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Comparative analyses of laying performance and follicular development characteristics between fat and lean broiler lines

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ABSTRACT The deposition of high levels of fat in broiler breeder hens can have a profound impact on follicular development and laying performance. This study was formulated with the goal of comparing egg production and follicular development characteristics at different laying stages in the Northeast Agricultural University broiler lines divergently selected for abdominal fat content (**NEAUHLF**). The egg production was analyzed using the birds from the 19th to 24th generations of NEAUHLF; the follicular development characteristics were analyzed by hematoxylin-eosin staining and quantitative real-time polymerase chain reaction using the birds from the 24th generation of NEAUHLF. The results showed that the age at first egg of lean hens was significantly earlier than that of fat hens in this study. While no significant differences in total egg output from the first egg to 50 wk of age were noted when comparing these 2 chicken lines, lean hens laid more eggs from the first egg to 35 wk of age relative to fat hens, whereas fat hens laid more eggs from wk 36 to 42 and 43 to 50 relative to their lean counterparts. No differences in ovarian morphology and small yellow follicle (**SYF**) histological characteristics were noted when comparing these 2 chicken lines at 27 wk of age. At 35 and 52 wk of age, however, lean hens exhibited significantly lower ovarian weight, ovarian proportion values, numbers of hierarchical follicles, hierarchical follicle weight, and SYF granulosa layer thickness as compared to fat hens, together with a significant increase in the number of prehierarchical follicles relative to those in fat hens. Gene expression analyses suggested that follicle selection was impaired in the fat hens in the early laying stage, whereas both follicle selection and maturation were impaired in the lean hens in the middle and late laying stages. Overall, these data highlight that fat deposition in broiler hens can have a range of effects on follicular development and egg production that are laying stage-dependent.

KEY WORDS: broiler hen, fat deposition, egg production, follicular development, laying stage

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INTRODUCTION

As a result of intensive selection efforts for 70+ yr focused on growth rates and feed efficiency, broiler chickens are among the most efficient animal production systems globally (Siegel, 2014; Carney et al., 2022). The rapid growth of these chickens, however, entails the deposition of excessively high levels of body fat, particularly in the abdominal region, and this can adversely impact broiler performance in part by reducing feed efficiency in chicks and impairing breeding hen laying performance (Abdalla et al., 2018; Zhang et al., 2020; Chen et al., 2021). Most studies focused on this issue to date have sought to examine the effects of obesity on egg production through the establishment of a diet-induced model of obesity in hens. In contrast, there has been relatively little research focused on the effects of genetically determined obesity on the laying performance of broiler hens. In their study of diet-induced obesity, Chen et al. (2006) found that higher levels of body fat were associated with a decrease in egg production attributed to abnormal ovarian morphology. In a separate report, researchers separated 41-wk-old hens of similar body weights into a lean and a fat group that were

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respectively subjected to feeding restriction and overfeeding, resulting in a significantly lower egg production rate in the fat group relative to the lean group (Mohiti-Asli et al., 2012). Yu et al. (1992) further determined that excessive energy intake in broiler breeder hens was associated with the impairment of egg production together with abnormal ovarian morphological characteristics at 35 and 50 wk of age, including higher rates of internal ovulation, ovarian regression, and hierarchical follicle atresia. In an early report studying 2 broiler lines exhibiting significant differences in abdominal fat content based on the selection of plasma very low-density lipoprotein (**VLDL**) levels, researchers noted a significant increase in egg production for hens from the lean line as compared to those from the fat line through 34 wk of age (Hocking and Whitehead, 1990). These same authors also determined that the lean line exhibited egg production that was significantly greater than that of the fat line from 30 to 36 wk of age under both restricted and free-feeding conditions (Hocking et al., 1992).

Hen egg production is primarily shaped by the processes that govern follicular growth and development, including primitive follicle recruitment, prehierarchical follicle development, follicle selection, and hierarchical follicle maturation (Johnson, 2015). When chicks first emerge from the shell, many primitive follicles are already distributed in the ovary, although they remain dormant until reaching sexual maturity (Hughes, 1963). In sexually mature hens, a subset of these primitive follicles is activated whereupon they slowly grow in batches through a process known as primitive follicle recruitment (Onagbesan et al., 2009). The primordial follicles that did not receive the activation signal remained in a "dormant state" until atresia (Chen et al., 2006). Over time, the primitive follicles that were recruited develop into prehierarchical follicles, which include small white follicles (**SWF**), large white follicles (**LWF**), and small yellow follicles (**SYF**) (Onagbesan et al., 2009). The follicle selection process then governs the selection of a dominant follicle from among the pool of SYFs for entry into the hierarchical stage (Johnson, 2015), with this process being closely tied to granulosa cell development in SYFs (Johnson and Woods, 2009). During the laying period, there are 4 to 6 hierarchical follicles distributed within the ovaries of hens that are classified (F1−F6) based on their weight, diameter, and ovulation order (Johnson and Woods, 2009). The process of hierarchical follicle maturation is characterized by the hepatic biosynthesis of yolk-targeted VLDL (**VLDLy**) and other yolk precursors and their deposition within oocytes (Elkin et al., 2012).

Previous work conducted by our team using the 14th to 18th generations of the Northeast Agricultural University (Harbin, China) broiler lines divergently selected for abdominal fat content (**NEAUHLF**) revealed that lean hens presented with a significantly earlier age at first egg (**AFE**) and a higher egg number (**EN**) as of 40 wk of age relative to fat hens (Zhang et al., 2018). However, this prior study did not address whether any laying stage-specific differences in egg production differences were evident between these 2 broiler lines, nor did they evaluate any potential differences in the follicular growth and development process between these lines. As such, the present study was developed with the goal of comparing the egg production and follicular development characteristics of the birds from NEAUHLF across different laying stages.

MATERIAL AND METHODS

Ethical Statement

The Guidelines for the Care and Use of Experimental Animals established by the Ministry of Science and Technology of the People's Republic of China were used to guide the present study, which received approval from the Laboratory Animal Management Committee and the Institutional Biosafety Committee of Northeast Agricultural University (Harbin, China) (approval#: 2006-39).

Experimental Animals

In this study, the hens from the 19th generation (G_{19}) to the 24th generation (G_{24}) of NEAUHLF were used for the analysis of egg-laying traits, and the hens from G_{24} of NEAUHLF were used for the analysis of abdominal fat traits and follicular development-related traits. These NEAUHLF lines have undergone selection since 1996 based on abdominal fat percentage (**AFP**) values and VLDL concentrations. Briefly, the G_0 generation of NEAUHLF came from the same grandsire line originating from the Arbor Acres broiler, which was then divided into 2 lines according to their VLDL concentration at 7 wk of age. From G_1 to G_{27} generations, birds from each line were raised in 2 hatches, with free access to feed and water. Plasma VLDL concentrations were measured for all male birds at 7 wk, and AFP of the male birds in the first hatch was measured after slaughter at 7 wk. Sibling birds from the families with lower (lean line) or higher (fat line) AFP than the average value of the population were selected as candidates for breeding, considering the body weight **(BW)** at 7 wk and plasma VLDL concentration of male birds in the second hatch and egg production of female birds in both hatches. The selection procedure have been described in detail previously (Guo et al., 2011). The AFP of fat and lean male birds at 7 wk of age in the G_{24} generation differed by 13.25 times.

