybrids identification, diversity studies, editing of genome, identification of parasite, and genetic development (6). These characteristics beside the quantitative traits are associated with genes, therefore many researcher dependents on the genes in their studies (7).

Many Studies have been observed many candidate genes are correlated with growth traits in animals (8), among which Insulin like growth factor group candidate genes that impact on growth and production in livestock(9,10).

Insulin like growth factor-1 (IGF-1) gene is a multi-promoter gene that has complex biological functions and plays a role in cells differentiation and proliferation as well as cellular DNA synthesis (11), as well as it is similar to insulin structurally. In 1978 at Zurich University, isolated the factors that is like in activity with insulin by Rinderknecht and his colleagues, which distinguish their ability to absence of cross-reactivity with insulin antibodies comparing with insulin (12).

In spite of the similarity between insulin and IGF1, insulin plays a major in regulating anabolic activities such as mediating glucose homeostasis and protein and lipid synthesis, whereas IGF1 mainly mediates long-term action such as cells fate and survival, decrease free fatty acid flowing into the liver, development of bones furthermore, it has a direct and indirect in lower impact of hepatic glucose, promotes the oxidation of free fatty acid within muscle, improving the signaling and sensitivity to insulin, (13,14). In addition, IGF1 is increases the bone density, muscle mass and the bones matrix directly.

Intestinal microbes, growth hormone (GH) and insulin stimulation are considered to be the main factors that leads to induce of IGF-1 secretions. From liver hepatocytes, IGF-1 is output mainly as an endocrine hormone, moreover, a paracrine/autocrine manner in target tissues and its release and manufacture are controlled by GH primarily when GH is released into the blood stream and then stimulates the liver to produce IGF-1(15,14). IGF1 relates with high affinity to IGF1R, activating both the mitogen activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) signaling pathways in target tissue (16, 17).

There are six kinds of protein (IGFBP) that can bind to IGF group genes and 98% of IGF1 is bind to one of these proteins approximately. IGFBP-3 is the most found of these proteins, about 80% of all IGF group binding (18).

To provides insights into cell physiology or cell groups at same time, should be research on gene expression of target organism. Gene expression is the synthesis of a functional gene

product using the information that provided by deoxyribonucleic acid (DNA) (19). From DNA, RNA is synthesized via the transcription process, which is a part of gene expression process. therefore, the studies of gene expression provide insights into cellular responses at a given point in time. There are many factors can influence on the results of gene expression such as; gender, age, tissue specify, and the time of sample collection (20). Could be explained the phenotypic variations between the organisms by knowledge of the small regulatory changes mechanism that related with gene expression alterations (21). By the measuring the differences in gene expression among individual's groups, the clinical researches has taken advantages from these properties to prove their results.

The research of gene expression changes is increased for many reasons; 1st, the costs that related with gene expression analysis is reduced by more than 50% (22). 2nd, increasing of new methods to measure it from a variety of tissues and growing of sample numbers available from repositories of tissue (23). Recently, the methods to analyze it are easier and accessible to use (22). Therefore, the current study examines growth factor (IGF-1) gene expression via quantitative real time PCR in order to investigate the role of growth factor exert in Fallow deer (*Dama dama*) production.

Material and methods

1-Animal and samples collection

The experimental period was performed from May 2021 to April 2022 at Institute of genetic engineering and biotechnology for post graduate studies/ Baghdad University/ Baghdad. In total, thirty-five Iraqi Fallow deer (*Dama dama*) from both sexes and different ages were investigated. The samples were collected from middle and south of Baghdad City, especially 20 samples from Reserve Kingdom of Deer in the middle of Baghdad and 15 samples from Al-Mahawil reserve.

The specialized Veterinary team from Veterinary Medicine College/Al-Qasim Green University, the blood samples were taken from the jugular vein. 100 µl of each blood sample was added to 300 µl of Shield within Eppendorf tube. Following, it was kept in cooled thermally insulated box (4^oC) and then transport to lab with minimum disturbance and shaking. All the tubes were kept in -2ºC to use it for molecular analysis in lab. By using metric tape (± 1 cm), morphometric measurements of Fallow deer were record.

