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Original Article

Investigation of Egg Weight, Ovarian Follicles Morphology and Growth Differentiation Factor 9 mRNA Expression in Potchefstroom Koekoek Chicken Breed

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ABSTRACT

The *growth differentiation factor 9* (*GDF9*) gene plays a vital role in the growth and maturation of ovarian follicles in laying hens. However, its messenger ribonucleic acid (mRNA) expression levels in preovulatory ovarian follicles of indigenous chickens remain poorly understood. The study aimed to identify the association between egg weight and egg quality traits, ovarian follicles morphology, and mRNA expression levels of the *GDF9* gene in pre-ovulatory ovarian follicles of the South African Potchefstroom Koekoek chicken breed. The correlation results showed that egg weight (EW) had a positively high significant correlation (p<0.01) with egg width (EWD), yolk weight (YW), shell surface area (SSA), albumen weight (AW), albumen ratio (AR) and egg volume (EV), and a positive significant correlation (p<0.05) with egg length (EL). The Student's T-test results revealed that the numbers of large yellow follicles were significantly lower (p<0.05) than those of small yellow follicles. ANOVA findings showed that there was a significant difference (p<0.05) in the average weight of the large yellow follicles. The quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR) findings indicated that there were significant differences (p<0.05) in the mRNA expression levels of the *GDF9* gene in preovulatory ovarian follicles of the Potchefstroom Koekoek chicken breed. The mRNA expression was more abundant in F1 and F4 than in other ovarian follicles.

INTRODUCTION

In poultry production, egg yield is one of the substantial economic traits which also affects the profit margins of the farmers (Qin *et al.*, 2015). The performance of chicken egg production is mostly determined during the stages of growth and development of ovarian follicles (Wang *et al.*, 2017; Mfoundou *et al.*, 2021). Indigenous chickens play a substantial role in food provision and as a source of income for rural households, as well as in the sociocultural life of the rural community (Mengesha & Tsega, 2011). Potchefstroom Koekoek chicken is a robust breed that is resistant to diseases and has a good temperament and carcass with eye-catching yellow skin color (Hlokoe *et al.*, 2022). However, Potchefstroom Koekoek has poor egg performance which can affect its profitability. The demand for eggs is high with the increasing human population, hence, it is essential to investigate the morphology of chicken ovarian follicles and the *GDF9* gene expression in the follicles since it plays an essential role in the growth of the follicles (Yang *et al.*, 2019). The *growth differentiation factor 9* (*GDF9*) gene has a great effect on laying hens since it affects the growth and development of the ovarian follicles and the growing ability of an egg (Huang *et al.*, 2015; Lou *et al.*, 2018). According to Huang *et al.* (2015), the association between several *GDF9* single nucleotide polymorphisms

(SNPs) and egg yield traits in Chinese indigenous chicken types indicated the important part of *GDF9* in the development of the hen ovaries.

Egg quality traits have been revealed as significant predictors of egg weight during breeding in three varieties of Japanese quail (Chimezie *et al.*, 2017) and Isa Brown egg layer chickens in Nigeria (Ukwu *et al.*, 2017). Previous research on chicken egg production traits has found that improving the chicken ovarian follicles enhanced the egg production traits of the Chinese dagu chicken genotype (Zhang *et al.*, 2012), of the Nigerian local chicken genotype (Gwaza *et al.*, 2016) and Egyptian Alexandria chicken genotype (Soliman *et al.*, 2020). Several studies have been carried out to examine the function of the GDF9 gene in the ovarian follicles of chicken (McDerment *et al.*, 2012; Qin *et al.*, 2015; Hlokoe *et al.*, 2022) and the expression of the GDF9 gene in chicken ovarian follicles of Single-comb White Leghorn hens (Johnson *et al.*, 2005), Chinese local chickens (Huang *et al.*, 2015) and Luhua and Dongxiang blue-shelled chickens (Liu *et al.*, 2018). However, the GDF9 mRNA expression in ovarian follicles of the South African Potchefstroom Koekoek chicken breed has not yet been known. Hence, the objectives of the present study were to 1) determine the relationship between egg weight and egg quality traits of the Potchefstroom Koekoek chicken breed, 2) identify the morphology of the ovarian follicles of the Potchefstroom Koekoek chicken breed and 3) determine the expression levels of GDF9 gene in preovulatory ovarian follicles (F4 to F1) of the Potchefstroom Koekoek chicken breed. This study will assist breeders with the genetic markers for improving the egg production performance of the Potchefstroom Koekoek chicken breed.

