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Original Article

Effects of Supplementation of Moringa Oleifera Leaf Powder on Some Reproductive Performance in Laying Hens

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■Keywords

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ABSTRACT

This study aimed to evaluate the effects of adding *Moringa oleifera* leaf powder (MOLP) to the basal diets of chickens at late laying period on clutch trait, reproductive organs, serum reproductive hormones and reproduction-related genes expression. A total of 350 hens (37 weeks old) with similar laying rate and clutch trait were randomly allocated into five groups, and fed 0 (Control, CON), 2.5 (MOLP2.5), 5, 7.5 and 10% MOLP supplemented diets for 6 weeks. The hens supplemented with 2.5% MLOP had the average clutch length, clutches and clutch intensity close to those in CON. The numbers and weight of hierarchal follicle were significantly increased at the supplementation 2.5% MOLP group. The estrogen concentration was highest in MOLP2.5 group and lowest in MOLP10 group. Expression levels of steroidogenesis-related genes of *StAR* and *Cyp19a1* were higher in MOLP2.5, MOLP5 and MOLP7.5 groups compared with that in the control. These findings suggested that dietary supplementation with 2.5% MOLP effectively increased hierarchal follicle numbers, estrogen level and gene expression of *StAR* and *Cyp19a1*, which has a potential beneficial effect on laying performance in laying chicken.

INTRODUCTION

Laying performance in chicken is primarily controlled by follicle development, which is characterized by the recruitment, growth, and follicle selection. Follicle selection determines the hierarchical follicle growth and laying performance. Follicle selection, a process from pre-hierarchical follicle to a hierarchical follicle defines the numbers of hierarchical follicle and hence the clutch trait (Johnson, 2012). Generally, the longer the clutch size, the better laying performance that layer chicken show. The clutch trait was influenced by a series of factor, such as breed (Wolc *et al.*, 2010), and nutrition (Jordan *et al.*, 2010). However, studies on the role of the phytogenic feed additives on chicken clutch trait are scarce. *Moringa oleifera lam. (M. oleifera)* is a popular tree in Asia, Africa and Arabia, widely used not only in human food but also in animal feed, because it is rich in protein and bioactive substances (Falowo *et al.*, 2018; Mahfuz *et al.*, 2019). Many studies have explored the role of *M. oleifera* on reproductive traits such as the reproductive hormone levels of rabbit (Adeyemi, 2014) and oocyte maturation of sheep (Barakat *et al.*, 2015). A study from Nayak *et al.*, (2016) found that the ethanolic extract of *M. oleifera* leaves protected the spermatogonial cells in pre-pubertal mice. Findings of Obembe *et al.*, (2018) showed that the extract of *M. oleifera* seed increased the number of spermatocytes and spermatids in rats. Physiology difference between females and males across many species. There are several reports on the role of *M. oleifera* in female animals. In mice, *M. oleifera*

had an effect of increasing size (Zeng *et al.*, 2019). In polycystic ovary syndrome (PCOS) model rats, *M. oleifera* was shown to improve follicle development (Amelia *et al.*, 2018). In sheep, *M. oleifera* extract combined with hormone supplementation improved maturation of oocytes (Barakat *et al.*, 2015).

During the late phase of the laying cycle, the laying persistency (Zhang *et al.*, 2019) is important for the goal of "500 eggs in 100 weeks". However, studies on the effect of *M. oleifera* on poultry largely focused on the egg number, feed intake and antioxidant status (Lu *et al.*, 2016; Cui *et al.*, 2018), little research has been performed on the role of *M. oleifera* in the clutch trait, follicle numbers and reproductive physiology, particularly in late laying period. Chicken is an important agricultural animal and a significant model for follicle development. Therefore, it is of great significance to fully understand the factors influencing reproductive performance, especially the follicle selection. Thus, this prompts us to investigate whether the follicle numbers of chicken could be increased by adding *M. oleifera* leaf powder (MOLP) into the basal diet. Furthermore, the physiological mechanisms underlying reproductive performance are also conducted.

MATERIAL AND METHODS

The experiment was carried out at Jiangsu Institute of Poultry Science, Yangzhou, China. The study protocol was reviewed and approved by Jiangsu University of Science and Technology Ethics Committee on Animal Research, Zhenjiang, China.

Moringa oleifera leaf powder preparation

M. oleifera fresh leaves were bought from Yunnan Daoshan Co. Ltd. Fresh leaves were air-dried with no direct sun exposure and kept at room temperature. After drying, the leaves were fine powdered. The analyzed nutrient structure of *M. oleifera* leaves were measured according to AOAC method (AOAC, 2004). The nutrient level was (on a dry matter basis): 7.96 MJ/kg metabolic energy, 27.6% crude protein, 19.26% crude fiber, 5.9% ether extract, 6.19% crude ash, 2.2% calcium content, 0.4% phosphorus contents, 1.83% lysine content, 0.25% methionine content. Total phenolics and total flavonoids were determined according to Meda *et al.* (2005), and expressed as mg of gallic acid equivalents/g of extract (GAEs) and mg of quercetin equivalent/g of extract (QEs), respectively. The values were 44.37 GAE mg/g and 23.78 QE mg/g, respectively.

