Effect of dietary concentrations of metabolizable energy and neutral detergent fiber on productive performance, egg quality, fatty liver incidence, and hepatic fatty acid metabolism in aged laying hens

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ABSTRACT The objective of the current experiment was to investigate the effect of dietary concentrations of ME and neutral detergent fiber (**NDF**) on productive performance, egg quality, fatty liver incidence, and hepatic fatty acid metabolism in aged laying hens. A total of three hundred twenty 75-wk-old Hy-Line Brown laying hens were allotted to 1 of 4 dietary treatments with 8 replicates. Each replicate consisted of 10 consecutive cages with 1 hen per cage. The experiment was conducted using a completely randomized design with 2 × 2 factorial arrangement consisting of 2 levels of ME (normal [commercially recommended AME_n levels; 2,730 kcal/kg] and low [50 kcal/kg reduction in AME_n; 2,680 kcal/kg]) and 2 levels of NDF (low [9.01 and 9.61%; normal-ME and low-ME diets, respectively] and high [12.57 and 13.42%; normal-ME and low-ME diets, respectively]) in the diet. The diets and water were provided to hens on an ad libitum basis for 12 wk. Results indicated that no interactions between dietary concentrations of ME and NDF were observed for all measurements except for egg yolk color, eggshell thickness, and 2 hepatic gene expressions (i.e., carnitine palmitoyl transferase 1A and malic enzyme). For the main effects, increasing NDF concentrations in diets increased ($P < 0.05$) feed intake without affecting other productive performance. Hens fed normal-ME and high-NDF diets showed the darkest ($P < 0.05$) egg yolk color among those fed treatment diets, showing an interaction ($P < 0.05$). Increasing NDF concentrations in low-ME diets did not influence eggshell thickness, but those in normal-ME diets increased eggshell thickness in laying hens, showing an interaction ($P < 0.05$). For the main effects, increasing concentrations of dietary NDF or ME reduced ($P < 0.05$) hepatic fat concentrations with decreasing expressions in several genes related to fatty acid synthesis. In conclusion, increasing NDF concentrations in commercially-recommended ME diets decrease hepatic fat concentrations in aged laying hens, and therefore, may have a preventative effect on the fatty liver development in aged laying hens.

Key words: aged laying hen, egg quality, fatty liver incidence, metabolizable energy, neutral detergent fiber

2023 Poultry Science 102:102497

https://doi.org/10.1016/j.psj.2023.102497

INTRODUCTION

Laying hens are typically raised until 70 to 80 wk of age in the commercial situation (Bain et al., 2016). The reduction in egg production and egg quality as hens become aged is one of the major reasons for depopulation and introduction of new flocks (England and Ruhnke, 2020). Therefore, extending the production period by improving egg production and quality in aged laying hens is necessary to increase economic benefits and sustainability in the layer industry (England and Ruhnke, 2020).

Fatty liver hemorrhagic syndrome (**FLHS**) is known to deteriorate the productive performance and egg quality in laying hens (Galea, 2011). Excessive fat accumulation structurally weakens the liver and causes massive hepatic hemorrhage, which leads to various health problems and even death (Shini et al., 2019). The FLHS is the most significant reason of fatalities in caged laying hens (Shini et al., 2019). In particular, FLHS occurs more frequently in aged laying hens than in younger hens (Dong and Tong, 2019). Therefore, the prevention of hepatic fat accumulation is essential to improve egg

Received November 7, 2022.
Accepted January 8, 2023.

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production and health in aged laying hens, which would subsequently enhance economic outcomes in egg production.

The lipid metabolism, especially for fat synthesis, in poultry occurs in the liver. Therefore, impaired fat utilization and increased fat synthesis in the liver can facilitate excessive fat accumulation, leading to the FLHS development (Zaefarian et al., 2019). It is appreciated that FLHS is caused by various factors, including internal and external conditions, and in particular, nutritional factor such as high energy and nutrients in diets significantly contributes to FLHS development (Gao et al., 2019; Zhu et al., 2021). Increasing concentrations of dietary fiber in layer diets may be a potential means to decrease the incidence of FLHS because of its low energy value and possible antinutritional effects (Desbruslais et al., 2021). It is reported that increasing concentrations of dietary fiber decreased the liver weight and fat concentrations in growing chickens (Akiba and Matsumoto, 1978). Moreover, dietary fiber is known to have a positive effect on digestive system development, immune function, and microbial balance in poultry (Jha et al., 2019; Okrathok et al., 2022). However, information regarding the interactive effect of different concentrations of energy and fiber in diets on hepatic fat metabolism is lacking in laying hens, especially for aged laying hens.

The objective of the current experiment was to investigate the effect of different concentrations of ME and neutral detergent fiber (**NDF**) in diets on productive performance, egg quality, fatty liver incidence, and hepatic fat metabolism in aged laying hens.

MATERIALS AND METHODS

Birds, Diets, and Experimental Design

All experimental procedures were reviewed and approved by the Animal Care and the Use Committee at Chung-Ang University.