MATERIALS AND METHODS

Ethical approval

Ethical approval was obtained from the University of Limpopo Animal Research Ethics Committee (ULAREC) before the commencement of the study. All procedures were performed following the standards and protocols set by the ULAREC.

Study area

The study was implemented at the University of Limpopo Experimental farm, South Africa. The farm is located about 10 km northwest of the Turfloop campus. The study area has ambient temperatures that range between 20 and 36 °C in the summer (November to January) and between 5 and 25 °C during the winter (May to July). The University of Limpopo lies at a latitude of 27.55 °S and a longitude of 24.77 °E. The mean annual rainfall is less than 400 mm (Kutu & Asiwe, 2010). The laboratory work was conducted at the Department of Biochemistry, Microbiology and Biotechnology Laboratory, University of Limpopo and at Inqaba Biotechnology laboratory, in Pretoria, South Africa.

Experimental birds, management, and study design

A total of 50 Potchefstroom Koekoek chickens were purchased at the age of 19 weeks from Monare Poultry Farm at Mohwelere in Lebowakgomo, Limpopo province, South Africa. The laying diet was purchased from Angels feeds in Polokwane, South Africa. The chickens were raised following the ordinary husbandry practices of feeding systems, housing, vaccination and health care as described by Alabi (2012). The chickens were housed under intensive production conditions. The chicken house was cleaned seven days before the chickens arrived and disinfected with Virokill disinfectants to avoid transmission of pathogenic diseases to the chickens. The biosecurity protocols were followed in the area, where the footbaths with disinfectant were placed at the door for disinfecting before entering the chicken house. The drinkers and feeders were obtained at NTK in Polokwane, South Africa. The chickens were fed the egg-laying diet from 19 to 30 weeks, and water was provided ad libitum. The chicken stress pack was given to the chickens in drinking water on arrival after transportation to relieve stress. A completely randomized design was used to select six laying chickens to be slaughtered to collect ovarian follicles for determining the morphology and three laying chickens to be slaughtered for gene expression experiments. Messenger ribonucleic acid (mRNA) expression levels of *GDF9* were examined from F4 to F1 preovulatory ovarian follicles.

Egg collection

A total of 300 eggs were randomly collected from 50 Potchefstroom Koekoek chickens at 20 weeks of age and for three (3) weeks to measure the physical egg quality traits. The collected eggs were transported to the laboratory to measure the external and internal egg quality traits.

External egg quality traits measurements

The egg weight (g) was measured and the recorded external egg quality traits were egg length (cm), egg

width (cm), egg shape index (%) and shell weight (g). External egg quality traits were measured as described by Olawumi & Ogunlade, (2008). Briefly, egg weight was measured using an electronic scale (Medidata®) with a precision of 0.01 g, whereas egg length and width were determined with a digital vernier calliper (Mitutoyo®) with an accuracy of 0.01 mm. Shell weight was determined by weighing the shell on the electronic scale. Other external egg quality traits including egg shape index, shell surface area, unit surface shell weight and shell ratio were calculated using formulas as described by Markos *et al.* (2017).

$$\text{Shape index (\%)} = \frac{\text{egg width}}{\text{egg length}} \times 100$$

$$\text{Shell surface area (cm}^2\text{)} = 3.9782 \times \text{egg weight}^{0.75056}$$

$$\text{Unit surface shell weight (g/cm}^2\text{)} = \frac{\text{shell weight}}{\text{shell surface area}}$$

$$\text{Shell ratio (\%)} = \frac{\text{shell weight}}{\text{egg weight}} \times 100$$

$$\text{Egg volume (cm}^3\text{)} = [0.6057 - (0.018 - \text{egg width})] \times \text{egg length} \times (\text{egg width})^2$$

