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Dietary Gamma-Oryzanol and Vitamin E Tocotrienols Mitigates Negative Impacts on Productivity, Egg Quality, and Immune- and Health-Related mRNA Abundance in Laying Hens Reared under High Cage Density

Tossaporn Incharoen [1,*], Wirot Likittrakulwong [2], Riantong Singanusong [1], Rangsun Charoensook [1], Wandee Tartrakoon [1] and Juan J. Loor [3]

[1] Faculty of Agriculture Natural Resource and Environment, Naresuan University, Phitsanulok 65000, Thailand
[2] Animal Science Program, Faculty of Food and Agricultural Technology, Pibulsongkram Rajabhat University, Phitsanulok 65000, Thailand
[3] Department of Animal Sciences, University of Illinois, Urbana 61801, USA
* Correspondence: tossaporni@nu.ac.th

Simple Summary: In order to increase the total egg production per housing unit, many egg producers endeavor to increase the number of laying hens per cage at maximum capacity. A high cage density has been associated with detrimental impacts, including decreased productivity and egg quality as well as an increased stressful condition. Recently, gamma-oryzanol and vitamin E tocotrienols are powerful natural antioxidant due to its capacity to prevent lipid peroxidation and the resulting oxidative stress. The research was conducted to evaluate the effect of laying diets supplemented with or without these antioxidants on productivity, egg quality, and immune- and health-related mRNA abundance in hens reared in different cage densities. The results suggest dietary gamma-oryzanol and vitamin E tocotrienols at a level 200 ppm, either individually or in combination could improve performance and egg and shell quality as well as regulate mRNA abundance of immune- and stress-related genes. Thus, we concluded that dietary antioxidants should be part of a nutritional strategy to mitigate the negative impacts on laying hens reared under high cage density conditions.

Abstract: A 4×2 factorial experiment was conducted to evaluate the potential of feeding dietary gamma-oryzanol and vitamin E tocotrienols in hens reared in 840 (low cage density; LCD) or 420 cm^2/hen (high cage density; HCD). A total of 120 hens were allocated into eight groups with five replicates. Diets were a control (CON) or the control diet supplemented with 200 ppm gamma-oryzanol (GO-200), 200 ppm vitamin E tocotrienols (VE-200) or 200 ppm gamma-oryzanol + 200 ppm vitamin E tocotrienols (GE-400). Results showed that HCD-housed hens decreased ($P < 0.01$) egg performance and quality. Average egg weight (AEW), egg mass and FCR improved in all supplemented treatments. Results on the AEW, FCR, and eggshell qualities of birds kept at the HCD revealed the best responses in the GE-400 group ($P < 0.01$). Among hens kept on an HCD, there was a noticeable decrease in HMGCR mRNA abundance in the VE-200 group, while the highest IFN-γ mRNA abundance was found in hens fed the GO-200 diet ($P < 0.05$). Thus, this study suggested that dietary GO or VE at 200 ppm either individually or in combination could improve performance and egg quality as well as regulate the abundance of immune- and stress-related genes.

Keywords: gamma-oryzanol; vitamin E tocotrienols; rice bran; high cage density; laying hen

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1. Introduction

Eggs are a high-quality protein source for humans that contains several essential amino acids, consequently helping build and maintain the body's muscle mass. With the increase in the public demand for eggs, commercial layer producers tend to operate at an intensive scale to increase maximal hen-day egg production. However, it well known that animal-rearing conditions under intensive production systems create stressful conditions. Environmental stressors including temperature, humidity, and stocking density are the main factors affecting animal welfare, health, and productivity [1]. Geng et al. [2] reported that stocking density in rearing spaces has become one of the most important environmental and management factors for modern intensive animal husbandry. Nevertheless, in order to increase the total egg production per housing unit, many egg producers endeavor to decrease the payback period and increase their net income by increasing the number of hens per cage at maximum capacity [3]. With increasing cage density (342 to 690 cm^2/hen), egg performance and ME efficiency of egg production decreased significantly in hens kept in cages at a density of 342 cm^2 per hen [4]. A dense environment also has been associated with detrimental impacts including decreased egg production and egg mass [5], decreased laying rate, and increased levels of noxious gas emissions from the litter [6]. A study carried out by Wang et al. [7] revealed that laying hens reared in high stocking density had reduced laying rate and decreased eggshell indices such as shell color, strength, and thickness. Physiologically, birds kept at high stocking density may be sensitive to oxidative stress [8,9]. Incharoen et al. [10] noted that nutritional modification might be a key factor to ameliorate stress from high stocking density in laying hens. Thus, addition of specific antioxidants to the diet could be one efficient approach to alleviate the negative impact of stress [11].