A total of three hundred twenty 75-wk-old Hy-Line Brown laying hens (initial BW = 1.98 ± 0.02 kg) with similar productive performance were allotted to 1 of 4 dietary treatments with 8 replicates. Each replicate comprised 10 consecutive cages with 1 hen per cage (24 × 36 × 39 cm), such that the density was guaranteed to be approximately 0.09 m^2/hen. The experiment was conducted using a completely randomized design with 2 × 2 factorial arrangement consisting of 2 concentrations of ME (normal and low) and 2 concentrations of NDF in diets. A normal-ME diet (2,730 kcal/kg AME_n) was formulated according to the recommendations of the Hy-Line Brown Management Guide (2016; Table 1). The low-ME diet was developed by reducing the AME_n of the normal-ME diet by 50 kcal/kg (2,680 kcal/kg AME_n). Within each ME level, the high-NDF diet (12.57 and 13.42% NDF for normal-ME and low-ME diets, respectively) was produced to contain 40% higher NDF concentrations than the low-NDF diet (9.01 and 9.61% NDF for normal-ME and low-ME diets, respectively). Different concentrations of NDF were achieved by modifying inclusion levels of wheat bran, distillers dried grains with solubles (**DDGS**), rice bran, sesame oil meal, and corn germ meal. The NDF and acid detergent fiber (**ADF**) concentrations in the feed were analyzed using an ANKOM 200 Fiber Analyzer (ANKOM Technology, Macedon, NY) based on the modified method described by Van Soest et al. (1991).

However, other nutrient concentrations in all treatment diets were equalized and met the requirement estimates for Hy-Line Brown laying hens (Hy-Line, 2016). All hens were raised according to the recommendation of environmental management in Hy-Line Brown laying hens (Hy-line, 2016). The experimental diets and water were provided to hens on an ad libitum basis for 12 wk. The average room temperature and relative humidity were maintained at approximately 21.6 ± 2.3°Cand 55.5 ± 10.4%, respectively. The lighting was provided for 16 h per d throughout the experiment.

Productive Performance and Egg Quality

Productive performance including hen-day egg production, egg weight, egg mass, and broken and shell-less egg production rate was recorded daily. The mortality was recorded daily throughout the experiment. Feed intake (**FI**) and feed conversion ratio (**FCR**) were calculated every 4 wk. The FCR was calculated by dividing FI with egg mass. The egg mass was calculated by multiplying the egg production rate (%) by the average weight of eggs (g) and divided by 100. The data for productive performance were then summarized for 12 wk from 75 to 86 wk of age.

Egg quality was assessed using randomly collected samples of 10 eggs per replicate with 5 eggs per day for 2 consecutive day at 4 wk intervals during the 12-wk feeding trial. Eggshell color was evaluated by using an eggshell color fan (Samyangsa, Kangwon-do, Republic of Korea; where on a scale of 1 to 15, with 1 being a very light and pale and 15 being a very dark brown). Eggshell strength, eggshell thickness (without shell membrane), egg yolk color, and Haugh unit were determined using a digital egg tester (DET-6000, Nabel Co., Ltd., Kyoto, Japan), based on the detailed procedure reported previously (Kim et al., 2022).

Sample Collection

At the end of the experiment (i.e., 86 wk of age), 1 hen per replicate with a BW close to the average of each replicate (i.e., 8 birds per treatment) was euthanized by CO_2 asphyxiation, and then promptly dissected.

The liver tissue was used for the analysis of fatty liver incidence and expression of genes related to fatty acid synthesis and oxidation. Regarding the fatty liver color score, the liver attached on the body was pictured to measure the subjective fatty liver color score using a scale from 1 to 5 (1 = dark red; 5 = light yellowish red). The liver hemorrhagic score was also analyzed using a scale

Table 1. Composition and nutrient concentrations of experimental diets.

Items	2,680 kcal ME/kg (Low-ME)		2,730 kcal ME/kg (Normal-ME)	
	Low NDF	High NDF	Low NDF	High NDF
Ingredients (%)				
Corn	62.43	52.44	63.30	53.29
Soybean meal, 46% CP	18.50	10.65	18.94	10.90
Corn gluten meal	2.43	2.84	2.50	3.96
Wheat	0.70	0.53	0.50	-
Tallow	-	1.03	0.70	1.40
Wheat bran	1.80	6.50	-	5.51
DDGS	-	5.00	-	4.02
Rice bran	-	-	-	1.94
Seasame oil meal	-	2.20	-	1.16
Corn germ meal	-	5.00	-	4.00
Monodicalcium phosphate	1.21	1.01	1.21	1.03
Limestone	11.10	11.21	11.10	11.21
54% L-Lysine H_2SO_4	0.15	0.36	0.14	0.36
98.5% L-Threonine	0.02	0.06	0.02	0.06
Liquid DL-Methionine	0.17	0.17	0.18	0.17
L-tryptophan	0.04	0.08	0.01	0.09
Celite	0.55	0.02	0.50	-
NaCl	0.30	0.30	0.30	0.30
50% Choline	0.10	0.10	0.10	0.10
Antioxidant	0.05	0.05	0.05	0.05
$NaHCO_3$	0.15	0.15	0.15	0.15
Vitamin premix[1]	0.15	0.15	0.15	0.15
Mineral premix[2]	0.15	0.15	0.15	0.15
Total	100.00	100.00	100.00	100.00
Calculated energy and nutrient concentration				
AME_n, kcal/kg	2,680	2,680	2,730	2,730
CP, %	15.00	15.00	15.00	15.00
Lysine, %	0.78	0.78	0.78	0.78
Methionine + Cysteine, %	0.65	0.65	0.65	0.65
Methionine, %	0.39	0.39	0.39	0.39
Threonine, %	0.57	0.57	0.57	0.57
Tryptophan, %	0.17	0.17	0.17	0.17
Total Calcium, %	4.40	4.40	4.40	4.40
Available phosphorus, %	0.33	0.33	0.33	0.33
Crude fat, %	2.91	4.34	3.57	4.81
NDF, %	9.61	13.42	9.01	12.57
ADF, %	3.23	4.22	3.06	3.97
Analyzed nutrient concentration				
NDF, %	9.35	12.94	8.78	12.10
ADF, %	3.05	4.11	2.85	3.73