Internal egg quality traits measurements

Internal egg quality traits measured were egg yolk weight (g) and albumen weight (g). Internal egg quality traits were measured using the methods described by Monira *et al.* (2003) and Fayeye *et al.* (2005). Briefly, each egg was delicately broken, taking care not to rupture the membranes that cover the egg yolk and albumen. After carefully separating the egg yolk and the albumen with an egg yolk separator, the weight of the egg yolk was assessed using an electronic scale. The albumen weight was estimated by subtracting the yolk and shell weights from the total weight of the egg. Other internal egg quality traits, such as albumen ratio, yolk ratio, yolk/albumen ratio, and egg volume, were calculated using the Ashraf *et al.* (2016) methods.

Albumen weight (g) = egg weight – (yolk weight + shell weight)

$$\text{Albumen ratio (\%)} = \frac{\text{albumen weight}}{\text{egg weight}} \times 100$$

$$\text{Yolk ratio (\%)} = \frac{\text{yolk weight}}{\text{egg weight}} \times 100$$

$$\text{Yolk / albumen} = \frac{\text{yolk weight}}{\text{albumen weight}} \times 100$$

Collection of ovarian follicles morphology

A total of six (6) hens aged 30 weeks were randomly selected from 50 chickens for slaughter. The hens were deprived of feed for a period of 8 to 12 hours for gut clearance. Chickens were slaughtered following the procedure of Mfoundou *et al.* (2021). In short, the chickens were sacrificed by cutting the throat, carotid arteries, jugular veins, oesophagus and trachea without splitting the head. Then they were dissected, and ovarian follicles were collected as described by Nassar *et al.* (2017). Briefly, the large yellow follicles (LYF) above 10 mm in diameter were harvested from the ovaries. The LYF were sorted by sizes from F6 to F1 using the digital Vernier calliper, with the F1 follicle as the largest follicle and individually weighed using the electronic weighing scale. The weight of the F1 follicles was recorded per chicken. The number of the LYF and of the small yellow follicles (SYF) of about 5-10 mm in diameter on the stroma were also recorded.

Total RNA extraction and analysis of *GDF9* mRNA expression

A total of three (3) laying chickens were randomly selected and slaughtered for harvesting the ovarian follicles for determining the *GDF9* mRNA expression levels. After slaughter, the ovarian follicles with sizes F4 to F1 were dissected for RNA extractions to compare chicken *GDF9* gene expression between the follicles, following the procedures of Nassar *et al.* (2017) and stored in dry ice at -80°C immediately. Total RNA was extracted from frozen ovarian follicle tissues using RNeasy mini kit according to the manufacturer's instructions (Qiagen®, USA). The extracted RNA samples were sent to the Inqaba Biotechnology laboratory. The reverse transcription of mRNA to form complementary DNA (cDNA) was performed using LunaScript RT Supermix kit (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions in a total volume of 20 ul containing 200 ng of total RNA. Following the synthesis of cDNA, q-PCR was employed as described by Pennetier *et al.* (2004). The qPCR program included an initial denaturation step at 95°C for 60 seconds (sec), {denaturation at 95°C for 15 sec, annealing at 25°C for 2 minutes (min), and extension at 60°C for 30 sec} x 30 cycles. Briefly, 1 ul of cDNA, 0.25 µM of forward and reverse primers were attained from the National Center for Biotechnology Information (NCBI) using the Primer Premier 5 software design (listed in Table 1) and 1X Luna Universal qPCR Master mix (New England Biolabs, Ipswich, MA, USA) were added to a 96-well plate. The reactions were then run on CFX96 Real-Time PCR System (Bio-Rad) following a standard two-step PCR program as recommended by the Luna Universal qPCR Master Mix manual. Three technical replicates were run for each cDNA sample. The mRNA expression of the *GDF9* gene was assessed based on

the quantification cycle (Cq) value. β-actin expression was used as an internal control. The products were analyzed using gel electrophoresis for relative gene expression detection and the $2^{-\Delta\Delta Ct}$ method was used to quantify the gene expression levels as detailed by Livak & Schmittgen (2001).