Animals

A total of 350 birds of the F1 generation from Wenchang Chicken and Rugao Yellow Chicken, both of which are China local chickens, were randomly divided into 5 groups with 5 replicates. The hens were reared in a single cage in an environmentally controlled house. The hens were provided with food and water *ad libitum* and exposed to a 16L:8D light regime throughout the experimental period. The differences in clutch trait and laying rate among the five groups before the start of the experiment were insignificant. After 1-week acclimation, the feeding experiment lasted for 6 weeks. The hens were fed with the basal diet (Control group, CON), the basal diet + 2.5% MOLP (MOLP2.5 group), the basal diet + 5% MOLP (MOLP5 group), the basal diet + 7.5% MOLP (MOLP7.5 group), and the basal diet + 10% MOLP (MOLP10 group), respectively. The diets were corn-based basal rations formulated to meet the nutrient requirements of laying hens according to the Management Guide of National Research Council (NRC, 1994). The composition and the nutrient levels of the basal diet are listed in Table 1.

Clutch performance evaluation

Hen-day egg production and hen mortality were recorded daily. Clutch length is the number of eggs in a clutch. The interval days between two clutches are delay days. Then clutches, average clutch length, maximal clutch length, delay days and maximal delay days were recorded. Clutch intensity was calculated by dividing the clutch length by the sum of clutch length and delay days per clutch.

Sample collection and measurement

At the end of experiment, two hens were randomly selected per replicate and slaughtered after being fasted for 12 h. Blood was obtained from the jugular vein into heparin-coated tubes before the birds were euthanized. Serum was isolated by centrifugation at 3,000 rpm for 10 min and immediately stored at –20°C until hormone analysis. The reproductive organs and tissues including ovary, follicle, oviduct and uterus were separated from the corpse. The numbers of hierarchical follicles (with a diameter >8 mm) and pre-hierarchical follicles (named as small yellow follicle, SYF, with a diameter between 4 and 8 mm) were counted. The weight of the ovary excluding all SYFs, hierarchical follicle and post-ovulatory follicles were measured. All hierarchical follicles and the whole oviduct and uterus were weighted. The length of the

Table 1 – Ingredients and nutrient levels of experiment diet.

Ingredients	*M. oleifera* leaf powder content (%)				
	0	2.5	5	7	10
Corn	64.42	63.93	63.44	62.96	62.47
Soybean mean	23.52	22.06	20.59	19.12	17.66
Moringa oleifera leaf powder	-	2.50	5.00	7.50	10.00
Shell powder	6.70	6.70	6.70	6.70	6.70
NaCl	0.30	0.30	0.30	0.30	0.30
Calcium hydrogen phosphate	0.88	0.85	0.82	0.80	0.77
Limestone	2.11	1.99	1.88	1.76	1.64
Zeolite powder	1.63	1.23	0.83	0.42	0.01
50% Choline Chloride	0.17	0.17	0.17	0.17	0.17
DL-Methionine	0.12	0.12	0.12	0.13	0.13
Vitamin/Trace mineral premix*	0.15	0.15	0.15	0.15	0.15
Phytase	0.007	0.007	0.007	0.007	0.007
Nutrient level (analyzed)1	0	2.5	5	7	10
Metabolizable energy, MJ/kg	11.087	11.087	11.087	11.087	11.087
Crude Protein, %	16.000	16.000	16.000	16.000	16.000
Ca, %	3.350	3.350	3.350	3.350	3.350
Available Phosphorus, %	0.320	0.320	0.320	0.320	0.320
Methionine, %	0.370	0.370	0.370	0.370	0.370
Lysine, %	0.785	0.790	0.796	0.801	0.806
Crude Fiber, %	2.418	2.502	2.586	2.670	2.753

Values are expressed on an air-dry basis.

* Premix provided per kilogram of diet: vitamin A (retinyl palmitate), 7,715 IU; vitamin D3(cholecalciferol), 2,755 international chick units; vitamin E (dl-tocopheryl acetate), 8.8 IU; vitamin K (menadione sodium bisulfate complex), 2.2mg; vitamin B12(cobalamin), 0.01mg; menadione (menadione sodium bisulfate complex), 0.18mg; riboflavin, 4.41mg; pantothenic acid (d-calcium pantothenate), 5.51mg; niacin, 19.8mg; folic acid, 0.28mg; pyridoxine (pyridoxine hydrochloride), 0.55mg; manganese (manganese sulfate), 50mg; iron (ferrous sulfate), 25mg; copper (copper sulfate),2.5mg; zinc (zinc sulfate), 50mg; iodine (calcium iodate), 1.0mg; selenium (sodium selenite), 0.15mg.