All birds from both lines had free food and water access from hatching to 2 wk of age, and were subjected to equivalent amounts of feed restriction from 3−53 wk of age. All hens were raised under identical environmental conditions. All hens were fed a commercial corn-soybean-based diet that met the nutritional requirements of broilers recommended by the National Research Council (NRC, 1994). From 16 to 53 wk of age, the female birds were housed in individual metal cages. Each bird was raised in a single cage from the chicken farm of Acheng Animal Husbandry Base of Northeast Agricultural

University (Harbin, China), and the eggs were collected from the individuals.

Analyses of Egg-Laying Traits

Age at first egg and EN at different laying stages were measured for hens from both the lean and fat lines from G_{19} to G_{24}. Specifically, measurements of AFE and EN were performed using 1,693 hens from the first egg to 50 wk of age, 1,674 hens from the first egg to 35 wk of age, 1,674 hens from 36 to 42 wk of age, and 1,672 hens from 43 to 50 wk of age.

Analyses of Abdominal Fat Traits and Ovarian Morphology

In total, 205 G_{24} hens were slaughtered after fasting for 12 h. Abdominal fat traits were assessed by measuring BW and abdominal fat weight (**AFW**) (including abdominal fat pad and fat adhered to the gizzard), which were used to calculate AFP (AFW/BW).

Analyses of ovary morphology, ovary weight (**OW**), and ovary percentage (**OP**; OW/BW) were performed. Specific ovarian characteristics monitored during these analyses included the following: (1) atretic hierarchical follicles, characterized by irregularly shaped follicles with dark yellow or brown coloration and loose surrounding connective tissue (Chen et al., 2006); (2) internal ovulation, as evidence by the presence of yolk or yolk-like liquid in the abdominal cavity (Chen et al., 2006); and (3) follicular grading, which was performed by excising the ovary and separating SWFs (1−3.9 mm), LWFs (4−4.9 mm), SYFs (5−8 mm), and hierarchical follicles (F6−F1, >9 mm) (Johnson and Woods, 2009). The numbers of each follicle type were quantified, and the weight of hierarchical follicles was assessed. If the weights of the 2 hierarchical follicles differed by less than 1 g, they were considered to be the same rank in the hierarchy (Hocking, 1996).

Histological Analyses of SYFs

All SYFs were collected from 10 G_{24} hens at 27, 35, and 52 wk of age ($n = 5$ hens per line at each time point), fixed with 4% paraformaldehyde, paraffin-embedded, and cut into 5 μm sections. These sections were then stained with hematoxylin and eosin (**H&E**) (Solarbio, Beijing, China). One section per SYF was selected to examine the structure of the SYF wall via microscopy (Nikon, Japan). Three visual fields were selected at random for each section, and images were collected with the microscope imaging system. For each visual field, 3 locations were selected at random, and SYF granular layer thickness was quantified using Image J 1.46R (NIH, Bethesda, MD).

RNA Isolation

Liver, SYF, and hierarchical follicle samples were harvested from 8 G_{24} hens at 27, 35, and 52 wk of age ($n = 4$ hens per line at each time point). These tissues were rinsed with 0.75% NaCl, snap-frozen with liquid nitrogen, and stored at −80°C. TRIzol (Invitrogen, CA) was used to extract total tissue RNA from 100 mg of each sample based on the provided directions with subsequent sample dilution using RNase-free water. Ultraviolet spectrophotometry (Eppendorf, Hamburg, Germany) was used to assess the quality of the extracted RNA, and only RNA samples with an OD_{260}/OD_{280} ratio from 1.8 to 2.1 were used in subsequent qPCR analyses. After preparation, RNA was stored at −80°C.

Quantitative Real-Time PCR

For each sample, 1 μg of total RNA was used to prepare cDNA with the ImProm-Ⅱ Reverse Transcription System (Promega, WI) based on the provided directions. Then quantitative Real-time PCR (qPCR) reactions were performed in triplicate with a FastStart Universal SYBR Green Master kit (Roche, Switzerland) and the following settings: 95°C for 10 min; 40 cycles of 95°C for 1 min, and 60°C for 1 min. Mean threshold cycle (**Ct**) values were used to compare gene expression levels among samples. Analyzed genes in the present study included follicle-stimulating hormone receptor (**FSHR**), antimullerian hormone (**AMH**), and bone morphogenetic protein 15 (**BMP15**) in SYFs, acetyl CoA carboxylase (**ACC**), fatty acid synthase (**FAS**), apolipoprotein B100 (**apoB100**), and apolipoprotein VLDL-Ⅱ (**apoVLDL-Ⅱ**) in the liver, and very low-density lipoprotein receptor (**VLDLR**) in the hierarchical follicles. TATA-box binding protein (**TBP**) was used to normalize gene expression, and relative expression levels were compared via the $2^{-\Delta CT}$ method (Schmittgen and Livak, 2008), where $\Delta CT = CT_{\text{target gene}} - CT_{TBP}$. Primers used for the present study are compiled in Table S1.

Statistical Analysis

The degree to which all data conformed to a normal distribution was assessed. Normally distributed data (BW, AFW, AFP, OW, OP, numbers of SWFs, LWFs, SYFs, and hierarchical follicles, hierarchical follicle weight, granulosa layer thickness of SYFs and gene expression levels) from the G_{24} generation were compared with independent samples t tests. Normally distributed data (AFE, EN) with large sample numbers from G_{19} to G_{24} were analyzed with a generalized linear model (**GLM**) approach. Model (1) was used to analyze data for each separate generation, with line (L) and hatch (H) as fixed effects in this model. Model (2) was used to analyze combined data from multiple generations, with line (L), generation (G), the interaction of

line and generation ($L \times G$), and hatch (H) as fixed effects:

$$Y = \mu + L + H + F(L) + D(F, L) + e \quad (1)$$

$$Y = \mu + L + G + L \times G + H + F(L) + D(F, L) + e \quad (2)$$

In both models, Y was the dependent variable, μ was the population mean, F (L) was a random effect of the family nested within the line, D (F, L) was a random effect of the dam nested within the line and the family, and e was the random error. Significant differences between the least squares means (**LSM**) of phenotype of the fat and lean lines were calculated. JMP Pro 14 (SAS Institute Inc., NC) was used for all statistical testing, with $P < 0.05$ as the cut-off for significance.

RESULTS

Laying Stage-specific Differences in the Laying Performance of Hens from the Fat and Lean Lines

Initially, AFE and EN values for hens from the lean and fat lines from G_{19} to G_{24} were compared at different laying stages. As shown in Table 1, the AFE of the lean line was significantly earlier than that of the fat line in both single- and combined-generation analyses ($P < 0.05$ or $P < 0.01$). When measured from the first egg to 50 wk of age, the EN of the lean line was significantly higher than that of the fat line in G_{22} ($P < 0.05$). No difference in EN over this interval was observed when comparing the lean and fat lines in G_{19}, G_{20}, G_{21}, G_{23}, G_{24}, and G_{19} to G_{24} combined (Table 2). In contrast, when measured from the first egg to 35 wk of age, the EN for the lean line was significantly greater than that for the fat line in G_{20}, G_{21}, and G_{22} ($P < 0.01$, Table 2). In a combined analysis of G_{19} to G_{24}, the EN for the lean line was significantly greater than that for the fat line as of 35 wk of age ($P < 0.01$, Table 2). Conversely, from 36 to 42 wk of age, the EN of the fat line was significantly greater than that of the lean line in G_{23} and G_{24} ($P < 0.05$, Table 2). In a combined analysis of G_{19} to G_{24}, the EN of the fat line from 36 to 42 wk of age was significantly greater than that of the lean line ($P < 0.01$, Table 2). Similarly, from 43 to 50 wk of age, the EN of the fat line remained significantly higher than that of the lean line in G_{23} and G_{24} ($P < 0.05$ or $P < 0.01$, Table 2), with a trend towards higher EN values for the fat line in G_{20} ($P = 0.071$, Table 2), but significant lower EN values for the fat line in G_{22} ($P < 0.05$, Table 2). In a combined analysis of G_{19} to G_{24}, the EN of the fat line was significantly elevated as compared to that of the lean line from 43 to 50 wk of age ($P < 0.05$, Table 2). As such, despite the absence of any differences in EN values between the fat and lean lines in a combined generation

Table 1. Comparison of the age at first egg (AFE) between fat and lean hens in different generations.