Table 1 – Primer information used for *GDF9* and β-actin gene amplification.

Gene	Primer	Sequence	Annealing temperature (°C)
GDF9	Forward Reverse	TACGCCACCAAGGAGGGAA AGCAAATCCACCGAGTGAAAGT	25
β-actin	Forward Reverse	GAGAAATTGTGCGTGACATCA CCTGAACCTCTCATTGCCA	25

Statistical analysis

Statistical Package for Social Sciences version 26.0 (IBM SPSS, 2020) was used to analyze the data. Pearson's correlations were employed to examine the correlation between egg quality traits. Descriptive statistics such as means and standard error were used to identify the morphology of ovarian follicles. Student's t-Test was used when the target gene and control gene were compared after confirming normal distributions of mRNA expression. ANOVA was used to examine the differences in the relative expression levels of *GDF9* in preovulatory ovarian follicles (F4 to F1). All the statistical analysis was performed at the 5% significance level for statistically significant and 1% for highly statistically significant. Significant differences between the means were separated using Duncan multiple range test. The following model was employed to determine the differences in mRNA expression levels among ovarian follicles:

$Y_{ij} = \mu + S_i + e_{ij}$

Where,

Y_{ij}: The j^{th} observation of the i^{th} ovarian follicle (mRNA expression level),

μ: The overall mean,

S_i: The effect of the i^{th} ovarian follicle (i = F1, F2, F3. F4) and

e_{ij}: Residual error.

RESULTS

Descriptive statistics

Descriptive statistics was utilized to determine the summary of egg weight and egg quality traits and the results are displayed in Table 2. The egg weight ranged from 32.05g to 46.65g, while measured egg quality traits ranged between 0.08 to 0.15.

Table 2 – Summary of egg quality traits of the Potchefstroom Koekoek chicken breed.

Traits	Mean ± SE	Minimum	Maximum
EW (g)	41.36 ± 0.22	32.05	46.65
EL (mm)	51.99 ± 0.11	41.00	58.84
EWD (mm)	40.82 ± 0.05	37.62	43.55
YW (g)	15.44 ± 0.07	13.05	18.03
SW (g)	6.18 ± 0.04	4.27	8.51
SI (%)	78.64 ± 0.20	63.94	97.44
SSA (cm2)	64.96 ± 0.26	53.69	71.16
USSW (g/cm2)	0.10 ± 0.001	0.08	0.14
SR (%)	15.06 ± 0.13	12.45	22.51
AW (g)	19.73 ± 0.17	13.36	24.53
AR (%)	47.47 ± 0.20	39.42	54.31
YR (%)	37.48 ± 0.14	32.21	42.26
Y/A (%)	79.71 ± 0.62	59.32	99.58
EV (cm3)	3593765.667 ± 15923.41	2652629.09	4324544.98

EW: egg weight, EL: egg length, EWD: egg width, YW: yolk weight, SW: shell weight, SI: shell surface index, S.S.A: shell surface area, USSW: unit shell surface weight, SR: shell ratio, AW: albumen weight, AR: albumen ratio, YR: yolk ratio, Y/A: yolk/albumen, EV: egg volume and SE: Standard error.

Phenotypic correlation between egg quality traits of Potchefstroom Koekoek chicken breed

Pearson's correlation was employed to examine the relationship (Table 3) between egg quality traits of the Potchefstroom Koekoek chicken breed. The results showed that EW had a positively high significant correlation (p<0.01) with EWD, YW, SSA, AW, AR and EV, a negatively high statistically significant relationship (p<0.01) with SR, YR and Y/A. The findings further revealed that EW had a positive significant correlation (p<0.05) with EL and a negatively high statistically significant correlation (p<0.05) with USSW. The outcomes also showed that EW had no statistically significant correlation (p>0.05) with SW and SI.

