Dietary supplementation with natural astaxanthin from *Haematococcus pluvialis* improves antioxidant enzyme activity, free radical scavenging ability, and gene expression of antioxidant enzymes in laying hens

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ABSTRACT The objective of this study was to evaluate the effects of natural astaxanthin (**ASTA**) from *Haematococcus pluvialis* on production performance, egg quality, antioxidant enzyme activity, free radical scavenging ability, and gene expression of antioxidant enzymes in laying hens. Nongda No. 3 laying hens (n = 450) were randomly allocated to 1 of 5 dietary treatments. Each treatment had 6 replicates of 15 hens each. All birds were assigned to a corn-soybean meal-based diet containing 0, 20, 40, 80, or 160 mg/kg ASTA for 4 wk. With increasing dietary ASTA, no significant effects were observed on egg weight, feed consumption, feed efficiency, laying rate, Haugh unit, or eggshell strength. Yolk color darkened linearly with increasing dose of ASTA ($P < 0.05$). Glutathione peroxidase activity was improved in the kidney with dietary ASTA at levels of 40 mg/kg. Total superoxide dismutase (**SOD**) was significantly increased in the liver, kidney, and plasma with dietary ASTA supplementation at 40 mg/kg. With increasing dietary ASTA, the scavenging abilities of hydroxyl radicals and superoxide anions were linearly increased ($P < 0.05$), and the malondialdehyde content decreased linearly ($P < 0.05$). Compared with the control group, mRNA expression of Cu-Zn SOD (*SOD1*), Mn SOD (*SOD2*), and nuclear factor E2-related factor 2 (***NRF2***) in the liver and kidney was significantly increased in the 40 mg/kg ASTA group ($P < 0.05$). The level of *GPX4* mRNA in the liver and kidney was significantly increased with ASTA supplementation at 40 and 80 mg/kg ($P < 0.05$). The results demonstrate that dietary ASTA improves free radical scavenging ability and antioxidant enzyme activity, which may be related in part to the upregulated mRNA expression of genes encoding antioxidant enzymes and *NRF2*.

Key words: antioxidant capacity, egg quality, laying hen, natural astaxanthin, *NRF2*

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INTRODUCTION

Natural astaxanthin (**ASTA**), an oxygenated derivative of carotenoid, has received much attention recently for its various beneficial characteristics: it inhibits lipid peroxidation (Naguib, 2000), it is antiatherogenic (Tanaka et al., 1995) and antiaging (Nootem et al., 2018), and it has antioxidant capacity (Zhao et al., 2019). The antioxidant ability of ASTA is 10 times higher than that of β-carotene and 300 times higher than that of α-tocopherol (Higuera-Ciapara et al., 2006; Rao et al., 2009). Dietary levels of ASTA from algae have been shown to darken egg yolk in a dose-dependent manner (Walker et al., 2012), but there has not been reported in the literature concerning the dose–response relationship of antioxidant capacity in laying hens, especially for ASTA from *Haematococcus pluvialis*.

Astaxanthin is found in marine life, such as *H. pluvialis*, phytoplankton, shellfish, shrimp, and crabs (Thana et al., 2008; Gassel, 2011; Li et al., 2020). Of these, *H. pluvialis* contains the most natural ASTA and the content of ASTA is reported to reach 500 mg/g of stem cell weight (Kobayashi, 2003). Previously, ASTA was utilized successfully to increase pigmentation of poultry production (Takahashi et al., 2004). Dietary supplementation with ASTA also improves broiler chicken

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meat quality (Perenlei et al., 2014). The addition of ASTA and palm tocos into hens' diets did not affect performance or egg quality except for egg yolk color (Walker et al., 2012). Whether dietary ASTA affects the antioxidant capacity of laying hens remains unknown.

In a previous study, supplementation of ASTA prevented lipid peroxidation at the end of a 4-week feeding trial that supplemented ASTA-rich *H. pluvialis* extract at 0.03% (Yang et al., 2011). Dietary supplementation with ASTA at 400 mg/kg improved pigmentation and increased total antioxidant capacity (Li et al., 2018). However, whether the effect of dietary ASTA from *H. pluvialis* on the antioxidant capacity of laying hens is dose dependent remains unclear. Because ASTA is beneficial for health and appears to be well absorbed from the diet by laying hens, we proposed to improve the antioxidant capacity in laying hens by feeding ASTA.

The objective of this study was to investigate the effects of dietary ASTA from *H. pluvialis* on production performance, egg quality, free radical scavenging ability, antioxidant enzyme activities, and gene expression involved in antioxidant enzymes in laying hens.

MATERIALS AND METHODS

Animal Care and Use

All experimental protocols were approved by the Animal Care and Use Committee of Beijing University of Agriculture.