	Lean line		Fat line		
Generation	n	AFE (d)	n	AFE (d)	P-value[1]
G_{19}	140	203.38 ± 0.81[2]	139	209.21 ± 0.83	<0.001
G_{20}	125	189.23 ± 1.43	138	197.42 ± 1.36	<0.01
G_{21}	135	190.26 ± 1.07	132	193.36 ± 1.10	<0.05
G_{22}	127	188.61 ± 0.83	115	197.58 ± 0.85	<0.001
G_{23}	152	197.21 ± 0.81	124	201.3 ± 0.85	<0.01
G_{24}	155	186.94 ± 0.84	121	192.76 ± 0.97	<0.001
G_{19}-G_{24}	834	192.72 ± 0.41	769	198.50 ± 0.42	<0.001

[1]*P*-values refered to the effect of line on AFE.
[2]Data were presented as Means ± SEM.Abbreviations: AFE, age at first egg; LSM, least squares mean; SEM, standard error of the mean.

Table 2. Comparison of egg number (EN) between fat and lean hens in different generations.

		Total			First egg to 35 wk of age			36 to 42 wk of age			43 to 50 wk of age		
Generation	Line	n	LSM ± SEM	P-value[1]	n	LSM ± SEM	P-value[1]	n	LSM ± SEM	P-value[1]	n	LSM ± SEM	P-value[1]
G_{19}	Lean	140	83.11 ± 1.30	0.596	138	35.66 ± 0.73	0.757	138	24.06 ± 0.52	0.154	137	24.12 ± 0.76	0.815
	Fat	139	81.89 ± 1.45		138	35.34 ± 0.73		139	22.98 ± 0.53		135	24.38 ± 0.78	
G_{20}	Lean	128	83.14 ± 1.83	0.63636	125	27.06 ± 0.88	<0.01	124	26.83 ± 0.63	0.4	128	30.48 ± 0.93	0.071
	Fat	141	82.27 ± 1.65		135	23.00 ± 0.86		137	27.57 ± 0.60		140	32.90 ± 0.91	
G_{21}	Lean	138	85.79 ± 1.43	0.416	138	30.21 ± 0.89	<0.01	138	31.81 ± 0.62	0.266	137	28.08 ± 0.81	0.16
	Fat	139	84.12 ± 1.66		138	26.47 ± 0.90		138	32.81 ± 0.59		136	29.74 ± 0.83	
G_{22}	Lean	128	93.38 ± 1.73	<0.05	127	30.85 ± 0.99	<0.001	126	31.59 ± 0.71	0.365	128	30.9 ± 1.07	<0.05
	Fat	117	84.44 ± 2.13		117	24.55 ± 1.01		116	32.52 ± 0.72		117	27.62 ± 1.06	
G_{23}	Lean	154	78.42 ± 1.41	0.16	153	23.81 ± 0.74	0.368	152	27.97 ± 0.68	<0.05	154	25.69 ± 0.81	<0.05
	Fat	159	82.19 ± 1.55		156	22.89 ± 0.70		157	30.18 ± 0.63		159	28.13 ± 0.74	
G_{24}	Lean	155	88.50 ± 1.04	0.142	154	26.52 ± 0.75	0.218	155	30.16 ± 0.57	<0.05	151	29.8 ± 0.72	<0.01
	Fat	155	91.39 ± 1.52		155	25.18 ± 0.78		154	32.23 ± 0.59		150	33.56 ± 0.74	
G_{19} to G_{24}	Lean	843	85.77 ± 0.78	0.47	835	29.13 ± 0.39	<0.001	833	28.66 ± 0.24	<0.01	835	28.25 ± 0.30	<0.05
	Fat	850	84.97 ± 0.77		839	26.33 ± 0.38		841	29.63 ± 0.24		837	29.29 ± 0.30	

[1]*P*-values refered to the effect of line on EN.Abbreviations: EN, egg number; LSM, least squares mean; SEM, standard error of the mean; wk, week.

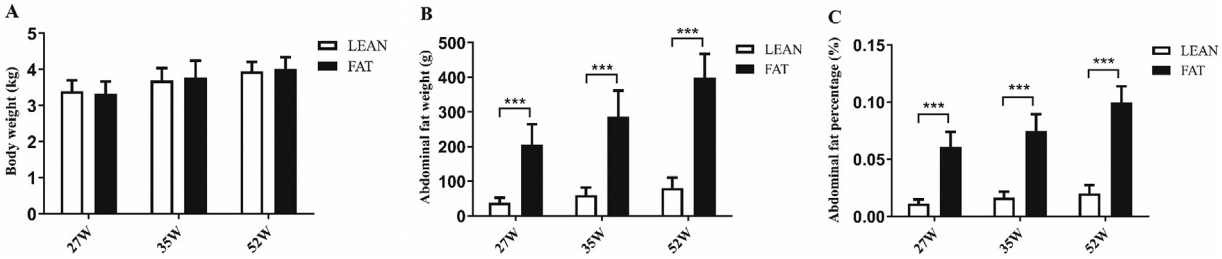

Figure 1. Comparison of body weight, abdominal fat weight, and abdominal fat percentage between fat and lean hens at 27, 35, and 52 wk of age in G_{24} generation. (A) Comparison of body weight between fat and lean hens. (B) Comparison of abdominal fat weight between fat and lean hens. (C) Comparison of abdominal fat percentage between fat and lean hens. The result was expressed as means ± SEM. ***$P < 0.001$. Abbreviations: W, week of age; LEAN, lean line; FAT, fat line; SEM, standard error of the mean.

analysis conducted for the entire laying period, significant differences in these values were evident at different laying stages. Strikingly, the egg production of the lean line was greater than that of the fat line during the early laying stage (first egg to 35 wk of age), but lower than that of the fat line during the middle (36 to 42 wk of age) and late (43 to 50 wk of age) laying stages.

Abdominal Fat Trait Analyses at Different Laying Stages

The BW, AFW, and AFP of each female hen from G_{24} were measured at 27, 35, and 52 wk of age. The AFW and AFP of fat females were significantly higher than those of lean females ($P < 0.01$, Figure 1). BW values did not differ significantly between lean and fat females at any time point (Figure 1).