Morphology of chicken ovarian follicles

Descriptive statistics such as means and standard error were used to identify the morphology of ovarian follicles. Student's T-test was used to determine the differences in the number of LYF and SYF of Potchefstroom Koekoek chicken breed (Table 4). The results revealed that the numbers of large yellow follicles were significantly lower (p<0.05) than those of small yellow follicles.

Table 4 – The average number of ovarian follicles of the Potchefstroom Koekoek chicken breed.

Ovarian follicles		
LYF (Mean ± SE)	SYF (Mean ± SE)	*p*-value
6.83 ± 0.40^{b}	10.17 ± 1.08^{a}	0.02

LYF: large yellow follicles (>10 mm in diameter), SYF: small yellow follicles (5 to 10 mm in diameter), and Means with the same superscripts are not significantly different (p>0.05)

Table 3 – Phenotypic correlation between egg quality traits of Potchefstroom Koekoek chicken breed.EW: egg weight, EL: egg length, EWD: egg width, YW: yolk weight, SW: shell weight, SI: shell surface index, S.S.A: shell surface area, USSW: unit shell surface weight, SR: shell ratio, AW: albumen weight, AR: albumen ratio, YR: yolk ratio, Y/A: yolk/albumen, EV: egg volume, ns: not significant (p>0.05), * Significant (p<0.05) and **Significant (p<0.01).

Traits	EW	EL	EWD	YW	SW	SI	SSA	USSW	SR	AW	AR	YR	Y/A	EV
EW (g)														
EL (mm)	0.368*													
EWD (mm)	0.70**	0.11ns												
YW (g)	0.72**	0.32*	0.45*											
SW (g)	0.18ns	0.06ns	0.41*	-0.03ns										
SI (%)	-0.04ns	-0.89**	0.34*	-0.10ns	0.11ns									
SSA (cm2)	1.00**	0.37*	0.69**	0.72**	0.18ns	-0.05ns								
USSW (g/cm2)	-0.37*	-0.16ns	0.01ns	-0.42*	0.84**	0.14ns	-0.37*							
SR (%)	-0.51**	-0.21ns	-0.10ns	-0.51**	0.75**	0.14ns	-0.51**	0.99**						
AW (g)	0.95**	0.33*	0.61**	0.52**	-0.00ns	-0.04ns	0.94**	-0.52**	-0.63**					
AR (%)	0.73**	0.25*	0.38*	0.22ns	-0.25*	-0.06ns	0.73**	-0.64**	-0.71**	0.92**				
YR (%)	-0.58**	-0.16ns	-0.46*	0.14ns	-0.32*	-0.04ns	-0.58**	0.02ns	0.11ns	-0.73**	-0.78**			
Y/A (%)	-0.72**	-0.24ns	-0.43*	-0.09ns	-0.01ns	0.04ns	-0.72**	0.39*	0.48*	-0.89**	-0.96**	0.92**		
EV (cm3)	0.75**	0.57**	0.88**	0.52**	0.36*	-0.14ns	0.75**	-0.07ns	-0.18ns	0.66**	0.44*	-0.45*	-0.47*	

Figure 1 – Preovulatory ovarian follicles of the Potchefstroom Koekoek chicken breed.

Analysis of variance (ANOVA) was used to determine the differences in the average weights of the large yellow follicles of the Potchefstroom Koekoek chicken breed (Table 5). The findings showed that there was a significant difference (p<0.05) in the average weight of the large yellow follicles. The average weight of F1 was significantly higher (p<0.05) than the average weight of all the large yellow follicles but not significantly different (p>0.05) from that of F2, while the average weight of F2 was not significantly different (p>0.05) from that of F3. The results further revealed that the average weight of F4 was significantly different (p<0.05) from that of F3 and not significantly different (p>0.05) from that of F5, while the average weight of F5 was not significantly different from the average weight of F6. Lastly, the average weight of F6 was the lowest among the large yellow follicles.