Experimental Materials

H. pluvialis was purchased from Jingzhou Natural Astaxanthin Ltd. (Hubei, China), and its ASTA content was 1.5%.

Birds and Diets

Four hundred and fifty Nongda No. 3 laying hens were randomly allocated to 1 of 5 dietary treatments. There were 5 cages of 3 birds per replicate. All birds were fed a basal diet for 1 wk and then assigned to a corn-soybean meal-based diet containing 0, 20, 40, 80, or 160 mg/kg ASTA for 4 wk. The composition and nutritional level of the basal diet are shown in Table 1.

Sample Collection

At the end of the 4-week feeding trial, 3 eggs per replicate were collected for assessment of egg quality. The eggs had no defects (cracks or breaks) and egg weights were close to the average egg weight for each replicate. One bird from each replicate was chosen randomly and killed by cervical dislocation. Immediately thereafter, blood samples were collected in the anticoagulants tube by bleeding the left jugular vein and centrifuged at 4°C at 3,000 × *g* for 10 min. Liver and kidney samples were collected via dissection and frozen at −80°C until analysis.

Performance and Egg Quality Measurement

Egg production rate was calculated as follows: egg production rate = number of eggs/(total birds × days). Feed efficiency was calculated weekly. An egg analyzer (Orka Food Technology Ltd., Ramat Hasharon, Israel) was used to measure Haugh units, albumen height, and yolk color. Yolk color was defined according to the Roche yolk color fan, where 1 represents bright yellow and 15 represents dark yellow. An egg force reader (Orka Food Technology Ltd.) was used to measure eggshell strength. The weights of egg white and egg yolk were measured after the egg white and yolk were separated completely by using an egg yolk separator.

Measurement of Antioxidant Enzyme Activity

The activities of total superoxide dismutase (**T-SOD**) and glutathione peroxidase (**GSH-Px**), the ability to scavenge superoxide anion radicals and hydroxyl radicals, and concentration of malondialdehyde (**MDA**) were measured using the protocol in the test kit purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). The activity of T-SOD was measured using the xanthine/xanthine oxidase method based on inhibition of nitroblue tetrazolium formazan. The activity of GSH-Px was measured using H_2O_2 as a substrate in the presence of reduced glutathione. GSH-Px activity was expressed as micromoles of oxidized NADPH per minute. Malondialdehyde concentration was assayed by using the absorbance of thiobarbituric acid at 532 nm. The values of scavenging ability used the absorbance of the chromogenic substrate at 550 nm.

Quantitative PCR Analysis

Comparative mRNA levels of *SOD1*, *SOD2*, *GPX4*, and *NRF2* were measured in the liver and kidney of laying hens. The TRIzol Reagent Kit (#15596026, Thermo Fisher Scientific, Shanghai, China) was used to extract total RNA from the liver and kidney according to the manufacturer's protocol. The concentration of RNA was measured using a spectrophotometer (Genova Nano, Jenway, Stone, United Kingdom) by defining the absorbance at 260 and 280 nm (A260/A280). Reverse transcription was performed according to the protocol in the test kit purchased from Thermo Fisher Scientific (#K1621). The StepOnePlus Real-Time PCR system (Applied Biosystems, Shanghai, China) was used to analysis quantitative PCR. The primers of the selected genes are shown in Table 2. Samples were run in triplicate. The relative gene expression levels were calculated

Table 1. The composition and nutritional level of the basic diet (air-dried basis).

Ingredients	Content (%)	Nutrient level	Content (%)
Corn	63.30	Metabolizable energy (MJ/kg)	10.96
Soybean meal	23.75	Crude protein (%)	16.10
Cottonseed meal	1.00	DL-Methionine (%)	0.368
DL-Methionine	0.10	L-Lysine (%)	0.750
Limestone	8.70	Total calcium (%)	3.51
$CaHPO_4$	1.80	Total phosphorus (%)	0.62
NaCl	0.35	Available phosphorus (%)	0.44
Premix1[1]	1.00		
Total	100.00		

[1]Premix provided per kg of diet: vitamin A 13,000 IU; vitamin D_3 6,000 IU; vitamin E 20 IU; vitamin K 2 mg; vitamin B_1 1 mg; vitamin B_2 9 mg; vitamin B_6 6 mg; vitamin B_{12} 0.006 mg; folic acid 0.3 mg; calcium pantothenate 6 mg; niacin 20 mg; biotin 0.2 mg; Cu 10.04 mg; Fe 60 mg; Mn 95.4 mg; Zn 103.5 mg; I 0.4 mg; Se 0.3 mg.

by using the $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen, 2001), where Ct is the cycle threshold, with *ACTB* (β-actin) as the reference gene.

