INVESTIGATING EGG WEIGHT AND EXPRESSION OF *GROWTH DIFFERENTIATION FACTOR 9* GENE IN PREOVULATORY OVARIAN FOLLICLES OF POTCHEFSTROOM KOEKOEK CHICKEN GENOTYPE

BY

VICTORIA RANKOTSANE HLOKOE

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SUPERVISOR: PROF TL TYASI

CO-SUPERVISORS: Dr B GUNYA

: PROF VG MBAZIMA

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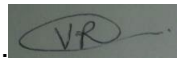
DECLARATION

I declare that this mini-dissertation hereby submitted to the University of Limpopo for the degree of Masters of Science (Animal Production) is my original work and has not been submitted by me for a degree at this or any other university, this is my work in design and execution, and that all materials contained herein have been duly acknowledged.

Signature…..  …… Date…..03/04/2023……

Student name: Victoria Rankotsane Hlokoe

LIST OF PUBLICATIONS

My role in the paper is indicated. The * indicates the corresponding author.

Chapter 2

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DEDICATIONS

This dissertation is dedicated to my family, particularly my parents Wilson Mashaphe Hlokoe and Angelina Raisibe Hlokoe, who have always been there for me and played a significant role in ensuring that I succeed in life. This is to express gratitude for their efforts in raising me to be the scholar that I am today; it is only with their help that I have succeeded.

ABSTRACT

Poultry eggs are an essential source of animal protein, and their consumption has expanded drastically in recent decades. The study aimed to determine the relationship between egg weight and egg quality traits, ovarian follicles morphology, and mRNA expression levels of the *growth differentiation factor 9* gene in pre-ovulatory ovarian follicles of the Potchefstroom Koekoek chicken genotype. A total of 300 eggs were collected to measure the egg weight (EW) and egg quality traits viz. egg length (EL), egg width (EWD), egg shape index (SI), shell weight (SW), egg yolk weight (YW), albumen weight (AW), shell surface area (SSA), unit surface shell weight (USSW), shell ratio (SR), albumen ratio (AR), yolk ratio (YR), yolk/albumen (Y/A) and egg volume (EV). Six chickens aged 30 weeks were slaughtered to harvest ovarian follicles for morphological examination, and three chickens were slaughtered to harvest follicles for evaluating the gene expression levels. Pearson's correlation, Student's T-test and Analysis of variance (ANOVA) were used for data analysis. The correlation results showed that EW had a highly positive significant association ($P < 0.01$) 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 association ($P < 0.05$) with EL and a negatively high statistically significant association ($P < 0.05$) with USSW. The findings also showed that EW had no statistically significant association ($P > 0.05$) with SW and SI. The Student's T-test results revealed that the numbers of large yellow follicles were significantly different ($P < 0.05$) from those of small yellow follicles. The ANOVA findings showed that there was a significant difference ($P < 0.05$) in the average weight of the large yellow follicles. The Quantitative real-time PCR 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 genotype. The mRNA expression was most abundant in F1 and F4. The results also showed significant differences ($P < 0.05$) in the *GDF9* mRNA expression between F2 and F3, with significant amounts in F2 than F3. In conclusion, the results indicate that the improvement of EWD, YW, SSA, AW, AR, EV and EL might enhance egg weight in Potchefstroom Koekoek chicken breed. The average weight of the large yellow follicles ranges from 1.15g to 10.28g in Potchefstroom Koekoek at 30 weeks of age. The *GDF9* gene expression findings suggest that *GDF9* gene play an important role in the growth and development of ovarian follicles of the Potchefstroom Koekoek chicken genotype. The findings

might potentially aid with *GDF9* gene expression levels for selection during breeding to improve Potchefstroom Koekoek chicken eggs. However, more research on the expression of the *GDF9* gene in additional South African indigenous chicken breeds is needed.

Keywords: Analysis of variance, correlation, egg length, egg width, yolk weight

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LIST OF ABBREVIATIONS

GDF9	*Growth differentiation factor 9 gene*
GC	Granulosa cells
mRNA	Messenger ribonucleic acid
TGF-b	Transforming growth factor-b
SYF	Small yellow follicle
LYF	Large yellow follicle
SNPs	Single nucleotide polymorphisms
INHB	Inhibin B
INHA	Inhibin A
FSH	Follicle stimulating hormone
cDNA	Complementary deoxyribonucleic acid
RT	Reverse transcription
RT-qPCR	Quantitative real time polymerase chain reaction
RNA	Ribonucleic acid
NCBI	National center for biotechnology information
ACTB	Beta actin gene
EW	Egg weight
EL	Egg length
EWD	Egg width
YW	Yolk weight
SW	Shell weight
SI	Shell index
SSA	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
SE	Standard error
SAS	Statistical analysis system

CHAPTER 1

INTRODUCTION

1.1. Background

Layer poultry farming is the practice of raising egg-laying birds for commercial egg production (Mbuza *et al.*, 2016). Indigenous poultry farming is critical to the provision of food and income for rural households, as well as to the sociocultural life of the rural community (Mengesha and Tsega, 2011). Although indigenous chickens grow slowly and lay smaller eggs, they have good mothering ability and are good brooders, great foragers, hardy, and have inherent immunity against common diseases (Dessie *et al.*, 2011). One of the most essential characteristics of native chickens is their capacity to survive extreme environmental circumstances and poor husbandry procedures such as watering, feeding, temperature, and handling with little production loss (Padhi, 2016).

