Dietary supplemental microalgal astaxanthin modulates molecular profiles of stress, inflammation, and lipid metabolism in broiler chickens and laying hens under high ambient temperatures

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ABSTRACT This research was to determine effects of supplemental dietary microalgal astaxanthin (**AST**) on hepatic gene expression and protein production of redox enzymes, heat shock proteins (**HSPs**), cytokines, and lipid metabolism in broilers (**BR**) and laying hens (**LH**) under high ambient temperatures. A total of 240 (day old) Cornish male BR and 50 (19 wk old) White Leghorn Shavers LH were allotted in 5 dietary treatments with 6 and 10 cages/treatment (8 BR or 1 LH/cage), respectively. The birds were fed corn-soybean meal basal diets supplemented with microalgal (*Haematococcus pluvialis)* AST at 0, 10, 20, 40, and 80 mg/kg diet for 6 wk. Supplemental AST to the BR diet linearly decreased ($P < 0.10$, $R^2 = 0.18$–0.36) hepatic mRNA levels of several redox status-controlling genes, heat shock protein 70 (***HSP70***), heat shock transcription factor 1 (***HSTF1***), c-Jun N-terminal kinase 1 (***JNK1***), tumor necrosis factor-α, and sterol regulatory element-binding protein 1 (***SREBP1***). The supplementation linearly elevated ($P = 0.04$, $R^2 = 0.20$) diacylglycerol acyltransferase 2 (***DGAT2)*** mRNA level and produced quadratic changes ($P < 0.10$, $R^2 = 0.15$–0.47) in mRNA levels of glutathione S-transferase (***GST***), serine/threonine kinase (***AKT1***), P38 mitogen-activated protein kinase (***P38MAKP***), lipid metabolism–controlling genes, and the protein production of HSP90 and P38MAPK in the liver. Supplementing AST to the LH diets linearly decreased ($P < 0.10$, $R^2 = 0.18$–0.56) mRNA levels of *GST*, *HSF1*, *JNK1*, and interleukin 10; lipogenesis genes; and JNK1 protein production. However, supplemental dietary AST produced quadratic changes ($P < 0.10$, $R^2 = 0.26$–0.72) in mRNA levels of most antioxidant-, stress-responsive, and lipid metabolism–related genes in the liver of LH. In conclusion, supplemental dietary AST affected the hepatic gene expression and protein production related to redox status, heat stress and inflammation, and lipid metabolism in both BR and LH. The impacts varied with the chicken type and demonstrated linear and quadratic regressions with the inclusion levels of AST.

Key words: astaxanthin, gene, poultry, redox status

2020 Poultry Science 99:4853–4860
https://doi.org/10.1016/j.psj.2020.05.022

INTRODUCTION

Our previous research investigated effects of defatted and full-fatted microalgae as a new, alternative protein supplement on the nutrient metabolism and enriching meat and eggs with n-3 fatty acids (Austic et al., 2013; Ekmay et al., 2014, 2015; Gatrell et al., 2015). Meanwhile, microalgae are known as a rich source of phytochemicals including flavonoids, phenols, and other bioactive components (Buono et al., 2014). Astaxanthin (**AST**) (3,3′-dihydroxy-β, β-carotene-4,4′-dione) is a carotenoid without vitamin A activity, abundant in many species of microalgae, and found to have clinical applications (Jyonouchi et al., 1995; Fassett and Coombes, 2009). AST is best known with its high antioxidant activity (Miki, 1991; Naguib, 2000), with other potent biologic and pharmacological effects (Ikeuchi et al., 2007; Pashkow et al., 2008). High ambient temperature, in particular, in the summer, is emerging as a major threat to animal health and performance in poultry production (Lara and Rostagno, 2013). Although previous research determined effects of high ambient temperature or heat stress on health and performance of broilers (**BR**) or laying hens (**LH**) (Mashaly et al., 2004; Lu et al., 2007), few studies have systematically explored its

Received January 15, 2020.
Accepted May 22, 2020.

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effects on responses of metabolic or stress-related gene expression to different dietary supplements such as AST (Quinteiro-Filho et al., 2010; Willemsen et al., 2011; Zhang et al., 2012). Previously, we ran 2 feeding experiments to attest if supplemental microalgal AST was bioavailable and safe to BR and LH under high ambient temperatures and how the phytochemical affected their health status, production performance, and meat or egg quality (Magnuson et al., 2018; Sun et al., 2018). In the present study, we selected liver samples collected from those 2 studies as the most vital and representative metabolic site in the body to explore how the supplemental AST affected the gene expression and protein production of major redox enzymes, heat shock and stress-responsive proteins (**HSPs**), cytokines, and lipid metabolism in the BR and LH at high ambient temperatures.

MATERIALS AND METHODS

Animal Care and Diets

Our animal protocol was approved by the Institutional Animal Care and Use Committee of Cornell University. Two feeding experiments—one BR and one LH—were conducted as previously described (Magnuson et al., 2018; Sun et al., 2018). The experimental diets for both trials were corn-soybean meal-based and were supplemented with microalgal (*Haematococcus pluvialis*) AST (Heliae Development, LLC, Gilbert, AZ) at 0, 10, 20, 40, and 80 mg/kg diet. All diets were formulated to be isocaloric and isonitrogenous based on analyzed values of the main ingredients to meet the recommendations for BR and LH (NRC, 1994) (Supplementary Tables 1 and 2). Both trials lasted for 6 wk, and all birds had free access to water and feed supplied in mash form throughout the experiments.