[1]Calculated value.

oviduct and the uterus was measured. Finally, the ovarian cortical tissues excluding the pre-hierarchical follicles and hierarchical follicles were immediately collected and freeze dried after measurement. The tissues were stored at –80 °C before being analyzed for gene expression.

Estrogen and progesterone level analysis

The estradiol (E2) and progesterone (P4) in serum were measured using ELISA method according to the manufacture of Jiancheng Company (Jiancheng, Nanjing, China). A microplate reader (Tecan Group, Männedorf, Switzerland) set at 450 nm was used to measure absorbance values. All samples were measured triplicate and at appropriate dilutions.

Gene expression analysis

Total 100 mg tissues of ovarian cortical tissues were extracted by Trizol regent (Takara, Dalian, China). Concentration and quality of RNA were determined by Nano Drop (Nano Drop technologies, Wilmington, USA). RNA integrity was assessed by agarose gel electrophoresis. RNA would be adopted for subsequent analyses only when it had a OD260/OD280 ratio close to 2.0. Total RNA was reverse transcribed into cDNA using Reverse Transcription Kit (Takara, Dalian, China). The cDNA was analyzed by quantitative real-time PCR (RT-qPCR) using the SYBR Premix Ex Taq (Takara, Dalian, China). The reaction system contains 20 uL with 1uL cDNA, 10 μL SYBR Premix Ex Taq, 1 μL each of the primers (10 μM), and 7 μL ddH2O. The reaction conditions were set as follows: 95°C for 5 min, 95°C for 30 sec, 60°C for 30 sec, 72°C for 1 min, in total 40 cycles; extension at 72°C for 5 min. The primers for genes *Estrogen receptors* α (*ESR1*), *Estrogen receptors* β *(ESR2*), *Aromatase* (*Cyp19a1*) and *Steroidogenic acute regulatory protein* (*StAR*) are listed in Table 2. β-*actin* was used as a reference gene. Samples were run in triplicates. Relative mRNA expression was calculated as $2-\Delta\Delta CT$ values (Livaka, 2001).

Statistical analysis

All values are represented as the mean and the standard error of the mean (SEM). Statistical significance was determined with One-way ANOVA followed by Tukey multiple comparison test to calculate the interrelation between the groups, using SPSS software (SPSS 22.0 for Windows, SPSS Inc., Chicago, United State). Probability values below *0.05* and *0.01* were considered significant and extremely significant,

Table 2 – Primer sequences used in current study.

Gene1	Primer sequence (5'-3')	Product size (bp)	Accession No.
β-*actin*	F: CAGCCATCTTTCTTGGGTAT R: CTGTGATCTCCTTCTGCATCC	167	NM_205518.1
ESR1	F: ACCAACCTTGCAGACAGAGA R: CTAACCAGGCACATTCCAGC	115	NM_205183.2
ESR2	F: ACATCTGCCCAGCTACCAAT R: TCTTTTACACGGGTTGCAGC	214	NM_204794.2
Cyp19a1	F: GGCCTCTCATTTCACATGGG R: GCTTGCTCCCAAATCGAGAA	189	NM_001001761.3
StAR	F: GGTGGACAACATGGAGCAGA R: GAGCACCGAACACTCACAAA	159	NM_204686.3

[1] *ESR1*: Estrogen Receptor 1; *ESR2*: Estrogen Receptor 1; *Cyp19a1*: Cytochrome P450 Family 19 Subfamily A Member 1; *StAR*: Steroidogenic Acute Regulatory Protein.

respectively. The effects of MOLP supplementation at various levels were evaluated for linear and quadratic effects. Plotting was accomplished using Prism software (GraphPad Software Inc).

RESULTS

Effect on laying rate and clutch trait

Table 3 shows the effects of different MOLP level on laying rate and clutch trait. The laying rate significantly decreased in a line with the MOLP level ($p<0.05$). Compared with the control group, the clutches significantly increased in MOLP7.5 and MOLP10 group ($p<0.01$) and exhibited a linear response ($p<0.001$). A similar trend in clutch intensity was observed where the higher level of MOLP supplemented and the lower clutch intensity performed. No significant difference of clutches was observed in CON group, MOLP2.5 and MOLP5 group. Although clutch length was decreased with supplementation of MOLP, it was significantly longer in CON and MOLP2.5 than both that in MOLP7.5 and MOLP10 ($p<0.001$).