The Potchefstroom Koekoek is a South African indigenous chicken genotype produced at Potchefstroom Agricultural College in the North West province of South Africa by crossing White Leghorn, Black Australorp, and Barred Plymouth Rock (Grobbelaar *et al.*, 2010). It is a free-range dual-purpose chicken genotype that is primarily bred for meat and egg production in rural communal farms (Mtileni *et al.*, 2012). This chicken genotype features sex-specific plume color and designs, making sexing of day-old chicks easier (Heit, 2017).

1.2. Problem statement

According to Ajayi (2010), Potchefstroom Koekoek is an indigenous genotype that performs well in rural regions, has good self-sustainability, disease resistance, and a wonderful temperament. However, Potchefstroom Koekoek hens have poor egg quality traits (egg length, egg width, yolk weight, shell weight, shell surface index, shell surface area, unit shell surface weight, shell ratio, albumen weight, albumen ratio, yolk ratio, yolk/albumen and egg volume), egg weights, and egg number, resulting in lower farmer profit margins (Huang *et al.*, 2015). Hence, egg production traits of this chicken genotype require genetic improvement. *GDF9* gene has been discovered to have an influence in regulating the chicken ovarian follicular development and ovulation, with a higher effect in laying hens (Yan *et al*., 2020). *GDF9* gene is one factor secreted by the oocyte with a significant role in controlling ovarian function in female reproduction, altering both the cell fate of the somatic granulosa cells and the quality and growing

ability of the egg (Lou *et al*., 2018). The improved egg quality and yield might help to increase the profit margins of the farmers and food security (Huang *et al.*, 2015).

1.3. Rationale

Previous research on chicken egg production traits found that improving the chicken ovarian follicles enhanced the egg production traits of the Chinese Dagu chicken genotype (Zhang *et al*., 2012), of Nigerian local chicken genotype (Gwaza *et al*., 2016) and of Egyptian Alexandria chicken genotype (Soliman *et al*., 2020). Adeolu and Oleforuh-Okoleh (2011) discovered the link between egg quality traits and egg weight in a south-eastern Nigerian chicken genotype. Additionally, the study suggested that improving egg quality traits through breeding could improve egg weights. According to Johnson *et al*. (2005), *GDF9* gene messenger ribonucleic acid (mRNA) was found to be expressed in highest concentration in small yellow follicles in Single-comb White Leghorn hens. Another study identified *GDF9* gene expression in ovarian follicles at the primary and preovulatory stages in chickens (Hayashi *et al.*, 2009). The *GDF9* gene is important for egg production traits as well as ovarian follicle growth (Huang *et al.*, 2015; Qin *et al.*, 2015). According to Juengel *et al.* (2004), the *GDF9* gene is located in the ovaries and has a beneficial effect on the proliferation of granulosa cells (GC) in chickens. In laying hens, the *GDF9* gene stimulates and improves GC proliferation (Johnson *et al.*, 2005). Johnson (2015) identified the *GDF9* gene as one of the genes involved in steroidogenesis and the proliferation of chicken granulosa cells. This demonstrates the importance of the *GDF9* gene in the development of chicken ovarian follicles.

The *GDF9* gene expression in the ovarian follicles of the South African Potchefstroom Koekoek chicken genotype is unknown. As a result, the current study may assist Potchefstroom Koekoek chicken farmers, researchers, and agricultural advisers in understanding the relationship between egg quality traits and selecting egg quality traits that can be used to improve egg weights during breeding. This investigation may yield information on the morphology of ovarian follicles, which is the initial step toward genetic improvement. The study may also reveal *GDF9* gene expression levels that can be used to aid in selection during breeding to improve Potchefstroom Koekoek chicken eggs.

1.3.1. Aim

The aim of the study was to evaluate the association between egg quality traits and egg weight, ovarian follicles morphology and expression levels of *growth differentiation factor 9* gene in pre-ovulatory ovarian follicles of the Potchefstroom Koekoek chicken genotype.

1.3.2. Objectives

The objectives of the study were to:

i. Determine the relationship between egg weight and egg quality traits of the Potchefstroom Koekoek chicken genotype.
ii. Identify the morphology of the ovarian follicles of Potchefstroom Koekoek chicken genotype.
iii. Determine the expression levels of *GDF9* gene in preovulatory ovarian follicles (F4 to F1) of the Potchefstroom Koekoek chicken genotype.