Experiment 1 The broiler experimental design and protocol were described by Sun et al. (2018). Briefly, Cornish male chicks (day old, total = 240) were purchased from Moyer's Chicks (Quakertown, PA). The birds were weighed and allotted to experimental diets in a completely randomized design with 6 replicate cages/treatment and 8 birds/cage. The birds were kept in an environmentally controlled room with 2:22 h dark:light cycles for the entire experimental period. The temperature schedule was followed according to the industrial guide (Cobb-Vantress, 2013) (first wk: 34°C, second wk: 31°C, and third wk: 27°C). At the start of wk 4 of the experiment, the high ambient temperature was applied to BR by rising up room temperature 10°F (5.6°C) above the recommended temperature (fourth wk: 32.5°C, fifth wk: 30°C, and sixth wk: 28.3°C).

Experiment 2 The LH experimental design and protocol were described by Magnuson et al. (2018). Briefly, White Leghorn Shavers (19 wk old, total = 50, donated by Kreher Farmers, Clarence, NY) were used in this study. All hens were weighed individually and randomly assigned to experimental diets in a completely randomized design with 10 cages/treatment and 1 hen/cage. The hens were kept in an environmentally controlled room maintained with 8:16 h dark:light cycles at 25°C and 55% relative humidity. At the start of wk 4 of the experiment, the temperature was elevated gradually over several days to a set point of 28°C to induce high ambient temperature to the hens until the end of the experiment.

Sample Collection

At the end of each experiment (6 wk), 5 hens from each treatment (close to the group average body weight) were euthanized via asphyxiation with CO_2. After euthanization, hens were eviscerated, and a portion from the liver tissue was dissected out, immediately frozen in liquid nitrogen, and stored at −80°C until the molecular analysis.

Quantitative Real-Time PCR

Abundance of mRNA of genes involved in redox status including glutathione reductase (**GR**), glutathione peroxidase 1 (**GPX1**), glutathione S-transferase (**GST**), and superoxide dismutase 1 (**SOD1**), heat shock and stress response proteins (heat shock protein 70 [**HSP70**], heat shock protein 90 [**HSP90**], heat shock transcription factor 1 [**HSF1**], serine/threonine kinase AKT1 [**AKT1**], mitogen-activated protein kinase [**P38AMPK**], and c-Jun N-terminal kinase 1 [**JNK1**]), inflammation (interleukin 6 [**IL6**], interleukin10 [**IL10**], and tumor necrosis factor-α [**TNF-α**]), and lipid metabolism (fatty acid synthase [**FAS**], acyl Co-A carboxylase [**ACC**], stearoyl CoA desaturase 1 [**SCD1**], sterol regulatory element-binding protein1 [**SREBP1**], diacylglycerol acyltransferase 2 [**DGAT2**], carnitine palmitoyl acyltransferase [**CPT1**], and peroxisome proliferator-activated receptor-α [**PPAR-α**]) were determined. The primer sequences used for these assayed genes are shown in Supplementary Table 3. Total mRNA was isolated and purified using TRIzol Reagent (Life Technologies, Carlsbad, CA) from the liver tissue (20 mg). Total RNA was analyzed both qualitatively and quantitatively using SpectraDrop Micro-Volume Microplate (SpectraMax Plus 384 Absorbance Plate Reader, Molecular Devices, LLC). The reverse transcription was performed using a cDNA synthesis kit (Applied Biosystems, Grand Island, NY) according to kit guidelines.

The subsequent quantification was carried out by real-time polymerase chain reaction (**qPCR**) (7900 HT; Applied Biosystems) following the established method (Haunshi et al., 2017). The $2^{-\text{delta delta Ct }(\Delta\Delta Ct)}$ equation was used to quantify the expression levels of RNA (Livak and Schmittgen, 2001).

Immunoblotting Analysis

Protein from 50-mg liver tissue was extracted by homogenization using protein lysis buffer. The homogenates

then centrifuged for 15 min at 14,000 × *g* at 4°C. The protein contents of the resulting supernatants were then determined by bicinchoninic acid assay (Thermo Fisher Scientific, Rockford, IL). Liver homogenates (75-μg protein) were then used for measuring protein amounts of HSP90, SOD1, P38 MAPK, JNK1, and AKT1 following the established method (Ekmay et al., 2015). The relative densities of the protein bands were quantified using the ImageJ software (NIH) and normalized to glyceraldehyde 3-phosphate dehydrogenase as a loading control (Yan et al., 2012).

Statistical Analysis

Data were analyzed by one-way analysis of variance (**ANOVA**) and linear and quadratic regression models using SPSS statistical software (Ver. 20.0 for Windows, SPSS, Inc., Chicago, IL). Cage was considered to be the experimental unit (n = 5 cages per dietary treatment). Mean differences were tested using Tukey's honestly significant difference test. For all the analyses, pooled SEs were listed. Data were expressed as mean ± SE, and $P < 0.05$ was considered statistically significant and $P < 0.10$ was included to show a trend of the treatment effects.