Abbreviations: DDGS, distillers dried grains with solubles; NDF, neutral detergent fiber; ADF, acid detergent fiber.

[1]Provided per kg of the complete diet: vitamin A, 10,000 IU (retinyl acetate); vitamin D3, 4,500 IU (cholecalciferol); vitamin K3, 3.0 mg (menadione dimethylpyrimidine); vitamin B1, 2.50 mg; vitamin B2, 6.50 mg; vitamin B6, 3.20 mg; vitamin B12, 18.0 μg; biotin, 180 μg; folic acid, 1.9 mg; niacin, 60 mg.

[2]Provided per kg of the complete diet: cobalt, 1,200 μg ($CoSO_4$); copper, 19.0 mg ($CuSO_4$); iron, 72 mg ($FeSO_4$); iodine, 1.5 mg ($Ca[IO_3]_2$); manganese, 144.0 mg (MnO); selenium, 360 μg (Na_2SeO_3); zinc, 120 mg ($ZnSO_4$).

from 0 to 5 (0 = normal liver; 5 = large and massive hemorrhages; Diaz et al., 1999; Choi et al., 2012). These subjective measurements were taken by 3 investigators using a blind test. The standards for fatty liver color and liver hemorrhagic scores were established based on pictures of the liver of laying hen (Choi et al., 2012). In addition, the Commission Internationale de l'Eclairage (**CIE**) color scale for the lightness (**L***), redness (**a***), and yellowness (**b***) were also measured using a colorimeter (model CR-10, Konica Minolta, Tokyo, Japan). After the liver was pictured and dissected, then its weight was measured for the relative liver weight to total BW. The small pieces of the liver were then taken from the middle portion of the liver to the 1.7-mL microtube and immediately transferred to a liquid nitrogen, and stored at −80°C for the analyses of total fat concentrations and expression levels of genes related to fatty acid synthesis and oxidation. Total fat concentrations in the liver were analyzed using the acid-hydrolyzed ether extraction (method 996.01; AOAC, 2007).

The spleen, crop, proventriculus, gizzard, small intestine, and large intestine were also collected, and the content of each organ was washed with distilled water and then weighed to measure the relative organ weight as a percentage of total BW (Pitargue et al., 2019).

Analyses of Gene Expression Related to Fatty Acid Synthesis and Oxidation Metabolism

The expression levels of genes related to fatty acid synthesis and oxidation in the liver were analyzed using the modified method of Pitargue et al. (2019). In short, RNA from the liver tissue samples was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). Following

Table 2. Primers used for quantitative RT-PCR.

Primer name[1]	Primer sequence[2] (5′-3′)	Product size (bp)	GenBank accession number
GAPDH	F: tgctgcccagaacatcatcc R: acggcaggtcaggtcaacaa	142	NM_204305
CPT1A	F: tctcatgctttggaaagaa R: attcgctgttcagaagagtt	147	NM_001012898
PPAR-α	F: gtaagctctcagaaactttgtt R: ccgctttccataatctgttc	145	NM_001001464
FASN	F:ttcccaaaaagggatcaaca R: agttcttcagagcatcgttt	132	NM_205155.3
SREBP-1	F: gccctctgtgcctttgtcttc R: actcagccatgatgcttcttcc	130	AY_029224
ACC	F: aatggcagctttggaggtgt R: tctgtttgggtgggaggtg	117	NM_205505
SCD	F: gatactacaagccctcagtg R: cattgagccctaaggtgtag	132	NM_204890
PPAR-γ	F: ccagcgacatcgaccagtt R: ggtgatttgtctgtcgtctttcc	145	AF_163811
Malic enzyme	F: tgccagcattacggtttagc R: ccattccataacagccaaggtc	175	NM_204303

[1]*GAPDH*, glyceraldehyde-3-phosphate; *CPT1A*, carnitine palmitoyl transferase 1A; *PPAR-α*, peroxisome proliferator-activated receptor-α; *FASN*, fatty acid synthase; *SPEBP-1*, sterol response element-binding protein 1; *ACC*, acetyl-CoA carboxylase; *SCD*, stearoyl-CoA desaturase; *PPAR-γ*, peroxisome proliferator-activated receptor-γ.
[2]F, forward; R, reverse.

extraction, 2 μg of diluted RNA was reverse-transcribed to cDNA by using the RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA), according to manufacturer's instructions, and the cDNA was stored at −20°C until further analysis.