Analyses of Ovarian Morphology at Different Laying Stages

Ovarian morphological characteristics were next compared between lean and fat G_{24} hens at 27, 35, and 52 wk of age. With the exception of LWF number and F5 follicle weight, no significant differences in ovarian morphology were noted when comparing lean and fat hens at 27 wk of age (Table 3). In contrast, OW, OP, the number of hierarchical follicles, and the weight of each hierarchical follicle for the lean line were significantly decreased relative to those for the lean line at 35 and 52 wk of age ($P < 0.05$ or $P < 0.01$, Table 3), while significantly increased numbers of SWFs, LWFs, SYFs, and total prehierarchical follicles were evident for the fat line relative to the lean line ($P < 0.05$ or $P < 0.01$, Table 3). This suggests that in the early laying stage, the follicle selection and maturation processes in lean and fat hens may be comparable, whereas follicle selection and maturation in the lean line may be hindered relative to the fat line in the middle and late laying stages.

SYF Histologic Analyses at Different Laying Stages

To more fully explore differences in follicular selection between lean and fat hens, SYF histological characteristics were compared between these 2 chicken lines in G_{24} hens at 27, 35, and 52 wk of age. H&E staining revealed

Table 3. Comparison of ovary morphology of fat and lean hens at different laying stages in G_{24} generation.

	27 wk of age			35 wk of age			52 wk of age		
Traits	Lean ($n = 35$)	Fat ($n = 32$)	*P*-value	Lean ($n = 39$)	Fat ($n = 29$)	*P*-value	Lean ($n = 35$)	Fat ($n = 35$)	*P*-value
Ovary weight (g)	56.30 ± 2.41[1]	59.55 ± 3.20	0.42	60.14 ± 1.93	74.71 ± 2.55	<0.01	50.44 ± 2.08	64.8 ± 2.37	<0.001
Fractional ovarian weight (%)	1.65 ± 0.06	1.78 ± 0.10	0.22	1.64 ± 0.06	2.00 ± 0.07	<0.01	1.28 ± 0.06	1.63 ± 0.06	<0.001
Number of hierarchical follicles	7.11 ± 0.25	7.22 ± 0.25	0.77	6.31 ± 0.23	8.10 ± 0.26	<0.001	5.51 ± 0.18	6.80 ± 0.32	<0.01
Number of prehierarchical follicles	36.34 ± 2.73	31.00 ± 3.06	0.2	52.95 ± 1.87	43.55 ± 2.19	<0.01	57.11 ± 2.45	41.69 ± 2.24	<0.001
Number of SWF	20.94 ± 1.68	18.47 ± 1.85	0.326	25.49 ± 1.17	20.55 ± 1.51	<0.01	29.57 ± 1.45	21.94 ± 1.38	<0.01
Number of LWF	11.14 ± 1.06	6.63 ± 0.78	<0.01	13.23 ± 0.7	10.57 ± 0.72	<0.01	16.46 ± 1.23	10.77 ± 0.93	<0.01
Number of SYF	4.26 ± 0.43	5.91 ± 0.74	0.061	15.31 ± 0.96	12.79 ± 1.00	<0.05	11.09 ± 0.78	8.97 ± 0.68	<0.05
F1 follilce weight (g)	12.47 ± 0.29	12.33 ± 0.24	0.695	15.00 ± 0.19	15.57 ± 0.26	0.09	15.29 ± 0.63	16.99 ± 0.27	<0.05
F2 follilce weight (g)	10.01 ± 0.36	10.03 ± 0.29	0.516	11.44 ± 0.28	12.78 ± 0.37	<0.01	11.24 ± 0.50	13.35 ± 0.49	<0.01
F3 follilce weight (g)	6.91 ± 0.31	7.55 ± 0.29	0.160	7.22 ± 0.32	9.68 ± 0.43	<0.001	6.08 ± 0.45	8.98 ± 0.56	<0.001
F4 follilce weight (g)	4.28 ± 0.22	4.91 ± 0.27	0.072	3.87 ± 0.27	6.87 ± 0.49	<0.001	2.92 ± 0.29	5.77 ± 0.49	<0.001
F5 follilce weight (g)	2.23 ± 0.16	3.31 ± 0.33	<0.05	1.93 ± 0.18	4.92 ± 0.35	<0.001	1.66 ± 0.22	3.17 ± 0.43	<0.01
F6 follilce weight (g)	2.08 ± 0.6	2.08 ± 0.21	0.994	1.29 ± 0.16	2.83 ± 0.31	<0.001	1	1.4	——[2]
Atretic hierarchical follicles	2/35	6/32	0.102	2/39	6/29	0.056	4/35	3/35	0.5
Internal ovulation	2/35	4/32	0.294	2/39	3/29	0.36	6/35	11/35	0.132

[1]Data were presented as Means ± SEM.

[2]The horizontal line indicated that statistical analysis cannot be conducted because at 52 wk of age, only 1 hen contained F6 follicle in the fat and lean line, respectively.Abbreviations: SWF, small white follicle; LWF, large white follicle; SYF, small yellow follicle; SEM, standard error of the mean; wk, week.