RESULTS

Experiment 1

Supplementing different concentrations of AST (**x**) to the BR diets caused linear decreases ($P < 0.007$ to 0.05, R^2 = 0.18 to 0.36) in hepatic mRNA levels (**Y**) of GR (Y = 0.899–0.008x), *GPX1* (Y = 0.717–0.007x), *SOD1* (Y = 0.972–0.006x), *HSP70* (Y = 1.05–0.01x), *HSTF1* (Y = 1.11–0.006x), *JNK1* (Y = 1.07–0.007x), and *TNF-α* (Y = 0.914–0.006x), respectively (Table 1). In comparison, the supplementations of AST to the BR diets produced quadratic changes (P = 0.08, 0.004, and 0.04, R^2 = 0.15, 0.35, and 0.36) in hepatic mRNA levels of *GST* (Y = 1.12 + 0.038x–0.001x^2), *AKT1* (Y = 9.19 + 0.033x–0.00x^2), and *P38MAKP* (Y = 1.25 + 0.024x–0.00x^2), respectively.

Supplementing different concentrations of AST to the BR diets caused linear increase (P = 0.04, R^2 = 0.20) in hepatic mRNA levels of *DGAT2* (Y = 1.03 + 0.008x) but linear decrease (P = 0.03, R^2 = 0.21) in hepatic mRNA levels of *SREBP1* (Y = 0.871–0.004x). In comparison, the supplementation of AST to the BR diets produced quadratic changes ($P < 0.03$ to 0.05, and R^2 = 0.23, 0.25, and 0.26) in hepatic mRNA levels of *ACC* (Y = 1.12 + 0.058x–0.001x^2), *CPT1* (Y = 0.776 + 0.025x–0.00x^2), and *PPAR-α* (Y = 1.02 + 0.018x–0.00x^2), respectively (Table 2).

Supplementing different concentrations of AST to the BR diets had no effect on hepatic protein production of SOD1 and JNK1 but produced quadratic changes (P = 0.09 and 0.06, R^2 = 0.44 and 0.47) in hepatic protein production of HSP90 (Y = 1.16–0.015x + 0.00x^2) and P38MAPK (Y = 0.968–0.013x + 0.00x^2), respectively (Figure 1).

Experiment 2

Supplementing different concentrations of AST to the LH diets caused linear decreases ($P < 0.01$ to 0.06, R^2 = 0.18 to 0.34) in hepatic mRNA levels of *GST* (Y = 1.60–0.010x), *HSF1* (Y = 1.34–0.008x), *JNK1* (Y = 1.19–0.009x), and *IL10* (Y = 0.661–0.008x), respectively (Table 3). In comparison, the supplementations of AST to LH diets produced quadratic changes ($P < 0.00$ to 0.09, R^2 = 0.26 to 0.72) in hepatic mRNA levels of *GR* (Y = 0.891 + 0.016x + 0.00x^2), *SOD1* (Y = 0.865 + 0.055x–0.001x^2), *HSP70* (Y = 0.760 + 0.081x–0.001x^2), *HSP90* (Y = 0.896 + 0.002x + 0.00x^2), *AKT1* (Y = 1.00 + 0.039x–0.001x^2), and *P38MAPK* (Y = 1.06 + 0.026x–0.00x^2), respectively. Hens fed the 20-mg AST/kg had higher ($P < 0.05$) mRNA level of *GPX1* than those fed the control and other diets.

Supplementing different concentrations of AST to the LH diets caused linear decreases (P = 0.06, 0.005, and 0.08, R^2 = 0.18, 0.36, and 0.16) of hepatic mRNA levels of *ACC* (Y = 0.809–0.005x), *SCD1* (Y = 0.978–0.006x), and *DGAT2* (Y = 0.906–0.004x), respectively. In comparison, the supplementations of AST to the LH diets produced quadratic changes ($P < 0.007$ to 0.06, R^2 = 0.28 to 0.42) in hepatic mRNA levels of *FAS* (Y = 0.917–0.021x + 0.00x^2), *SREBP1* (Y = 1.03 + 0.027x + 0.00x^2), *CPT1* (Y = 1.22 + 0.137x–0.00x^2), and *PPAR-α* (Y = 1.08 + 0.024x + 0.00x^2), respectively (Table 4).

Supplementing different concentrations of AST to the LH diets had no effect on hepatic protein levels of HSP90, SOD1, or P38MAPK but produced a linear decrease (P = 0.01, R^2 = 0.56) of hepatic JNK1 (Y = 0.819–0.008x) (Figure 2).

DISCUSSION

The most significant findings from our study were dose-dependent, chicken type-specific responses of hepatic antioxidant status-, lipid metabolism-, and stress-related genes and proteins to graded levels of supplemental dietary microalgal AST to diets for BR and LH raised at high ambient temperatures. Our premise for this research was that exposing the BR and LH to chronic high ambient temperatures would induce oxidative stress and inflammation and subsequently dysregulate their antioxidant defense, stress control, and lipid metabolism (Lara and Rostagno, 2013). Although AST is a well-known potent antioxidant (Miki, 1991; Naguib, 2000), previous research did not examine effects of supplemental dietary AST on the aforementioned metabolic cascade associated with high ambient temperatures in the BR and LH at the gene expression or protein production level (Lei et al., 2009; Rimoldi et al., 2015). Thus, we performed this study to characterize the dose effects of supplemental AST on those molecular

Table 1. Effects of different concentrations of dietary supplemental microalgal AST on the relative mRNA levels of redox enzymes, heat shock and stress responsive proteins, and cytokines in liver tissue of broiler chicks.[1]