Table 3 – Effects of dietary supplementation of moringa oleifera leaf powder (MOLP) on clutch trait of laying hens.

Variables	Moringa oleifera leaf powder (%)1					S.E.M*	*p*-value		
	0	2.5	5	7.5	10		MOLP	Linear	Quadratic
Initial laying rate	79.94	78.75	78.56	79.11	79.24	0.852	0.332	--	--
Initial average clutch length	4.94	4.93	4.90	5.03	4.96	1.260	0.409	--	--
Laying rate (%)	78.91a	76.90ab	74.51b	73.95b	73.42b	0.901	0.069	0.012	0.218
Clutches	7.51c	7.51c	7.97bc	8.30ab	8.97a	0.15	0.001	<0.001	0.991
Average clutch length	4.77a	4.53a	4.25ab	3.47c	3.66bc	0.10	<0.001	<0.001	0.661
Maximal clutch length	9.77a	8.75ab	8.83ab	7.13c	7.57bc	0.24	0.003	<0.001	0.560
Delay days	1.32b	2.04a	1.51ab	1.30b	1.52ab	0.09	0.085	0.611	0.396
Maximal delay days	1.53b	1.98ab	2.23ab	2.24ab	2.50a	0.11	0.062	0.005	0.509
Clutch intensity	0.75a	0.74ab	0.71bc	0.69c	0.69c	0.06	<0.001	<0.001	0.681

* S.E.M, pooled standard error of mean.

a-c Mean value with a row not sharing the same superscript are significantly differ at $p<0.05$.

Effect on reproductive organs and tissues

The effects of diets supplemented with MOLP on reproductive organs and tissues are listed in Table 4. There was no significant difference in terms of oviduct and uterus length or weight and small yellow follicle numbers among groups. Feeding MOLP increased hierarchical follicle numbers and birds in MOLP2.5 group performed more follicle numbers compared to birds in other treatment groups and control. The follicle weight in MOLP2.5 was highest among the treatment groups except MOLP7.5.

Effect on hormone level

Regarding the results of the E2 and P4, the levels of E2 in CON and MOLP2.5 groups were significantly higher than those in MOLP5, MOLP7.5 and MOLP10 groups ($p=0.025$). It seems that feeding MOLP at appropriate levels increased P4 level (Table 5).

Effect on gene expression

Figure 1 shows the reproductive-related gene expression change at different MOLP supplementation level. The expression level of *ESR2* was significantly

Table 4 – Effects of dietary supplementation of moringa oleifera leaf powder (MOLP) on ovarian performance of laying hens.

Variables	Moringa oleifera leaf powder (%)1					S.E.M1	*p*-value		
	0	2.5	5	7.5	10		MOLP	Linear	Quadratic
Ovary weight (g)	4.25	4.09	3.84	3.97	3.96	0.901	0.719	0.602	0.720
Hierarchical follicle number	3.50b	4.50a	4.10ab	4.30ab	3.90ab	0.14	0.218	0.542	0.063
Hierarchical follicle weight	21.34b	29.08a	23.19b	26.79ab	23.07b	1.05	0.128	0.427	0.121
SYF	9.40	9.70	9.30	10.50	9.50	0.62	0.06	0.820	0.820
OUL	43.80	43.30	44.20	44.50	44.54	0.79	0.215	0.200	0.444
OUW	40.84	37.63	37.53	36.39	41.18	0.99	0.183	0.937	0.079

Data are means of 2 birds per replicate (n=10); SYF, small yellow follicle with diameter 4-10 mm. OUL, oviduct and uterus length. OUW, oviduct and uterus weight.

* S.E.M, pooled standard error of mean.

a-b Means value with a row not sharing the same superscript are significantly differ at $p<0.05$.

Table 5 – Effects of dietary supplementation of moringa oleifera leaf powder (MOLP) on estrogen and progesterone level.

Variables(ng/L)	Moringa oleifera leaf powder (%)1					S.E.M*	*p*-value		
	0	2.5	5	7.5	10		MOLP	Linear	Quadratic
Estrogen	169.83a	179.97a	155.55ab	150.84ab	126.28b	5.83	0.025	0.004	0.330
Progesterone	6.44b	7.34ab	8.33a	7.17ab	5.04b	0.77	0.767	0.590	0.238

Data are means of 2 birds per replicate (n=10);

* S.E.M, pooled standard error of mean.

a-b Mean value with a row not sharing the same superscript are significantly differ at $p<0.05$.

higher in MOLP10 group (*p<0.05*) compared to that in other groups. Both expression levels of *Cyp19a1* and *StAR* were significantly increased from hens fed the diet with 2.5%, 5% and 7.5% MOLP than those from the control and fed the diet with 10% MOLP (*p<0.05*). No significant difference expression level of *ESR1* gene was observed among the groups.