Quantitative real-time PCR (**qRT-PCR**) was performed using CFX Connect Real-time PCR Detection System (Bio Rad Laboratories, Hercules, CA). The primers of the selected genes (i.e., carnitine palmitoyltransferase 1A, ***CPT1A***; peroxisome proliferator-activated receptor-α, ***PPAR-α***; fatty acid synthase, ***FASN***; sterol response element-binding protein 1, ***SREBP-1***; acetyl-CoA carboxylase, ***ACC***; stearoyl-CoA desaturase, ***SCD***; peroxisome proliferator-activated receptor-γ, ***PPAR-γ***; malic enzyme) for fatty acid oxidation and synthesis were produced. The primer sequences are listed in Table 2. Thermal conditions for performing qRT-PCR are as follows: initial incubation at 95°C (2 min); 40 cycles of denaturation at 95°C (30 s), annealing at the annealing temperature for each primer (30 s), and extension at 72°C (30 s); and termination by final incubation at dissociation temperatures of 95°C (10 s), 65°C (60 s), 97°C (1 s), and 37°C (30 s). The relative quantification of gene-specific expression was calculated using the $2^{-\Delta\Delta Ct}$ method after normalization to the gene expression of glyceraldehyde-3-phosphate dehydrogenase (***GAPDH***). The gene expression analysis was conducted at the BT research faility center, Chung-Ang University.

Statistical Analysis

All data were analyzed by ANOVA as a completely randomized design using the PROC MIXED procedure (SAS Institute Inc., Cary, NC). Each replicate was considered an experimental unit. The model included the effects of dietary levels of ME, NDF, and their interaction. All data were checked for normal distribution and outliers using the UNIVARIATE procedure of SAS (Steel et al., 1997). The LSMEANS procedure was used to calculate the treatment means and the PDIFF option was used to separate the means if the difference was significant. In order to adjust individual variations in productive performance and egg quality among aged hens at the start of the experiment, pre-trial productive performance and egg quality values were determined and used as the covariance in the statistical model. The statistical significance was set at $P < 0.05$.

RESULTS

Productive Performance and Egg Quality

No interactions between dietary levels of ME and NDF were observed for all productive performance including hen-day egg production, egg weight, broken and shell-less egg production, egg mass, FI, and FCR in aged laying hens (Table 3). For the main effects, dietary concentrations of ME did not affect all productive performance. However, feeding high-NDF diets to aged hens increased ($P < 0.05$) FI than feeding low-NDF diets, regardless of dietary concentrations of ME.

In egg quality, no interactions between dietary levels of ME and NDF were observed for eggshell color, eggshell strength, and Haugh unit (Table 4). However, significant interactions ($P < 0.05$) were identified for egg yolk color and eggshell thickness. High-NDF diets increased ($P < 0.05$) egg yolk color to a darker extent in normal-ME diets than in low-ME diets. In addition, increasing NDF concentrations in low-ME diets had no effects on eggshell thickness, whereas increasing NDF concentrations in normal-ME diets increased ($P < 0.05$) eggshell thickness. For the main effects, feeding low-ME diets to hens decreased ($P < 0.05$) egg yolk color and eggshell thickness, regardless of dietary concentrations of NDF. High-NDF diets increased ($P < 0.01$) egg yolk color as compared to low-NDF diets.

Table 3. Effect of dietary concentrations of ME and neutral detergent fiber (NDF) on productive performance of aged laying hens.[1]

		Productive performance					
Item		Hen-day egg production (%)	EW (g)	Broken and shell-less egg (%)	EM (g/hen/d)[2]	FI (g/hen/d)	FCR (g/g)
ME[3]	NDF[4]						
Low	Low	88.5	65.8	0.25	58.2	131	2.25
	High	88.1	65.6	0.25	58.1	133	2.29
Normal	Low	88.5	66.0	0.18	58.5	130	2.23
	High	88.4	66.0	0.19	58.4	132	2.26
SEM (n = 8)		1.37	0.52	0.098	0.85	0.8	0.030
Main effect							
ME							
Low		88.3	65.9	0.25	58.2	132	2.27
Normal		88.5	66.0	0.18	58.5	131	2.25
SEM (n = 16)		1.04	0.37	0.091	0.62	0.6	0.020
NDF							
Low		88.5	65.9	0.21	58.4	131	2.24
High		88.3	66.0	0.22	58.3	133	2.28
SEM (n = 16)		1.01	0.38	0.085	0.62	0.6	0.020
Effect (*P*-value)							
ME		0.914	0.815	0.154	0.724	0.389	0.418
NDF		0.871	0.967	0.921	0.923	0.023	0.225
ME × NDF		0.889	0.909	0.913	0.994	0.716	0.864

Abbreviations: EW, egg weight; EM, egg mass; FI, feed intake; FCR, feed conversion ratio.