	AST mg/kg diet						*P* value[2]			R^2	
Gene	0	10	20	40	80	SEM	ANOVA	Linear	Quadratic	Linear	Quadratic
Redox enzymes											
GR	1.00	0.70	0.56	0.63	0.26	0.111	0.282	0.050	0.607	0.179	0.190
GPX1	1.00	0.58	0.44	0.38	0.24	0.079	0.037	0.007	0.099	0.339	0.440
GST	1.00	1.27	1.61	1.94	0.89	0.185	0.481	0.683	0.084	0.008	0.149
SOD1	1.00	0.94	0.81	0.79	0.49	0.065	0.196	0.013	0.866	0.261	0.262
Heat shock and stress proteins											
HSP70	1.00	0.86	0.52	0.87	0.28	0.133	0.150	0.045	0.741	0.186	0.191
HSP90	1.00	0.90	0.88	0.94	0.56	0.135	0.658	0.161	0.829	0.106	0.109
HSF1	1.00	1.23	0.79	1.13	0.54	0.084	0.061	0.046	0.364	0.185	0.220
AKT1	1.00	1.06	1.16	1.78	0.74	0.104	0.011	0.611	0.004	0.013	0.347
P38MAPK	1.00	1.97	1.16	1.77	0.61	0.137	0.006	0.065	0.045	0.176	0.355
JNK1	1.00	1.29	0.78	0.87	0.44	0.072	0.005	0.007	0.197	0.356	0.422
Cytokines											
IL6	1.00	0.65	0.39	0.41	0.45	0.096	0.281	0.163	0.101	0.105	0.240
IL10	1.00	0.77	0.52	0.83	0.77	0.116	0.673	0.669	0.395	0.013	0.069
TNF-α	1.00	0.69	0.75	0.80	0.37	0.077	0.050	0.015	0.984	0.335	0.335

Abbreviations: AKT1, serine/threonine kinase AKT1; AST, astaxanthin; GPX1, glutathione peroxidase 1; GR, glutathione reductase; GST, glutathione S-transferase; HSP70, heat shock protein 70; HSP90, heat shock protein 90; HSF1, heat shock transcription factor1; IL6, interleukin6; IL10, interleukin 10; JNK1, c-Jun N-terminal kinase 1; P38AMPK, P38 mitogen-activated protein kinase; SOD1, superoxide dismutase 1; TNF-α, tumor necrosis factor-α.

[1]Values are expressed as the mean of 5 birds/treatment for all the variables. The values are normalized to the control.

[2]Data were analyzed using linear and quadratic regression models of SPSS.

responses and to compare differences in those responses between the BR and LH. Quantitatively, the graded levels of supplemental dietary AST (0–80 mg/kg) produced the following 5 types of effects on the molecular biomarkers: 1) linear increases, 2) linear decreases, 3) quartic changes, 4) isolated differences, and 5) no changes in both BR and LH. Specifically, the AST dose-dependent effect on any given biomarkers varied with the functional group of the gene or protein and the poultry type.

Supplemental AST resulted in linear decreases in the hepatic mRNA levels of *GPX1*, *GR*, and *SOD1*in the BR but only quadratic or isolated (single dose effect) changes of these genes in the LH. However, hepatic mRNA levels of *GST* responded to supplemental AST in an opposite way to *GPX1*, *GR*, and *SOD1* between the 2 types of poultry (quadratic in the BR and linear decrease in the LH). Seemingly, regulations of hepatic *GPX1*, *GR*, and *SOD1* gene expression by supplemental AST were relatively simple or unilaterally in the BR but with more sophisticated or feedback mechanism in the LH. The unique regulations of hepatic *GST* mRNA levels by supplemental AST might reflect a special or different coordination among these antioxidant enzyme gene expressions between the BR and LH. Although the linear decreases in the hepatic mRNA levels of *GPX1*, *GR*, and *SOD1* were consistent with the respective enzyme activity changes reported earlier in the BR fed the 5 levels of supplemental AST (Sun et al., 2018), the detected mRNA level changes of these genes did not match the corresponding decreases in the GR, SOD1, and GPX1 activities in the liver of LH (Magnuson et al., 2018). We postulated a coordination between the enrichment of AST as an extrinsic antioxidant and intrinsic antioxidant enzyme activities to maintain a redox balance (Magnuson et al., 2018), but differences in the aforementioned antioxidant gene expression responses to the supplemental AST between the BR and LH remain a future research question (Felver-Gant et al., 2012).

Table 2. Effects of different concentrations of dietary supplemental microalgal AST on the relative mRNA levels of lipid metabolism controlling genes in liver tissue of broiler chicks.[1]

	AST mg/kg diet						*P* value[2]			R^2	
Gene	0	10	20	40	80	SEM	ANOVA	Linear	Quadratic	Linear	Quadratic
FAS	1.00	1.02	1.50	1.88	1.09	0.203	0.717	0.779	0.240	0.004	0.080
ACC	1.00	1.63	1.78	2.44	0.94	0.218	0.324	0.677	0.038	0.009	0.225
SCD1	1.00	1.92	2.31	1.08	1.30	0.260	0.663	0.609	0.683	0.016	0.026
SREBP1	1.00	0.63	0.75	0.71	0.54	0.058	0.032	0.031	0.392	0.213	0.243
DGAT2	1.00	0.95	1.18	1.37	1.65	0.116	0.337	0.042	0.776	0.200	0.204
CPT1	1.00	1.06	0.56	1.50	0.34	0.127	0.013	0.174	0.052	0.086	0.247
PPAR-α	1.00	1.21	1.18	1.46	0.86	0.073	0.180	0.281	0.036	0.058	0.257

Abbreviations: ACC, acyl Co-A carboxylase; AST, astaxanthin; CPT1, carnitine palmitoyl acyltransferase 1; DGAT2, diacylglycerol acyltransferase 2; FAS, fatty acid synthase; PPAR-α, peroxisome proliferator activated receptor-α; SCD1, stearoyl CoA desaturase 1; SREBP1, sterol regulatory element–binding protein1.