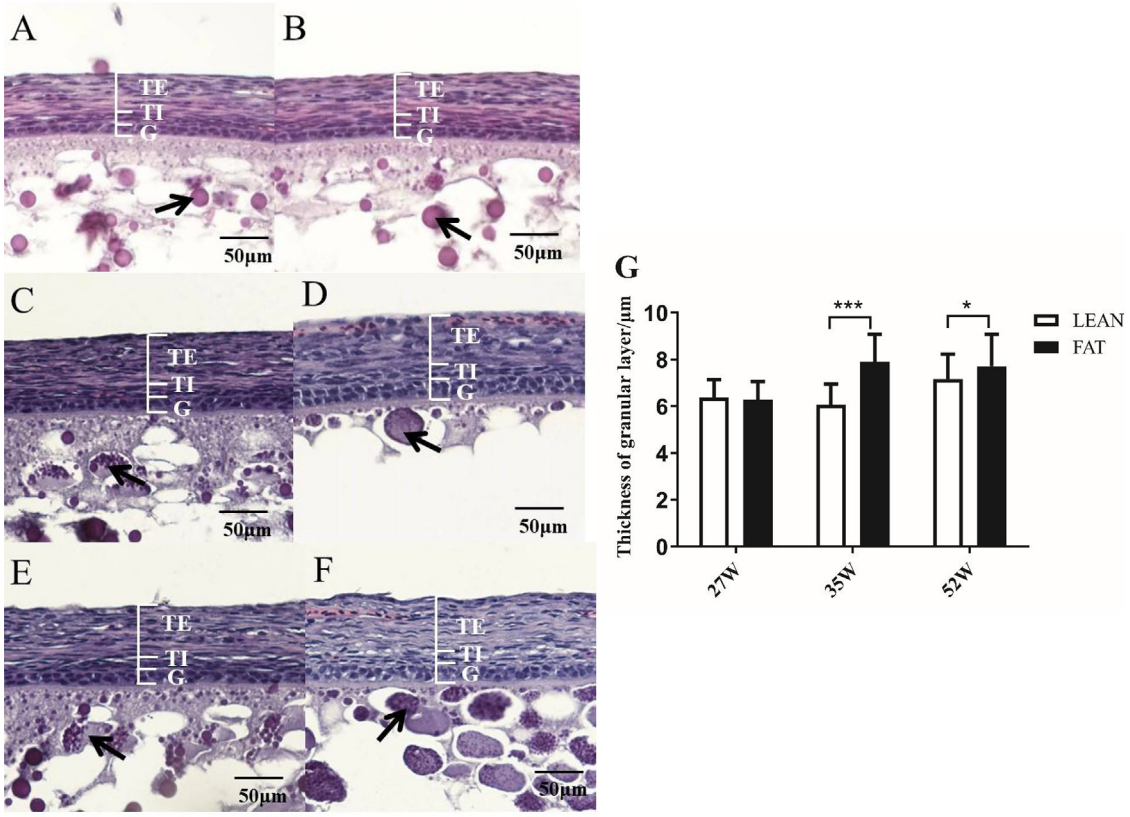

Figure 2. Histological analysis of small yellow follicle (SYF) between fat and lean hens at 27, 35, and 52 wk of age in the G_{24} generation. (A) Follicular wall of SYF of lean line at 27 wk of age. (B) Follicular wall of SYF of the fat line at the age of 27 wk of age. (C) Follicular wall of SYF of lean line at 35 wk of age. (D) Follicular wall of SYF of the fat line at 35 wk of age. (E) Follicular wall of SYF of lean line at 52 wk of age. (F) Follicular wall of SYF of the fat line at 52 wk of age. (G) Comparison of granulosa layer thickness of SYF between fat and lean hens at 27, 35, and 52 wk of age. The arrow represents the yolk. The result was expressed as means ± SD. Scale bar: 50 μm. $^{*}P < 0.05$, $^{***}P < 0.001$. Abbreviations: wk, week; G, granular layer; TI, intimal layer; TE, adventitia layer. W, week of age; LEAN, lean line; FAT, fat line; SD, standard deviation.

an intact follicle wall structure of SYF in both lines at all time points, with a clear boundary between the granular and membrane layers, regular granulosa cell arrangement, and no evidence of atresia (Figures 2A−F). No significant differences in SYF granulosa thickness were evident between the 2 lines at 27 wk of age (Figures 2A, B, and G). At 35 and 52 wk of age, however, SYFs from the fat line exhibited significantly greater granular layer thickness as compared to the lean line ($P < 0.05$, Figures 2C−G), indicating that granulosa cell development in SYFs from the lean line was impaired relative to that of the fat line. These data also suggest the absence of any differences in follicle selection between these lines in the early laying stage, whereas follicle selection may be impaired to some extent in lean hens in the middle and late laying stages.

Analyses of Expression Patterns of Genes Associated with Follicle Selection and Maturation at Different Laying Stages

Next, the mRNA levels of genes associated with follicle selection were analyzed in SYF samples, including the positive regulatory genes *FSHR* and *BMP15* as well as *AMH,* a negative regulator of follicle selection. In addition, hierarchical follicle maturation-related genes associated with VLDLy synthesis and deposition (Liver: *ACC, FAS, apoB100*, and *apoVLDL-II*; Hierarchical follicle: *VLDLR*) were analyzed in G_{24} hens at 27, 35, and 52 wk of age. Follicle selection-associated genes *FSHR* and *BMP15* expression levels in SYFs from the fat line were significantly reduced relative to those from the lean line at 27 wk of age ($P < 0.05$ or $P < 0.01$, Figures 3A−B). At 35 wk of age, *FSHR* expression levels in SYFs from the lean line were significantly reduced relative to those from the fat line ($P < 0.01$, Figure 3A). At 52 wk of age, *FSHR* expression level in SYFs from the lean line trended lower compared to those from the fat line ($P = 0.067$, Figure 3A). These data suggested that follicle selection in the fat line might be hindered relative to the lean line during the early laying stage, whereas follicle selection in the lean line may be impaired relative to the fat line in the middle and late laying stages.

When analyzing liver samples, significant reductions in the expression of the VLDLy synthesis-related genes *ACC, FAS, apoB100*, and *apoVLDL-II* were noted in samples from the lean line at 27 wk of age ($P < 0.05$ or $P < 0.01$, Figures 4A−D). At 35 wk of age, *apoB100* expression level in the liver from the lean line trended downwards relative to that from the fat line ($P = 0.099$, Figure 4C). At 52 wk of age, significantly reduced *ACC, FAS, apoB100*, and *apoVLDL-II* expression was evident

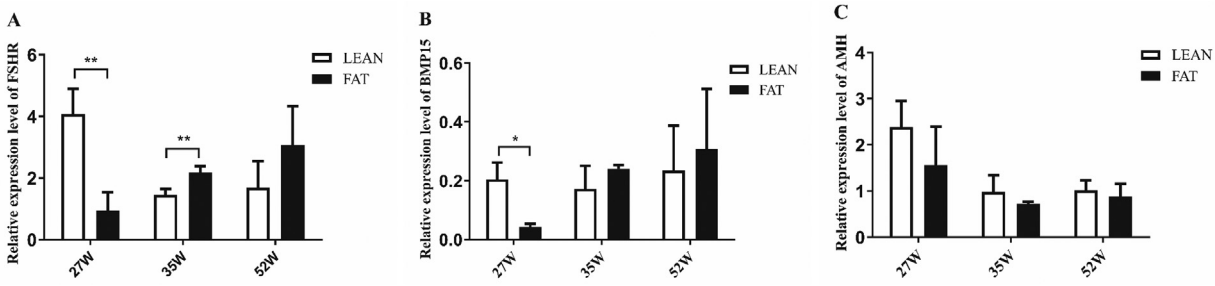

Figure 3. Comparison of expression levels of follicular selection-related genes in the small yellow follicle (SYF) between fat and lean hens at 27, 35, and 52 wk of age in the G_{24} generation. (A) The expression of the *FSHR* gene in SYF. (B) The expression of the *BMP15* gene in SYF. (C) The expression of the *AMH* gene in SYF. The result is expressed as means ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$. Abbreviations: wk, week; SYF, small yellow follicle; W, week of age; LEAN, lean line; FAT, fat line; SD, standard deviation; *FSHR*, follicle-stimulating hormone receptor; *BMP15*, bone morphogenetic protein 15; *AMH*, antimullerian hormone.

in liver samples from the lean line relative to the fat line ($P < 0.05$ or $P < 0.01$, Figures 4A−D). These data suggest that lean hens exhibit impaired hepatic VLDLy synthesis relative to the fat line during the early, middle, and late laying stages.

When individual hierarchical follicles were analyzed, significantly lower VLDLy deposition-related gene *VLDLR* expression was evident in F2, F5, and F6 follicles from the fat line relative to those from the lean line at 27 wk of age ($P < 0.05$, Figure 5A). At 35 wk of age, significantly reduced *VLDLR* mRNA levels were evident in F2, F3, F4, and F5 follicles from the lean line relative to those from the fat line ($P < 0.05$, Figure 5B). At 52 wk of age, the *VLDLR* expression level in the F3 follicle from the lean line trended downwards relative to that from the fat line ($P = 0.071$, Figure 5C). These data suggested that hens from the fat line exhibited impaired VLDLy deposition ability in the hierarchical follicles in the early laying stage relative to lean hens, but that this difference was reversed in the middle and late laying stages.