1.3.3. Hypotheses

i. There is no relationship between egg weight and egg quality traits of Potchefstroom Koekoek chicken genotype.
ii. Preovulatory ovarian follicles (F4 to F1) of the Potchefstroom Koekoek chicken genotype have the same expression levels of *GDF9*.

CHAPTER 2

LITERATURE REVIEW

Chicken ovarian follicles morphology and growth differentiation factor 9 gene expression in chicken ovarian follicles: review.

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2.1. Introduction

Egg-laying production efficiency is a major economic trait in the global poultry industry. Excellent production is determined by the amount of ovarian follicles destined for ovulation or atresia, as well as the oviduct's ability to convert the ova into a hard-shelled egg. A well-organized follicular hierarchy is essential for increased egg laying performance. Yang *et al.* (2019) and Mfoundou *et al.* (2021) demonstrated that geese egg production is connected to follicular development. *Growth differentiation factor 9* (*GDF9*) gene is a member of the transforming growth factor-b (TGF-b) superfamily and is found in females (Huang *et al.*, 2015). *GDF9* genes have been shown to affect ovarian follicular growth and ovulation in chickens, with a strong influence in laying hens. The *GDF9* gene is one of the factors produced by the oocyte that plays a crucial role in controlling ovarian function in female reproduction, influencing both the cell fate of somatic granulosa cells and the egg's quality and growth potential (Lou *et al.*, 2018).

It is also important in determining follicular physiological roles and is a key gene in regulating reproductive traits in various species. *GDF9* gene play a role in the synthesis of steroids by granulosa cells, according to Li *et al.* (2019), albeit the influence differs across animals. The growing human population increases the need for eggs, therefore, there is a need to analyse the morphology, development, and expression of the *GDF9* gene in the follicles because it plays a significant role in follicle development (Yang *et al.*, 2019). The growth, development, and function of chicken ovarian follicles determines laying performance (Johnson, 2015). The laying of eggs begins with the development of follicles in the chicken ovaries in a systematic manner until the eggs are generated. As a result, without the progressive stages of ovarian follicle growth and development, the egg production performance of chickens will suffer (Wang *et al.*, 2017; Li *et al.*, 2019).

The role of the *GDF9* gene in chicken ovarian follicles has been studied (Otsuka *et al.*, 2011; McDerment *et al.*, 2012; Qin *et al.*, 2015), as has the expression of the *GDF9* gene in Chinese local chicken ovarian follicles (Huang *et al.*, 2015) and Single-comb White Leghorn hens (Johnson *et al.*, 2005). The study's literature review will focus on the following topics: several indigenous egg-laying chicken genotypes (Potchefstroom Koekoek, Venda, Botchveld, and Ovambo), morphological description of chicken ovarian follicles, and the *GDF9* gene and its association with chicken egg production.

2.2. Some of the indigenous egg-laying chicken genotypes

2.2.1. Potchefstroom Koekoek chicken genotype

The Potchefstroom Koekoek (Figure 2.1) is a chicken genotype developed by researcher at Potchefstroom Agricultural College in the 1950s (Dessie and Gatachew, 2016). Potchefstroom Koekoek is an indigenous chicken genotype created by crossing Black Australorp, Bared Plymouth Rock, and White Leghorn, according to Tyasi *et al.* (2019). The term "Koekoek" refers to the genotype's color patterns. The feather coloration is sex-linked, making it useful throughout the breeding process. The chicken genotype is well adapted to tropical settings, allowing it to live under hot climatic circumstances. It is a dual-purpose chicken genotype that is best suited for free-range agricultural operations (Dessie and Gatachew, 2016). The chicken genotype is self-sustaining, disease resistant, and has an outstanding disposition. It was designed for features such as egg production and carcass with appealing yellow skin color (Mutibvu *et al.*, 2019). Furthermore, as illustrated in Figure 2.01, the genotype has black and white stripped feathers and yellow legs, and the hens are broody and make good sitters (Magothe *et al.*, 2012). The hens can produce approximately 198 eggs per year on average, with an egg weight of approximately 55.78 g. (Mtileni *et al.*, 2012). Large eggs with brown shells and bright yellow to orange yolks can be laid by the hens (Heit, 2017). It is a very popular genotype for meat and egg production among South African rural farmers and neighbouring countries (Mtileni *et al.*, 2012).

Figure 2.1. Potchefstroom Koekoek chicken genotype.

2.2.2. Venda chicken genotype

Dr Naas Coetzee initially described Venda chicken, which was named after the previous Venda province, which is now part of the Limpopo province in South Africa (Norris and Ngambi, 2006). Venda chickens have multi-coloured feathers with black, white, and red dominating, rose-colored combs, and five-toed feet (Figure 2.2). The genotype is huge and capable of laying large, coloured eggs with an average of 70 eggs per year and a weight of 53 g. (Mtileni *et al.*, 2011). Venda chicken genotype possesses good mothering ability, broodiness, and high survivorship, as well as the capacity to tolerate hard weather conditions and disease resistance (Ngambi *et al.*, 2013). They are excellent scavengers and can survive for long periods of time without food. They eat a variety of foods, including seeds, domestic waste, insects, lizards, and small rodents (Mabelebele *et al.*, 2014).