Statistical Analysis

All data were analyzed using SPSS 22.0 (IBM Corp., Armonk, NY). One-way ANOVA with orthogonal linear and quadratic contrasts followed by Tukey's multiple comparison test was used to examine statistical differences among treatments. Statistical significance was defined at $P < 0.05$.

RESULTS

Performance and Egg Quality

The effects of dietary natural ASTA addition on performance and egg quality are shown in Table 3. We found no significant effects ($P > 0.05$) of increasing ASTA supplementation on egg weight, feed consumption, feed efficiency, laying rate, Haugh unit, or eggshell strength; yolk color was darkened linearly ($P < 0.05$) by ASTA supplementation.

Antioxidant Enzyme Activity and Free Radical Scavenging Ability

The effects of dietary ASTA addition on antioxidant enzyme activity and free radical scavenging ability in the liver are shown in Table 4. The activity of T-SOD in the liver increased quadratically with increasing dietary ASTA addition ($P < 0.05$). In the 40 mg/kg ASTA group, compared with the control group, T-SOD activity increased significantly in the liver ($P < 0.05$). With increasing dietary levels of ASTA, the ability to scavenge hydroxyl radical and superoxide anion increased linearly ($P < 0.05$), and MDA content decreased linearly ($P < 0.05$).

The effects of dietary ASTA addition on antioxidant enzyme activity and free radical scavenging ability in the kidney are shown in Table 5. With increasing levels of dietary ASTA, the activity of T-SOD in the kidney

Table 2. Primer sequences used for quantitative real-time PCR.

Gene	Primer sequence	Fragment size (bp)	Accession number
SOD1[1]	Forward: CACGGTGGACCAAAAGATGC Reverse: GATGCAGTGTGGTCCGGTAA	123	NM_205064.1
SOD2[2]	Forward: ACTGTTGTGCGACAAAGGGA Reverse: CACAAAGTGTGCGTTTCCACT	143	NM_204211.1
GPX4[3]	Forward: AGAATGGCGGACGAGTGG Reverse: ACCGCGGTCTTTCCTCATTT	146	NM_001346448.1
NFE2L2 (*NRF2*)[4]	Forward: ACGCTTTCTTCAGGGGTAGC Reverse: GTTCGGTGCAGAAGAGGTGA	170	NM_205117.1
β-Actin	Forward: ATCCGGACCCTCCATTGTC Reverse: AGCCATGCCAATCTCGTCTT	120	NM_205518.1

[1]*SOD1* means Cu-Zn superoxide dismutase.
[2]*SOD2* means Mn superoxide dismutase.
[3]*GPX4* means glutathione peroxidase 4.
[4]NFE2L2 (*NRF2*) means nuclear factor E2-related factor 2.

Table 3. Effect of dietary astaxanthin supplementation on performance and egg quality.

Item	Time (week)	Natural astaxanthin (mg/kg)					SEM	*P*-value		
		0	20	40	80	160		ANOVA	Linear	Quadratic
Egg weight (g/egg)	1–2	60.26	60.43	60.76	60.27	60.86	0.13	0.436	0.260	0.994
	3–4	61.52	61.97	62.06	61.59	61.95	0.11	0.399	0.534	0.403
	1–4	60.89	61.21	61.41	60.93	61.40	0.10	0.280	0.285	0.628
Feed consumption (g/d)	1–2	138.86	139.06	138.00	140.51	140.60	0.61	0.643	0.278	0.532
	3–4	133.75	136.49	135.00	135.02	134.21	0.60	0.688	0.904	0.291
	1–4	136.30	137.77	136.50	137.77	137.40	0.51	0.843	0.564	0.803
Feed efficiency (kg/kg)	1–2	2.52	2.50	2.50	2.57	2.52	0.02	0.702	0.536	0.957
	3–4	2.34	2.36	2.32	2.30	2.28	0.01	0.361	0.066	0.486
	1–4	2.43	2.43	2.41	2.43	2.40	0.01	0.893	0.590	0.741
Laying rate (%)	1–2	91.99	92.38	91.91	91.83	92.86	0.50	0.971	0.757	0.714
	3–4	93.57	94.60	93.17	94.52	94.08	0.39	0.700	0.523	0.832
	1–4	92.78	93.49	92.54	93.17	93.69	0.39	0.889	0.603	0.729
Haugh unit		81.21	82.42	83.04	82.50	83.35	0.43	0.605	0.174	0.614
Yolk color[1]		10.89^{d}	11.83^{c}	12.84^{b}	14.50^{a}	14.89^{a}	0.29	<0.001	<0.001	0.489
Eggshell strength (N/cm^2)		42.03	42.09	42.67	43.02	42.52	0.44	0.960	0.575	0.734