Recently, gamma-oryzanol in the rice bran layer has been identified as a potent natural antioxidant due to its capacity to prevent lipid peroxidation and the resulting oxidative stress [12]. It contains a mixture of ferulic acid esters and phytosterols (sterols and triterpenic alcohols) [13, 14]. Antioxidant components of gamma-oryzanol such as 24-methylenecycloartanyl ferulate, cycloartenyl ferulate, campesteryl ferulate, and β-sitosteryl ferulate, are able to inhibit lipid peroxidation and free radical production and scavenge the free radicals from the body [15]. Ferulic acid in particular not only scavenges free radicals, but also increases the activity of enzymes that are responsible for scavenging free radicals and inhibits enzymes that catalyze the production of free radicals [16]. Furthermore, the structure of gamma-oryzanol components is similar to that of cholesterol and can reduce oxidative stress and maintain the functionality of cells [17].

As a lipid-soluble nutrient, vitamin E takes on a vital function as a peroxyl radical-scavenging antioxidant and inhibitor of lipid peroxidation by breaking chain propagation [18]. Natural vitamin E consists of 8 different analogues: α-, β-, γ- and δ-tocopherol; and α-, β-, γ- and δ-tocotrienol [19]. Among these, α-tocopherol has been mainly used as a supplement in livestock feed. Previous studies revealed that compared with a basal diet without vitamin E supplementation, dietary α-tocopherol acetate (200 to 500 mg/kg) enhanced the antioxidant capacity in hens [20, 21, 22]. Zhao et al. [23] reported that dietary natural tocopherol at a dosage of 100 mg/kg enhanced laying performance and tocopherol deposition as well as egulated serum cholesterol concentrations and improved antioxidant status. Although tocopherol is the most acceptable to use in domestic animal production, Serbinova et al. [24] reported higher antioxidant activity with tocotrienol than with α-tocopherol against lipid peroxidation in rat liver microsomes. Thus, it appears that vitamin E tocotrienol has greater potency. Furthermore, tocotrienols possess powerful antioxidant, neuroprotective, anti-cancer, and cholesterol regulatory activities that often differ from the properties of tocopherols [25].

We hypothesized that supplementing gamma-oryzanol and vitamin E tocotrienols or their combination would be an effective nutritional strategy to lessen the detrimental impact of high cage density in laying hens. Hence, the current research was conducted to evaluate the effect of diets supplemented with or without GO and VE on the productivity, egg quality, and immune- and health-related mRNA abundance in laying hens reared at different cage densities.

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2. Materials and Methods

2.1. Animal, diet and management

Hy-Line Brown layers purchased from a commercial farm in Phitsanulok province were used. The gamma-oryzanol (98.0% purity) and vitamin E tocotrienols (60.0 % total tocotrienols and 30.0 % total tocopherols) products were extracted from the rice bran of Oryza sativa Linne (Gramineae) and obtained from Oryza Oil & Fat Chemical Co., Ltd., Japan. All animals were reared in wire cages in tunnel-ventilated houses equipped with an evaporative cooling system to control the ambient temperature. Throughout the duration of the experiment, the average temperature remained consistent at 28±2 °C, accompanied by a relative humidity range of 60-65%. LED artificial lighting was at a consistent photoperiod (17L:7D). At 54-weeks of age, a total of 120 laying hens with identical body weight and egg uniformity were allocated into 8 groups with 5 replicates per group (3 layers/replicate). A completely randomized experiment designed with a 4×2 factorial arrangement of treatments was used. Dietary treatments were a basal diet without supplementation (CON) and three other diets with the same chemical composition as CON, but supplemented with 200 ppm gamma-oryzanol (GO-200), 200 ppm vitamin E tocotrienols (VE-200) or 200 ppm gamma-oryzanol + 200 ppm vitamin E tocotrienols (GE-400). The first 4 groups of hens were kept in wire cages with a low density of 840 cm^2/hen (LCD). Laying hens in the other 4 treatments were confined to a wire cage with a high density of 420 cm^2/hen (HCD). During the 54 to 62 weeks of age, all hens had free access to clean drinking water and feed. Diets were formulated in accordance with the nutrient requirement recommendation of NRC [26] (Table 1).

Table 1. Feed ingredients and nutrient composition of a basal diets s (%, as-is basis unless noted).

