Dietary naringin supplementation on laying performance and antioxidant capacity of Three-Yellow breeder hens during the late laying period

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ABSTRACT In this study, the effects of 3 graded dietary levels (0.1%, 0.2%, and 0.4%) of naringin were studied in Three-Yellow breeder hens during the late laying period (55–62 wk). A total of 480 Three-Yellow breeder hens (54-wk-old) were randomly divided into 4 groups (6 replicates of 20 hens): basal diet group (C), and basal diets supplemented with 0.1%, 0.2%, and 0.4% of naringin (N1, N2, and N3), respectively. Results showed that dietary supplementation with 0.1%, 0.2%, and 0.4% of naringin for 8 wk increased the laying rate and egg mass, enhanced egg yolk color, and decreased the feed egg ratio (P < 0.05). Meanwhile, compared with hens in C group, there were more preovulatory follicles and higher ovarian index while, compared with hens in C group, there were more preovulatory follicles and higher ovarian index as well as an enhanced ovarian somatic cell proliferation in hens of N2 and N3 groups (P < 0.05). With 0.2% and 0.4% naringin, glutathione concentration, the activity of catalase and total superoxide dismutase, and the total antioxidant capacity of ovarian tissues and serum increased (P < 0.05), while the contents of malondialdehyde and hydrogen peroxide decreased (P < 0.05). Moreover, compared to C group, the transcription levels of antioxidant genes in ovarian tissues increased in hens from N2 and N3 groups (P < 0.05). In conclusion, supplementation with 0.2% and 0.4% naringin both could improve the laying rate, ovarian and serum antioxidant capacity of Three-Yellow breeder hens during the late laying period.

Key words: naringin, Three-Yellow breeder hen, late laying period, laying performance, antioxidant capacity

INTRODUCTION

Three-Yellow chicken is one of yellow broilers in China, which is famous for its delicious meat and high carcass quality. Normally, egg laying rate of Three-Yellow chicken reaches the peak (≥70%) at about 26 wk of age and lasts for few weeks, however, the laying rate is less than 55% at 50 wk of age. Moreover, the hen only produces about 77 eggs per year on average (Xu and Chen, 2003). Short laying period and low laying performance seriously reduce the number of breeding eggs and the commercial value of Three-Yellow chickens. Therefore, the exploration of strategies to prolong the laying period is necessary for improving the economic benefits of Three-Yellow chickens.

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During the late laying period of hens, the egg laying rate declines sharply (Dai et al., 2020) due to age-related changes in hormone (Liu et al., 2013), ovarian dysfunction (May-Panloup et al., 2016), liver function failure (Huang et al., 2021a), etc. Numerous studies on laying hens demonstrated that ovarian function decline is one of the most crucial factors causing reductions in laying performance (Alvarez and Hocking 2012; Hao et al., 2020). It is demonstrated that at the late laying stage of the hens, decreased number of preovulatory follicles and growing follicles result in declines in laying performance and the level of serum estrogen (Zhang et al., 2021; Wu et al., 2022; Yao et al., 2022).

Growing evidences demonstrate that ovarian functions are associated with serum hormones, organism physiological state, ovarian redox state (Wu et al., 2017; Ren et al., 2018; Huang et al., 2021b), etc. In mammals, oxidative stress could increase the percentage of atretic follicles and decrease the secretion of reproductive hormones (Sharma et al., 2015; Yu et al., 2019). Similar to mammals, ovarian dysfunction induced by oxidative stress was found in laying hens and resulted in the decrease of laying performance and egg quality (Wang...
et al., 2016; Abbas et al., 2020; Wang et al., 2021a). Li et al. (2020) reported that oxidative stress caused by heat stress induces ovarian cells apoptosis, and leads to declines in the number of follicles and laying rate.

Recently, plant-derived extracts including polyphenols, carotenoids and flavonoids as antioxidants were added to the dietary in order to enhance the laying performance and egg quality by improving the resistance to oxidative stress of hens (Liu et al., 2014; Yuan et al., 2016; An et al., 2019). Naringin is one kind of flavonoids that is widely distributed in foods of plant sources such as vegetables and fruits, especially in the pomelo, grapefruit, and citrus rutaceae (Zeng et al., 2019). Studies on mice showed that naringin has effects of anti-inflammatory, antiapoptotic, neuroprotective, and antioxidant (Chtourou et al., 2016; Kim et al., 2018; Lim et al., 2018; Wang et al., 2021b). A previous study in broilers showed that naringin could improve liver antioxidant capacities to against thiram-induced tibial dyschondroplasia (Jiang et al., 2020). Goliomytis et al. (2015) demonstrated that naringin could improve the antioxidant capacity of breast and thigh meat in broilers. Besides, Hager-Theodorides et al. (2021) reported that naringin could up-regulated transcription levels of antioxidant genes, thereby improving antioxidant capacity of hens. However, the effects of naringin on laying performance of Three-Yellow breeder hens in the late laying stage remain poorly understood.