[a–d]Means within a row with no common superscripts differ significantly ($P < 0.05$).
[1]The yolk color is defined according to Roche yolk color fan; colors range from 1 to 15, where 1 represents bright yellow and 15 represents dark yellow.

increased quadratically ($P < 0.05$). Compared with the 40 mg/kg ASTA group, the activity of GSH-Px in the kidney was significantly decreased in the 160 mg/kg ASTA group ($P < 0.05$). The ability to scavenge hydroxyl radical and superoxide anion increased linearly ($P < 0.05$), and the level of MDA decreased linearly ($P < 0.05$), with increasing dietary supplementation of ASTA.

The effects of dietary ASTA addition on antioxidant enzyme activity and free radical scavenging ability in the plasma are shown in Table 6. The activity of T-SOD increased linearly with increasing dietary ASTA ($P < 0.05$). No significant effect on T-SOD was observed in the 160 mg/kg treatment compared to the control ($P > 0.05$). With increasing dietary supplementation of ASTA, the MDA content decreased linearly ($P < 0.05$), and the ability to scavenge hydroxyl radical and superoxide anion increased linearly ($P < 0.05$).

Antioxidant and NRF2 *mRNA Expression*

The effects of dietary ASTA supplementation on the mRNA expression of *SOD1*, *SOD2*, *GPX4*, and *NRF2* in the liver are shown in Table 7 and Figure 1. Compared with the control, the mRNA expression of *SOD1*, *SOD2*, and *GPX4* increased quadratically ($P < 0.05$). The mRNA expression of *SOD1*, *SOD2*, and *GPX4* in the 160 mg/kg group was dramatically decreased compared with the 40 mg/kg group ($P < 0.05$). The mRNA expression of *NRF2* was markedly increased with increasing dietary ASTA supplementation ($P < 0.05$).

The effects of dietary ASTA supplementation on the mRNA expression of *SOD1*, *SOD2*, *GPX4*, and *NRF2* in the kidney are shown in Table 8 and Figure 2. The mRNA expression of *SOD1*, *SOD2*, and *GPX4* significantly increased in the 40 mg/kg ASTA group compared with the control ($P < 0.05$). The mRNA expression of *SOD1*, *SOD2*, and *GPX4* in the kidney was markedly decreased in the 160 mg/kg group compared with the 40 mg/kg group ($P < 0.05$). The mRNA expression of *NRF2* increased linearly with increasing dietary ASTA addition ($P < 0.05$).

DISCUSSION

This study was designed to investigate the effect of dietary ASTA (from *H. pluvialis*) on production performance, egg quality, free radical scavenging ability, antioxidant enzyme activity, and gene expression of antioxidant enzymes and *NRF2* in laying hens. A previous study showed that feeding 1.35% ASTA (from algae) had no effects on production performance or egg quality except for egg yolk color (Walker et al., 2012). In the current study, we added ASTA from *H. pluvialis* to the

Table 4. Antioxidant enzyme activity and free radical scavenging ability parameters with different levels of dietary natural astaxanthin supplementation in liver.

Item	Natural astaxanthin (mg/kg)					SEM	*P*-value		
	0	20	40	80	160		ANOVA	Linear	Quadratic
T-SOD[1] (U/mgprot)	277.04^{c}	$304.31^{b,c}$	361.42^{a}	328.01^{b}	291.14^{c}	7.25	<0.001	0.160	<0.001
GSH-Px[2] (U/mgprot)	22.94	23.91	25.34	24.01	23.11	0.37	0.268	0.867	0.042
Hydroxyl radicals scavenging ability (U/mgprot)	28.10^{c}	32.28^{b}	36.69^{a}	37.74^{a}	39.74^{a}	0.94	<0.001	<0.001	0.118
Superoxide anion scavenging ability (U/mgprot)	174.84^{d}	206.26^{c}	285.95^{b}	299.50^{b}	327.76^{a}	11.52	<0.001	<0.001	0.054
Malondialdehyde (U/mgprot)	5.38^{a}	4.23^{b}	$3.88^{b,c}$	$3.57^{c,d}$	3.30^{d}	0.15	<0.001	<0.001	0.012

[a–d]Means within a row with no common superscripts differ significantly ($P < 0.05$).
[1]T-SOD means total superoxide dismutase.
[2]GSH-Px means glutathione peroxidase.

Table 5. Antioxidant enzyme activity and free radical scavenging ability parameters with different levels of dietary natural astaxanthin supplementation in kidney.