Figure 2.2. Venda chicken genotype.

2.2.3. Boschveld chicken genotype

Boschveld (Figure 2.3) is an indigenous chicken genotype established by crossing three native genotypes, Venda, Matabele, and Ovambo, according to Bosch (2011). The genotype is self-sufficient, can wander around in search of food, and is well adapted to harsh environmental conditions. According to Dessie *et al.* (2011), the Boschveld chicken genotype was bred to be disease resistant, to grow faster, and to function well in a free-range system with homemade meals and in scavenging conditions. The chicken genotype is huge and hardy, with brown and white feathers. The hens are good brooders with a strong maternal instinct and may reproduce successfully in tough conditions. The hens can lay medium brown-shelled eggs and an average of 200 eggs per annum.

Figure 2.3. Boschveld chicken genotype.

2.2.4. Ovambo chicken genotype

The Ovambo chicken genotypes originate from Namibia's northern region and Ovamboland. They can function successfully in a low-management environment (Bett *et al.*, 2013). The Ovambo chicken genotype (Figure 2.4) is a small chicken genotype with various color patterns that help them disguise and protect themselves from predators. Due to their small size, they may fly and perch on top of trees to avoid predators. The ovambo chicken breed has dark to black feathers with white or orange stripes (Grobbelaar *et al.*, 2010). These chicken genotypes are known as layers and can live in tough environments. At 16 weeks, their average body weight is approximately 1.32 kg, and at 20 weeks, it is 1.54 kg (Bett *et al*., 2013).

Figure 2.4. Ovambo chicken genotype.

2.3. Morphological characterization of chicken ovarian follicles

The performance of chicken egg production is mostly determined by the various stages of growth and development of ovarian follicles, which are classified into two types: pre-hierarchical and hierarchical follicles (preovulatory follicles) (Wang *et al.*, 2017). Folliculogenesis is the process through which ovarian follicles develop from primordial follicles to a well-developed follicular hierarchy, including cell proliferation and multiplication as well as differentiation prior to ovulation or follicle degeneration (Yu *et al.*, 2016). Many small and large white follicles (approximately 2-5mm in diameter) and 5 to 6 small yellow follicles (SYF) (about 5-10mm in diameter) comprise

the pre-hierarchical follicles. Hierarchical follicles, also known as preovulatory follicles, number 5-6 and are larger than 10mm in diameter (Wang *et al.*, 2017).

The ovaries of the layers are made up of an order of yellow yolky follicles known as preovulatory follicles ranging from F5 to F1 (Figure 5) and many thousands of small follicles from which large yolky follicles are conscripted (Apperson *et al.*, 2017). Follicles evolve from a category of small yellow follicles into the preovulatory hierarchy approximately once a day throughout the laying cycle, according to Johnson (2014). The chosen follicle will progress more quickly from F6 to F1 follicle until ovulation occurs. According to Wang *et al.* (2014), the preovulatory ovarian follicles develop in the following order: F1 as the largest follicle, which is ready to be ovulated next, F2 as the second largest follicle, followed by others until F6 as the smallest.

According to Yang *et al.* (2019), during the egg-laying season, Yangzhou geese had more follicles than Zhejiang geese and Carlos geese. There were differences in the quantity and weight of follicles between the three breeds, which could be related to egg production. Zhang *et al.* (2015) found that commercial Hy-line hens had more hierarchical follicles and heavier ovary weights than Chinese indigenous hens. The proliferation of granulosa cells was said to stimulate pre-hierarchical follicles to enter hierarchical growth in geese, which means that the thicker the granulosa cell layer, the more pre-hierarchical follicles mature and the higher egg production (Yang *et al.*, 2019). The expression levels of the genes fluctuate at various phases of follicle development; hence, it is vital to highlight the expression of the *GDF9* gene at various stages of follicle development (Pablo *et al.*, 2018).