DISCUSSION

Egg production is a key measure of poultry reproductive performance. Follicle growth and development play a

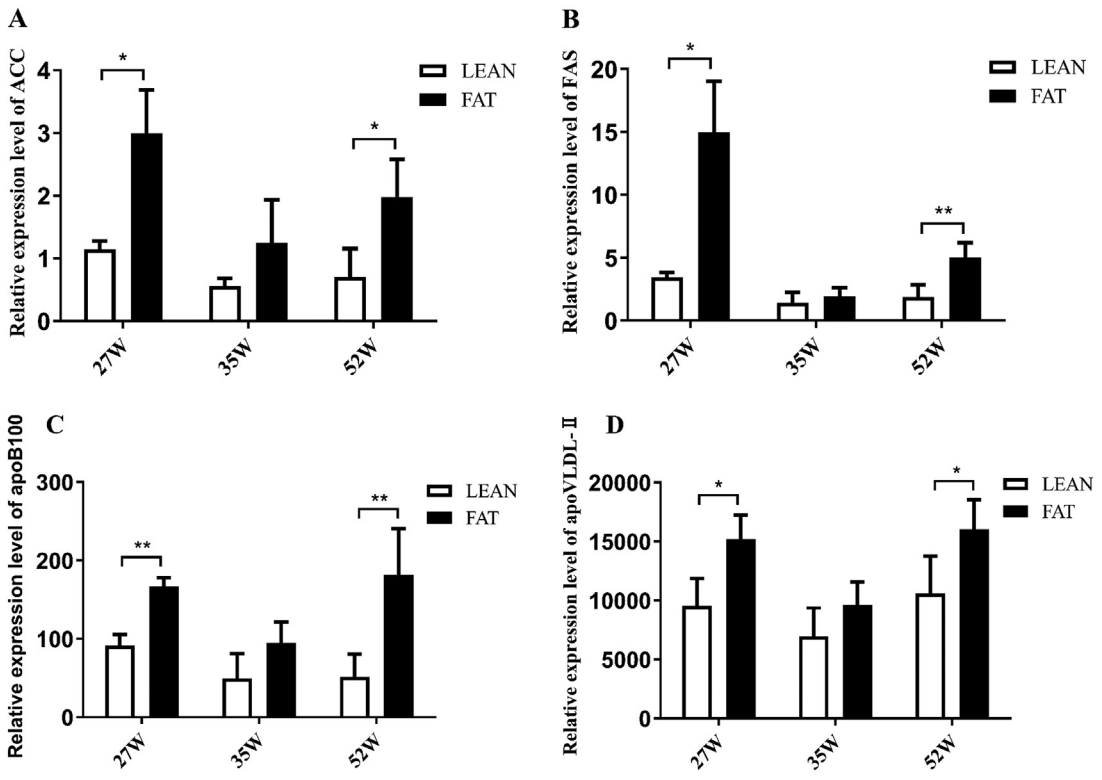

Figure 4. Comparison of expression levels of VLDLy synthesis-related genes in the liver between fat and lean hens at 27, 35, and 52 wk of age in G_{24} generation. (A) The expression of *ACC* in the liver. (B) The expression of *FAS* in the liver. (C) The expression of *apoB100* in the liver. D) The expression of *apoVLDL-Ⅱ* in the liver. The result is expressed as means ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$. Abbreviations: wk, week; *ACC*, acetyl CoA carboxylase; *FAS*, fatty acid synthase; *apoB100*, apolipoprotein B100; *apoVLDL-Ⅱ*, apolipoprotein VLDL-Ⅱ; W, week of age; LEAN, lean line; FAT, fat line; SD, standard deviation.

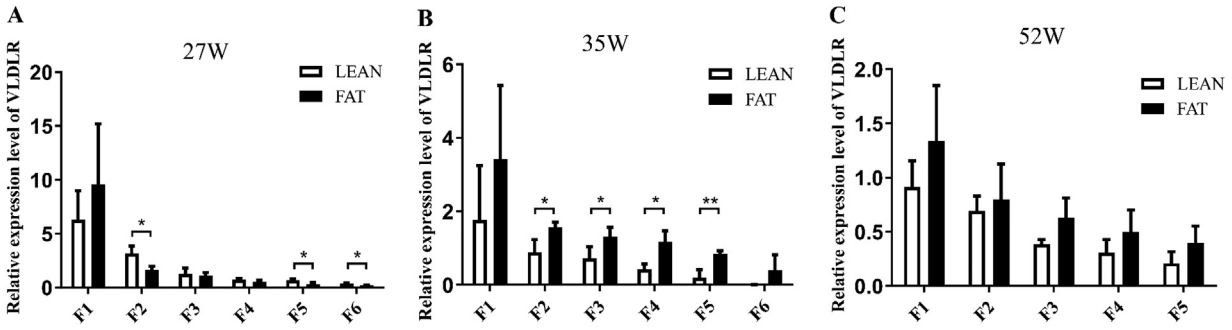

Figure 5. Comparison of expression levels of *VLDLR* in hierarchical follicles between fat and lean hens at 27, 35, and 52 wk of age in G_{24} generation. (A) The expression of *VLDLR* in each of the hierarchical follicle of fat and lean hens at 27 wk of age. (B) The expression of *VLDLR* in each of the hierarchical follicle of fat and lean hens at 35 wk of age. (C) The expression of *VLDLR* in each of the hierarchical follicle of fat and lean hens at 52 wk of age. Note: No F6 follicles were found in the ovaries of fat and lean hens at 52 wk of age. The result is expressed as means ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$. Abbreviations: wk, week; *VLDLR*, very low density lipoprotein receptor; W, week of age; LEAN, lean line; FAT, fat line; SD, standard deviation; F1, F1 follicle; F2, F2 follicle; F3, F3 follicle; F4, F4 follicle; F5, F5 follicle; F6, F6 follicle.

key role in determining egg production (Yoshimura and Barua, 2017). Excessively high levels of fat deposition can affect the egg-laying performance of hens (Chen et al., 2006; Mohiti-Asli et al., 2012). While prior work from our group demonstrated that the egg production of lean hens was significantly improved relative to that of fat hens from the first egg to 40 wk of age for 14th to 18th generations of NEAUHLF (Zhang et al., 2018), it remained uncertain as to whether there were laying stage-specific differences in egg production and any differences in follicular growth and development between these 2 broiler lines. Here, we similarly found that lean hens exhibited significantly higher egg production relative to fat hens from the first egg to 40 wk of age from the 19th to 24th generations (data not shown), consistent with data from the data generated using chickens from the 14th to 18th generations. While no differences in egg production over the entire laying period (first egg to 50 wk of age) were noted when comparing the fat and lean lines, significant differences in egg production were noted in different laying stages. Strikingly, lean hens laid more eggs than fat hens in the early laying stage, whereas the opposite was true in the middle and late laying stages. Moreover, differences in follicular development were noted between these chicken lines, including significantly altered prehierarchical and hierarchical follicle numbers, hierarchical follicle weight, SYF thickness, and follicle selection- and maturation-related gene expression. As this study utilized unique divergent chicken lines, these results suggested that selection aimed at reducing abdominal fat deposition can improve laying performance in the early laying stage but adversely impact laying performance in the middle and late laying stages.

Differences in AFE between Fat and Lean Hens

When evaluating hens, AFE is a particularly important laying-related trait. Several different variables have been shown to shape AFE, including sufficient age, weight, and fat deposition (Leeson and Summers, 1983; Brody et al., 1984; Renema et al., 1999). Early work from Bornstein et al. found that every 10 g/kg increase in abdominal fat content was associated with an average AFE that was 10.3 d sooner such that efforts to increase the abdominal fat content in hens would be conducive to an earlier average AFE (Bornstein et al., 1984). Under free-feeding conditions, however, hens exhibiting a higher abdominal fat content were found to exhibit a later AFE (Hocking, 1996). We previously found that the average AFE of the lean line was 2 d earlier than that of the fat line from G_{14} to G_{18} (Zhang et al., 2018). These data suggest that both diet-induced and hereditary obesity can contribute to AFE delays. In the present report in which fat hens had an AFP roughly 4 times that of lean hens (Figure 1C), the AFE for this fat line was 1 wk later than that for the lean line (Table 1), in line with the findings of Hocking (1996) and Zhang et al. (2018), but in contrast with the work published by Bornstein et al. (1984). Overall, these data suggested that efforts to increase hen abdominal fat content can contribute to an earlier AFE within a particular range, but that excessively high levels of abdominal fat can ultimately result in AFE delays.