	Natural astaxanthin (mg/kg)						*P*-value		
Item	0	20	40	80	160	SEM	ANOVA	Linear	Quadratic
T-SOD[1] (U/mgprot)	376.56^{c}	472.19^{a,b}	527.45^{a}	476.46^{a,b}	443.16^{b}	11.06	<0.01	0.007	<0.001
GSH-Px[2] (U/mgprot)	27.23^{b}	30.88^{a,b}	32.83^{a}	31.24^{a,b}	27.23^{b}	0.61	0.002	0.912	<0.001
Hydroxyl radicals scavenging ability (U/mgprot)	66.19^{c}	78.56^{b,c}	99.81^{b}	166.34^{a}	180.25^{a}	8.96	<0.001	<0.001	0.036
Superoxide anion scavenging ability (U/mgprot)	189.76^{d}	230.94^{c,d}	272.22^{c}	343.83^{b}	397.96^{a}	14.91	<0.001	<0.001	0.239
Malondialdehyde (U/mgprot)	6.08^{a}	5.18^{a,b}	4.65b^{b,c}	4.09^{c}	3.78^{c}	0.18	<0.001	<0.001	0.218

[a–d]Means within a row with no common superscripts differ significantly ($P < 0.05$).
[1]T-SOD means total superoxide dismutase.
[2]GSH-Px means glutathione peroxidase.

hens' diet, which did not affect egg weight, feed consumption, feed efficiency, laying rate, Haugh unit, or eggshell strength. Dietary ASTA deepened the egg yolk color, which may be attributed to the direct deposition of ASTA, a natural pigment, into the egg yolk. Astaxanthin can be precipitated in egg yolk, making the eggs appear dark yellow or red.

Under normal physiological conditions, free radicals and antioxidants show a dynamically balanced relationship in vivo (Poprac et al., 2017). Once this balance is disrupted, a series of free radical chain reactions occur and induce different degrees of oxidative damage in the body (Djordjevic, 2004), such as structural damage to proteins and peroxidation of lipids (Kurashige et al., 1990; Djordjevic, 2004; Zorov et al., 2014). Both SOD and GSH-Px play important roles in preventing oxidative stress (Mates et al., 1999; Papas et al., 2019). Superoxide dismutase can efficiently scavenge free radicals in vivo, and GSH-Px is able to reduce the level of reactive oxygen species by promoting decomposition of H_2O_2 in vivo (Shang et al., 2018). In a previous study, feeding 25 mg/kg ASTA to rats improved activities of SOD, catalase, and GSH-Px in rats with gastric ulcer (Yang et al., 2009). In the current study, addition of dietary ASTA enhanced activities of antioxidant enzymes in laying hens, suggesting that ASTA could eliminate excess oxygen free radicals in vivo by improving the antioxidant enzyme system of laying hens.

Malondialdehyde is a major oxidation product of peroxidized polyunsaturated fatty acids and an important indicator of lipid peroxidation (Freeman and Crapo, 1981; El-Farrash et al., 2019). In a previous study, injection of ASTA into Sprague Dawley rats reduced MDA activity in serum (Xuan et al., 2016). Treatment with oral ASTA (25 mg/kg) decreased MDA content in Long-Evans male rats (Alam et al., 2017). Our results were similar to the previous results in that MDA content in laying hens was decreased with increasing dietary ASTA, indicating that increasing the concentration of ASTA effectively inhibited lipid peroxidation and free radical scavenging ability of laying hens. These results indicate that ASTA can scavenge free radicals by self-oxidization or enhancement of the antioxidant enzyme activity, reducing lipid peroxidation.

Our results showed that although dietary ASTA up to 40 mg/kg can enhance the activity of endogenous antioxidant enzymes, activities in the high-dose groups (80 and 160 mg/kg ASTA) were not increased, showing an inconsistency. The decrease in endogenous antioxidant enzyme activity may be attributed to the fact that high doses of ASTA can resist oxidative stress-induced cell injury through its own oxidation. Previous studies have shown that ASTA can scavenge free radicals by self-oxidation (Liang et al., 2009), which may be related to the degradation of carotenoids to transfer excited state electrons of singlet oxygen to the carotenoid chain (Liang et al., 2009; Fleischmann et al., 2020; Kumar et al., 2020). Moreover, the unique structure of the ASTA terminal ring moiety improves antioxidant activity by binding to the phospholipid (Goto et al., 2001). In the high-dose ASTA groups, ASTA could effectively eliminate reactive oxygen species in vivo and prevent

Table 6. Antioxidant enzyme activity and free radical scavenging ability parameters with different levels of dietary natural astaxanthin supplementation in plasma.