GDF9 gene mRNA expression in preovulatory ovarian follicles

Quantitative RT-PCR was conducted to evaluate the mRNA expression level of the *GDF9* gene in preovulatory ovarian follicles (F4-F1) of the Potchefstroom Koekoek chicken breed. The Student's t-test was used to compare the target gene and control gene after confirming normal distributions. The findings indicated that there were significant differences (p<0.05) in the mRNA expression levels of *GDF9* gene in preovulatory ovarian follicles of Potchefstroom Koekoek chicken breed. The mRNA expression was most abundant in F1 and F4. However, there was no significant difference (p>0.05) in the *GDF9* mRNA expression between F1 and F4. The Quantitative real-time PCR results also showed significant differences (p<0.05) in the *GDF9* mRNA expression between F2 and F3 (Figure 2).

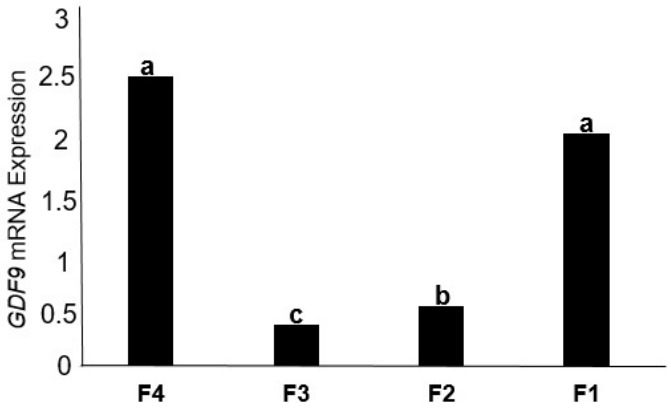

Figure 2 – Levels of *GDF9* mRNA expression in preovulatory ovarian follicles (F4-F1) of the Potchefstroom Koekoek chicken breed.

Table 5 – Average weight (Mean ± SE) of the large yellow follicles of Potchefstroom Koekoek chicken breed.

Large yellow follicles (g)						
F1	F2	F3	F4	F5	F6	p-value
10.28 ± 1.12a	8.65 ± 1.04ab	7.01 ± 0.80b	4.00 ± 0.70c	2.43 ± 0.42cd	1.15 ± 0.20d	0.0001

F1: Follicle 1, F2: Follicle 2, F3: Follicle 3, F4: Follicle 4, F5: Follicle 5 and F6: Follicle 6 and means in the same row with different superscripts are significantly different (p<0.05).

DISCUSSION

Egg weight can be evaluated and predicted using egg quality traits during chicken breeding (Ukwu *et al.*, 2017). In this study, Pearson's correlation was initially employed to investigate the relationship between egg weight and egg quality traits in the Potchefstroom Koekoek chicken breed. The data demonstrated that, with the exception of shell weight and shell index, all of the egg quality traits had a significant correlation with egg weight. The study's findings are consistent with those of Ukwu *et al.* (2017) in Nigerian Isa Brown egg layer chickens, Dzungwe *et al.* (2018) in French broiler Guinea fowl, and Saroj *et al.* (2020) in indigenous Sakini chickens, who found that egg weight had a significant correlation with egg width, yolk weight, shell surface area, albumen weight, albumen ratio, egg volume, and egg length. The findings differ from those of Singh *et al.* (2020) in indigenous chickens of India, who found that egg weight had no significant correlation with yolk weight, and the variances could be due to breed differences. The current study's findings imply that increasing egg width, yolk weight, shell surface area, albumen weight, albumen ratio, egg volume, and egg length may improve egg weight in the Potchefstroom Koekoek chicken breed. According to Maiwashe *et al.* (2002), when traits are positively correlated, it can be suggested that they are controlled by the same gene.