2- RNA extraction and PCR program

The protocol for RNA extraction by Direct-zol™ RNA Mini Prep Kit, that prepared by ZYMO/ USA with Shield ™. It is including Direct-zol™ RNA Prewash1, Direct-zol™ RNA MiniPrep kit size, RNA wash buffer2, DNA Digestion Buffer, DNase I3 (lyophilized), DNase/RNase-free Water and Zymo-Spin™ IIC Columns. The concentration of RNA was determined via Nanodrop. It was used to measure the optical density at wave length (260 nm and 280 nm). The primer sequence of IGF1 (Exon 2), and GAPDH genes are shown in table (1).

Table (1): The specific primer of IGF1 (Exon 2) and GAPDH genes

Primer		Sequence	Tm (°C)	GC (%)	Accession No.
IGF1 (exon2)	Reverse	5'-CACAGCCTATTATCCCACTCT-3'	62	47.61	(24) (NC_007303.4)
	Forward	5'-CTTTTGACCCTATGAACCA-3'	54	44.44	
GAPDH	Forward	5'-TGCCCAGAACATCATCCC-3'	56.04	56	(XM_043440341.1)
	Reverse	5'-TCGATCCTCCACCACACGA-3'	55.41	58	

Total RNA was reversely transcribed to cDNA using Prime Script^{TM} RT reagent kit that prepared by TaKaRa/Japan. It is involving Prime Script^{TM} RT Enzyme Mix, RNase Free dH_2O and EASY Dilution Buffer (for Real Time PCR). The thermal cycler was programmed by using the conditions shown in table (2). Prepare the reaction mixture (12 µl of 5 × Prime Script RT master mix, up to 10µl of Total RNA and total 10µl*2 of RNase Free dH2O) on ice. The optimum GAPDH concentration and IGF1 (exon2) forward and reverse primers (10 picoml) and the annealing temperature is 60°C for both genes.

Table (2): Thermal cycler steps of conditions cDNA reverse transcription

	Stage 1	Stage 2	Stage 3
Temperature	37°C	85°C	85°C
Time	15 min.	30 sec.	30 sec.

3- Quantitative Real-Time PCR (qRT-PCR)

The primers, template cDNA and SYBR green kappa master mix were mix to preparing qRT-PCR reaction. The appropriate volume of reaction mixture was 20µl. The protocol was programmed according to thermal profile for target gene and GAPDH gene shown in table (3).

Table (3): Thermal conditions of IGF1 (exon2) and GAPPDH gene expression

Step	Temp. (°C)	Time	Cycle
Reverse transcription	42°C	10 min	Hold
Initial denaturation	95°C	3 min	Hold
Denaturation	95°C	30 sec	40
Annealing	60°C	40 sec	
Extension	72°C	30 sec	

The amplification accuracy of PCR product was observed by the value of cycle threshold (Ct) for the reactions. The improvement of RT-PCR test accuracy through including housekeeping gene to comparison between the samples. GAPDH (Glyceraldehyde-3- phosphate dehydrogenase) as a common housekeeping gene (internal control) for samples is widely used in real time PCR to be used in calculating ΔCt value. All total RNA levels were normalized to GAPDH.

4- Statistical analysis

The data were processed by one-way classification (Analysis of Variance, ANOVA) by using Completely Randomized Design (CRD). The results were obtained in all measurements were presented in the tables as mean values and standard error of mean. To detect the impact of

different factor in the present study, the Statistical Analysis System (Stat Soft Inc., Tulsa, OK, USA) - SAS (25) Program and General Linear Model - GLM procedure were used. Duncan's multiple range test (26) (Analysis of Variation-ANOVA) and Last Square Means (LSM) were used, to compare the significance difference between the mean values at $p < 0.05$.

The regression (β) and straight linear equation as well as correlation (r) between different variables in current study were estimated also, via the formulas;

$$\beta = \frac{n\Sigma xy - \Sigma x \Sigma y}{n\Sigma x^2 - (\Sigma x)^2}$$

$$r = \frac{n(\sum xy) - (\sum x)(\sum y)}{\sqrt{[n(\sum x^2) - (\sum x)^2]\,[n(\sum y^2) - (\sum y)^2]}}$$

A. Statistical model of IGF1 (exon2) expression

$$Y_{ijklm} = \mu + A_i + S_j + N_k + P_l + e_{ijklm}$$

Where; Y_{ijklm} = the observed value, μ = Overall mean of traits, A_i = Effect of age (least than 1, 1-3, more than 3 year), S_j = Effect of sex (female and male), N_k = Effect of Antlers (antlers, no antlers), P_l = Effect of parity (0, 1st, 2nd, 3rd) and e_{ijklm} = The random error.