	Natural astaxanthin (mg/kg)						*P*-value		
Item	0	20	40	80	160	SEM	ANOVA	Linear	Quadratic
T-SOD[1] (U/mgprot)	161.93^{c}	175.39^{a,b}	190.21^{a}	183.09^{a,b}	195.24^{c}	3.15	0.012	0.451	<0.001
GSH-Px[2] (U/mgprot)	2,793.20	2,868.93	3,037.86	2,895.15	2,831.07	37.73	0.305	0.700	0.068
Hydroxyl radical scavenging ability (U/mgprot)	3,103.51^{c}	3,449.95^{b,c}	3,849.80^{a,b}	3,972.50^{a}	4,061.99^{a}	82.21	<0.001	<0.001	0.080
Superoxide anion scavenging ability (U/mgprot)	169.75^{c}	185.94^{b,c}	195.2^{b,c}	212.54^{a,b}	228.64^{a}	4.92	<0.001	<0.001	0.782
Malondialdehyde (U/mgprot)	6.34^{a}	5.91^{a,b}	5.01^{b,c}	4.39^{c}	4.01^{c}	0.19	<0.001	<0.001	0.682

[a–c]Means within a row with no common superscripts differ significantly ($P < 0.05$).
[1]T-SOD means total superoxide dismutase.
[2]GSH-Px means glutathione peroxidase.

Table 7. Effects of adding astaxanthin in diets on antioxidation-related mRNA expression in liver.

	Natural astaxanthin (mg/kg)						*P*-value		
Item	0	20	40	80	160	SEM	ANOVA	Linear	Quadratic
SOD1[1]	0.97^{c}	1.34^{b}	1.76^{a}	1.14^{b,c}	1.18^{b,c}	0.06	<0.001	0.308	<0.001
SOD2[2]	0.99^{c}	1.17^{c}	1.66^{a}	1.39^{b}	1.36^{b}	0.05	<0.001	<0.001	<0.001
GPX4[3]	0.93^{b}	1.08^{b}	1.44^{a}	1.32^{a}	0.99^{b}	0.04	<0.001	0.030	<0.001
NRF2[4]	0.97^{d}	1.49^{c}	2.39^{b}	2.81^{a}	2.84^{a}	0.14	<0.001	<0.001	<0.001

[a–d]Means within a row with no common superscripts differ significantly ($P < 0.05$).
[1]*SOD1* means Cu-Zn superoxide dismutase.
[2]*SOD2* means Mn superoxide dismutase.
[3]*GPX4* means glutathione peroxidase 4.
[4]NFE2L2 (*NRF2*) means nuclear factor E2-related factor 2.

oxidative stress-induced cell injury mainly by self-oxidation, thus reducing the need for endogenous antioxidant enzymes. In the low-dose ASTA groups, the lower supplementation level may not have been sufficient to effectively scavenge free radicals by being oxidized, and the dynamic balance of the reactive oxygen level and antioxidant ability in vivo would be maintained mainly by improving the endogenous antioxidant enzymes activities.

The increase in the activities of antioxidant enzymes may be related to increased gene expression (Tiedge et al., 1997). A previous study showed that the mRNA expression levels of *SOD2* and catalase in mice could be increased by the addition of 0.03% ASTA (Yang et al., 2011). In the current study, mRNA expression of *SOD1*, *SOD2*, and *GPX4* in the plasma and tissues was increased by dietary supplementation with ASTA; furthermore, activity of antioxidant enzymes was increased by upregulated mRNA expression of the genes encoding antioxidant enzymes. Interestingly, we found that high-dose ASTA decreased the mRNA expression levels of antioxidant enzymes compared with the 40 mg/kg ASTA group. The decrease in mRNA expression suggests that the high-dose ASTA group might resist oxidative stress-induced cell damage through its own oxidation (Liang et al., 2009). The present results indicate that dietary ASTA enhances antioxidant enzyme activities at least in part by upregulating the mRNA expression of the genes encoding the enzymes.