In the present study, it was designed to investigate the effects of dietary supplemented with naringin on laying performance, egg quality and antioxidant capacity on Three-Yellow breeder hens in the late laying stage. The results of this study would be helpful to prolong laying lifespan and provide theoretical basis for improving the egg production of Three-Yellow breeder hens.

**MATERIALS AND METHODS**

**Birds and Experimental Design**

All animal experiments in this study were performed in accordance with the recommendations of Council for International Organizations of Medical Sciences for animal use and were approved by the Animal Care and Use Committee on the Ethics of Animal Experiments of Guangxi University (NO. GXU2020-011).

A total of 480 healthy Three-Yellow breeder hens were raised in a local farm in Yulin (Guangxi Zhuang Autonomous Region, China). All hens were randomly allocated into control and naringin groups (0.1%, 0.2%, and 0.4%) with 6 replicates of 20 birds per replicate at 54 wk of age. Before that, a 2-wk pre-experiment was conducted to ensure that all hens used in this study were in the same status of health and laying rate. The hens in control group (C) were fed with basal diet (Table 1), which met all NRC requirements. The hens in 3 naringin groups (N1, N2, and N3) received a basal diet supplemented with 0.1%, 0.2%, and 0.4% of naringin, respectively. Naringin (purity ≥ 95%) used in this study was purchased from ChengDu ConBon Biotech Co. Ltd., (Chengdu, China).

| Ingredients | Content (%) | Nutrient levels | Content (%) |
|-------------|-------------|-----------------|-------------|
| Corn        | 63.50       | ME/(MJ/kg)      | 11.49       |
| Soybean meal| 21.50       | Crude protein   | 16.27       |
| Soybean oil | 2.50        | Crude fat       | 4.66        |
| Limestone   | 8.00        | Crude fibre     | 1.62        |
| Dicalcium phosphate | 1.50 | Calcium | 3.39 |
| NaCl        | 0.35        | Total phosphorus| 0.54        |
| DL-Methionine| 0.15       | Methionine      | 0.40        |
| Premix      | 2.5         | Lysine          | 0.69        |
| Total       | 100.00      |                 |             |

The premix provided the following (per kilogram of diet): vitamin A, 10,800 IU; vitamin D3, 2160 IU; vitamin E, 27 IU; vitamin K3, 1.4 mg; vitamin B1, 1.8 mg; vitamin B2, 8 mg; niacin, 32 mg; pantothenic acid, 11 mg; vitamin B6, 4.1 mg; folate acid, 1.08 mg; vitamin B12, 0.010 mg; biotin, 0.18 mg; Fe, 72 mg; Zn, 72 mg; Mn, 90 mg; Cu, 7 mg; I, 0.9 mg; and Se, 0.27 mg.

All hens were housed in an individual wire cages (width: 30 cm, depth: 40 cm, height: 40 cm) with temperature between 23°C and 26°C, the relative humidity between 65 and 75%, and illumination at 16 h/D. Each of the 20 hens formed one experimental unit and shared a common feed trough. Hens were provided ad libitum access to water and feed. Eggs were collected at 10 am and 4 pm every day.

**Sample Collection and Analytical Determination**

**Laying Performance** The total number of eggs and the weight of each egg in each replicate were recorded every day. According to the production records, laying performance parameters include egg laying rate, egg mass, and average egg weight were calculated. The average daily feed intake (ADFI) was determined weekly by subtracting the final feed weight of each replicate from the initial feed weight. The feed egg ratio was calculated and recorded weekly (daily feed consumption/average egg weight).

**Egg Quality** At the midterm and the end of the experiment, 72 normal eggs were randomly selected (18 eggs from each group, 3 eggs per replicate) to evaluate egg quality traits. The eggshell thickness (mm) was measured using a micrometer (SanLiang Precision instrument Co. Ltd., Guangdong, China) at 3 different locations (sharp end, middle, and blunt end) of the egg. Eggshell strength (Pa), albumen height (mm), yolk color, egg yolk weight (g), and Haugh units were determined using an EA-01 egg analyzer (ORKA Food Technology, Ltd., Ramat Hasharon, Israel) according to the instructions of the manufacturer. The longitudinal and transverse diameters of the egg were measured via a vernier caliper (BiaoKang Science and Technology Co. Ltd., Shenzhen, China).