[1]Data are least squares means of 8 observations per treatment. The data for productive performance were then summarized for 12 wk from 75 to 86 wk of age.

[2]EM = egg production (%) × EW (g) / 100.

[3]Normal-ME diets contained commercially recommended levels of ME (2,730 kcal AME_n/kg); low-ME diets contained less ME by 50 kcal ME/kg (2,680 kcal AME_n/kg).

[4]High-NDF diets contained 40% higher NDF concentrations than low-NDF diets.

Relative Organ Weight

No interactions between dietary levels of ME and NDF for all relative organ weights were observed (Table 5). For the main effects, high-NDF diets decreased ($P < 0.05$) the relative weights of the liver, but increased ($P < 0.05$) the relative weights of the gizzard as compared to low-NDF diets. However, dietary concentrations of NDF did not affect the relative weights of other organs. Similarly, dietary concentrations of ME had no effects on the relative weights of any of the organs.

Fatty Liver Indicator

No interactions between dietary levels of ME and NDF for all indicators for fatty liver development were identified (Table 6). For the main effects, high-NDF

Table 4. Effect of dietary concentrations of ME and neutral detergent fiber (NDF) on egg quality of aged laying hens.[1]

		Egg quality				
Item		Eggshell color[2]	Egg yolk color	Haugh unit	Eggshell strength (kg/cm^2)	Eggshell thickness (μm)
ME[3]	NDF[4]					
Low	Low	11.7	7.6[a]	83.1	3.34	393[b]
	High	11.3	8.3[c]	84.0	3.45	390[b]
Normal	Low	11.8	7.9[b]	82.0	3.44	393[b]
	High	11.6	8.8[d]	83.1	3.53	401[a]
SEM (n = 8)		0.11	0.08	0.79	0.051	2.3
Main effect						
ME						
Low		11.5	8.0	83.5	3.39	391
Normal		11.7	8.3	82.6	3.48	397
SEM (n = 16)		0.08	0.06	0.50	0.036	1.6
NDF						
Low		11.8	7.8	82.5	3.39	393
High		11.5	8.5	83.5	3.49	396
SEM (n = 16)		0.08	0.05	0.60	0.036	1.6
Effect (*P*-value)						
ME		0.125	<0.001	0.112	0.075	0.018
NDF		0.334	<0.001	0.247	0.063	0.209
ME × NDF		0.513	0.045	0.898	0.804	0.021

[a-d]Means in the same column with different superscripts are different ($P < 0.05$).

[1]Data are least squares means of 8 observations per treatment.

[2]Eggshell color was measured by the eggshell color fan scale.

[3]Normal-ME diets contained commercially recommended levels of ME (2,730 kcal AME_n/kg); low-ME diets contained less ME by 50 kcal ME/kg (2,680 kcal AME_n/kg).

[4]High-NDF diets contained 40% higher NDF concentrations than low-NDF diets.

Table 5. Effect of dietary concentrations of ME and neutral detergent fiber (NDF) on relative organ weight of aged laying hens.[1]

		Relative organ weight (%)						
Item		Liver	Spleen	Crop	Proventriculus	Gizzard	Small intestine	Large intestine
ME[2]	NDF[3]							
Low	Low	2.49	0.11	0.33	0.30	1.20	2.70	0.59
	High	2.27	0.10	0.32	0.31	1.31	2.73	0.66
Normal	Low	2.59	0.10	0.29	0.33	1.20	2.84	0.67
	High	2.23	0.10	0.32	0.31	1.26	2.80	0.70
SEM (n = 8)		0.123	0.010	0.017	0.014	0.037	0.088	0.035
Main effect								
ME								
Low		2.38	0.10	0.32	0.31	1.25	2.71	0.63
Normal		2.41	0.10	0.31	0.32	1.23	2.82	0.69
SEM (n = 16)		0.084	0.007	0.012	0.010	0.025	0.061	0.024
NDF								
Low		2.54	0.10	0.31	0.32	1.20	2.77	0.63
High		2.25	0.10	0.32	0.31	1.28	2.76	0.68
SEM (n = 16)		0.084	0.007	0.012	0.010	0.025	0.061	0.024
Effect (*P*-value)								
ME		0.809	0.774	0.247	0.536	0.434	0.207	0.076
NDF		0.020	0.827	0.844	0.578	0.025	0.926	0.144
ME × NDF		0.592	0.317	0.254	0.305	0.546	0.708	0.596

[1]Data are least squares means of 8 observations per treatment.
[2]Normal-ME diets contained commercially recommended levels of ME (2,730 kcal AME_n/kg); low-ME diets contained less ME by 50 kcal ME/kg (2,680 kcal AME_n/kg).
[3]High-NDF diets contained 40% higher NDF concentrations than low-NDF diets.

diets decreased ($P < 0.05$) L*, a*, and b* values in the liver; however, dietary concentrations of ME did not influence those color values. The hemorrhagic score was also unaffected by dietary levels of ME and NDF. For the main effects, however, high-NDF diets decreased ($P < 0.01$) total fat concentrations in the liver but low-ME diets increased ($P < 0.05$) total fat concentrations in the liver.