[1]Values are expressed as the mean of 5 birds/treatment for all the variables. The values are normalized to the control.

[2]Data were analyzed using linear and quadratic regression models of SPSS.

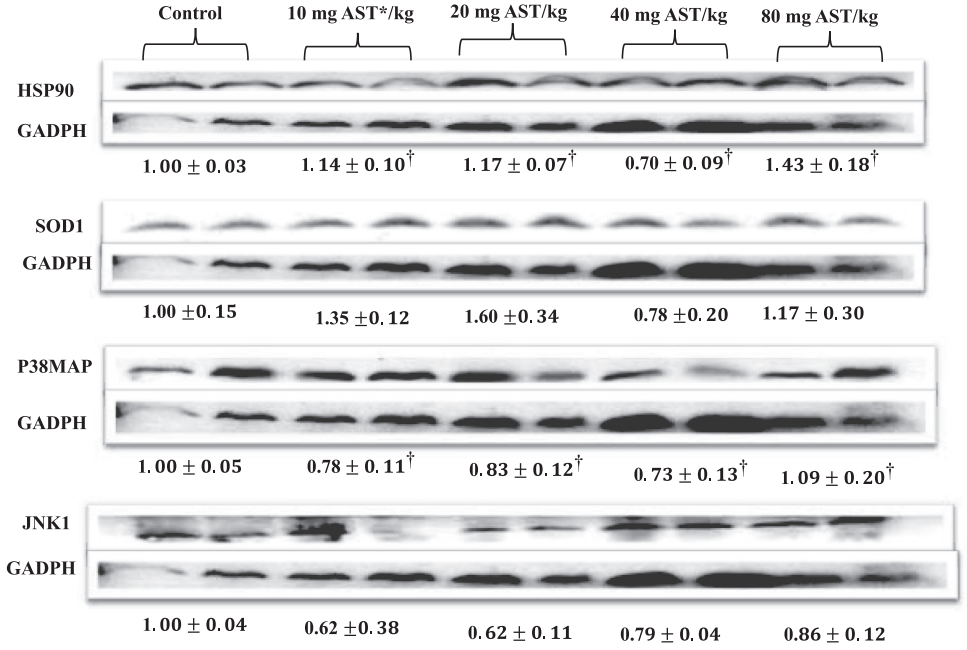

Figure 1. Western blot analysis of HSP90, SOD1, P38 MAPK, and JNK1 levels in the liver tissue of BR fed supplemental AST for 6 wk. Values below the protein band were relative densities and are expressed as means ± SE (n = 2 chickens per dietary treatment). Values are expressed as a ratio to GADPH and then normalized to the control. †$P < 0.10$. Blots are representative of 2 independent replicate gels. *AST effect. Abbreviations: GADPH, glyceraldehyde 3-phosphate dehydrogenase; HSP90, heat shock protein 90 ($P = 0.09$, $R^2 = 0.44$); JNK1, c-Jun N-terminal kinase 1; P38 MAPK, P38 mitogen-activated protein kinase ($P = 0.06$, $R^2 = 0.47$); SOD1, superoxide dismutase 1.

Supplemental AST lead to linear decreases of *HSTF1* and *JNK1* and quadratic changes of *AKT1* and *P38MAPK* mRNA levels in the liver of both BR and LH. However, the supplementation produced different hepatic mRNA level changes of heat shock proteins or other stress-responsive proteins and cytokines (*HSP70*, *90*, *IL-10*, and *TNF-α*) between the 2 types of poultry. In addition, supplemental AST produced quadratic changes in hepatic protein production of HSP90 and P38MAPK in the BR, but a linear decrease of hepatic JNK1 in the LH. As mentioned previously, exposure to high ambient temperatures might generate excessive reactive oxygen species which, in turn, disrupt cell integrity and function through denaturation of cytoskeletal proteins (Murphy, 2009). Consequently, HSPs might bind and stabilize cytoskeletal proteins (Concannon et al., 2003), and the oxidative stress–elevated proinflammatory cytokines could upregulate mitogen-

Table 3. Effects of different concentrations of dietary supplemental microalgal AST on the relative mRNA levels of redox enzymes, heat shock and stress responsive proteins, and cytokines in liver tissue of laying hens.[1]