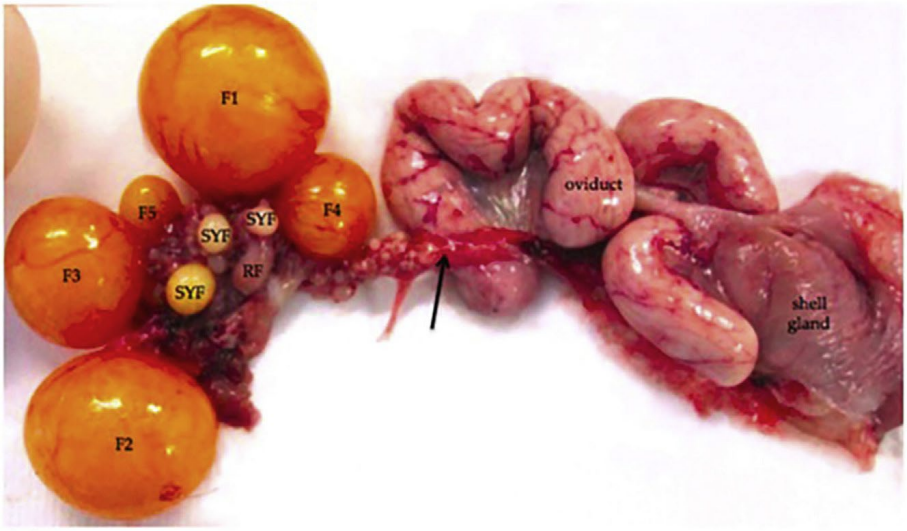

Figure 2.5. Chicken ovarian follicles (Apperson *et al.*, 2017). Five preovulatory follicles are present (F1–F5). Small yellow follicles (SYF) and a degenerating post-ovulatory follicle (RF) are visible. The black arrow symbols nerves and blood vessels. The oviduct and shell gland are labelled.

2.4. *Growth differentiation factor 9* gene and its association with egg production in chickens

The *GDF9* gene is found in the oocytes and granulosa cells of ovarian follicles in chickens and is a key regulator of folliculogenesis and ovulation rate (Otsuka *et al.*, 2011). McDerment *et al.* (2012) discovered that the *GDF9* gene plays a substantial role in the growth and maturation of ovarian follicles in chickens, with a probable effect on egg production in laying chickens. The association between various *GDF9* single nucleotide polymorphisms (SNPs) and egg production qualities in Chinese indigenous chicken varieties, according to Huang *et al.* (2015), underlined the critical function of *GDF9* in the growth of hen ovaries. The literature on chicken ovaries has revealed that the expression of *GDF9* is mainly throughout follicular growth and it has an influence on the proliferation of pre-hierarchical granulosa cells (Qin *et al*., 2015).

Besides controlling folliculogenesis, *GDF9* also regulates the other genes that are expressed in granulosa cells. The Gremlin protein had its expression improved by *GDF9* in murine granulosa cells as well as inhibin B (INHB) and steroidogenesis acute controlling protein, which also had improved expression whereas inhibin A (INHA) had its expression reduced by *GDF9* (Ernst *et al*., 2018; Sanfins *et al*., 2018). The *GDF9* expression pattern was closely associated with ovarian growth in *Schizothorax*

prenanti (fish). Compared with other non-gonadal tissues, the ovary showed more expression of the *GDF9* mRNA. Although the *GDF9* mRNA is expressed at high levels in the ovary, its expression shows a stage-specific pattern during ovarian development, and it is expressed at greater levels during the start of primary oocytes from oogonium or vitellogenesis during oocyte maturation (Yan *et al*., 2020).

It was also discovered that in mice, a lack of *GDF9* expression hampered follicle formation and resulted in infertility. *GDF9* can inhibit the biological effects of follicle stimulating hormone (FSH) in undifferentiated granulosa cells, regulate granulosa cell development, and prevent premature luteinization of granulosa cells (Lou *et al.*, 2018). According to Johnson *et al.* (2005), the *GDF9* gene mRNA is most abundant in little yellow follicles of Single-comb White Leghorn hens. Another study identified *GDF9* gene expression in follicles from the primary to preovulatory stages in chickens (Hayashi *et al.*, 2009) and mice (Lou *et al.*, 2018). According to Wang *et al.* (2013), normal expression of the *GDF9* gene allows for the down regulation of inhibin A, allowing follicles to pass through the primary stage of growth. Furthermore, it promotes the growth of preantral follicles by inhibiting granulosa cell death. Oocytes demonstrated irregular development in experiments that included the shutdown of *GDF9* expression in mammals, and with the deactivation of *GDF9*, folliculogenesis was disrupted in the main stage of growth, leading to the non-formation of mature follicles, ovulations, and, eventually, pregnancies (Castro *et al.*, 2016; Sanfins *et al.*, 2018).

2.5. Conclusions

The review of the literature concentrated on various indigenous egg laying chicken genotypes (Potchefstroom Koekoek, Venda, Boschveld, and Ovambo), morphological description of chicken ovarian follicles, and the *growth differentiation factor 9* gene and its association with chicken egg production. According to the literature, increased egg production in chickens is dependent on progressive developmental phases and the formation of ovarian follicles. The *GDF9* gene has been demonstrated to have an important role in folliculogenesis and granulosa cell proliferation in chickens, resulting in enhanced follicle development, and that a lack of *GDF9* expression may affect follicle growth and lead to infertility.

CHAPTER 3

METHODOLOGY AND ANALYTICAL PROCEDURES

3.1. Study area

The study was carried out at the University of Limpopo Experimental farm, South Africa. The farm is situated about 10 km northwest of the Turfloop campus. The study area has ambient temperatures that range between 20 and 36 ºC in summer (November to January) and between 5 and 25 ºC during winter (May to July). University of Limpopo lies at latitude 27.55 ºS and longitude 24.77 ºE (Kutu and 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.