B. Quantitative Real Time PCR analysis:

The analysis of qRT-PCR were summarized according to the formula (27);

ΔCT (test) = CT for target gene – CT for internal control (reference gene)

The folding expression value was calculated according to formula;

$$2^{-\Delta Ct} = \text{Normalized expression ratio}$$

Results

1- qRT-PCR

The presence of low copies of the target genes indicate to the high Ct values thus, lead to low in gene expression and vice versa (28,29).

2- Fold expression of GAPDH (housekeeping) and IGF1(exon2) genes

IGF1 is expressed in every body tissues nearly and regulate the bioactivities variety including cell differentiation, proliferation, and survival (16). According to our knowledge, the current study was the first study that investigates the gene expression quantity of IGF1 gene in Iraqi Fallow deer.

In all study groups, mean Ct values of GAPDH gene are (25.23±0.27) (Table 4). The results were observed decreasing of candidate gene expression value in compare with housekeeping gene level ($\bar{x}$ ± S.E), which were (25.23 ± 0.27) in GAPDH gene while, (17.51 ± 1.65) in target gene, that record in table (4).

Table (4): Overall means of gene expression and parameters in Iraqi Fallow deer

Parameters	No.	Mean ± S.E
GAPDH Ct	35	25.23±0.27
IGF1 (exon2) Ct	35	17.51 ± 1.65
IGF1(exon2) ΔCt	35	-0.970 ± 1.02
IGF1(exon2) Folding	35	1.126 ± 1.12

Figure (1 and 3) illustrates the distribution of GAPDH and IGF1(exon2) gene expression levels in study samples. Ct values for GAPDH gene was ranged from (22 to 27) and (20 to 27) was appeared for candidate

gene according to the photograph that taken directly via Sacace qPCR machine illustrated. Dissociation curves for all study samples via qPCR was taken directly by Sacace qPCR machine for GAPDH gene (Tm=79.5°C to 81°C) and IGF1 (exon2) gene (Tm=80°C to 81°C) (Figures 2 and 4). The perfect peak appears with the primer temperature about (60ºC) for both genes. Both figures (2 and 4) were showed distinct single peaks for all samples.

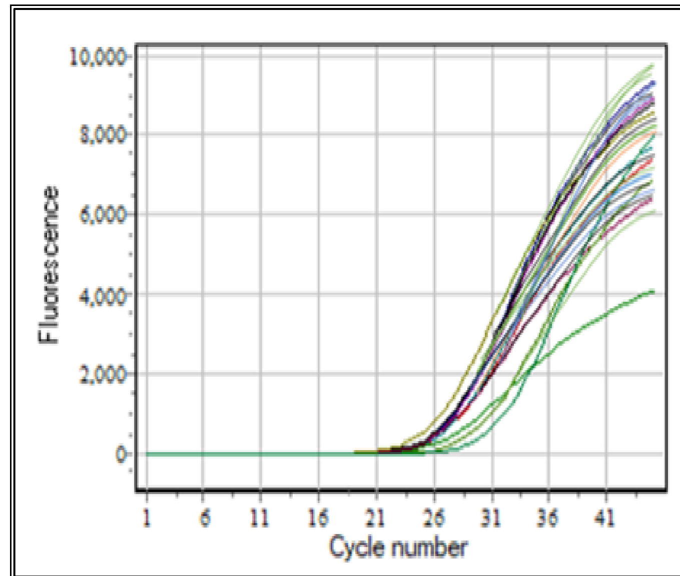

Figure (1): GAPDH amplification for all samples by qRT-PCR. Ct values was taken directly from Sacace qPCR machine and ranged from the photograph. Ct values ranged from 22 to 27.