**Sample Collection** At the midterm and the end of the experiment, 3 hens in each replicate were randomly selected (18 hens per group) and weighed. Blood samples were collected from the wine vein, than centrifuged at 3,500 rpm for 10 min. The serum samples were stored at -80°C for antioxidant parameters analysis.
At the end of the experiment, 2 hens in each replicate (12 hens per group) were selected randomly. The hens were slaughtered by jugular exsanguination postanesthesia. Ovaries without follicular follicles (diameter >12 mm) were weighed. Ovaries without follicles over 1 mm in diameter immediately snap frozen in liquid nitrogen for the analysis of the antioxidant parameters, and the extraction of RNA and protein.

Morphological Observation of the Ovary

Ovaries without follicles over 1 mm in diameter were dissected and fixed in 4% neutral paraformaldehyde solution at 4°C for more than 48 h. After that, ovarian tissues were dehydrated in a graded ethanol and cleared in xylene. Subsequently, ovarian tissues were embedded in paraffin, sectioned into 5 μm thickness using a microtome (Leica Biosystems, Buffalo Grove, IL). The sections were deparaffinized in xylene and rehydrated in a graded dilutions of ethanol, and then stained with hematoxylin and eosin (HE).

Ovarian Somatic Cell Proliferation Analysis

Ovarian tissues were homogenized in ice-cold RIPA and then centrifuged at 12,000 rpm for 20 min at 4°C to obtain the supernatant. Protein concentration in supernatant was determined using a BCA protein assay kit (AR1189, Boster Biological Technology Co. Ltd., Wuhan, China). Around 20 μg protein from each sample were electrophoresed on a 10% SDS-PAGE, and then transferred to a polyvinylidene difluoride (PVDF) membrane. After blocked with 5% skimmed milk at room temperature for 2 h, the blots were probed with corresponding primary antibodies β-actin (1:1000; BM3873), PCNA (1:1000; BM0104) and CDK2 (1:1000; PB0562) purchased from Boster Biological Technology Co. Ltd., (Wuhan, China) with and goat anti-mouse (1:5000; BA1050) antibodies and diaminobenzidine-conjugated goat anti-rabbit (1:5000; BA1054) secondary antibody. Secondary horseradish peroxidase-conjugated goat anti-rabbit (1:5000; BA1054) and goat anti-mouse (1:5000; BA1050) antibodies were purchased from Boster Biological Technology Co. Ltd., (Wuhan, China). Immunological signals were detected by enhanced chemiluminescence (ECL) Kit using a ChemiScope 3400 Mini machine. The band intensities were quantified using Quantity one software and the results were normalized to β-actin.

Ovarian and Serum Antioxidant Capacity Determination

Ovarian tissues were homogenized in PBS, and centrifuged at 3,000 rpm for 10 min at 4°C to obtain 10% ovarian tissue homogenate. The protein concentration in homogenate was determined via a BCA protein assay kit (AR1189, Boster Biological Technology CO. Ltd., Wuhan, China). The activities of total superoxide dismutase (T-SOD; A015-1), catalase (CAT; A007-1), and total antioxidant capacity (T-AOC; A015-1), and the concentration of glutathione (GSH; A006-2), the malondialdehyde (MDA; A003-1) and hydrogen peroxide (H2O2; A064-1) in ovarian tissue homogenate and serum were determined using specific detection kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Table 2. Primers used for qRT-PCR.

| Gene name | Accession number | Primer sequence (5’-3’) | Product length/bp |
|-----------|------------------|-------------------------|------------------|
| SOD1      | NM_205064.2      | F:GGCAATGTTGCTGCAAAGGG  | 133              |
|           |                  | R:CCCGCTTACCCAGGTCATCA  |                  |
| GPX1      | NM_001277853.3   | F:AGTACATCATCTGCTGCCG   | 137              |
|           |                  | R:CTCGATGTCGTCCTGCAGTT  |                  |
| CAT       | NM_001031215.2   | F:TCAGGAGATGTGCAAGCTT   | 109              |
|           |                  | R:TCTTACAACACGCTTGGGT   |                  |
| GLRX      | NM_205160.2      | F:GGATCCTGCCCCCTTACTGAA | 137              |
|           |                  | R:GTTGCTGCCCTTGTGGT    |                  |
| GSR       | XM_040671422.1   | F:TCTGACTGAGGCTCTCGAG   | 150              |
|           |                  | R:AACCTGGGTACACCGGAT    |                  |
| PRDX3     | XM_040674879.1   | F:CAAGGGGAAATACCTGTGCT  | 118              |
|           |                  | R:CACCGGTGGACATCATCGT   |                  |
| β-actin   | NM_205518.1      | F:ACACCGACACCCGCTGTAGAA | 136              |
|           |                  | R:TGCTGCTGACACCTGATACC  |                  |

Abbreviations: CAT, catalase; GLR, glutaredoxin; GPX1, glutathione peroxidase 1; GSR, glutathione reductase; PRDX3, peroxiredoxin 3; SOD1, superoxide dismutase 1.