3.2 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 University of Limpopo Animal Research Ethics Committee (AREC) number AREC/14/2021: PG.

3.3 Experimental birds, management, and study design

In this study, the Potchefstroom Koekoek chicken genotype were used. A total of 50 Potchefstroom Koekoek hens at the age of 19 weeks were obtained from Monare Poultry Farm in Mohwelere, Limpopo province, South Africa. The chickens were raised under intensive production system in accordance with the normal husbandry procedures such as feeding systems, housing, immunization, and health care as explained by Alabi (2012). To avoid the spread of harmful infections to the hens, the chicken house was cleaned seven days before arrival and disinfected with Virokill. The stress pack was provided to the chickens in drinking water on arrival after transportation to relieve stress. 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 egg laying mash was bought from Angels feeds in Polokwane, South Africa, while the drinkers and feeders were bought at NTK in Polokwane, South Africa. The chickens were fed egg laying mash from 19 weeks to 30 weeks, and water was provided *ad libitum*. 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 studies. Messenger ribonucleic acid (mRNA) expression levels of *GDF9* were examined from F4 to F1 preovulatory ovarian follicles.

3.4 Egg collection

A total of 300 eggs were randomly collected from 50 Potchefstroom Koekoek chickens at 20 weeks of age for a period of ten (10) weeks to measure the physical egg quality traits. A total of 30 eggs per week were randomly selected from the chickens. The collected eggs were taken to the laboratory at room temperature to measure the external and internal egg quality traits.

3.5 External egg quality traits measurements

The egg weights (g) were measured and the external egg quality traits that were measured involve egg length (cm), egg width (cm), egg shape index (%) and shell weight (g). External egg quality traits were measured as described by Olawumi and 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 measured 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. They were measured every week after egg collection to avoid long periods of storage. 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).

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

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

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

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

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

3.6 Internal egg quality traits measurements

Internal egg quality traits measured were egg yolk weight (g) and albumen weight (g) (g). These traits were measured using the methods described by Monira *et al.* (2003)

and Fayeye *et al.* (2005). Briefly, each egg was delicately split open, taking care not to rupture the membranes that cover the egg yolk and albumen. After carefully separating the egg yolk and 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)

Albumen ratio (%) = $\frac{\text{albumen weight}}{\text{egg weight}}$ x100

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

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

3.7 Collection of ovarian follicles for morphology examinations

A total of six (6) hens aged 30 weeks were randomly selected from 50 chickens for slaughtering as recommended by Nassar *et al.* (2017). 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). Briefly, the chickens were sacrificed by cutting the throat, carotid arteries, jugular veins, oesophagus and trachea. 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 size from F6 to F1 using a digital Vernier calliper, with F1 follicle as the largest follicle and individually weighed using an electronic weighing scale. The weight of the F1 follicles were recorded per chicken. The number of the LYF were also counted. The number of the small yellow follicles (SYF) of about 5-10 mm in diameter on the stroma was recorded.

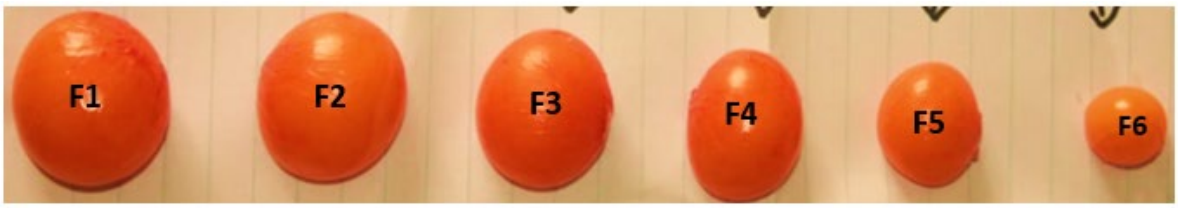

Figure 3.1. Preovulatory ovarian follicles of Potchefstroom Koekoek chicken genotype.

3.8 Total RNA extraction and analysis of *GDF9* mRNA expression

A total of three (3) chickens were randomly selected as recommended by Johnson *et al.* (2005) and Ocłoń and Hrabia (2021), and slaughtered for harvesting the ovarian follicles for determining the *GDF9* mRNA expression levels. After slaughter, the ovarian follicles with size F4 to F1 were dissected out for RNA extractions for comparing chicken *GDF9* gene expression between the follicles, following the procedures of Nassar *et al*. (2017) and stored in dry ice at -80ºC immediately. Total RNA was extracted from frozen ovarian follicle tissues using RNeasy Mini Kit according to manufacturer's instructions (Qiagen®, USA). The extracted RNA samples were sent to Inqaba Biotechnology laboratory. Quantitative for Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR). The reverse transcription of mRNA to form complementary DNA (cDNA) was performed using LunaScript RT Super mix kit (New England Biolabs, Ipswich, MA, USA) according to manufacturer's instructions in a total volume of 20ul containing 200 ng total RNA. Following the synthesis of cDNA, q-PCR was employed for cDNA amplification as described by Pennetier *et al*. (2004). The qPCR program comprised 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 that were attained from National Center for Biotechnology Information (NCBI) using the Primer premier 5 software design (listed in Table 3.1) 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 suggested by Luna Universal qPCR Master Mix manual. Three technical replicates were run for each cDNA sample. The mRNA expression of the *GDF9* gene was evaluated based on quantification cycle (Cq) value. *β-actin* expression was used as an internal control. The products were analysed 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 and Schmittgen, (2001).