Hepatic Gene Expression in Fat Metabolism

The expression levels of genes related to fatty acid oxidation (*CPT1A* and *PPAR-α*) and synthesis (*FASN, SREBP-1, ACC, SCD, PPAR-γ*, and malic enzyme) were analyzed to study hepatic fat metabolism in aged laying hens as affected by dietary levels of ME and NDF (Table 7). In genes related to fatty acid oxidation, an interaction ($P < 0.05$) between dietary levels of ME and NDF was found for *CPT1A* expression. Increasing NDF concentrations in normal-ME diets increased ($P < 0.05$) *CPT1A* expression, but this observation was not found for low-ME diets. No interaction was observed for *PPAR-α* expression, but for the main effects, low-ME diets decreased ($P < 0.05$) *PPAR-α* expression, whereas high-NDF diets increased ($P < 0.01$) *PPAR-α* expression.

Table 6. Effect of dietary concentrations of ME and neutral detergent fiber (NDF) on fatty liver incidence of aged laying hens.[1]

		Fatty liver incidence					
		Liver color [2]					
Item		Score	L*	a*	b*	Hemorrhagic score	Total fat concentration (% DM)
ME[3]	NDF[4]						
Low	Low	3.00	33.3	22.8	13.9	2.46	48.1
	High	2.52	30.0	19.6	10.1	1.95	31.3
Normal	Low	2.92	33.4	21.0	13.1	2.17	29.9
	High	2.63	29.6	18.4	9.5	1.54	25.8
SEM (n = 8)		0.318	1.58	0.87	1.87	0.388	3.32
Main effect							
ME							
Low		2.76	31.6	21.0	12.0	2.21	39.7
Normal		2.77	31.5	19.7	11.3	1.85	27.9
SEM (n = 16)		0.218	1.08	0.59	1.28	0.266	2.35
NDF							
Low		2.96	33.4	21.9	13.5	2.31	39.0
High		2.57	29.8	19.0	9.8	1.75	28.5
SEM (n = 16)		0.218	1.08	0.59	1.28	0.266	2.35
Effect (*P*-value)							
ME		0.977	0.919	0.083	0.682	0.351	0.012
NDF		0.216	0.026	0.002	0.045	0.138	0.002
ME × NDF		0.763	0.893	0.732	0.938	0.873	0.061

[1]Data are least squares means of 8 observations per treatment.
[2]Liver color was measured by the score scale and CIE color system.
[3]Normal-ME diets contained commercially recommended levels of ME (2,730 kcal AME_n/kg); low-ME diets contained less ME by 50 kcal ME/kg (2,680 kcal AME_n/kg).
[4]High-NDF diets contained 40% higher NDF concentrations than low-NDF diets.

Table 7. Effect of dietary concentrations of ME and neutral detergent fiber (NDF) on the relative expression of genes related to fatty acid metabolism in the liver of aged laying hens.[1]

		Gene expression[2]							
		Fatty acid oxidation		Fatty acid synthesis					
		CPT1A	*PPAR-α*	*FASN*	*SREBP-1*	*ACC*	*SCD*	*PPAR-γ*	Malic enzyme
ME[3]	NDF[4]								
Low	Low	1.00[ab]	1.00	1.00	1.00	1.00	1.00	1.00	1.00[a]
	High	0.68[ab]	1.43	0.04	0.81	0.33	0.30	0.27	0.21[b]
Normal	Low	0.54[b]	1.25	0.40	2.66	0.57	0.65	0.54	0.27[b]
	High	1.39[a]	1.51	0.67	1.65	0.23	0.28	0.18	0.12[b]
SEM (n = 8)		0.28	0.15	0.32	0.76	0.19	0.26	0.16	0.11
Main effect									
ME									
Low		0.84	1.22	0.57	0.91	0.67	0.65	0.67	0.60
Normal		0.97	1.38	0.61	2.16	0.40	0.47	0.36	0.20
SEM (n = 16)		0.20	0.13	0.21	0.52	0.15	0.16	0.10	0.07
NDF									
Low		0.77	1.13	1.06	1.83	0.79	0.83	0.77	0.64
High		1.04	1.47	0.13	1.23	0.28	0.29	0.23	0.17
SEM (n = 16)		0.20	0.13	0.22	0.53	0.15	0.16	0.10	0.07
Effect (*P*-value)									
ME		0.647	0.021	0.903	0.099	0.048	0.048	0.044	0.001
NDF		0.338	<0.001	0.004	0.429	0.003	0.008	<0.001	<0.001
ME × NDF		0.040	0.346	0.774	0.583	0.263	0.233	0.173	0.004

[a,b]Means in the same column with different superscripts are different ($P < 0.05$).

[1]Data are least squares means of 8 observations per treatment.

[2]*CPT1A*, carnitine palmitoyl transferase 1A; *PPAR-α*, peroxisome proliferator-activated receptor-α; *FASN*, fatty acid synthase; *SPEBP-1*, sterol response element-binding protein 1; *ACC*, acetyl-CoA carboxylase; *SCD*, stearoyl-CoA desaturase; *PPAR-γ*, peroxisome proliferator-activated receptor-γ.