	AST mg/kg diet						*P* value[2]			R^2	
Gene	0	10	20	40	80	SEM	ANOVA	Linear	Quadratic	Linear	Quadratic
Redox enzymes											
GR	1.00	0.75	1.17	1.23	0.66	0.067	0.004	0.193	0.019	0.092	0.349
GPX1	1.00^{b}	2.15^{b}	4.96^{a}	1.62^{b}	2.38^{b}	0.362	0.006	0.915	0.267	0.001	0.077
GST	1.00	2.34	1.28	1.09	0.73	0.162	0.004	0.066	0.546	0.176	0.194
SOD1	1.00	0.88	2.12	1.89	1.01	0.149	0.003	0.988	0.002	0.000	0.429
Heat shock and stress proteins											
HSP70	1.00	0.93	1.70	2.68	0.26	0.272	0.041	0.428	0.008	0.035	0.366
HSP90	1.00	0.81	1.24	1.34	0.52	0.117	0.152	0.209	0.060	0.086	0.262
HSF1	1.00	1.36	1.26	1.11	0.58	0.108	0.169	0.024	0.243	0.252	0.311
AKT1	1.00	1.08	1.99	1.49	0.69	0.145	0.023	0.283	0.012	0.064	0.360
P38MAPK	1.00	1.26	1.60	1.31	0.58	0.089	0.000	0.015	0.000	0.288	0.716
JNK1	1.00	1.03	1.11	0.93	0.43	0.103	0.184	0.013	0.458	0.296	0.320
Cytokines											
IL6	1.00	0.77	1.26	0.97	0.49	0.166	0.481	0.140	0.833	0.117	0.120
IL10	1.00	0.33	0.15	0.41	0.14	0.107	0.004	0.037	0.094	0.219	0.341
TNF-α	1.00	1.20	1.01	1.30	0.81	0.074	0.271	0.284	0.124	0.063	0.188

Means with different superscript lowercase letters within the same row differ according to one-way ANOVA ($P < 0.05$).

Abbreviations: AKT1, serine/threonine kinase AKT1; AST, astaxanthin; GPX1, glutathione peroxidase 1; GR, glutathione reductase; GST, glutathione S-transferase; HSP70, heat shock protein 70; HSP 90, heat shock protein 90; HSF1, heat shock transcription factor1; IL6, interleukin 6; IL10, interleukin 10; JNK1, c-Jun N-terminal kinase 1; P38AMPK, mitogen-activated protein kinase; SOD1, superoxide dismutase 1; TNF-α, tumor necrosis factor-α.

[1]Values are expressed as the mean of 5 birds/treatment for all the variables. The values are normalized to the control.

[2]Data were analyzed using linear and quadratic regression models of SPSS.

Table 4. Effects of different concentrations of dietary supplemental microalgal AST on the relative mRNA levels of lipid metabolism controlling genes in liver tissue of laying hens.[1]

	AST mg/kg diet						*P* value[2]			R^2	
Gene	0	10	20	40	80	SEM	ANOVA	Linear	Quadratic	Linear	Quadratic
FAS	1.00	0.71	0.33	0.56	0.38	0.071	0.003	0.015	0.062	0.288	0.424
ACC	1.00	0.55	0.51	0.76	0.39	0.078	0.041	0.061	0.614	0.181	0.193
SCD1	1.00	1.12	0.61	0.72	0.55	0.064	0.004	0.005	0.246	0.356	0.407
SREBP1	1.00	1.30	1.29	1.62	0.78	0.097	0.061	0.258	0.007	0.070	0.398
DGAT2	1.00	0.57	1.014	0.69	0.57	0.069	0.035	0.077	0.865	0.163	0.165
CPT1	1.00	2.01	4.74	3.63	3.32	0.416	0.022	0.157	0.023	0.108	0.348
PPAR-α	1.00	1.18	1.72	1.33	0.88	0.102	0.075	0.325	0.034	0.054	0.280

Abbreviations: ACC, acyl Co-A carboxylase; AST, astaxanthin; CPT1, carnitine palmitoyl acyltransferase 1; DGAT2, diacylglycerol acyltransferase 2; FAS, fatty acid synthase; PPAR-α, peroxisome proliferator activated receptor-α; SCD1, stearoyl Co-A desaturase 1; SREBP1, sterol regulatory element binding protein 1.

[1]Values are expressed as the mean of 5 birds/treatment for all the variables. The values are normalized to the control.

[2]Data were analyzed using linear and quadratic regression models of SPSS.

activated protein kinases (**MAPKs**) such as P38MAPK and JNK and then induce AKT1 to protect cells from oxidative damage (Wang, 2000; Zhang et al., 2016). Meanwhile, upregulation of anti-inflammatory cytokine IL10 might inhibit the induced inflammation (Dokka et al., 2001). The linear decreases of hepatic *HSTF1*, *JNK1*, *HSP70*, *IL-10*, and *TNF-α* mRNA levels and (or) hepatic JNK1 protein in the BR and (or) LH can be interpreted as positive effects of supplemental AST on protection against the high ambient temperature–associated oxidative stress and inflammation (Concannon et al., 2003). However, there seems to have no plain assertion to explain the quadratic effects of supplemental AST on the gene expression and protein production of hepatic *AKT1*, P38MAPK, and HSP90 or the mixed and different responses of some of the heat shock and stress responsive proteins between the 2 types of poultry.

Supplementing dietary AST to BR diets resulted in linear decreases in the mRNA levels of *SREBP1*, the key lipogenesis controlling transcription factor, linear increases in the hepatic mRNA levels of lipid anabolism controlling gene *DGAT2*, and quadratic responses in the hepatic mRNA levels of *ACC* and the key lipolysis-controlling gene and transcription factor *CPT1* and *PPAR-α*. Conversely, supplementing AST to LH diets caused linear decreases in the hepatic mRNA levels of lipogenesis controlling genes (*ACC*, *SCD1*, and *DGAT2*), quadratic responses of hepatic *FAS*, *SREBP1*, *CPT1*, and *PPAR-α* mRNA levels. Overall, supplemental AST seemed to show an upregulation of gene expression related to lipid catabolism or a