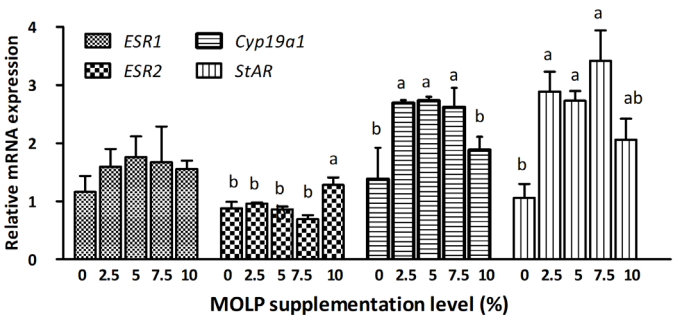

Figure 1 – Effects of moringa oleifera leaf power on the expression level of steroidogenesis-related genes in laying hens.0

Data are means of 2 birds per replicate (n=10); Means not sharing the same letter at the same gene of experiment are significant different ($p<0.05$). *ESR1*: Estrogen Receptor 1; *ESR2*: Estrogen Receptor 1; *Cyp19a1*: Cytochrome P450 Family 19 Subfamily A Member 1; *StAR*: Steroidogenic Acute Regulatory Protein.

DISCUSSION

This is the first study to evaluate *M. oleifera* leaf powder on the clutch trait in laying hen. Clutch related to oviposition was influenced by a series of factors, such as breed, age, environment, and nutrition (Akil *et al.*, 2015). Previous studies mainly focused on the laying rate, but less on the clutch trait at *M. oleifera* addition experiment. Lu *et al.*, (2016) found that dietary supplementation with 5% MOLP improved yolk color but without adverse effects on laying rate in Hy-Line Grey commercial layers. Study of Chen *et al.*, (2020) showed that the dosage of *M. oleifera* leaves less than 1% in Hy-Line brown laying hens had no side effects on laying performance. Our data showed that the effect of MOLP on laying rate is close to previous studies, it is improper to adding high level of MOLP in layer diets, the suitable supplementation level in China local chicken should be less than or equal to 2.5%. Our results revealed that the higher supplementation level of MOLP, the greater its influence on the clutch trait. Generally, the clutch length decreased, the delay days increased. A study from Jordan *et al.*, (2010) found that the unbalanced protein intake of hens delayed the ovulation time about 1h, and the clutch trait was influenced. Although the protein level between groups were the same, the higher the level of MOLP added, the higher the protein sourced from MOLP. Protein in MOLP is more difficult to absorb and digest than soybean protein as shown in previous studies that the protein digestibility of *moringa oleifera* leaf is about 41.42% (Mune *et al.*, 2016), which was lower than that in soybean meal, which is about 60% (Saki *et al.*, 2009). It was deduced that protein with different origins affect apparent digestibility, thereby indirectly affecting reproductive performance. Furthermore, the crude fiber of diets in the current study increased as the supplementation level of MOLP changed. It has been proved that the higher addition of high fiber

ingredients in layer lowered the N digestibility (Holt *et al.*, 2006), thereby affecting protein digestibility in the treatment groups. However, in the current study, we also found that the clutch trait and laying rate showed no significant difference between MOLP2.5 (low level) and CON group, suggesting that lower supplementation level of MOLP in laying chicken is feasible.

Some studies proved that diet nutrition could affect follicle number or weight of poultry. Capsaicin increased large yellow follicle (LYF, >8mm) weight but decreased SYF weight (3mm <diameter <8 mm) (Liu *et al.*, 2020). Daidzein treatment ISA hens significantly increased the number of SYF (6-8mm) and large white follicle (LWF, 2-4mm) (Liu *et al.*, 2008). The weight of LWF (>8mm) and SYF (3-8mm) of Longyan laying duck showed difference among different crude protein treatment (Ruan *et al.*, 2018). A study by Long *et al.*, (2017) showed that the number of SYFs (4-10mm) was significantly increased with octacosanol supplementation. The number of follicles is more important for clutch intensity, while weight of follicle could be more important for yolk weight. Our results demonstrated that MOLP supplementation increased the numbers of hierarchical follicle, moreover, the number was significantly increased at MOLP2.5 group.