Item	Quantity
Feed ingredients (%)	
Corn	51.0
Cassava meal	6.3
Palm oil	2.6
Soybean meal (45% CP)	23.2
Fish meal (57% CP)	6.0
Calcium carbonate	8.8
Dicalcium phosphate	1.5
Vitamin-mineral premix[1]	0.3
DL-Methionine	0.2
Salt	0.1
Total	100.0
Nutrient composition[2]	
Metabolizable energy (kcal/kg)	2,800
Crude protein (%)	18.04
Ether extract (%)	5.36
Crude fiber (%)	2.97
Calcium (%)	4.20
Available phosphorus (%)	0.47

[1]Vitamin-mineral premix provided per kilogram of diet: vitamin A (trans-retinyl acetate), 12,000 IU; vitamin D3 (cholecalciferol), 3000 IU; vitamin E (allrac-tocopherol-acetate), 12 mg; vitamin K3 (bisulphate menadione complex), 3.6 mg; vitamin B1, 1.4 mg; vitamin B2, 5.4 mg; vitamin B6, 4.2 mg; vitamin B12 (cyanocobalamin), 0.02 mg; nicotinic acid, 9 mg; pantothenic acid, 9 mg; folic acid, 0.6 mg; biotin, 45 mg; choline chloride, 210 mg; selenium, 0.18 mg; cobalt, 0.3 mg; iodine, 1.08 mg; iron, 54 mg; zinc sulfate, 60 mg; manganese oxide, 96 mg;

copper sulfate, 12 mg. [2]The nutrient values were calculated based on the analyzed nutrient values according to NRC [26].

2.2. Laying performance and egg quality measurements

All eggs were carefully collected twice daily (at 6:00 A.M. and 6:00 P.M.) from each cage and counted per replication. We also recorded the weight of each collected egg on a daily basis, while monitoring the remaining feed on a weekly basis. Parameters analyzed included hen-day production (HDE), average egg weight (AEW), average daily feed intake (ADFI), egg mass, and feed conversion ratio (FCR). Additionally, we collected 10 eggs from each group on a weekly basis to assess eggshell breaking strength (ESBS), eggshell thickness (EST), eggshell ratio (ESR), yolk ratio (YR), albumen ratio (AR), albumen height (AH), yolk color (YC) and Haugh unit (HU). These parameters were evaluated using a TA-XT2 Plus Analyzer (Stable Microsystems, UK) following the methods described by Likittrakulwong et al. [27].

2.3. Sample Collection

At 62 weeks of age, blood samples were collected from five hens from each group. They were taken by venipuncture from the wing vein, and blood was saved into collection tubes using a sterile syringe, kept in blood collection tubes, and stored at 4 ºC in a refrigerator. Blood samples were mixed with an anticoagulant solution [ethylene diamine tetraacetic acid; (EDTA)] and then used for mRNA abundance analysis of hydroxyl-3-methyl-glutaryl coenzyme A reductase (HMGCR) and heat shock protein 70 (HSP-70) [10]. After blood collection, hens were sacrificed under mild anesthesia. Whole visceral organs were pulled out of the abdomen and placed on a clean aluminum tray. Using sterile equipment, the spleen was removed and cut into a small pieces of 4-5 mm thickness, rapidly frozen in liquid nitrogen, and kept at -80 ˚C until mRNA abundance analysis of interleukin-12 subunit beta (IL-12β) and interferon gamma (IFN- γ). Approximately 30 mg of spleen tissue from each treatment were homogenized with the TissueRuptor homogenizer (Qiagen GmbH, Hilden, Germany) in 350 µl of RLT buffer (RNeasy Mini RNA isolation kit, Qiagen GmbH, Hilden, Germany) and stored at -80˚C for RNA extraction.

2.4. mRNA abundance analysis

Total RNA was isolated using the RNeasy Mini RNA isolation kit (Qiagen GmbH, Hilden, Germany) and eluted in 50 µl RNase-free water. The concentration of total RNA was measured using a nanodrop Quawell UV–VIS Spectrophotometer Q5000 (Quawell Technology, Inc., San Jose, CA, USA). One µg of total RNA from each sample was used for first-strand cDNA synthesis, which was performed using the RevertAidTM first strand cDNA synthesis kit (Fermentas, Burlington, Canada), following the manufacturer's recommendations. One µl of first-strand cDNA from each sample was used as the template for semi-quantitative RT-PCR analysis. PCR amplification was performed using specific primers [28, 29, 30] (Table 2). Quantitative Real-time RT-PCR (qPCR) was performed as previously described by Incharoen et al. [10] to measure the levels of HMGCR, HSP70, IL-12β, IFN-γ and beta-actin (internal control) mRNA. The reactions were performed in triplicate in a MyGo Pro real-time PCR instrument (IT-IS Life Science Ltd., Mahon, Cork, Ireland). The relative mRNA abundance was analyzed using MyGoPro qPCR software (IT-IS Life Science Ltd., Mahon, Cork, Ireland). Results of real-time PCR were analyzed by the 2-ΔΔCt method [31]. The mRNA abundance of these genes was normalized to beta-actin.

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Table 2. Specific primers used in the current trial.