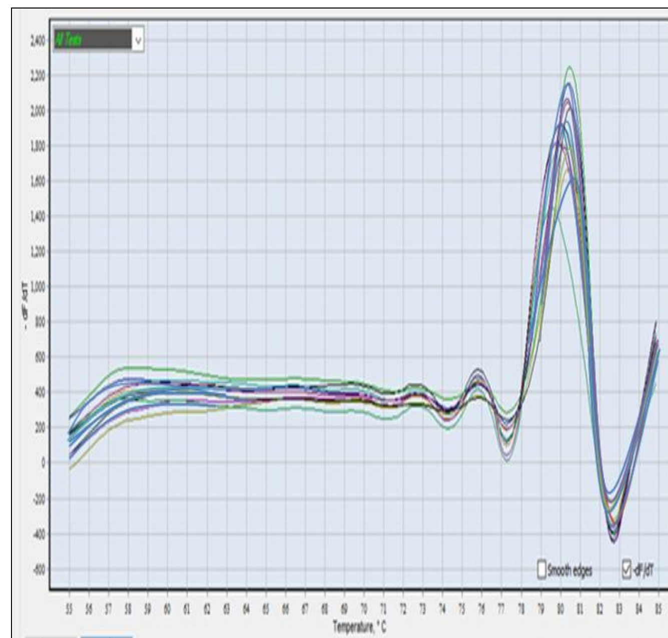

Figure (2): GAPDH dissociation curves for all study samples by q PCR. Mt (Melting temperature) was ranged between 79.5°C to 81°C. The photograph was taken directly from Sacace qPCR machine.

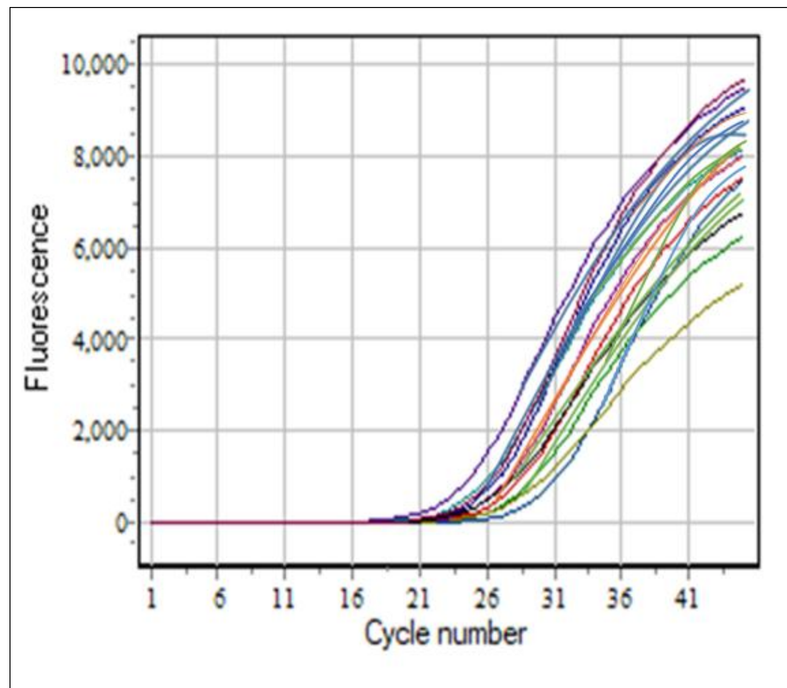

Figure (3): IGF1 (exon2) amplification for all samples by qPCR. Ct values was taken directly from Sacace qPCR machine and ranged from the photograph. Ct values ranged from 20 to 27.