Quantitative Real-Time PCR (qRT-PCR) Analysis

Total RNA was isolated from ovarian tissues using RNAiso Plus (AJ11050A, TaKaRa Biotechnology, Dalian, China). RNA concentration was quantified depending on the absorbance at the wavelength of 260/280 nm using a NanoDrop 1000 UV spectrophotometer. 1.0 μg RNA was immediately used for reverse transcription using HiScript III RT SuperMix for qPCR (+gDNA wiper) reagent kits (R323-01, Vazyme Biotech Co., Ltd, Nanjing, China). qRT-PCR was performed using ChamQ Universal SYBR qPCR Master Mix (Q711-03, Vazyme Biotech Co., Ltd, Nanjing, China) according to the instructions of the manufacturer. Comparisons of transcription levels were determined using specific detection kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the instructions of the manufacturer. Comparisons of transcription levels were determined using specific detection kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The sequences for forward and reverse primers are listed in Table 2.
Statistical Analysis

All data were analyzed using SPSS 19.0 (SPSS, Inc., Chicago, IL). Differences in naringin diet supplementation were tested using one-way analysis of variance (ANOVA) for independent samples, followed by Duncan’s multiple comparison tests. Polynomial contrasts for the linear and quadratic responses were used to determine the effect of different naringin levels. All data are presented as the means with standard error of mean (SEM). Values were considered to be statistically significant at $P < 0.05$.

RESULTS

Appearance, Body Weight, and Laying Rate

The results showed that dietary supplemented with naringin had no remarkable effect on the appearance of Three-Yellow breeder hens (Figure 1A). Meanwhile, dietary supplemented with naringin did not alter the body weight of Three-Yellow breeder hens ($P > 0.05$; Figure 1B). Dietary naringin supplementation had no effect on laying rate of hens from 55 to 57 wk of age ($P > 0.05$), but there was a linear increase in laying rate of hens.

Figure 1. Appearance, body weight, and laying rate of Three-Yellow breeder hens. $^{a,b}$Mean values with different superscripts letters denote statistically significantly difference ($P < 0.05$, $n = 6$). Abbreviations: C, basal diet group; N1, N2 and N3, basal diets supplemented with 0.1%, 0.2% and 0.4% of naringin, respectively; SEM, standard errors of mean.
hens at 58 wk ($P = 0.014$). Compared with C group, laying rate in the 3 treatment groups increased significantly ($P < 0.05$). During 59 to 60 wk, laying rate increased quadratically ($P < 0.05$). From 61 wk of age, laying rate was a significantly quadratic increase in hens from 3 treatment groups, compared with those in C group. Meanwhile, laying rate of hens in N2 and N3 groups were significantly higher than that in N1 group ($P < 0.05$; Figures 1C and 1D).

**Egg Quality**

As shown in Table 4, the results of egg quality at the midterm and the end of the experiment indicated that dietary supplemented with naringin had no significant effect on egg quality parameters include egg shape index, eggshell thickness, eggshell strength, albumen height, egg yolk weight, and Haugh unit ($P > 0.05$). After feeding with naringin for 8 wk, the egg yolk color was quadratically improved ($P < 0.05$). No significant difference was found in egg yolk color among 3 doses of naringin groups ($P > 0.05$).