A previous study has shown that although the hens had reached the age of sexual maturity, excessive fat deposition had led to the failure of mature follicle into the oviduct, resulting in a later average AFE (Renema et al., 1995). In a similar vein, obese hens have been found to exhibit higher rates of follicular atresia and internal ovulation at the beginning of egg production that ultimately delay the AFE (Hocking, 1996; Ferreira et al., 2016). In these hens, follicular atresia and internal ovulation occur as a consequence of uncoordinated ovulation of hens at the start of egg production (Renema et al., 1995). This uncoordinated ovulatory activity, in turn, is the result of a lack of synchronous ovary and oviduct function such that even when the ovary contains mature follicles, the oviduct is not ready to receive them due to physical compression and/or incomplete development (Yu et al., 1992). In the present study, we found that oviduct weight and oviduct percentage of the fat line were significantly decreased relative to those of the

lean line at 27 wk of age (Table S2), and hierarchical follicular atresia and internal ovulation were more common among fat hens at 27 wk of age relative to lean hens (Table 3). Together, we speculated that delayed AFE in fat hens may be a consequence of compression of the oviduct caused by a greater amount of abdominal fat, impairing their ability to receive mature follicles and thus contributing to hierarchical follicle atresia and internal ovulation, ultimately leading to a later AFE.

Comparisons of Egg Production and Follicular Development in Fat and Lean Hens in the Early Laying Stage

Excessive fat deposition has repeatedly been demonstrated to adversely affect egg production (Hocking et al., 1992; Chen et al., 2006; Pan et al., 2014). In line with these prior reports, fat hens exhibited significantly reduced egg production relative to lean hens from the first egg to 35 wk of age (Table 2). Follicular growth and development are key mediators of the egg production process (Yoshimura and Barua, 2017), with particularly important roles for follicular selection and maturation in this context (Johnson, 2015). Normally during the laying period, hens maintain the ovulation process by having 1 SYF per day that is selected for entry into the grade stage (Johnson, 2015). If more SYFs are selected, this will result in a longer laying period and a higher level of overall egg production (Johnson and Woods, 2009; Johnson, 2015). This suggests that the degree to which the follicular selection process occurs normally is a key determinant of egg production in hens. Following the selection of SYFs for grading stage entry, yolk precursors (primarily VLDLy) produced in the liver in response to estrogen will be deposited in the selected follicles, promoting their maturation and ovulation (Griffin et al., 1992; Cui et al., 2020). As such, normal hierarchical follicle maturation also shapes the laying performance of hens. In an effort to determine whether the reduced egg production observed in fat hens in the early laying stage was attributable to differences in the follicle selection and maturation process, the morphological characteristics of the ovaries of fat and lean hens were meticulously compared. At 27 wk of age, no significant differences in any analyzed ovarian morphological characteristics were evident when comparing these 2 lines, OW, OP, the number of hierarchical follicles, the weight of each hierarchical follicle (except for the F5 follicle), and the number of SYFs (Table 3). As such, follicle selection and maturation appear not to differ between these fat and lean chicken lines in the early laying stage.

Granulosa cell development in SYFs is closely associated with the follicle selection process (Johnson, 2015). To evaluate potential differences in follicle selection during the early stage of the egg production process in greater detail, SYFs from fat and lean hens were collected for histological analyses. H&E staining revealed an absence of any differences in SYF granulosa layer thickness between these 2 lines at 27 wk of age (Figures 2A, B, and G), further suggesting an absence of any early differences in follicle selection between these 2 lines. Follicle selection-related gene expression is another important factor that regulates the follicle selection process. After its secretion from the pituitary gland, FSH can regulate follicle selection by binding to its cognate receptor, FSHR (Wang et al., 2017). Elevated levels of FSHR expression in a given SYF indicate that that follicle will be selected for entry into the graded development stage (Woods and Johnson, 2005). The TGFβ superfamily protein BMP15 is a critical positive regulator of follicular selection (Liu et al., 2021), as exogenous BMP15 administration can promote *FSHR* expression in SYFs (Stephens and Johnson, 2016). Conversely, the secretion of AMH by granulosa cells can suppress FSHR expression and the sensitivity of SYFs to FSH, thus impairing the follicle selection process (Durlinger et al., 2001). It was reported that obese women exhibited reduced granulosa cumulus cell FSHR expression, contributing to slower follicular development (Xu et al., 2019). Here, a reduction in *FSHR* mRNA levels was observed in the fat line relative to the lean line at 27 wk of age (Figure 3A), in line with the observations reported earlier (Xu et al., 2019). There was a significant decrease in ovarian BMP15 expression at 56 wk of age in AA broilers with a lower abdominal fat percentage relative to those with a higher abdominal fat percentage (Wang et al., 2021). At 27 wk of age, however, significantly reduced *BMP15* expression was instead evident in the fat line relative to the lean line in the present study (Figure 3B). These inconsistent results may be attributable to differences in utilized broiler strains, tissue samples, or laying stages at the time of analysis. Overall, these results of gene expression suggested that follicular selection in fat hens was impaired relative to that in lean hens in the early laying stage. The reason for the apparent inconsistency between the results of analyses of gene expression and ovarian morphology and SYF histology remains uncertain but may be related to the greater amount of time necessary for phenotypic differences in follicular development to manifest relative to changes in gene expression. Given that fat hens exhibited a later AFE than lean hens, this suggests that the higher abdominal fat content in fat hens contributed to reduced *FSHR* and *BMP15* expression in SYFs, in turn suppressing follicle selection and delaying the AFE, contributing to lower egg production in the early laying stage.

Hierarchical follicle maturation in hens is closely related to VLDLy synthesis, transport, and deposition (Walzem et al., 1999; Schneider, 2016). During the laying period, estrogen stimulates VLDLy synthesis in the liver after which it is transported through the blood to the ovaries. As mediators of fatty acid synthesis, FAS and ACC are important regulators of VLDLy biosynthesis (Gong et al., 2023). ApoVLDL-II inhibits LPL-mediated lipolysis during the process of VLDLy transport, thereby ensuring its ovarian delivery (Schneider et al., 1990). ApoB100 binding to VLDLR present on the oocyte surface facilitates VLDLy deposition in hierarchical follicles (Schneider et al., 1990). The c.2177G>C

mutation in the chicken *VLDLR* coding region interferes with the expression of this gene, leading to impaired yolk deposition and hierarchical follicle maturation, ultimately compromising egg production (Bujo et al., 1995). A previous study observed significantly lower hepatic *FAS* and *ACC* mRNA levels in hens with a high abdominal fat content as a result of free feedings as compared to hens with a lower level of abdominal fat content as a result of restricted feeding, with corresponding upward trends in *apoB-100* and *apoVLDL-II* mRNA levels relative to those in hens with low abdominal fat content (Richards et al., 2003). Here, significantly reduced hepatic *ACC, FAS, apoB100*, and *apoVLDL-II* expression was observed in the lean line relative to the fat line (Figures 4A−D), in contrast with the findings reported by Richards et al. (2003). This discrepancy may be a consequence of differences in the utilized experimental animals, as Richards et al. (2003) studied diet-based changes in abdominal fat content, whereas the present study focused on hens with significant differences in abdominal fat content as a result of multiple generations of selective breeding. *VLDLR* mRNA levels in F2, F5, and F6 follicles from the fat line were also significantly reduced relative to those from the lean line at 27 wk of age (Figure 5A). This suggests that hepatic VLDLy synthetic ability in fat hens was more robust than that in lean hens, whereas VLDLy absorption by the hierarchical follicles of fat hens was impaired relative to that in lean hens. This may account for the absent difference in hierarchical follicle maturation at the early laying stage between these 2 chicken lines.