The hormone level of E2 and P4 was further investigated in the present study. Some *in vivo* experiments indicated that E2 and P4 were influenced by phytogenic feed additives. Long *et al.*, (2017) showed that E2 and P4 was significantly increased with octacosanol supplementation. As the daidzein addition level increased, the serum estrogen level increased and reached the upper level at the highest daidzein addition level (Ni *et al.*, 2012). Extensive *in vitro* culture granulosa cells studies have proved that plant flavonoids have an impact on progesterone or estrogen synthesis, along with genes related to reproductive regulation. The progesterone level increased with the flavonoids of epimedium supplemented time *in vitro* culture granulosa cells from pre-hierarchical follicle (Guo *et al.*, 2020). The progesterone levels *in vitro* culture hierarchical granulosa cells were significantly increased under genistein addition level at 1 nM after 48 h (Xiao *et al.*, 2019). *M. oleifera* leaf contain abundant flavonoids such as quercetin (Wang *et al.*, 2021), which has been shown to influence the ovarian function in many animals such as rat, rabbit and hen (Naseer *et al.*, 2017; Yang *et al.*, 2018; Sirotkin *et al.*, 2019). In laying hens, the estrogen and progesterone levels were both increased at quercetin with a purity of 97% supplemented groups (Yang *et al.*, 2018). As quercetin sourced *M. oleifera* as well as other flavonoids take part in the hormone synthesis, the mechanism requires further examination. It is well known that P4 is mainly produced from hierarchical follicles by Δ4 pathway. In the current study, progesterone level in MOLP2.5, MOLP5 and MOLP7.5 were increased, but no significant difference was observed, which is in agreement with many studies mentioned above in terms of phytogenic feed additives.

The ovary used in the current study, excluded hierarchical follicles and pre-hierarchical follicles with a diameter of 4-8mm. It is well understood that avian estrogen is mainly produced from the small follicles. Estrogen increased *FSHR* mRNA expression level in vitro granulosa cells culture (Baba *et al.*, 2017) and follicle development and activation in chicks (Zhao *et al.*, 2017). The increase of estrogen in the present study may sensitize the hypothalamic-pituitary axis to the positive feedback the FSH (Dunn *et al.*, 2003) and thus promoter follicle development. Estrogen also plays a physiological role in the secretion of yolk precursors from the liver and promote the yolk precursors to incorporate into the developing yolky follicles (Stephens *et al.*, 2016). In the current study, the number and weight hierarchical follicle at MOLP2.5 group was significantly increased, maybe in part related to the higher level of estrogen. Reproduction is a complex and highly regulated process, involving a wide variety of hormone regulation network, transcription regulator and nutritional level, and is influenced by different breed and different physiological status.

In addition to hormone change, mRNA relative expression levels of gene *ESR1*, *ESR2*, *StAR* and *Cyp19a1* were detected. Data showed that all genes were affected by MOLP except *ESR1*. Previous studies showed that gene *ESR2* and *StAR* at 0.3% *M. oleifera* seed powder level were significantly increased than that in the control group in Japanese Laying Quail under heat stress (Abou-Elkhair *et al.*, 2020). *StAR* gene expression level increased with the flavonoids of epimedium supplemented time *in vitro* culture granulosa cells from pre-hierarchical follicle (Guo *et al.*, 2020) and *ESR2* was significantly increased under genistein addition level at 1 nM after 48 h from granulosa cells of hierarchical follicle (Xiao *et al.*, 2019). *StAR*, an essential and limiting factor enzyme for progesterone synthesis, is responsible for transporting free cholesterol from intracellular into the mitochondrial inner. *Cyp19a1* encodes for the cytochrome P450 aromatase an enzyme that is responsible for the last step synthesis of estrogen (Wang *et al.*, 2017). Previous study showed

that higher expression level of *StAR* and *Cyp19a1* were related to the laying performance of ducks (Ren *et al.*, 2019). According to reproduction theories, estrogen is an important feedback regulator in the reproduction system (Bahr, 1991), and plays an important role in regulating follicle growth at gonadotropin-independent stage and gonadotropin-dependent stage. *ESR2* may be activated with the increasing level of estrogen and the induced estrogen-dependent activation of genes, thus the follicle numbers or clutch trait changed. Date in the present study suggested that expression levels of *StAR* and *Cyp19a1* were associated with the regulation of estrogen and progesterone secretion.

In conclusion, 2.5% MOLP supplementation is beneficial for laying hens and has potential effects on reproductive physiology in terms of hormone synthesis and gene regulation, and thus has a long-lasting major impact on laying performance. This finding provides an insight into hens' clutch trait and follicle development in response to MOLP and emphasis the requirement for further study into the phytogenic feed additive on chicken reproductive performance.

DISCLOSURE STATEMENT

The authors declare that there are no conflicts of interest.

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REFERENCES

Abou-Elezz Fouad Mohammed K, Sarmiento-Franco L, Santos-Ricalde R, Solorio-Sanchez JF. The nutritional effect of Moringa oleifera fresh leaves as feed supplement on Rhode Island Red hen egg production and quality. Tropical Animal Health and Production 2012;44:1035-1040.

Abou-Elkhair R, Abdo Basha H, Slouma Hamouda AW, Ajarem JS, Maodaa SN, Allam AA, *et al.* Effect of a diet supplemented with the moringa oleifera seed powder on the performance, egg quality, and gene expression in japanese laying quail under heat-stress. Animals (Basel) 2020;10:809.