**Table 3. Laying performance of Three-Yellow breeder hens.**

| Item                          | Groups          | C   | N1  | N2  | N3  | SEM | ANOVA | Linear Quadratic |
|-------------------------------|-----------------|-----|-----|-----|-----|-----|-------|------------------|
| 55−58 W Laying rate (%)       |                 |     |     |     |     |     | 0.012 | 0.003            | 0.124 |
| Egg mass (g/d/hen)            |                 | 49.05 | 49.98 | 50.45 | 50.45 | 0.393 |       |                  |      |
| Average egg weight (g)        |                 | 22.97 | 23.64 | 23.89 | 23.81 | 0.245 |       |                  |      |
| Feed egg ratio                |                 | 80.75 | 81.00 | 80.73 | 80.65 | 0.174 |       |                  |      |
| Feed egg ratio                |                 | 3.52  | 3.45  | 3.41  | 3.40  | 0.029 |       |                  |      |
| 59−62 W Laying rate (%)       |                 | 46.03 | 49.13 | 49.85 | 49.80 | 0.511 | <0.001 | <0.001            | 0.001 |
| Egg mass (g/d/hen)            |                 | 21.58 | 23.60 | 23.74 | 23.57 | 0.344 | <0.001 | <0.001            | 0.001 |
| Average egg weight (g)        |                 | 80.75 | 80.65 | 80.70 | 80.85 | 0.199 | 0.777  | 0.588            | 0.392 |
| Feed egg ratio                |                 | 3.74  | 3.51  | 3.42  | 3.46  | 0.051 | <0.001 | <0.001            | 0.003 |
| 55−62 W Laying rate (%)       |                 | 47.54 | 49.55 | 50.15 | 50.13 | 0.528 | <0.001 | <0.001            | 0.011 |
| Egg mass (g/d/hen)            |                 | 22.27 | 23.62 | 23.82 | 23.69 | 0.273 | <0.001 | <0.001            | 0.01  |
| Average egg weight (g)        |                 | 46.94 | 46.96 | 46.97 | 46.96 | 0.066 | 0.987  | 0.775            | 0.831 |
| Feed egg ratio                |                 | 3.63  | 3.51  | 3.42  | 3.46  | 0.042 | <0.001 | <0.001            | 0.010 |

Table 4. Egg quality of Three-Yellow breeder hens.

| Item                          | Groups          | C   | N1  | N2  | N3  | SEM | ANOVA | Linear Quadratic |
|-------------------------------|-----------------|-----|-----|-----|-----|-----|-------|------------------|
| 58 W Egg shape index          |                 | 1.29 | 1.28 | 1.28 | 1.28 | 0.015 | 0.895  | 0.914            | 0.891 |
| Eggshell thickness (mm)       |                 | 0.40 | 0.39 | 0.38 | 0.38 | 0.013 | 0.472  | 0.210            | 0.349 |
| Eggshell strength (N)         |                 | 42.58 | 42.48 | 42.50 | 42.58 | 1.658 | 1.000  | 0.993            | 0.930 |
| Albumen height (mm)           |                 | 5.40 | 5.41 | 5.41 | 5.42 | 0.086 | 0.998  | 0.969            | 0.854 |
| Egg yolk color                |                 | 6.78 | 6.89 | 6.94 | 7.00 | 0.379 | 0.945  | 0.549            | 0.918 |
| Egg yolk weight (g)           |                 | 16.17 | 16.18 | 16.21 | 16.24 | 0.391 | 0.998  | 0.892            | 0.919 |
| Haugh unit                    |                 | 77.16 | 77.23 | 77.21 | 77.19 | 0.659 | 1.000  | 0.977            | 0.927 |
| 62 W Egg shape index          |                 | 1.27 | 1.27 | 1.28 | 1.26 | 0.015 | 0.867  | 0.556            | 0.483 |
| Eggshell thickness (mm)       |                 | 0.33 | 0.33 | 0.35 | 0.35 | 0.011 | 0.329  | 0.115            | 0.775 |
| Eggshell strength (N)         |                 | 41.48 | 41.56 | 41.46 | 41.58 | 2.810 | 1.000  | 0.982            | 0.992 |
| Albumen height (mm)           |                 | 5.39 | 5.43 | 5.38 | 5.37 | 0.231 | 0.999  | 0.861            | 0.889 |
| Egg yolk color                |                 | 7.28  | 8.25b | 8.50b | 8.51b | 0.286 | <0.001 | <0.001            | 0.016 |
| Egg yolk weight (g)           |                 | 16.08 | 16.05 | 16.10 | 16.06 | 0.318 | 0.999  | 0.971            | 0.979 |
| Haugh unit                    |                 | 76.56 | 76.68 | 76.76 | 76.69 | 1.832 | 1.000  | 0.936            | 0.944 |

**Table 4.** Mean values within a row with different superscripts letters denote statistical significantly different ($P < 0.05$, $n = 6$). Abbreviations: ADFI, Average daily feed intake; C, basal diet group; N1, N2, and N3, basal diets supplemented with 0.1%, 0.2%, and 0.4% of naringin, respectively.