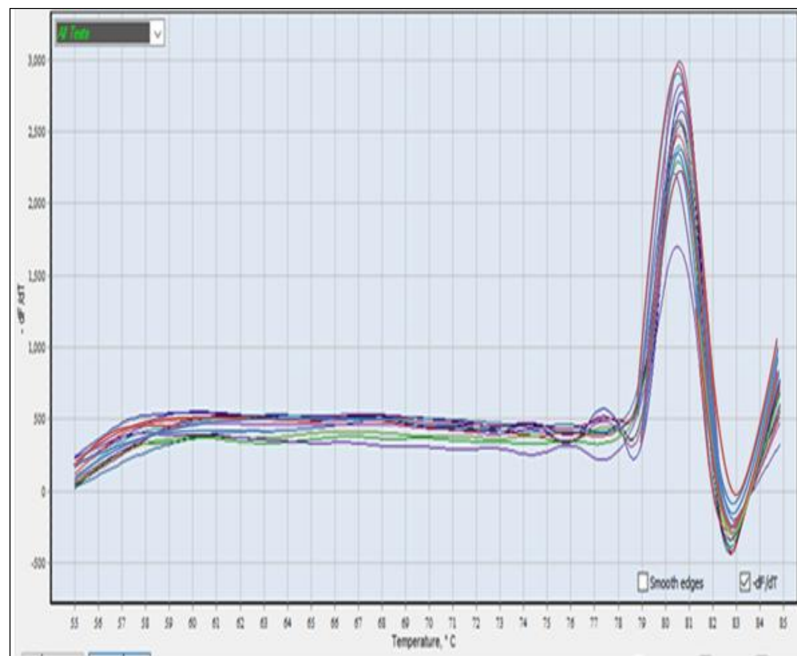

Figure (4): IGF1 (exon 2) dissociation curves for all study samples by q PCR. Mt (Melting temperature) ranged from 80°C to 81°C. The photograph was taken directly from Sacace qPCR machine.

3- The relationship between IGF1 (exon2) gene expression with some quantitative traits

The expression levels of target gene in Fallow deer (*Dama dama*) was determined through the real-time PCR method. The correlation between IGF1

(exon2) gene expression with Fallow deer parameters as; age, antler, sex, and parity are showed in table (5).

There is a significant association (P≤0.05) was observed between the expression of IGF1 (exon2) gene and sex (male and female) 1.27±0.11 and 1.006±0.08 respectively (Figure 5), (Table 5). These few variations in the expression values among the study groups provide *GAPDH* gene as useful control or reference gene. Both IGF-1 and -2 are bind to IGF-1R (30,31). These findings were similar with Dakheel and Al-anbari (9) when they suggested the significant differences (P≤0.05) of the effect IGF1R gene on sex and they reported the high expression was related to male and lower expression were related to female in their study on Fallow deer.

A significant effect (P≤0.05) were showed among groups of parity (0, 1st, 2nd and 3rd) with high expression are observed in 3rd parity (1.890 ± 0.12) (Figure 6). No studies are reported about the expression effect of IGF1 gene in deer population, that is perhaps due to the difficult studying on deer as well as lack availability of deer samples.

The effect of IGF1 gene expression levels are decreasing in antlers, in other words, it has been limited effects on the development of Fallow deer (*Dama dama*) antlers (11). Finally, among study groups, no difference was observed for IGF1(exon2) gene between either age or antlers production. Whatever, Yang et al., (24) suggested same results, when they observed the level of differences were not significant in two population of deer.

Table (5): Effect of different factors on least square means of IGF1 (exon2) gene expression

Factors	Level	No	Least square means ± SE
			Folding - IGF1
Age (years)	least than 1	11	1.07 ± 0.07
	1-3	8	1.14 ± 0.10
	More than 3	16	1.14 ± 0.08
Level of sig.		---	NS
Sex	Male	24	1.270 ± 0.11 a
	Female	11	1.006 ± 0.08 b
Level of sig.		---	*
Antlers	Antlers	14	1.073 ± 0.07
	No antlers	21	1.360 ± 0.10
Level of sig.		---	NS
Parity	0	20	1.078 ± 0.09 b
	First	4	0.885 ± 0.06 b
	Second	4	0.805 ± 0.09 b
	Third	7	1.890 ± 0.12 a
Level of sig.		---	*
Means having with the different letters in same column differed significantly. * (P≤0.05), NS: Non-Significant			