Pearson's correlation approach only reveals associations between the traits, not the differences in the ovarian follicles morphology (Nassar *et al.*, 2017). As a result, the study's second goal was to determine the morphology of Potchefstroom Koekoek chicken ovarian follicles at 30 weeks of age. The number of large and small yellow follicles, as well as the average weights of large yellow follicles, were determined. The results demonstrated that there were significant differences in the number of large and small yellow follicles. In the Potchefstroom Koekoek chicken breed, the average number of small yellow follicles was significantly higher than that of large yellow follicles. At 36 weeks of age, Nassar *et al.* (2017) found substantial variations in the number of large and small yellow follicles in Cairo L-2 strain and LBL strain birds. The average weight of small yellow follicles, on the other hand, was much lower than that of large yellow follicles. These variances could be attributed to age differences when the morphology was determined. The average weight of the large yellow follicles differed significantly as well, according to the findings. The average weight of F1 was substantially greater than the average weight of all large yellow follicles but not significantly different from that of F2, and the average weights of F2 and F3 were not significantly different. The study's findings are consistent with those of Nassar *et al.* (2017), who discovered substantial variations in the average weights of large yellow follicles in Cairo L-2 strain and LBL strain birds. The findings differ from those of Nie *et al.* (2022), who found a lower average number and weight of small and large yellow follicles in yellow-bearded chickens aged 50 weeks. Variations in the findings could be attributed to breed differences as well as the age at which the ovarian follicles morphology was detected. According to Wang *et al.* (2017) and Li *et al.* (2019), the performance of chicken egg production is dependent on the various stages of ovarian follicle growth and development.

The identification of the chicken ovarian follicles morphology does not show the mRNA expression of the gene that plays a role in follicles development and growth (Johnson *et al.*, 2005). Therefore, the study further evaluated the mRNA expression levels of the *GDF9* gene in the preovulatory ovarian follicles (F4-F1) of the Potchefstroom Koekoek chicken breed. Quantitative RT-PCR results showed significant differences in the mRNA expression levels of the *GDF9* gene in preovulatory ovarian follicles of the Potchefstroom Koekoek chicken breed. The *GDF9* mRNA was expressed in all the preovulatory ovarian follicles (F4-F1). However, the *GDF9* mRNA expression level was higher in F1 and F4, with no significant differences in the expression levels between the follicles. The results of the study are dissimilar to those of Johnson *et al.* (2005) in Single-comb White Leghorn hens, McDerment *et al.* (2012) in broiler breeder ovary, and Liu *et al.* (2018) in Luhua and Dongxiang blue-shelled chickens, which showed that the expression of *GDF9* mRNA is higher in the small yellow follicles compared with larger follicles. The differences could be attributed to breed variations. Liu *et al.* (2018) also reported that the *GDF9* gene was expressed in the preovulatory ovarian follicles (F6-F1) of the two breeds mentioned. An additional study showed that the expression of the *GDF9* gene was shown in follicles of the primary to preovulatory stages in Jinghai Yellow chicken (Lou *et al.*, 2018). The *GDF9* gene expression outcomes of the current study show that the *GDF9* gene plays an important role in the growth and development of ovarian follicles of the Potchefstroom Koekoek chicken breed. Liu *et al.* (2018) displayed that a high expression level of *GDF9* is one of the significant circumstances for sustaining the development of a

large number of ovarian follicles. The outcomes of the study increased the present understanding of the morphology of chicken ovarian follicles, and the *GDF9* gene and its role in the follicle development of chickens. However, additional studies are essential for functional validation.

CONCLUSIONS

The correlation findings revealed a link between egg weight and specific egg quality traits in the Potchefstroom Koekoek chicken breed. The results of the morphology of chicken ovarian follicles revealed substantial variances in the average number of small and large yellow follicles, as well as differences in the average weights among large yellow follicles. RT-qPCR outcomes revealed that *GDF9* mRNA expression was found in F1, F2, F3 and F4, with higher expression in F1 and F4. The present study will assist chicken breeders with the molecular markers for use in selection during breeding to enhance egg production performance of Potchefstroom Koekoek chicken breed. Further studies need to be conducted on the morphology of chicken ovarian follicles and expression of *GDF9* gene on some other South African indigenous chicken breeds.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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