**Table 3.** Mean values within a row with different superscripts letters denote statistical significantly different ($P < 0.05$, $n = 18$). Abbreviations: C, basal diet group; N1, N2, and N3, basal diets supplemented with 0.1%, 0.2%, and 0.4% of naringin, respectively.
**Ovarian Morphology, Index, and Cell Proliferation**

More preovulatory follicles were observed in hens supplemented with naringin compared to hens fed with basal diet (Figures 2A and 2B). The histomorphology of ovarian tissues showed that there were more growing follicles in ovaries from hens in 3 treatment groups, compared with those in C group (Figure 2C). The ovarian index of N2, and N3 groups were higher than that in the C group ($P < 0.05$). No significant difference was found in ovarian index between C and N1 group, or among 3 treatment groups (Figure 2D). Meanwhile, the expression levels of proteins related to cell proliferation PCNA and CDK2 in ovarian tissues of N2 and N3 groups were higher than those in C group ($P < 0.05$). A higher expression level of PCNA was observed in N1 group in comparison to C group ($P < 0.05$). However, no significant difference was detected in CDK2 expression between C and N1 group (Figures 2E and 2F).

**Ovarian Antioxidant Capacity**

Compared to the C group, the 3 treatment groups increased linearly in the activities of T-SOD and T-AOC of ovarian tissues, and decreased linearly in the concentration of $\text{H}_2\text{O}_2$ ($P < 0.05$). N2 and N3 dietary treatment groups increased the content of GSH, activity of CAT, and decreased the concentration of MDA in ovarian tissues ($P < 0.05$). Moreover, the N2 group enhanced the content of GSH, and the activity of CAT significantly compared with those in N1 groups ($P < 0.05$). No significant difference was found in the activity of CAT and the concentrations of GSH and MDA in ovarian tissues between C and N1, and N1 and N3 ($P > 0.05$, Table 5).

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**Figure 2.** Ovarian morphology, index, and protein expression of Three-Yellow breeder hens. Mean values with different superscripts letters denote statistical significantly difference ($P < 0.05$). (A) Ovary morphology in each group; (B) Numbers of the preovulatory follicles (with diameters over 12 mm) in each group ($n = 12$). (C) Ovarian histomorphology in each group, scale bar: 50 μm. (D) Ovarian index in each group ($n = 12$). (E) Western blot analysis of CDK2 and PCNA in ovary. F: Expression levels of proteins related to cell proliferation of ovarian tissues in each group. Abbreviations: C, basal diet group; N1, N2, and N3, basal diet supplemented with 0.1%, 0.2%, and 0.4% of naringin, respectively.
Compared with C group, expression levels of CAT, SOD1, GPX1, GLRX, and PRDX3 in 3 treatment groups enhanced significantly ($P < 0.05$). Furthermore, the transcription levels of GSR in N2 and N3 groups were significantly higher than that in C group ($P < 0.05$; Figure 3). However, no significant difference was detected in the expression of GSR between C and N1 groups ($P = 0.248$). No significant difference was found among the 3 treatment groups ($P > 0.05$).

**Serum Antioxidant Capacity**

After feeding with naringin for 4 wk, activity of T-AOC and CAT in serum of N2 and N3 group were increased linearly than those in other 2 groups ($P < 0.05$). However, after 4 wk of feeding, dietary supplementation with naringin did not alter the activity of T-SOD, and the contents of GSH, MDA, and H$_2$O$_2$ in serum ($P > 0.05$, Table 6).

At the end of the experiment, dietary supplemented with naringin of 3 doses were linearly improved the activities of T-SOD and T-AOC in serum ($P < 0.05$), and the concentration of MDA in serum was a decreased linearly ($P < 0.05$). There was no significant difference in parameters mentioned above among 3 doses of treatments ($P > 0.05$). In N2 and N3 groups, the contents of H$_2$O$_2$ in serum were significantly lower than that in C group ($P < 0.05$). No significant difference was found between N1 group and C group ($P = 0.289$). Dietary supplemented with 0.2% naringin (N2) and 0.4% naringin (N3) both linearly improved the content of GSH and the activity of CAT in serum ($P < 0.05$), while no significant changes of them was observed in them between N1 and C groups ($P > 0.05$, Table 6).

**Discussion**

During the late laying period, laying performance and egg quality of hens reduce dramatically (Lv et al., 2019). Recently, as antioxidants, many kinds of plant extracts are added to the diets in order to improve the egg production and prolong the laying lifespan of hens (Xie et al., 2019; Chen et al., 2021; Zhang et al., 2021). Naringin, as a natural flavonoid extract from plants, could benefit the health status of animals attributes to its properties of hormonal regulation, antioxidant, anti-inflammatory, antitoxicity, antineuroinflammation and metabolic regulation (Caglayan et al., 2018; Akintunde et al., 2020; Shirani et al., 2020).