Table 3.1. Primer information used for *GDF9* and *β-actin* mRNA expression

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

3.9 Statistical analysis

Statistical Package for Social Sciences version 26.0 (IBM SPSS, 2019) was used to analyse the data. Pearson's correlations were employed to examine the association 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 were performed at the 5% significance level for statistical significance and 1% for highly statistically significant. Significant differences among the means were separated using Duncan multiple range test. The following model was used to examine the differences of 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.

CHAPTER 4

RESULTS

4.1. Descriptive statistics of the egg quality traits of Potchefstroom Koekoek chicken genotype

Descriptive statistics was used to determine the summary of egg weight and egg quality traits (Table 4.1). Egg weight ranged from 32.05g to 46.65g while measured egg quality traits ranged between 4.27 to 8.51.

Table 4.1. Summary of egg quality traits of Potchefstroom Koekoek chicken genotype

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.

4.2. Phenotypic correlations between egg quality traits of Potchefstroom Koekoek chicken genotype

Pearson's correlation was employed to examine the relationships between egg quality traits of Potchefstroom Koekoek chicken genotype (Table 4.2). The results showed that EW had a positively high significant association ($P < 0.01$) 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 weight had a positive

significant association ($P < 0.05$) with EL and a negatively high statistically significant association ($P < 0.05$) with USSW. The outcomes also showed that EW had no statistically significant association ($P > 0.05$) with SW and SI.

Table 4.2. Phenotypic correlation between egg quality traits of Potchefstroom Koekoek chicken genotype

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

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 ($P < 0.01$).

4.3. Morphology of chicken ovarian follicles

Descriptive statistics such as means and standard error was 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 genotype (Table 4.3). The results revealed that the numbers of large yellow follicles were significantly different ($P < 0.05$) from those of small yellow follicles. The average number of small yellow follicles were significantly higher ($P < 0.05$) than those of large yellow follicles.

Table 4.3. Average number of the small and large yellow follicles of Potchefstroom Koekoek chicken genotype

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 same superscripts are not significantly different ($P > 0.05$)

Analysis of variance (ANOVA) was used to determine the differences in the average weights of the large yellow follicles of Potchefstroom Koekoek chicken genotype (Table 4.4). The findings showed that there was a significant difference ($P < 0.05$) in the average weights 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 outcomes 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 significantly lower ($P < 0.05$) than the average weight of all the large yellow follicles.

Table 4.4. Average weight (Mean ± SE) of the large yellow follicles of Potchefstroom Koekoek chicken genotype

Large yellow follicles (g)						
F1	**F2**	**F3**	**F4**	**F5**	**F6**	**P-value**
10.28 ± 1.12^{a}	8.65 ± 1.04^{ab}	7.01 ± 0.80^{b}	4.00 ± 0.70^{c}	2.43 ± 0.42^{cd}	1.15 ± 0.20^{d}	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 ($P < 0.05$).

4.4 *GDF9* gene mRNA expression in preovulatory ovarian follicles

Quantitative real-time PCR was conducted to evaluate the mRNA expression level of *GDF9* gene in preovulatory ovarian follicles (F4-F1) of Potchefstroom Koekoek chicken genotype (Figure 4.1). The Student's T test was used when the target gene and control gene were compared 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 genotype. 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, with significant amounts in F2 than F3 (Figure 4.2).