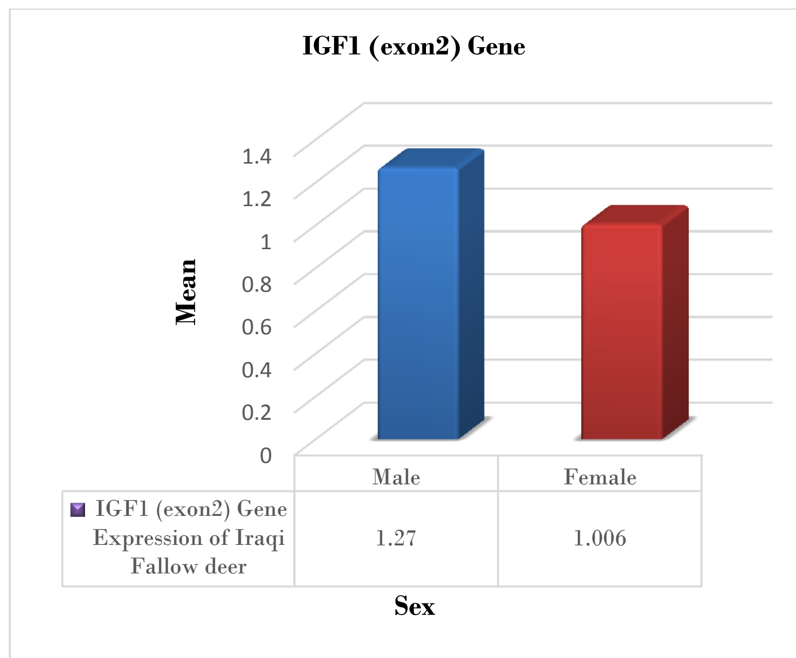

Figure (5): The levels of IGF1 (exon2) gene expression in different sex within study.

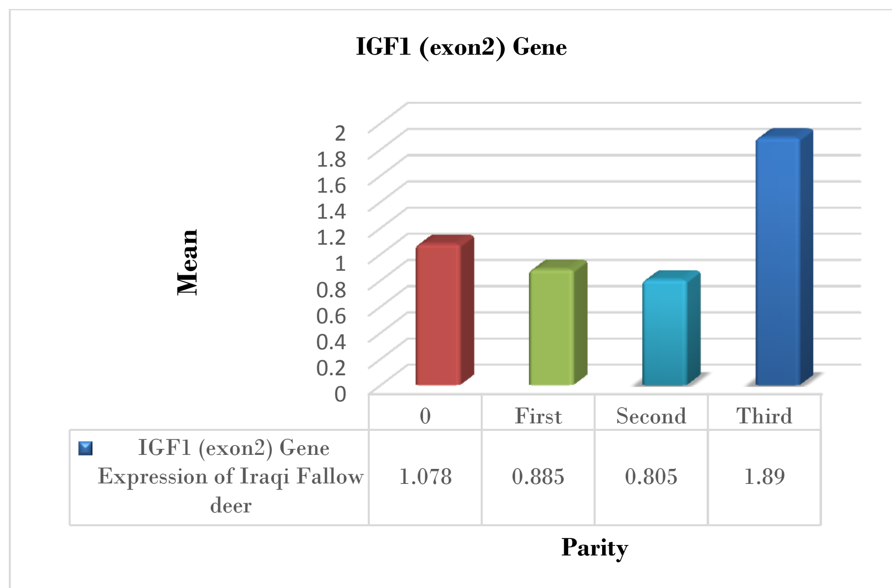

Figure (6): The levels of IGF1 (exon2) gene expression in different Parity within study.

4- Significant correlation between weight and body dimensions with folding of IGF1(exon2)

As reported in (Tab., 6) there is a significant correlation (P≤0.05) between IGF1 (exon2) gene and body weight of Iraqi Fallow deer (-0.25). However, the negative result of correlation coefficient (r) indicates to inverse relationship, in other words, increasing of IGF1(exon2) expression lead to reducing in body weight about 0.25 kg. Also, the results were mentioned a significant effect (P≤0.05) with negative value (-0.29) between IGF1 (exon2) and body length.

Conversely, IGF1 (exon2) correlation coefficient observed non-significant effects and negative impacts with heart girth and height at shoulders were (-0.14 and -0.12) respectively.

Table (6): Correlation coefficient between weight and body dimensions with folding of IGF1 (exon2).

Parameters	Correlation coefficient-r	Level of sig.
Body weight	-0.25	*
Body length	-0.29	*
Height at shoulder	-0.12	NS
Heart girth	-0.14	NS
*** (P≤0.05), NS: Non-Significant.**		

5- Relationship between simple, multiple regression and linear equation of parameters and IGF1 (exon2) folding gene.

The result that recorded within (Table 7), its refer to a negative significant of regression (P≤0.05) for body weight (kg) with IGF1(exon2) gene expression were (-0.0344) and the determination coefficient (R^2) was 0.18.

A negative significant regression (P≤0.05) was determined in present study between folding of candidate gene and body length (cm) (-0.0296) and the R^2 is recorded about (0.27).