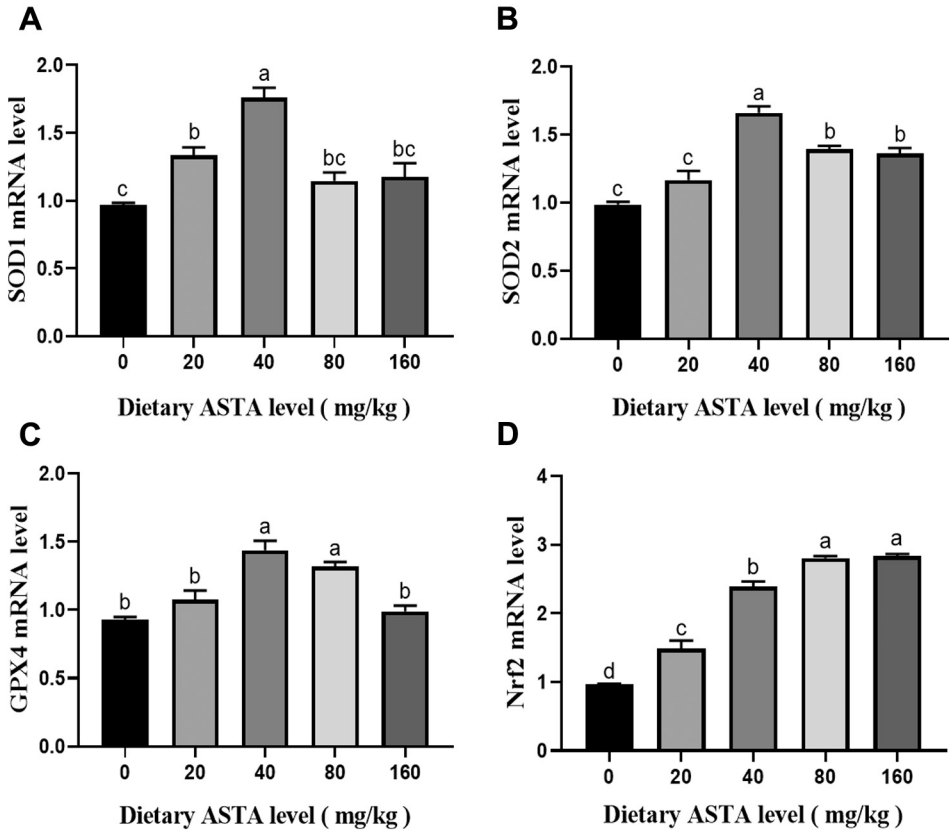

Figure 1. Effects of adding ASTA in diets on antioxidation-related mRNA expression in liver. The effect of adding ASTA (0, 20, 40, 80, 160 mg/kg) in diets in the liver, measured by quantitative PCR: (A) *SOD1*, (B) *SOD2*, (C) *GPX4*, and (D) *NRF2*. Values are expressed as means ± SEM of 6 birds per treatment. Means without a common letter differ ($P < 0.05$). Abbreviations: ASTA, astaxanthin; *GPX4*, glutathione peroxidase 4; *NRF2*, nuclear factor E2-related factor 2; *SOD1*, Cu-Zn superoxide dismutase; *SOD2*, Mn superoxide dismutase.

Table 8. Effects of adding astaxanthin in diets on antioxidation-related mRNA expression in kidney.

	Natural astaxanthin (mg/kg)						*P*-value		
Item	0	20	40	80	160	SEM	ANOVA	Linear	Quadratic
SOD1[1]	0.92[c]	2.42[b]	3.36[a]	2.62[b]	2.45[b]	0.16	<0.001	<0.001	<0.001
SOD2[2]	0.96[b]	1.23[b]	1.86[a]	1.79[a]	1.19[b]	0.08	<0.001	0.008	<0.001
GPX4[3]	0.97[b]	2.14[a]	2.58[a]	2.54[a]	1.25[b]	0.14	<0.001	0.065	<0.001
NRF2[4]	0.95[c]	2.03[b]	2.74[b]	3.79[a]	4.00[a]	0.23	<0.001	<0.001	0.100

[a–c]Means within a row with no common superscripts differ significantly ($P < 0.05$).
[1]*SOD1* means Cu-Zn superoxide dismutase.
[2]*SOD2* means Mn superoxide dismutase.
[3]*GPX4* means glutathione peroxidase 4.
[4]NFE2L2 (*NRF2*) means nuclear factor E2-related factor 2.

NRF2 is a member of the Cap'n'collar family of basic region-leucine zipper transcription factors that play an important role in the transcription factor that regulates the antioxidant response (Li and Kong, 2009). In normal physiological conditions, *NRF2* is bound to Kelch-like ECH-associated protein-1 on the cytoskeleton (Itoh et al., 2003; Kensler et al., 2007). Under oxidative stress, intracellular *NRF2* is released from Kelch-like ECH-associated protein-1, rapidly transfers into the nucleus, and binds to adenylate uridylate-rich element sequences, thus transcriptionally activating the expression of antioxidant genes (Itoh et al., 2003; Kwak et al., 2004; Kim et al., 2020). In a previous study, activation of *NRF2* increased the mRNA expression level of antioxidant enzyme genes. The addition of ASTA into adult retinal pigment epithelial cell line-19 cells upregulates the expression of *NRF2*, thus resisting oxidative stress-induced cell injury (Li et al., 2013). The expression of *NRF2* improves the defense capability of the antioxidant enzyme system via nuclear translocation (Qi et al., 2018). Our current results showed that expression of *NRF2* increased linearly with increasing levels of dietary ASTA, whereas mRNA expression of genes encoding antioxidant enzymes in high-dose ASTA groups did not increase and, in fact, decreased. This contradictory result may be explained by the fact that addition of ASTA at a high dose may improve antioxidant capacity mainly by self-oxidation, reducing the requirement for