Previous study on 1-day-old Ross broiler chickens showed that dietary addition of naringin (0.75 and 1.5 g/kg) for 42 d had no effect on the average body or carcase weight (Goliomytis et al., 2015). Similarly, Iskender et al. (2017) found that dietary supplemented with...
hesperidin (0.5 g/kg), naringin (0.5 g/kg), or quercetin (0.5 g/kg) did not affect the body weight of 28-wk-old Lohmann White laying hens. In line with these studies, our results showed that dietary addition of naringin (0.1%, 0.2%, and 0.4%) for 8 wk had no effect on body weight and appearance of Three-Yellow breeder hens during the late laying period. However, in our study, from the 4th wk of the feeding trials, the egg laying rate of N1, N2, and N3 groups were significantly higher than that in the C group. These results indicated that naringin as an additive to diet could improve the egg laying rate rather than enhancing the growth performance of Three-Yellow breeder hens in the late laying stage.

It is demonstrated that egg laying performance and egg quality could be influenced by dietary supplemented with additives extracted from plants (Xie et al., 2019). Wang et al. (2018) demonstrated that supplementing tea polyphenols or tea catechins (200 mg/kg) in diet for 10 wk improved the laying rate, egg mass, and feed conversion rate of 64-wk-old Hy-Line Brown laying hens. Amevor et al. (2021b) reported that the feed intake, laying rate and egg weight increased after dietary supplemented with quercetin (0.4 g/kg) for 10 wk in 52-wk-old Tianfu breeder hens. Study of Liu et al. (2014) showed that diet addition of genistein (40 and 400 mg/kg) improved egg laying rate significantly of 55-wk-old Ross 308 breeder hens. Consistent with these studies, we observed that naringin supplementation (0.1%, 0.2%, and 0.4%) in the feed of 54-wk-old Three-Yellow breeder hens increased the laying rate, egg mass, and feed egg ratio. Interestingly, a previous study reported that supplementing with naringin (0.75 and 1.5 g/kg) in 54-wk-old Lohmann Brown laying hens diet for 67 d had no effect on laying performance (Goliomytis et al., 2019). We speculate these differences may be due to the different varieties and laying stages of hens, and dissimilar doses of naringin used in the trials.

As great factors affecting hatching rate of breeding eggs, the quality and breakage rate of breeding eggs are closely related to eggshell strength and thickness. In hens, increased breakage rates caused by decreasing eggshell strength during the aging process result in declines in reproductive performance and commercial value (Qiu et al., 2020; Amevor et al., 2021b). Previous studies indicated that dietary flavonoids supplementation in hens had specific effects on egg quality. Liu et al. (2013) suggested that supplementing quercetin (0.2 and 0.4 g/kg) in diet of Hessian laying hens for 8 wk increased Haugh unit, eggshell strength, and eggshell thickness. Study of Liu et al. (2014) suggested that egg quality was not influenced by quercetin (0.2, 0.4, and 0.6 g/kg) supplementation. Similarly, Goliomytis et al. (2019) found that hesperidin or naringin (0.75 and 1.5 g/kg) supplementation had no effects on egg quality except yolk color of Lohmann Brown laying hens. In our present study, after 8 wk of dietary naringin supplementation, the parameters of egg quality did not change significantly, except egg yolk color. These results are in line with the results of a previous report that enhanced color of egg yolk caused by increased activity of antioxidant enzyme by neohesperidin dihydrochalcone additive in the Lohmann laying hens (Zhu et al., 2021). This may be related to the naringin and neohesperidin are both flavonoids that could increase the oxidation capacity of hens to prevent lutein from being oxidized and increase pigment deposition.

In hens, normal ovarian function plays a crucial role in the maintenance of laying performance. Ovarian index could partially reflect the reproductive function (Han et al., 2020). And the number of ovarian follicles determines the laying rate of the hen (Hao et al., 2020). Long et al. (2017) reported that dietary supplemented with octacosanol improved the laying rate of Hy-Line Brown laying hens by increasing the ovarian weight and the number of follicles in the ovary. In hens, high laying rate is always accompanied with high cell proliferation and low follicular atresia rate (Wang et al., 2021c). The research of Dai et al. (2021) on Qiling breeder hens suggested that dietary addition with hawthorn-leaves flavonoids (30 and 60 mg/kg) improved ovary relative weight and the laying rate. Previous study reported that ovarian granulosa cells apoptosis induced by oxidative stress