[3]Normal-ME diets contained commercially recommended levels of ME (2,730 kcal AME_n/kg); low-ME diets contained less ME by 50 kcal ME/kg (2,680 kcal AME_n/kg).

[4]High-NDF diets contained 40% higher NDF concentrations than low-NDF diets.

In genes related to fatty acid synthesis, increasing NDF concentrations in low-ME diets decreased ($P < 0.05$) malic enzyme expression, whereas increasing NDF concentrations in normal-ME diets did not influence its expression, showing an interaction ($P < 0.01$). However, no interactions were identified for other genes related to fatty acid synthesis. For the main effects, low-ME diets increased ($P < 0.05$) expressions of *ACC, SCD, PPAR-γ*, and malic enzyme but high-NDF diets decreased ($P < 0.01$) expressions of *ACC, SCD, PPAR-γ*, and malic enzyme. High-NDF diets decreased ($P < 0.01$) *FASN* expression, but different levels of ME in diets did not affect *FASN* expression.

DISCUSSION

The effects of dietary energy levels on productive performance and egg quality of laying hens have been documented; however, inconsistent results of dietary energy concentrations have been observed for productive performance and egg quality in laying hens (Wu et al., 2005; Jalal et al, 2006; Han and Thacker, 2011; Pérez-Bonilla et al., 2012; Ribeiro et al., 2014). In addition, several experiments associated with the effect of dietary fiber concentrations on productive performance, egg quality, and gut health of laying hens have been performed and the results have also been controversial (Roberts et al., 2007; Sousa et al., 2019; Desbruslais et al., 2021). In particular, information pertaining to the interactive effect of dietary concentrations of energy and fiber on productive performance, egg quality, and fatty liver incidence in aged laying hens is largely lacking.

In the current experiment, dietary levels of ME did not affect productive performance in aged laying hens. It is known that laying hens have the ability to adjust FI in response to different energy concentrations in diets (Classen, 2017), whereas multiple factors, such as genotype, diet compositions, feed forms, age, energy to protein ratio, and environment, can affect FI changes of hens in response to different energy concentrations in diets (Classen, 2017). Junqueira et al. (2006) reported no differences in FI by increasing concentrations of dietary ME in aged laying hens, which agrees with the current results. On the other hand, the results of the current experiment indicated that laying hens fed high-NDF diets, regardless of ME levels, exhibited a greater FI than those fed low-NDF diets although the difference in FI was very small. Dietary fiber is known to have a low-energy value and to exert anti-nutritional effects, such as decreased nutrient digestion and absorption, possibly due to facilitated passage rate of intestinal contents in animals (Jha et al., 2019; Desbruslais et al., 2021). However, it was reported that feeding diets containing high insoluble fiber increased the passage rate of intestinal contents, but this increase contributed to enhanced FI in laying hen (Jha et al., 2019). Thus, it is speculated that wheat bran and DDGS as used to increase NDF concentrations in treatment diets contained high amounts of insoluble fiber, which may be the reason for increased FI by high-NDF diets in this

experiment. However, no interactive effects of dietary ME and NDF levels were observed in this experiment, indicating that the current levels of ME and NDF in diets may have little effects on productive performance in aged laying hens.

Considering egg quality, significant interactions and main effects of dietary levels of ME and NDF were observed for the egg yolk color in aged laying hens. This observation is likely associated with inclusion of specific feed ingredients for producing different ME and NDF concentrations in treatment diets. Egg yolk color directly reflects dietary concentrations of carotenoids such as lutein and zeaxanthin that are contained at the high amount in DDGS and corn gluten meal (Shin et al., 2015; Grashorn, 2016). In the current experiment, inclusion levels of DDGS and corn gluten meal were increased to produce high-NDF diets, and their inclusion levels were also adjusted in different ME diets.

Normal-ME diets increased eggshell thickness and tended to increase eggshell strength in aged laying hens for the main effects. This result indicated that appropriate ME levels in diets are required to maintain eggshell quality because eggshell formation in the uterus requires high amounts of energy (Jonchère et al., 2012). Interestingly, there was an interaction for eggshell thickness between dietary levels of ME and NDF because increasing NDF levels in low-ME diets had no effects on eggshell thickness, but increased eggshell thickness in aged hens fed normal-ME diets. The reason for this interaction is not clear because information regarding how dietary levels of ME and fiber affect eggshell thickness in aged laying hens is largely limited. Both dietary Ca and P concentrations and their availability are known to be critical to improve eggshell thickness and strength in laying hens (Pelicia et al., 2009). Thus, although calculated total Ca and available P concentrations were equalized among all treatment diets in this experiment, it may be speculated that feed ingredients used in high-ME and high-NDF diets may have more available Ca and P than those used in other treatment diets.