Gene[1]	Sequence (5′-3′)	Annealing Temperature (°C)	Product Size (bp)	References
HMGCR	F:ATGCATGGCCTTTTTGTGGCCTCTCATCCA R:CTTGAGAAGATTGTGAGGAGACCAGCAATA	55 °C	242 bp	Beloor et al. [28]
HPS70	F:AATCTATCATCATGTCTGGCAAAGGGCCGG R:GCGGCCGATGAGACGCTTGGCATCAAAGAT	58 °C	220 bp	Beloor et al. [28]
IL-12β	F:TGTCTCACCTGCTATTTGCCTTAC R:CATACACATTCTCTCTAAGTTTCCACTGT	60 °C	82 bp	Brisbin et al. [29]
IFN-γ	F:ACACTGACAAGTCAAAGCCGC R:AGTCGTTCATCGGGAGCTTG	60 °C	129 bp	Brisbin et al. [29]
β-actin	F:CCACCGCAAATGCTTCTA R:GCCAATCTCGTCTTGTTTTATG	60 °C	96 bp	Sohn et al. [30]

[1]HMGCR: hydroxyl-3-methyl-glutaryl coenzyme A reductase; HSP70: heat shock protein70; IL-12β: interleukin-12 subunit beta; IFN-γ: interferon gamma.

2.5. Statistical Analysis

A two-way analysis of variance of the data on egg performance and quality using the general linear model procedure of SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) was performed according to a 4×2 factorial arrangement of treatments, including dietary supplementation and cage density as the main effects and the respective interactions. One-way ANOVA was used to determine differences in mRNA abundance. Statistically significant means were compared using Duncan's Multiple Range Test and a probability level of $P < 0.05$ was considered significant.

3. Results

3.1. Egg production response to dietary supplementation and cage density

Compared with the CON group, average egg weight (AEW) tended to be greater in the GO-200 and VE-200 groups, and was greater ($P < 0.01$) in the GE-400 group (Table 3). Egg mass was greater ($P < 0.01$) in all supplemented treatments compared with the CON group. In addition, FCR improved in the GO-200 and VE-200 groups, with the best responses ($P < 0.01$) detected in the GE-400 group. Data on average daily feed intake (ADFI) and hen-day egg production (HDE) did not differ among dietary treatments. Compared with the LCD condition, in HCD the hens experienced a decrease ($P < 0.01$) in ADFI, HDE, and egg mass, while there were no changes observed in AEW and FCR.

The ADFI, AEW, HDE, FCR, and egg mass of laying hens was influenced by the interaction between dietary supplementation and cage density. Except for the GE-400-fed birds, results pertaining to ADFI in birds raised under HCD conditions did not demonstrate a significant variance across the different dietary treatments. Birds in GE-400 exhibited the lowest ($P < 0.01$) ADFI compared with other groups. CON-fed hens maintained in HCD tended to have lower HDE and egg mass compared with other groups, and had the lowest values ($P < 0.01$) compared with the GO-200 group. Results of AEW in birds maintained under HCD revealed higher values in all groups that received dietary antioxidants, with the greatest response observed in the GE-400 group ($P < 0.01$). Additionally, all antioxidant-fed hens reared at HCD had better ($P < 0.01$) FCR and the GO-200 and GE-400 groups had the lowest FCR values.

3.2. Dietary supplementation and cage density influenced egg quality

Compared with the CON group, all of the supplemented groups had better responses in various egg quality parameters (except for the AR) including ESBS, EST, ESR, YR, AH, YC, and HU (Table 4). Furthermore, the highest response ($P < 0.01$) in these collected parameters was detected in the VE-200

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group. Compared with the LCD environment, the HCD-housed hens exhibited a significant reduction ($P < 0.01$) in ESBS, ESR, YR, AR, and AH. There was no effect on EST, YC, and HU. In addition, there was an interaction between dietary supplementation, specifically of gamma-oryzanol and vitamin E tocotrienols with cage density on ESBS, ESR, YR, AH, YC, and HU. Furthermore, compared with the CON group, HCD-housed hens fed GO-200 and GE-400 had a notable increase in ESR. Compared with the CON birds raised in the HCD environment, the GO-200 group exhibited the highest AH and the lowest YC. However, there were no significant differences observed among dietary treatments regarding EST, AR, and HU of laying hens housed in the HCD situation.

Table 3. Productivity of laying hens fed a basal diet supplemented with gamma-oryzanol and vitamin E tocotrienols as antioxidants during 54-62 week of ages.