Subsequently, no significant effect of IGF1 (exon2) folding was observed with heart girth (cm) and height at shoulder (cm) (-0.0318 and-0.0373) respectively. R^2 values of heart girth (cm) and height at shoulder (cm) (0.07 and 0.11) respectively.

Table (7): Simple and Multiple Regression and straight linear equation of parameters on folding IGF1 (exon2) gene

Parameters	Regression on folding	Equation	Level of sig.	R^2
Body weight (Kg)	-0.0344	Y^ = 1.186 -0.0344X	*	0.18
Body length (cm)	-0.0296	Y^ = 1.205 -0.0296X	*	0.27
Height at shoulder (cm)	-0.0373	Y^ = 1.136 -0.0373X	NS	0.11
Heart girth (cm)	-0.0318	Y^ = 1.371 -0.0318X	NS	0.07
Multiple	Y^ = 1.247 + 0.0014 -0.0398 -0.0031 + 0.0208X *			0.20
Means having with the different letters in same column differed significantly * (P≤0.05), NS: Non-Significant				

References

1. Long, J. L. (2003). Introduced Mammals of the World: Their History, Distribution and Influence (Cabi Publishing). 24-32.
2. Daszkiewicz, T.; Hnatyk, N.; Dąbrowski, D.; Janiszewski, P.; Gugolek, A.; Kubiak, D.; Smiecinska, K.; Winiarski, R.; Koba-Kowalczyk, M. (2015). A comparison of the quality of the Longissimus lumborum muscle from wild and farm-raised fallow deer (*Dama dama L.).* Small Rumin Research., 129(1):77-83.
3. Chakanya, C.; Dokora, A.E.M.; V. Muchenje, V. and Hoffman, L.C. (2016). The Fallow deer (*Dama spp*.); endangered or not? Zoology Garten*;* 85:160-172.
4. Serrano, M.P.; Maggiolino, A.; Lorenzo, J.M.; De-Palo, P.; Garcia, A.; Landete-Castillejos, T.; Gambin, P.; Cappelli, J.; Dominguez, R.; Perez-Barberia, F.J. and Gallego, L. (2019). Meat quality of farmed red deer fed a balanced diet: effects of supplementation with copper bolus on different muscles. Animal Journal*,* 13(4): 888-896.
5. Zmijewski, T.; Modzelewska-Kapitula, M.; Pomianowski, J. and Ziomek, A. (2020). Farmed-raised fallow deer (*Dama dama* L.) carcass characteristics and meat nutritional value. Journal Food Science Technology., 57(9): 3211-3220.
6. Camargo, G.M.F. (2018). The role of molecular genetics in livestock production.