No interactions between dietary levels of ME and NDF on all relative organ weights in aged laying hens were observed; however, for the main effects, high-NDF diets altered the relative weights of the liver and gizzard. In this experiment, aged laying hens fed high-NDF diets had a lower relative weight of the liver than those fed low-NDF diets. Most of previous experiments studying fatty liver diseases have been shown to increase the liver weight with increasing fat concentrations in poultry (Harms et al., 1977; Lee at el., 2010). Therefore, decreased liver weights by feeding high-NDF diets may be an indirect evidence for decreasing fatty liver development, which was supported by our observation that high-NDF diets decreased total fat concentrations in the liver. Moreover, increasing fiber intake is reported to stimulate the intestinal development by increasing feed retention in the intestine (Sousa et al., 2019). Increased weights of the gizzard were observed by feeding high-NDF diets in this experiment, but such an effect was not observed for other intestinal organs.

Laying hens frequently experience with the health problem in the liver due to increasing fat accumulation with age (Zaefarian et al., 2019). The FLHS is one of well-known liver problems in laying hens and it is characterized by enlarged and pale colored liver with marked hemorrhage (Zaefarian et al., 2019). Structurally and functionally weakened liver causes various health problems that often lead to the death of laying hens. It is reported that FLHS is considered the main reason of the mortality in cage-raised laying hens (Shini et al., 2019). According to Dong and Tong (2019), aged laying hens are more prone to FLHS than younger laying hens. However, in most of the previous studies, the FLHS was induced artificially by feeding high fat and energy diets to broiler chickens and young laying hens, such that the research regarding FLHS in aged laying hens is still limited. Therefore, one of the major purposes of the present experiment was to investigate the interactive effect of dietary concentrations of ME and NDF on FLHS development in aged laying hens.

Hepatic fat concentrations in aged laying hens were increased by both low-ME diets and low-NDF diets for the main effects, and feeding low-NDF and low-ME diets induced the highest fat concentrations in the liver, showing the tendency for the interaction. This result may indicate that increasing NDF concentrations in normal-ME diets are effective in decreasing hepatic fat accumulation, which may aid in preventing FLHS development in aged laying hens. This result agrees with previous experiments reporting that increasing concentrations of dietary fiber decreased hepatic fat retentions in poultry (Akiba and Matsumoto, 1982; Akiba et al., 1987; Bogusławska-Tryk et al., 2016). According to Akiba and Matsumoto (1982), dietary fiber can stimulate the secretion of lipoproteins from the liver and decrease de novo fatty acid synthesis in the liver. In the current experiment, feeding high-NDF diets increased hepatic expression of *PPAR-α* that is related to fatty acid oxidation, but decreased the expression of *FASN, ACC, SCD, PPAR-γ*, and malic enzyme that are related to fatty acid synthesis. Interactions between dietary levels of ME and NDF for expressions of *CPT1A* and malic enzyme were also observed. These altered fatty acid metabolisms in aged laying hens are likely the primary reason why high-NDF diets reduced hepatic fat concentrations in this experiment. To the best of our knowledge, this is the first experiment that shows the modification of hepatic fatty acid metabolism and reduction in hepatic fat concentrations by high-NDF diets in aged laying hens.

The observation that hens fed low-ME diets showed a greater fat concentration with less expression of *PPAR-α* and greater expression of *ACC, SCD, PPAR-γ*, and malic enzyme in the liver than those fed normal-ME diets was unexpected because decreased energy concentrations in diets generally reduce hepatic fat concentrations in poultry (Whitehead, 1979). The reason for this contrasting observation is difficult to be explained; however, it may be related to differences in dietary fat concentrations between low and normal-ME diets. In this

experiment, normal-ME diets contained more fat than low-ME diets because of higher ME concentrations. It is reported that higher fat concentrations in diets may decrease fatty acid synthesis but increase fatty acid oxidation in rats (Zhang et al., 2014). Moreover, increasing fat concentrations as a replacement of carbohydrates in diets were reported to reduce hepatic fat retention in laying hens, possibly through feed-back mechanisms (Haghighi-Rad and Polin, 1982). However, it should be noted that the effects of dietary ME and NDF concentrations on lipid metabolism were determined only based on hepatic fat concentrations and expression of some selected genes related to oxidation and synthesis of fatty acids in the current experiment. Further research is required to verify the alteration of hepatic lipid metabolism in aged laying hens by dietary ME and NDF concentrations at the transcriptomic and proteomic level.

CONCLUSION

Decreasing ME concentrations in diets impairs egg quality and aggravates hepatic fat accumulation in aged laying hens without affecting productive performance. However, increasing NDF concentrations in diets improves egg quality with no negative effects on productive performance in aged laying hens. In addition, increasing NDF concentrations in commercially-recommended ME diets decreases hepatic fat concentrations in aged laying hens by increasing fatty acid oxidation and decreasing fatty acid synthesis in the liver, which may aid in preventing FLHS development in aged laying hens.

ACKNOWLEDGMENTS

This research was carried out with the support of the Cooperative Research Program for Agriculture Science and Technology Development (Project No. PJ01256701), Rural Development Administration, Republic of Korea. This research was also supported by the Chung-Ang University Graduate Research Scholarship (Academic scholarship for College of Biotechnology and Natural Resources) in 2023.

DISCLOSURES

The authors declare no conflict of interest for the data presented in this experiment.

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