Item	Productivity parameter[1]				
	ADFI (g/b)	AEW (g)	HDE (%)	FCR	Egg mass (g/b/d)
Diet[2]					
CON	92.22	56.04^{b}	76.13	2.16^{c}	42.60^{b}
GO-200	86.67	57.45^{b}	78.15	1.93^{b}	44.88^{a}
VE-200	89.96	58.21^{ab}	77.80	1.99^{b}	45.27^{a}
GE-400	85.06	59.22^{a}	75.51	1.90^{a}	44.69^{a}
SEM[3]	1.59	0.28	1.03	0.02	0.55
Cage density[4]					
LCD	91.82^{a}	56.76	80.05^{a}	2.02	45.43^{a}
HCD	85.14^{b}	58.70	73.74^{b}	1.97	43.28^{b}
SEM[3]	1.42	0.74	0.69	0.01	0.67
Diet × Cage density					
CON × Low	96.81^{a}	54.58^{c}	80.31^{a}	2.21^{c}	43.83^{bc}
CON × High	87.62^{b}	57.50^{b}	71.94^{c}	2.12^{bc}	41.37^{c}
GO-200 × Low	89.81^{ab}	56.27^{bc}	78.92^{b}	2.02^{b}	44.41^{b}
GO-200 × High	83.53^{bc}	58.62^{ab}	77.37^{b}	1.84^{a}	45.35^{b}
VE-200 × Low	90.47^{ab}	57.88^{b}	83.11^{a}	1.88^{a}	48.10^{a}
VE-200 × High	89.45^{ab}	58.53^{ab}	72.49^{c}	2.11^{bc}	42.43^{bc}
GE-400 × Low	90.17^{ab}	58.30^{ab}	77.86^{b}	1.99^{b}	45.39^{b}
GE-400 × High	79.95^{c}	60.13^{a}	73.16^{bc}	1.82^{a}	43.99^{bc}
SEM[3]	1.16	0.33	0.72	0.02	0.42
P-value					
Diet	NS	<0.01	NS	<0.01	<0.01
Cage density	<0.01	NS	<0.01	NS	<0.01
Diet × Cage density	<0.01	<0.01	<0.01	<0.01	<0.01

[1]Each parameter of productivity was collected: ADFI = average daily feed intake; AEW = average egg weight; HDE = hen-day egg production; FCR = feed conversion ratio. [2]Diets were divided into 4 groups: CON = a basal diet without supplementation; GO-200 = a basal diet supplemented with 200 ppm gamma-oryzanol; VE-200 = a basal diet supplemented with 200 ppm vitamin E tocotrienols; GE-400 = a basal diet supplemented with 200 ppm gamma-oryzanol + 200 ppm vitamin E tocotrienols. [3]SEM: Standard error of means. [4]Animal were reared in different cage densities: LCD = low cage density (840 cm^2/bird); HCD = high cage density (420 cm^2/bird). [a-c]Means with different superscripts within each column are significantly different ($P < 0.01$).

3.3. Stress-, lipid metabolism-and immune-related genes

In the LCD conditions, the hens had minimal differences in mRNA abundance of HMGCR, HSP70, IL-12 β, and IFN- γ genes across all dietary treatments (Figures 1 and 2). However, HCD-housed hens had a decrease ($P < 0.05$) in HMGCR abundance within the VE-200 group compared with the CON group. Among hens kept in HCD-conditions, those fed GO-200 exhibited the highest level

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of IFN-γ mRNA abundance relative to other diets. However, there was no difference in abundance of HSP70 and IL-12 β in HCD-reared hens regardless of diet.

Table 4. Egg quality of laying hens fed a basal diet supplemented with gamma-oryzanol and vitamin E tocotrienols as antioxidants during 54-62 week of ages.