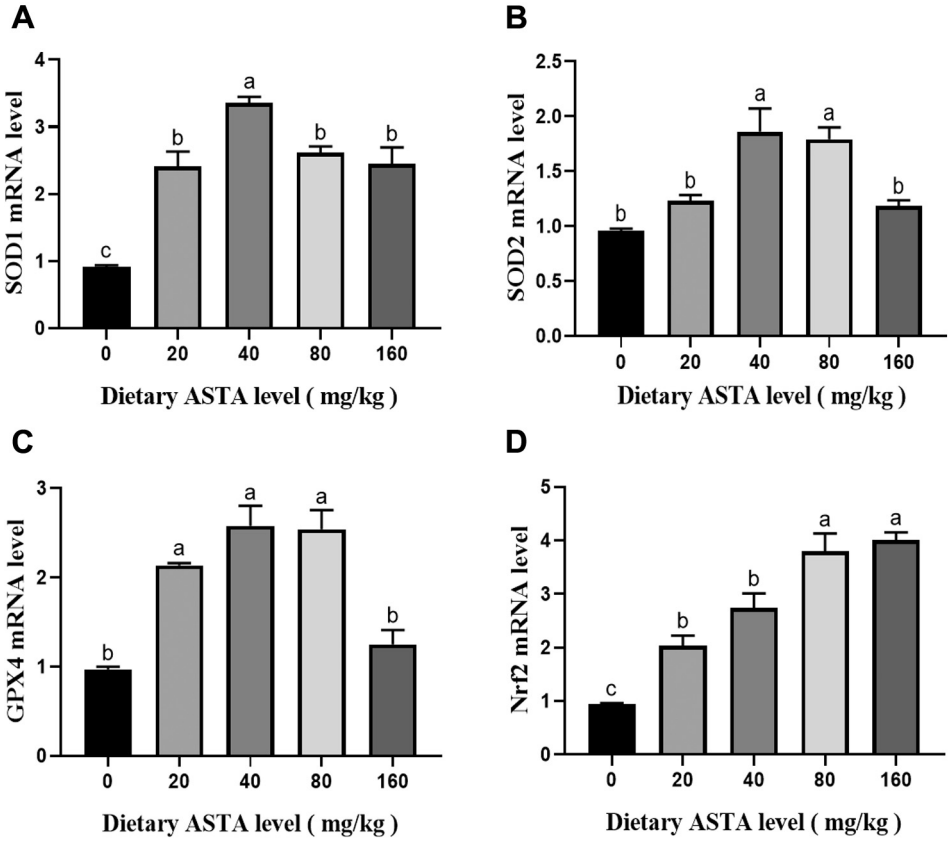

Figure 2. Effects of adding ASTA in diets on antioxidation-related gene mRNA expression in kidney. The effect of adding ASTA (0, 20, 40, 80, 160 mg/kg) in diets in the kidney, measured through quantitative PCR: (A) *SOD1*, (B) *SOD2*, (C) *GPX4*, and (D) *NRF2*. Values are expressed as means ± SEM of 3 birds per treatment. Means without a common letter differ ($P < 0.05$). Abbreviations: ASTA, astaxanthin; *GPX4*, glutathione peroxidase 4; *NRF2*, nuclear factor E2-related factor 2; *SOD1*, Cu-Zn superoxide dismutase; *SOD2*, Mn superoxide dismutase.

antioxidant enzymes (Liang et al., 2009). Another possible reason is that mRNA expression of genes encoding antioxidant enzymes may be regulated by factors other than *NRF2* (Mlakar et al., 2012; Liu et al., 2014). Addition of low-dose ASTA may improve antioxidant enzyme activities by upregulating *NRF2* mRNA expression.

In summary, we demonstrated that dietary supplementation of ASTA from *H. pluvialis* improved the free radical scavenging ability and reduced lipid peroxidation in laying hens. Low doses of ASTA supplemented in the diet protected against oxidative stress mainly by increasing the activity of antioxidant enzymes. High doses of ASTA in the diet may improve the antioxidant capacity mainly by being self-oxidized. Dietary ASTA may increase the activity and mRNA expression of antioxidant enzymes by upregulating mRNA expression of *NRF2*.

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DISCLOSURES

The authors have no conflicts of interest to declare.

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