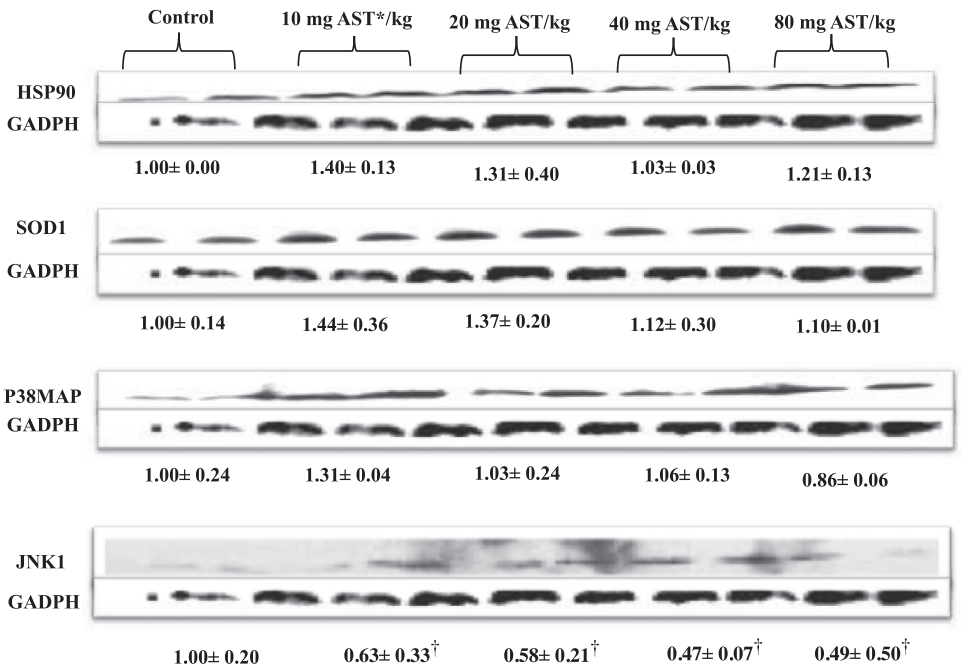

Figure 2. Western blot analysis of HSP90, SOD1, P38 MAPK, and JNK1 levels in the liver tissue of LH fed supplemental AST for 6 wk. Values below the protein band were relative densities and are expressed as means ± SE (n = 2 chickens per dietary treatment). Values are expressed as a ratio to GADPH and then normalized to the control. †$P < 0.10$. Blots are representative of 2 independent replicate gels. *AST effects. Abbreviations: GADPH, glyceraldehyde 3-phosphate dehydrogenase; HSP90, heat shock protein 90; JNK1, c-Jun N-terminal kinase 1 ($P = 0.01$, $R^2 = 0.56$); P38 MAPK, P38 mitogen-activated protein kinase; SOD1, superoxide dismutase 1.

downregulation of lipogenesis gene expression more apparently in the LH than in the BR. However, we were not able to sort out the potential confounding responses of poultry to the high ambient temperatures that might trigger mobilization of lipid store from the liver to generate energy to attenuate the adverse effects of heat production (Manoli et al., 2007). It has been shown that AST improved lipid metabolism via accumulation on the mitochondrial membrane, leading to an enhanced function of CPT1 (Takahashi et al., 2004). Aoi et al. (2008) hypothesized that reactive oxygen species generated from mitochondria during stress conditions oxidize CPT1 and inhibit its function. Thus, supplemental AST may regulate lipid metabolism by inhibiting the oxidative alteration of CPT1 (Takahashi et al., 2004). Apparently, the relationship between supplemental AST and other lipid metabolism–controlling enzymes needs further study.

The underlying mechanism for supplemental AST to modulate the gene expression of the aforementioned biomarkers related to redox status, heat stress, inflammation, and lipid metabolism remains unclear. Park et al. (1999) proposed that various carotenoids might influence transcription of different target genes. Other researchers have also investigated the role of AST and other carotenoids in regulating gene expression of mammalian species (Bertram and Bortkiewicz, 1995; Park et al., 1999). The present study extends our earlier effort to reveal that supplemental AST was bioavailable to BR and LH with strong intrinsic activities (Magnuson et al., 2018; Sun et al., 2018). In conjunction with the current molecular findings, all these discoveries point out the need for identification of characteristic carotenoid receptors to help understand the mechanisms behind impacts of carotenoids; the response disparity between different genes, proteins, and poultry types; and the lack of some pertaining protein response to go along with the gene expression changes induced by the supplemental AST in the present study.

In conclusion, supplemental dietary microalgal AST to the BR and LH raised at high ambient temperatures affected their gene expression related to redox status, heat stress and inflammation, and lipid metabolism. However, the impacts varied with the chicken types and the inclusion levels of microalgal AST. Our findings shall stimulate future research for a better understanding of the role and mechanism of supplemental microalgal AST in diets for LH and BR to cope with stress insults associated with high ambient temperatures.

ACKNOWLEDGMENT

This study was supported in part by DOE MAGIC grant DE-EE0007091, USDA grant 2019-69012-29905, Cornell University Hatch grants NYC-127419/127302, and Heliae Development, Gilbert, Arizona.

Conflict of Interest Statement: The authors have no conflicts of interest.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at http://doi.org/10.1016/j.psj.2020.05.022.

REFERENCES

Aoi, W., Y. Naito, Y. Takanami, T. Ishii, Y. Kawai, S. Akagiri, Y. Kato, T. Osawa, and T. Yoshikawa. 2008. Astaxanthin improves muscle lipid metabolism in exercise via inhibitory effect of oxidative CPT 1 modification. Biochem. Biophys. Res. Commun. 366:892–897.