Item	Egg quality[1]							
	ESBS (N)	EST (mm)	ESR (%)	YR (%)	AR (%)	AH (mm)	YC	HU
Diet[2]								
CON	37.51^{c}	0.33^{c}	10.55^{d}	25.30^{d}	64.15	5.30^{d}	7.29^{c}	68.65^{d}
GO-200	43.74^{b}	0.35^{b}	11.39^{b}	26.80^{b}	61.81	6.16^{b}	7.62^{b}	80.04^{b}
VE-200	48.93^{a}	0.37^{a}	11.70^{a}	27.70^{a}	60.60	6.53^{a}	7.97^{a}	83.14^{a}
GE-400	42.41^{b}	0.35^{b}	11.21^{c}	25.92^{c}	62.87	5.82^{c}	7.50^{b}	76.72^{c}
SEM[3]								
Cage density[4]								
LCD	44.23^{a}	0.36	11.30^{a}	26.61^{a}	62.09^{a}	6.05^{a}	7.59	76.83
HCD	39.57^{b}	0.35	11.13^{b}	26.25^{b}	62.62^{b}	5.85^{b}	7.60	77.44
SEM[3]								
Diet × Cage density								
CON × Low	42.46^{c}	0.31	10.13^{d}	26.76^{b}	63.11	4.96^{d}	6.62^{d}	62.44^{c}
CON × High	32.55^{e}	0.34	10.97^{c}	23.84^{d}	65.19	5.65^{c}	7.80^{b}	74.86^{bc}
GO-200 × Low	45.21^{a}	0.36	11.60^{ab}	26.80^{b}	61.60	6.31^{b}	7.89^{b}	81.27^{a}
GO-200 × High	42.26^{c}	0.34	11.18^{b}	26.81^{b}	62.01	6.00^{b}	7.35^{c}	78.80^{b}
VE-200 × Low	44.81^{a}	0.39	12.43^{a}	26.57^{bc}	61.00	7.19^{a}	8.48^{a}	87.64^{a}
VE-200 × High	43.05^{b}	0.35	10.99^{c}	28.83^{a}	60.18	5.88^{bc}	7.46^{bc}	78.65^{b}
GE-400 × Low	40.40^{d}	0.34	11.06^{bc}	26.32^{bc}	62.62	5.75^{c}	7.38^{c}	75.99^{bc}
GE-400 × High	44.42^{b}	0.36	11.37^{b}	25.52^{c}	63.11	5.88^{bc}	7.63^{b}	77.46^{b}
SEM[3]	0.03	0.37	0.04	0.07	0.25	0.05	0.08	0.04
P-value								
Diet	<0.01	<0.01	<0.01	<0.01	NS	<0.01	<0.01	<0.01
Cage density	<0.01	NS	<0.01	<0.01	<0.01	<0.01	NS	NS
Diet × Cage density	<0.01	NS	<0.01	<0.01	NS	<0.01	<0.01	<0.01

[1]Each parameter of egg quality was determined: ESBS = egg shell breaking strength; EST = egg shell thickness; ESR = egg shell ratio; YR = yolk ratio; AR = albumen ratio; AH = albumen height; YC = yolk color; HU = Haugh unit. [2]Diets were divided into 4 groups: CON = a basal diet without supplementation; GO-200 = a basal diet supplemented with 200 ppm gamma-oryzanol; VE-200 = a basal diet supplemented with 200 ppm vitamin E tocotrienols; GE-400 = a basal diet supplemented with 200 ppm gamma-oryzanol + 200 ppm vitamin E tocotrienols. [3]SEM: Standard error of means. [4]Animal were reared in different cage densities: LCD = low cage density (840 cm^{2}/bird); HCD = high cage density (420 cm^{2}/bird). [a-e]Means with different superscripts within each column are significantly different ($P < 0.01$).

4. Discussion

The reduction in ADFI, HDE, egg mass, ESBS, ESR, YR, and AH observed in laying hens raised under the HCD condition agreed with the recent study from Incharoen et al. [10] in which hens raised on HCD under heat stress displayed decreased egg performance and eggshell-breaking strength. Similarly, a lower HDE and daily egg mass [5], a reduction of ME efficiency in egg production [4], and a detrimental impact on laying rate and litter quality resulting in high levels of noxious gas emission were reported previously [6]. In broilers, there is evidence confirming that increasing stocking density decreased feed intake, body weight, weight gain, and FCR [32, 33]. Goo et al. [34] also reported that broilers reared under heat stress and high stocking density decreased performance with a negative impact on breast meat quality. During the starter period, studies have observed that high stocking density is associated with a reduction in feed intake and weight gain in White Pekin ducks [35] and

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geese [36]. Furthermore, research studies have provided scientific evidence that housing birds in a densely stocked environment can lead to elevated ambient temperatures surrounding the birds and lowering body heat dissipation resulting in heat stress conditions [33, 37]. Thus, this overwhelming evidence underscores that monitoring environmental factors and adjusting nutritional management practices accordingly is crucial to minimize harmful outcomes on health and welfare.