Adeyemi AA. Reproductive response of rabbits fed supplemental Moringa oleifera (Lam) leaf meal. Ibadan: Academic University of Ibadan; 2014.

Akil R, Zakaria AH. Egg laying characteristics, egg weight, embryo development, hatching weight and post-hatch growth in relation to oviposition time of broiler breeders. Animal Reproduction Sscience 2015;156:103-110.

Amelia D, Santoso B, Purwanto B, Miftahussurur M, Joewono H T. Effects of Moringa oleifera on Insulin levels and folliculogenesis in polycystic ovary syndrome model with insulin resistance. Immunology, Endocrine & Metabolic Agents in Medicinal Chemistry 2018;18:22-30.

AOAC. Official method of analysis of the Association of Official Analytical Chemists. 15th ed. Washington; 2004.

Baba T, Ting AY, Tkachenko O, Xu J, Stouffer RL. Direct actions of androgen, estrogen and anti-Müllerian hormone on primate secondary follicle development in the absence of FSH in vitro. Human Reproduction 2017;32:2456-2464.

Bahr JM. The Chicken ovary as a model of follicular development. Seminars in Reproductive Medicine 1991;9:352-359.

Barakat IA, Khalil WK, Al-Himaidi AR. Moringa oleifera extract modulates the expression of fertility related genes and elevation of calcium ions in sheep oocytes. Journal of Small Runimant Research 2015;130:67-75.

Chen Z-M, Chang W-H, Zheng A-J, Cai H-Y, Liu G-H. Tolerance evaluation of Moringa oleifera extract to Hailan brown laying hens. Journal of Animal Physiology and Animal Nutrition 2020;104(5):1375-1383.

Cui YM, Wang J, Lu W, Zhang H J, Wu SG, Qi GH. Effect of dietary supplementation with Moringa oleifera leaf on performance, meat quality, and oxidative stability of meat in broilers. Poultry Science 2018;97:2836-2844.

Dunn I, Lewis P, Wilson P, Sharp P. Acceleration of maturation of FSH and LH responses to photostimulation in prepubertal domestic hens by oestrogen. Reproduction 2003;126:217-225.

Falowo AB, Mukumbo FE, Idamokoro EM, Lorenzo JM, Afolayan AJ, Muchenje V. Multi-functional application of Moringa oleifera Lam. in nutrition and animal food products:A review. Food Research International 2018;106:317-334.

Guo Y, Li Y, Zhang S, Wu X, Jiang L, Zhao Q, Xue W, Huo S. The effect of total flavonoids of Epimedium on granulosa cell development in laying hens. Poultry Science 2020;99:4598-4606.

Holt JP, Johnston LJ, Baidoo SK, Shurson GC. Effects of a high-fiber diet and frequent feeding on behavior, reproductive performance, and nutrient digestibility in gestating sows. Journal of Animal Science 2006;84:946-955.

Johnson PA. Follicle selection in the avian ovary. Reproduction in Domestic Animals 2012;47:283-287.

Jordan D, Umar Faruk M, Lescoat P, Ali MN, Štuhec I, Bessei W, *et al.* The influence of sequential feeding on behaviour, feed intake and feather condition in laying hens. Applied Animal Behaviour Science 2010;127:115-124.

Livaka K, Schmittgenb T. Analysis of relative gene expression data using real-time quantitative PCR and the 2– ΔΔCT method. Methods 2001;25:402-408.

Liu HY, Zhang CQ. Effects of daidzein on messenger ribonucleic Acid expression of gonadotropin receptors in chicken ovarian follicles. Poultry Science 2008;87:541-545.

Liu J, Chen W, Abouelezz K, Ruan D, Wang S, Zhang Y, *et al.* Effects of capsaicin on laying performance, follicle development, ovarian anti-oxidant capacity in aged laying ducks. Poultry Science 2020;100:100901.

Long L, Wu SG, Yuan F, Zhang HJ, Wang J, Qi GH. Effects of dietary octacosanol supplementation on laying performance, egg quality, serum hormone levels, and expression of genes related to the reproductive axis in laying hens. Poultry Science 2017;96:894-903.

Lu W, Wang J, Zhang HJ, Wu SG, Qi GH. Evaluation of Moringa oleifera leaf in laying hens:effects on laying performance, egg quality, plasma biochemistry and organ histopathological indices. Italian Journal of Animal Science 2016;15:658-665.

Mahfuz S, Piao XS. Application of Moringa (Moringa oleifera) as Natural Feed Supplement in Poultry Diets. Animals (Basel) 2019;9:1-19.