**Table 6.** Antioxidant parameters in serum of hens.

| Item            | Groups | SEM  | ANOVA | Linear | Quadratic |
|-----------------|--------|------|-------|--------|-----------|
|                 |        |      |       |        |           |
| 58 W            |        |      |       |        |           |
| GSH (µmol/L)    | 2.29   | 2.37 | 2.42  | 2.44   | 0.228     | 0.913     | 0.485    | 0.822    |
| CAT (U/mL)      | 4.31b  | 4.64b| 5.02  | 5.04   | 0.165     | <0.001    | <0.001   | 0.189    |
| T-SOD (U/mL)    | 86.02  | 86.49| 86.60 | 86.74  | 1.128     | 0.927     | 0.529    | 0.855    |
| T-AOC (U/mL)    | 8.74a  | 9.28ab| 9.96b| 9.97b  | 0.422     | 0.013     | 0.002    | 0.374    |
| MDA (nmol/mL)   | 12.94  | 12.46| 12.14 | 12.04  | 0.622     | 0.457     | 0.131    | 0.673    |
| H$_2$O$_2$ (mmol/L) | 123.65 | 118.86 | 115.38 | 114.91 | 4.472     | 0.196     | 0.042    | 0.497    |
| 62 W            |        |      |       |        |           |
| GSH (µmol/L)    | 2.05a  | 2.13b| 2.77  | 2.74b  | 0.172     | <0.001    | <0.001   | 0.610    |
| CAT (U/mL)      | 4.10a  | 4.39ab| 4.75  | 4.73   | 0.175     | 0.001     | <0.001   | 0.209    |
| T-SOD (U/mL)    | 80.41  | 85.01| 85.11 | 85.77b | 1.637     | 0.007     | 0.003    | 0.096    |
| T-AOC (U/mL)    | 7.59a  | 8.88b| 9.25  | 9.31   | 0.445     | 0.001     | <0.001   | 0.057    |
| MDA (nmol/mL)   | 16.17  | 14.63| 13.16 | 13.12  | 0.654     | <0.001    | <0.001   | 0.112    |
| H$_2$O$_2$ (mmol/L) | 126.53 | 118.25 | 110.06 | 110.12b | 4.608     | 0.002     | <0.001   | 0.207    |

*Mean values within a row with different superscripts letters denote statistical significantly difference ($P < 0.05$, $n = 12$). Abbreviations: C, basal diet group; CAT, catalase; GSH, glutathione; H$_2$O$_2$, Hydrogen peroxide; MDA, malondialdehyde; N1, N2, and N3, basal diets supplemented with 0.1%, 0.2%, and 0.4% of naringin, respectively; T-AOC, total antioxidant capacity; T-SOD, total superoxide dismutase.
with aging resulted in ovarian follicle atresia (Amevor et al., 2021a). In this study, naringin supplementation at 0.2% and 0.4% both increased the number of preovulatory and growing follicles, ovarian index, and ovarian somatic cell proliferation of the Three- Yellow breeder hens at the late laying stage. We speculated that naringin could improve the antioxidant capacity of Three-Yellow breeder hens, thereby promoting ovarian somatic cell proliferation and alleviating the follicular atresic caused by oxidative stress. However, whether the results are consistent with our hypothesis needs further study.

Oxidative stress induced by the disruption of balance between oxidants and the antioxidant system could damage physiological functions of the ovary and result in reproductive failure (Xie et al., 2019). Studies in rats have shown that naringin could prevent cyclophosphamide-induced hepatotoxicity by increasing the activity of CAT, GSR, and GSH-Px, and the content of GSH in liver tissues (Akamo et al., 2021). Naringin have been reported to reduce MDA concentration in lamb carcass and prolong meat storage (Akamo et al., 2021). Naringin have shown that naringin could prevent clophosphamide-induced hepatotoxicity by increasing the activity of GSH-Px, ACOX1, PPARα, and HO-1 in mice. Wan et al. (2020) demonstrated that hesperetin inhibited oxidative stress and attenuated acetaminophen-induced hepatotoxicity via the up-regulation of HO-1 expression. Another study in broilers suggested that naringin up-regulated the expression of antioxidant genes. Boots et al. (2020) found that quercetin could improve the transcription levels of SOD2, CAT, and HO-1 in mice. This study, dietary supplemented with 0.2% and 0.4% naringin could improve the antioxidant capacity of the hens by enhanced the concentration of GSH, the activities CAT, T-AOC and T-SOD, and decreased the concentrations of MDA and H$_2$O$_2$ in the ovarian tissues and serum, as well as increased transcription levels of antioxidant genes were observed. Our results indicated that naringin supplementation improved the antioxidant capacity of the ovary and the serum of the hens.

In conclusion, this study provides the evidence that dietary supplemented with naringin has positive effects on laying rate, egg mass, feed egg ratio, and yolk color of hens, as well as the antioxidant capacity of ovary and the serum of Three-Yellow breeder hens during the late laying period. Taken together, a level of 0.2% or 0.4% naringin addition in the diet is more helpful than 0.1% for improving the laying performance of Three-Yellow breeder hens at the late laying stage.

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DISCLOSURES
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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