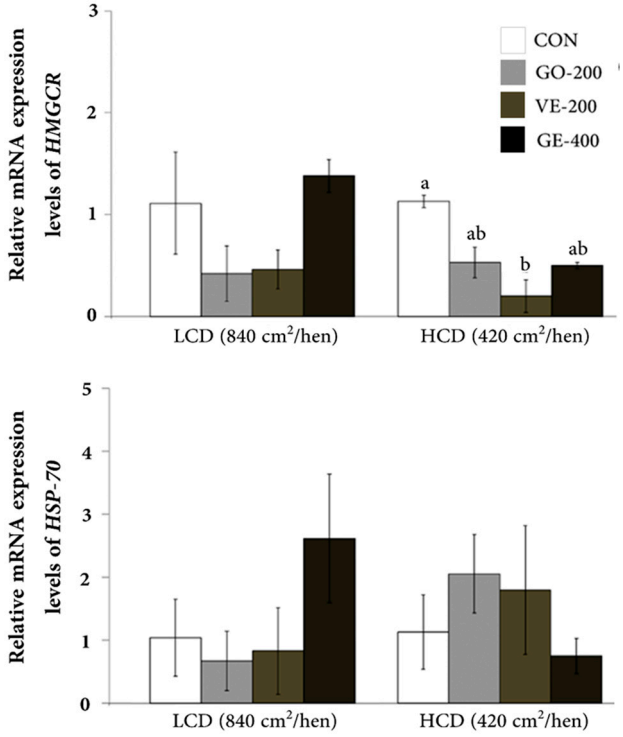

Figure 1. Relative quantification of HMGCR and HSP-70 in the blood of laying hens fed a basal diet supplemented with gamma-oryzanol and vitamin E tocotrienols as antioxidants during 54-62 weeks of age. Diets were divided into 4 groups: CON = a basal diet without supplementation; GO-200 = a basal diet supplemented with 200 ppm gamma-oryzanol; VE-200 = a basal diet supplemented with 200 ppm vitamin E tocotrienols; GE-400 = a basal diet supplemented with 200 ppm gamma-oryzanol + 200 ppm vitamin E tocotrienols. [a,b]Mean values with different small letters denote significant differences among experimental groups ($P < 0.05$). Animals were reared in different cage densities: LCD = low cage density (840 cm²/bird); HCD = high cage density (420 cm²/bird).

To our knowledge, there is no published research on the potential benefits of incorporating gamma-oryzanol and vitamin E tocotrienols, either individually or in combination, into poultry diets with regards to reducing the detrimental effects of oxidative stress caused by HCD conditions. However, Minatel et al. [12] demonstrated that gamma-oryzanol in the rice bran layer is a potent natural antioxidant due to its capacity to prevent lipid peroxidation and the resulting oxidative stress. In addition, López-Revuelta et al. [17] noted that the structure of gamma-oryzanol components is analogous to that of cholesterol, meaning that it can help reduce oxidative stress and support the normal functioning of cells. Previous studies have also reported that gamma-oryzanol has the potential to positively affect the immune system, lipid levels in blood, antioxidant capabilities, and better efficiency for the animal to avoid heat stress [38, 39, 40]. Functionally, due to its effectiveness as an antioxidant and inhibitor of lipid peroxidation by breaking chain propagation, tocopherol is the generic form of vitamin E used in feed [18]. Previous research provided evidence that hens fed a diet containing α-tocopherol acetate displayed stronger antioxidant capacity than control-fed birds [20, 21, 22]. Recently, Zhao et al. [23] reported that dietary tocopherol content (100 mg/kg diet) increased egg-laying performance and tocopherol deposition as well as regulated serum cholesterol concentration

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and improved antioxidant status. Despite these data, the study conducted in rat liver microsomes by Serbinova et al. [24], demonstrated that compared with α-tocopherol, tocotrienol had greater antioxidant properties in regards to lipid peroxidation. Thus, there is a clear benefit in generating more data on the relevance of tocotrienol in poultry diets.

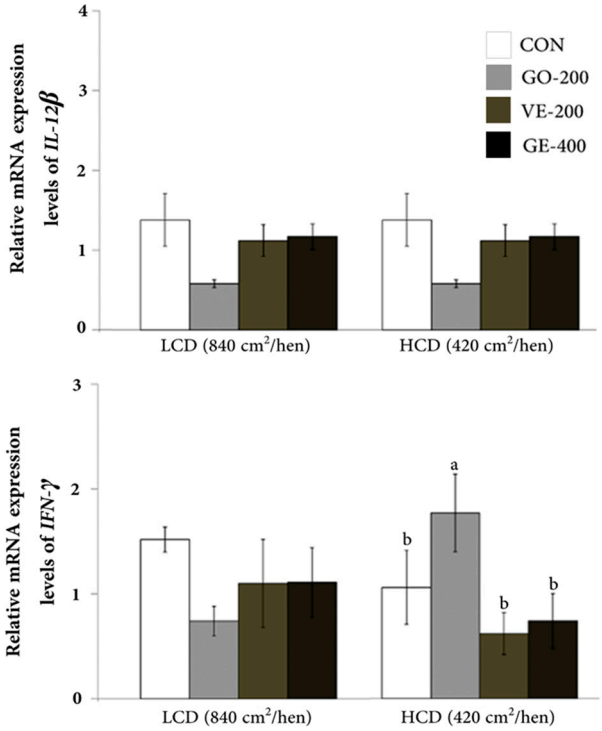

Figure 2. Relative quantification of IL-12β and IFN-γ in the spleen of laying hens fed a basal diet supplemented with gamma-oryzanol and vitamin E tocotrienols as antioxidants during 54-62 weeks of age. Diets were divided into 4 groups: CON = a basal diet without supplementation; GO-200 = a basal diet supplemented with 200 ppm gamma-oryzanol; VE-200 = a basal diet supplemented with 200 ppm vitamin E tocotrienols; GE-400 = a basal diet supplemented with 200 ppm gamma-oryzanol + 200 ppm vitamin E tocotrienols. [a,b]Mean values with different small letters denote significant differences among experimental groups ($P < 0.05$). Animals were reared in different cage densities: LCD = low cage density (840 cm²/bird); HCD = high cage density (420 cm²/bird).