Comparisons of Egg Production and Follicular Development in Fat and Lean Hens in the Middle and Late Laying Stages

Hocking and Whitehead (1990) previously reported significantly lower egg production for hens with a low level of abdominal fat content at 32 to 34 and 58 to 60 wk of age under restricted feeding conditions. Recent studies suggested that modern meat-type chickens were reducing body fat due to severe feed restriction and this could reduce egg production during the laying period (Hadinia et al., 2019; Artdita et al., 2021). A relaxation in the severity of feed restriction can increase fat pad deposition and egg production in broiler breeders (Zuidhof, 2018). Interestingly, a study has shown that feed restriction significantly decreased the egg production of lean-line hens but significantly increased that of fat-line hens from 34 to 54 wk of age (Li et al., 2011). In line with these results, the present study found that lean hens exhibited significantly reduced egg production during the middle and late laying stages (36−42 and 43−50 wk, respectively) (Table 2) under the restricted feeding condition.

Ovarian weight is a key determinant of hen egg production, and ovarian weight, in turn, is primarily dependent on the number of hierarchical follicles during the laying period (Yu et al., 1992). A previous study observed a reduction in the number of hierarchical follicles in hens exhibiting a low abdominal fat content as compared to that in hens exhibiting a high abdominal fat content at 33 and 62 wk of age (Hocking and Whitehead, 1990). A similar study noted a decrease in hierarchical follicle numbers in hens with lower abdominal fat content at 45 and 55 wk of age as compared to those with higher levels of abdominal fat (Mohiti-Asli et al., 2012). Here, OW, OP, and the number of hierarchical follicles in lean hens were significantly reduced as compared to those in fat hens at 35 and 52 wk of age (Table 3), in line with these prior reports. It is worth noting that the number of SYF in lean hens was significantly higher than that in fat hens at 35 and 52 wk of age (Table 3), but the number of hierarchical follicles and egg production were significantly lower than those in fat hens. These data suggested that follicle selection may be slower in the lean line in the middle and late laying stages, contributing to a reduction in egg production. There have been several reports demonstrating that the weight of hierarchical follicles reflects their degree of maturity (Raghu et al., 2002). The higher the weight of follicles of the same grade in different individuals' ovaries, the greater their maturity (Brady et al., 2021). In the present report, F2 to F6 follicles in lean hens weighed significantly less than those from fat hens at 35 wk of age, while at 52 wk of age, the F1 to F5 follicles of lean hens weighed significantly less than those of fat hens (Table 3). These results suggested that follicle maturation of lean hens may be slower than that of the fat hens in the middle and late laying stages.

Follicle selection is a process that is associated with granulosa cell growth and development and follicle selection-related genes expression in SYFs (Woods and Johnson, 2005; Johnson, 2015; Stephens and Johnson, 2016; Huang et al., 2021). In geese with lower laying performance, the SYF granular layer thickness is reduced relative to that of geese with high laying performance (Yang, 2018). Here, lean hens were found to exhibit a significant reduction in SYF granular layer thickness as compared to fat hens at 35 and 52 wk of age (Figures 2C−G), in line with prior findings in geese (Yang, 2018). These data suggested that granulosa cell development in SYFs of lean hens was impaired relative to that in fat hens. It was reported that ovarian *FSHR* expression in hens with a lower abdominal fat percentage was significantly elevated as compared to that in AA broilers with a higher abdominal fat percentage (Wang et al., 2021). In the present analysis, *FSHR* expression levels in SYFs of lean hens at 35 and 52 wk of age were significantly reduced or trended downwards as compared to those of fat hens (Figure 3A). The results of *FSHR* expression were inconsistent with them, and the reasons were unknown. Together, the ovarian morphology, SYF histology, and follicle-related gene expression data presented herein suggested that follicle selection in lean hens was somewhat impaired in the middle and late laying stages.

To better understand the degree to which the follicle maturation process was impaired in the middle and late

laying stages in lean hens, follicle maturation-associated gene expression was analyzed. At 35 wk of age, *apoB100* expression level in the liver from the lean line trended downwards relative to those from the fat line ($P = 0.099$, Figure 4C). At 52 wk of age, significant reductions in hepatic *ACC, FAS, apoB100*, and *apoVLDL-II* mRNA levels were evident in lean hens relative to fat hens (Figures 4A−D). Our results were in line with data published by Richards et al. (2003), who observed significantly reduced hepatic *ACC* and *FAS* expression in hens with lower abdominal fat content as compared to those with high abdominal fat content, and a corresponding downward trend in *apoB100* and *apoVLDL II* levels in these hens. In hierarchical follicles, significant reductions in *VLDLR* expression levels were evident in F2, F3, F4, and F5 from lean hens relative to fat hens at 35 wk of age (Figure 5B). Similarly, at 52 wk of age, the *VLDLR* expression level in the F3 follicle from the lean line trended downwards relative to that from the fat line ($P = 0.071$, Figure 5C). These data suggested that the hepatic synthesis of VLDLy and its deposition in the hierarchical follicles were both impaired in lean hens, contributing to impaired follicle maturation in the middle and late laying stages. In addition to the genetic aspect, feeding restriction should be another important reason leading to this result. In the middle and late laying stages, in order for the lean line to achieve follicle maturation rate similar to those of the fat line, more VLDLy needs to be synthesized in the liver. Fatty acids are important raw materials for synthesizing VLDL, and they could be synthesized from glucose through the pathway of glycolysis (Zaefarian et al., 2019). The transition from glucose to fatty acids mainly undergoes 3 processes: first, glucose catabolism produces acetyl-CoA; second, acetyl-CoA generates malonyl-CoA under the catalysis of ACC; third, malonyl-CoA generates fatty acids under the catalysis of FAS (Wakil and Abu-Elheiga, 2009). In this study, we have demonstrated that *de novo* fatty acid synthesis ability was impaired in the lean hens, evidenced by the decreased expression levels of *ACC* and *FAS* (Figures 4A and B). Therefore, to achieve a similar follicle maturation rate as fat hens, lean hen might need to consume far more diet in order to supply raw materials for VLDLy synthesis. Base on this, we speculated that feed restriction has a greater impact on follicle maturation of the lean line in the middle and late laying stages.Taken together, the lower egg production of the lean hens may be related to the obstruction of follicle selection and maturation in the middle and late laying stages. However, additional research will be essential to further determine how fat deposition regulates follicle selection and maturation in these hens.

In conclusion, the present results highlight novel laying stage-dependent effects of genetically determined body fat deposition on egg production in broiler breeder hens. Lower egg production was evident in fat hens in the early laying stage, potentially owing to their later AFE, whereas lean hens exhibited reduced egg production relative to fat hens in the middle and late laying stages that may be attributable to the impairment of follicle selection and maturation. Together, these results offer a new insight into the association between fat deposition and laying performance in avian species.

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DISCLOSURES

The authors declare that they have no conflict of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2023.103250.

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