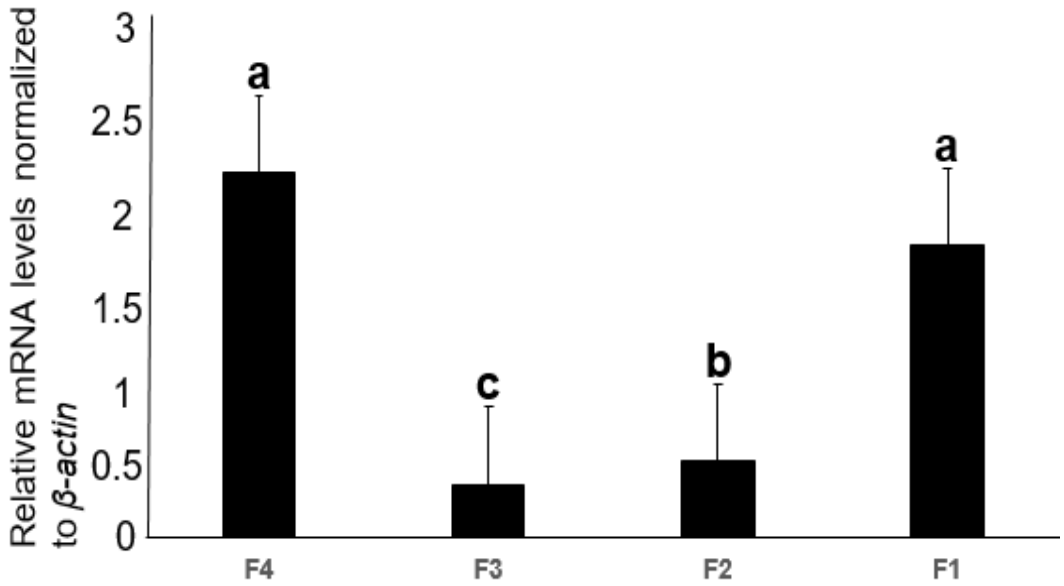

Figure 4.1. Levels of *GDF9* mRNA expression in preovulatory ovarian follicles (F4-F1) of Potchefstroom Koekoek chicken genotype.

CHAPTER 5

DISCUSSIONS, CONCLUSIONS AND RECOMMENDATIONS

5.1. Discussion

Egg weights can be best improved 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 genotype. The findings demonstrated that, with the exception of shell weight and shell index, all of the egg quality traits had a significant association 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 Dayanidhi *et al.* (2016) in indigenous Hansli hens, and the variations 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 during breeding may improve egg weight in the Potchefstroom Koekoek chicken genotype. According to Maiwashe *et al.* (2002), when traits are positively correlated, it suggests that they are controlled by the same gene.

Pearson's correlation analysis only reveals associations between the traits, not the variations 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 genotype, 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 morphologies were determined. According to the findings, the average weights of the large yellow follicles differed significantly as well. 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 play 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 Potchefstroom Koekoek chicken genotype. Quantitative RT-PCR results showed significant differences in the mRNA expression levels of *GDF9* gene in preovulatory ovarian follicles of Potchefstroom Koekoek chicken genotype. 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 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 may be due to breed variations. Liu *et al*. (2018) also reported that *GDF9* gene was expressed in preovulatory ovarian follicles (F6-F1) of the two breeds mentioned. Additional study reported that the expression of the *GDF9* gene has been discovered 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 shows that *GDF9* gene play an important role in the growth and development of ovarian follicles of Potchefstroom Koekoek chicken genotype. Liu *et al*. (2018) displayed that 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 present understanding of the morphology of

chicken ovarian follicles, and *GDF9* gene and its role in follicle development of chickens. However, additional studies are essential for functional validation.

5.2 Conclusions

Pearson's correlation was employed to assess the association between egg weight and egg quality traits. It is concluded that there is a positive link between egg weight and specific egg quality traits in the Potchefstroom Koekoek chicken genotype. The morphology of Potchefstroom Koekoek chicken ovarian follicles was determined using means and standard error of means. It is concluded that there are substantial variances in the average number of small and large yellow follicles, as well as variances in the average weights of large yellow follicles. The *GDF9* mRNA expression levels were determined using Quantitative real-time polymerase chain reaction (RT-qPCR). It is concluded that *GDF9* mRNA expression was found in F1, F2, F3 and F4, with higher expression in F1 and F4.

5.3 Recommendations

The recommendations of the current study are:

- Farmers should be educated on the relationship between egg weight and egg quality traits such as egg width, yolk weight, shell surface area, albumen weight, albumen ratio, egg volume, and egg length because they affect egg weight and can be used to increase egg weight in chickens.
- 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 breed.

CHAPTER 6

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APPENDIX

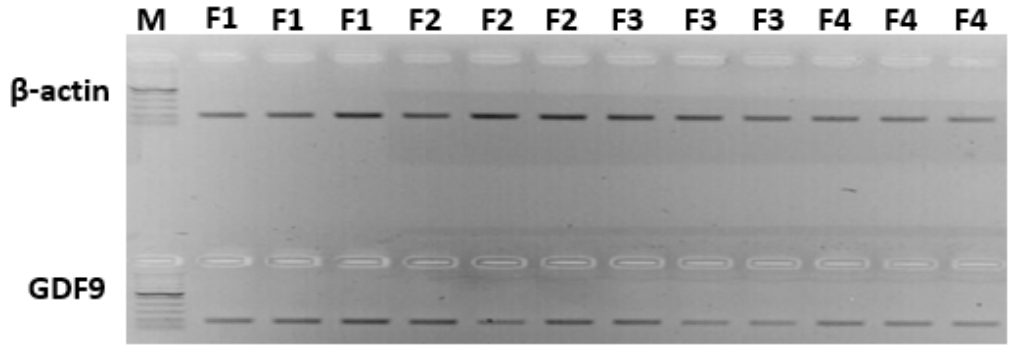

Figure 1. *GDF9* mRNA expression in preovulatory ovarian follicles (F4-F1) in Potchefstroom Koekoek chicken genotype. *β-actin* gene was used as an internal control.