Meda A, Lamien CE, Romito M, Millogo J, Nacoulma OG. Determination of the total phenolic, flavonoid and proline contents in Burkina Fasan honey, as well as their radical scavenging activity. Food Chemistry 2005;91:571-577.

Mune M, Nyobe EC, Bassogog CB, Minka S. A comparison on the nutritional quality of proteins from Moringa oleifera leaves and seeds. Agriculture 2016;2:1213618

Naseer Z, Ahmad E, Epikmen ET, Uçan U, Boyacioğlu M, Ipek E, *et al.* Quercetin supplemented diet improves follicular development, oocyte quality, and reduces ovarian apoptosis in rabbits during summer heat stress. Theriogenology 2017;96:136-141.

Nayak G, Honguntikar SD, Kalthur SG, D'Souza AS, Mutalik S, Setty MM, *et al.* Ethanolic extract of Moringa oleifera Lam. leaves protect the pre-pubertal spermatogonial cells from cyclophosphamide-induced damage. Journal of Ethnopharmacology 2016;182:101-109.

Ni Y, Wu J, Tong H, Huang Y, Lu L, Grossmann R, *et al.* Effect of dietary daidzein supplementation on egg laying rate was associated with the change of hepatic VTG-II mRNA expression and higher antioxidant activities during the post-peak egg laying period of broiler breeders. Animal Feed Science Technology 2012;177:116-123.

NRC. Nutrient requirements of poultry. Washington: National Academy Press; 1994.

Obembe OO, Raji Y. Effects of aqueous extract of seed on cadmium-induced reproductive toxicity in male Wistar rats. African Health Sciences 2018;18:653-663.

Ren J, Sun C, Chen L, Hu J, Huang X, Liu X, *et al.* Exploring differentially expressed key genes related to development of follicle by RNA-seq in Peking ducks (Anas Platyrhynchos). PloS One 2019;14:e0209061.

Ruan D, Fouad AM, Fan Q, Xia W, Wang S, Chen W, *et al.* Effects of dietary methionine on productivity, reproductive performance, antioxidant capacity, ovalbumin and antioxidant-related gene expression in laying duck breeders. The British Journal of Nutrition 2018;119:121-130.

Saki A, Abbasinezhad M, Ghazi S, Tabatabai M, Goodarzi M, Ahmadi A, *et al.* Protein pattern and urease activity of two types of soybean meal on protein digestibility and chicken performance. Journal of Applied Animal Research 2009;35:45-48.

Sirotkin AV, Štochmal'ová A, Alexa R, Kádasi A, Bauer M, Grossmann R, *et al.* Quercetin directly inhibits basal ovarian cell functions and their response to the stimulatory action of FSH. European Journal of Pharmacology 2019;860:e172560.

Stephens CS, Johnson PA. Bone morphogenetic protein 15 may promote follicle selection in the hen. General and Comparative Endocrinology 2016;235:170-176.

Surai PF. Polyphenol compounds in the chicken/animal diet: from the past to the future. Journal of Animal Physiology and Animal Nutrition 2014;98:19-31.

Wang F, Bao Y, Zhang C, Zhan L, Khan W, Siddiqua S, *et al.* Bioactive components and anti-diabetic properties of Lam. Critical Reviews in Food Science and Nutrition 2021;

Wang J, Gong Y. Transcription of CYP19A1 is directly regulated by SF-1 in the theca cells of ovary follicles in chicken. General and Comparative Endocrinology 2017;247:1-7.

Wolc A, Bednarczyk M, Lisowski M, Szwaczkowski T. Genetic relationships among time of egg formation, clutch traits and traditional selection traits in laying hens. Journal of Animal and Feed Sciences 2010;463:44.

Xiao YQ, Shao D, Tong HB, Shi SR. Genistein increases progesterone secretion by elevating related enzymes in chicken granulosa cells. Poultry Science 2019;98:1911-1917.

Yang JX, Chaudhry MT, Yao JY, Wang SN, Zhou B, *et al.* Effects of phyto-oestrogen quercetin on productive performance, hormones, reproductive organs and apoptotic genes in laying hens. Journal of Animal Physiology and Animal Nutrition 2018;102:505-513.

Zeng B, Luo J, Wang P, Yang L, Chen T, Sun J, *et al.* The beneficial effects of Moringa oleifera leaf on reproductive performance in mice. Food Science & Nutrition 2019;7:738-746.

Zhang Y, Ma W, Zhang Z, Liu F, Wang J, Yin Y, *et al.* Effects of Enterococcus faecalis on egg production, egg quality and caecal microbiota of hens during the late laying period. Archives of Animal Autrition 2019;73:208-221.

Zhao D, Lv C, Liu G, Mi Y, Zhang C. Effect of estrogen on chick primordial follicle development and activation. Cell Biology International 2017;41:630-638.