Animal Production Science, 59(2):201-206.
7. Fadhil, I.A. (2019). Genetic polymorphisms of CSN3 gene and its effect on some production traits. Iraqi Journal of Agriculture Science*,* 50(2):500-505.
8. Dakheel, M.H.; Fadhil, I.A. and Al-Sarai, T.Q. (2021). Polymorphism of β-lactoglobulin Gene and its Effect on Selection Program in Awassi sheep. Indian Journal of Ecology, 48 Special Issue (13): 58-61.
9. Dakheel, M.H. and Al-Anbari, N.N. (2020). Expression of Insulin Like Growth Factor One Restorer (IGF1R) Gene and Impact on Iraqi Fallow Deer Performance. Indian Journal of Ecology, 47(12): 275-280.
10. Ding, N.; Tian, D.; Li, X.; Zhang, Z.; Tian, F.; Liu, S.; Han, B.; Liu, D. and Zhao, K. (2022). Genetic Polymorphisms of IGF1 and IGF1R Genes and Their Effects on Growth Traits in Hulun Buir Sheep. Genes Journal, 13, 666.
11. Hu, W.; Li, M.; Hu, R.; Li, T. and Meng, X. (2015). MicroRNA-18b Modulates Insulin-Like Growth Factor-1 Expression in Deer Antler Cell Proliferation by Directly Targeting Its 3 Untranslated Region. DNA and Cell Biology Journal*,* 34 (4):282-289.
12. Al-Samerria, S. and Radovick, S. (2021). The Role of Insulin-like Growth Factor-1 (IGF-1) in the Control of Neuroendocrine Regulation of Growth. Cells, 10(10): 2664.
13. Halmos, T. and Suba, I. (2019). The physiological role of growth hormone and insulin-like growth factors. Orv Hetil., 160(45):1774-1783.
14. Hakuno, F. and Takahashi, S.I. (2018). IGF1 receptor signaling pathways. Journal of Molecular Endocrinology, *61*:69-86.
15. Jing, W.Q. and Li, F.C. (2010). Effects of dietary lysine on growth performance, serum concentrations of insulin-like growth Factor-I (IGF-I) and IGF-I mRNA expression in growing rabbits. Agriculture Science Chinical, 9: 887–895.
16. Vitale, G.; Pellegrino, G.; Vollery, M. and Hofland, L.J. (2019). Role of IGF-1 System in the Modulation of Longevity: Controversies and New Insights from a Centenarians' Perspective. *Front. Endocrinol.*, *10*.
17. AsghariHanjani, N. and Vafa, M. (2019). The role of IGF-1 in obesity, cardiovascular disease, and cancer. Medical Journal Islam Repub Iran., 33, 56.
18. Kheralla, Y.; Gotz, W.; Kawarizadeh, A.; Rath-Deschner, B. and Jager, A. (2010). IGF-I, IGF-IR and IRS1 expression as an early reaction of PDL cells to experimental tooth movement in the rat. Archives Oral Biology, 55: 215–222.
19. Perdew, G.H.; Vanden-Heuvel, J.P. and Peters, J.M. (2006). Regulation of gene Expression-Molecular mechanisms. Totowa, NJ: Humana Press.
20. Singh, K.P.; Miaskowski, C.; Dhruva, A.A.; Flowers, E. and Kober, K.M. (2018). Mechanisms and Measurement of Changes in Gene Expression. *Biological Research Nurse,* 20(4):369–382.
21. King, M.C. and Wilson, A.C. (1975). Evolution at two levels in humans and chimpanzees. *Science*, 188:107–116.
22. Kukurba, K.R. and Montgomery, S.B. (2015). RNA sequencing and analysis. *Cold* Spring Harbor Protocols, 951–969.
23. Shabihkhani, M.; Lucey, G.M.; Wei, B.; Mareninov, S.; Lou, J.J.; Vinters, H.V. and Yong, W.H. (2014). The procurement, storage, and quality assurance of frozen blood and tissue biospecimens in pathology, biorepository, and biobank settings. Clinical Biochemistry, 47:258–266.
24. Yang, F. F.; Zhang, P.; Huo, L. J.; Riaz, H.; Yang, L. G.; Zhang, S. J. and Xiong, J. J. (2014). The association of single nucleotide polymorphism in the IGF1, IGF2 and IGF1R with antler yield in sika deer. Pakistan Veterinary Journal, 34:469–473.
25. SAS. (2012). Statistical Analysis System, User's Guide. Statistical. Version 9.1th ed. SAS. Inst. Inc. Cary. N.C. USA.
26. Duncan, D.B. (1955). Multiple Rang and Multiple F-test. Biometrics, 11: 4-42.
27. Schmittgen, T.D.; Lee,E.J.; Jiang, J.; Sarkar, A.; Yang, L.; Elton, T.S. and Chen, C. (2008). Real-time PCR quantification of precursor and mature microRNA. Methods, 44:31-38.
28. Livak, K.J. and Schmittgen, T.D. (2008). Analyzing real-time PCR data by the comparative CT method. Nature Protocols, 3:1101-1108.
29. Nolan, T.; Hands, R. and Bustin, A. (2006). Quantification of mRNA using

real-time RT-PC. *Nature Protocols,* 1:1559-1582.

30. Demetriou, C.; Abu-Amero, S.; Thomas, A.C.; Ishida, M.; Aggarwal, R.; Al-Olabi, L.; Leon, L.J.; Stafford, J.L.; Syngelaki, A.; Peebles, D.; Nicolaides, K.H.; Regan, L.; Stanier, Ph. and Moore, G.E. (2014). Paternally Expressed, Imprinted Insulin-Like Growth Factor-2 in Chorionic Villi Correlates Significantly with Birth Weight.
31. Nuhiar, R. S., Salman, A. N., & AL-Rekaby, H. R. (2019). Association of transforming growth factor beta1 gene polymorphism with diabetes mellitus risk in Iraq patients. Iraqi Journal of Biotechnology, 18(2).