Decreased productivity and poor egg quality were observed in hens raised under HCD conditions, possibly due to reduced digestibility caused by heat stress [41]. However, there is some evidence suggesting that antioxidants delivered through supplementation in the diet can minimize oxidative stress [42, 43] leading to enhanced growth and feed efficiency, and optimizing nutrient utilization. Other reported that high dietary concentrations of gamma-oryzanols, tocotrienols, and other bioactive components in rice bran oil improved growth performance of broiler chickens [44, 45]. The present data demonstrating a significant impact on various aspects of egg production and quality, and AEW and FCR under HCD conditions in response to feeding gamma-oryzanol and vitamin E tocotrienols support and add to those findings. Hence, dietary supplementation with oryzanols and vitamin E tocotrienol could have a synergistic positive effect on hen's performance specifically by minimizing oxidative stress. As such, these compounds can improve nutrient digestibility and consequently enhance productivity during stressful periods. It is important to conduct further research in order to confirm and better understand the specific mechanisms whereby these nutrients have a positive impact on the animal.

The levels of mRNA transcription were confirmed using quantitative real-times RT-PCR. In our results, a significant effect of LCD situation on the expression levels of HMGCR, HSP70,

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IL-12β, and IFN-γ were not observed among 4 dietary groups. However, the expression levels of HMGCR and IFN - γ in the blood were significantly impacted by the HCD condition, whereas there was no significant difference for HSP70 and IL-12β.

The study of Sohn et al. [30] reported greater abundance of HMGCR, but not HSP70, in blood of chickens exposed to stress. A similar finding was reported by Incharoen et al. [10] where HMGCR abundance was lower in HCD-reared laying hens fed with dietary germinated paddy rice containing several bioactive compounds (vitamins, gamma-oryzanol, and γ-amino butyric acid) [46]. The lower HMGCR abundance in the HCD-housed hens that received gamma-oryzanol alone or combined with vitamin E tocotrienols (VE-200) could be taken as indication of a reduction in stressful conditions as reported by Sohn et al. [30]. This idea is further supported by data from Zavoshy et al. [47] where feeding vitamin E isomers (tocopherol and tocotrienols) and oryzanol (also contained in rice bran oil) led to lower total cholesterol and LDL-C levels by inhibiting HMG-CoA reductase, i.e., the rate-limiting enzyme in de novo cholesterol synthesis. Thus, based on our findings, providing dietary gamma-oryzanol and vitamin E tocotrienols may have a mitigating effect on the stress status caused by HCD conditions.

Interferon γ is a vital cytokine synthesized primarily by type 1 T helper cells and plays a crucial role in the activation of macrophages [48, 49]. In avian species, IFN-γ represents a natural component of the immune system [50] and its abundance has been detected in laying hen [10], duck [51] and goose [52]. Thus, the lower abundance of IFN-γ [53] in broiler chickens exposed to heat stress or in birds raised in environmental conditions with higher endotoxin levels underscore the usefulnes of this cytokine as a marker of stressful conditions in avian species [54]. Despite the lack of differences in the abundance of IFN-γ due to diet under LCD conditions, the fact that dietary GO-200 in hens raised in an HCD environment led to greater IFN-γ mRNA abundance suggests that nutrition may play a role in the function of this cytokine. Lee et al. [55] noted that high levels of IFN-γ have been associated with protective immune responses to parasitic infections. In fact, Gao et al. [51] suggested that IFN-γ has the potential to inhibit viral activity in ducks. Thus, the greater mRNA abundance of IFN-γ in birds fed gamma-oryzanol suggests that this compound might aid in mitigating the detrimental impacts of the HCD environment by enhancing the immune response.

5. Conclusions

Hens housed in 420 cm^2/hen HCD conditions produced lower productivity, egg quality, and eggshell hardness than hens kept in LCD conditions (840 cm^2/hen). However, dietary GO or VE at a level 200 ppm, either individually or in combination can improve performance and egg and shell quality as well as regulate mRNA abundance of immune- and stress-related genes. Thus, we conclude that dietary antioxidants should be part of a nutritional strategy to mitigate the negative impacts on laying hens reared under HCD conditions.

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Institutional Review Board Statement: Animal handling techniques and procedures were approved by the Naresuan University Agricultural Animal Care and Use Committee (approval number: 60 01 015), based on the Ethics of Animal Experimentation of the National Research Council of Thailand.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data presented in this research are available on request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

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