Austic, R. E., A. Mustafa, B. Jung, S. Gatrell, and X. G. Lei. 2013. Potential and limitation of a new defatted diatom microalgal biomass in replacing soybean meal and corn in diets for broiler chickens. J. Agric. Food Chem. 61:7341–7348.

Bertram, J. S., and H. Bortkiewicz. 1995. Dietary carotenoids inhibit neoplastic transformation and modulate gene expression in mouse and human cells. Am. J. Clin. Nutr. 62:1327S–1336S.

Buono, S., A. L. Langellotti, A. Martello, F. Rinna, and V. Fogliano. 2014. Functional ingredients from microalgae. Food Funct. 5:1669–1685.

Cobb-Vantress. 2013. Cobb Broiler Management Guide; L-1020-05. Cobb-Vantress, Siloam Springs, AR.

Concannon, C., A. Gorman, and A. Samali. 2003. On the role of Hsp27 in regulating apoptosis. Apoptosis 8:61–70.

Dokka, S., X. Shi, S. Leonard, L. Wang, V. Castranova, and Y. Rojanasakul. 2001. Interleukin-10-mediated inhibition of free radical generation in macrophages. Am. J. Physiol. Lung Cell. Mol. Physio. 280:L1196–L1202.

Ekmay, R., S. Gatrell, K. Lum, J. Kim, and X. G. Lei. 2014. Nutritional and metabolic impacts of a defatted green marine microalgal (Desmodesmus sp.) biomass in diets for weanling pigs and broiler chickens. J. Agric. Food Chem. 62:9783–9791.

Ekmay, R. D., K. Chou, A. Magnuson, and X. G. Lei. 2015. Continual feeding of two types of microalgal biomass affected protein digestion and metabolism in laying hens. J. Anim. Sci. 93:287–297.

Fassett, R. G., and J. S. Coombes. 2009. Astaxanthin, oxidative stress, inflammation and cardiovascular disease. Future Cardiol. 5:333–342.

Felver-Gant, J. N., L. A. Mack, R. L. Dennis, S. D. Eicher, and H. W. Cheng. 2012. Genetic variations alter physiological responses following heat stress in 2 strains of laying hens. Poult. Sci. 91:1542–1551.

Gatrell, S. K., J. Kim, T. J. Derksen, E. V. O'Neil, and X. G. Lei. 2015. Creating omega-3 fatty-acid-Enriched chicken using defatted green microalgal biomass. J. Agric. Food Chem. 63:9315–9322.

Haunshi, S., A. K. Burramsetty, T. Kannaki, K. R. Ravindra, and R. Chatterjee. 2017. Pattern recognition receptor genes expression profiling in indigenous chickens of India and White Leghorn. Poult. Sci. 96:3052–3057.

Ikeuchi, M., T. Koyama, J. Takahashi, and K. Yazawa. 2007. Effects of astaxanthin in obese mice fed a high-fat diet. Biosci. Biotechnol. Biochem. 71:893–899.

Jyonouchi, H., S. Sun, Y. Tomita, and M. D. Gross. 1995. Astaxanthin, a carotenoid without vitamin A activity, augments antibody response in cultures including T-helper cell clones and suboptimal doses of antigen. J. Nutr. 125:2483.

Lara, L. J., and M. H. Rostagno. 2013. Impact of heat stress on poultry production. Animals 3:356–369.

Lei, L., J. Yu, and E. Bao. 2009. Expression of heat shock protein 90 (Hsp90) and transcription of its corresponding mRNA in broilers exposed to high temperature. Br. Poult. Sci. 50:504–511.

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402–408.

Lu, Q., J. Wen, and H. Zhang. 2007. Effect of chronic heat exposure on fat deposition and meat quality in two genetic types of chicken. Poult. Sci. 86:1059–1064.

Magnuson, A., T. Sun, R. Yin, G. Liu, S. Tolba, S. Shinde, and X. Lei. 2018. Supplemental microalgal astaxanthin produced

coordinated changes in intrinsic antioxidant systems of layer hens exposed to heat stress. Algal Res. 33:84–90.
Manoli, I., S. Alesci, M. R. Blackman, Y. A. Su, O. M. Rennert, and G. P. Chrousos. 2007. Mitochondria as key components of the stress response. Trends Endocrinol. Metab. 18:190–198.
Mashaly, M., G. Hendricks, 3rd, M. Kalama, A. Gehad, A. Abbas, and P. Patterson. 2004. Effect of heat stress on production parameters and immune responses of commercial laying hens. Poult. Sci. 83:889–894.
Miki, W. 1991. Biological functions and activities of animal carotenoids. Pure Appl. Chem. 63:141–146.
Murphy, M. P. 2009. How mitochondria produce reactive oxygen species. Biochem. J. 417:1–13.
Naguib, Y. M. 2000. Antioxidant activities of astaxanthin and related carotenoids. J. Agric. Food Chemi. 48:1150–1154.
National Research Council. 1994. Nutrient Requirements of Poultry. 9th ed. Natl. Acad. Press, Washington, DC.
Park, J. S., B. P. Chew, T. S. Wong, J.-X. Zhang, and N. S. Magnuson. 1999. Dietary lutein but not astaxanthin or β-carotene increases pim-1 gene expression in murine lymphocytes. Nutr. Cancer 33:206–212.
Pashkow, F. J., D. G. Watumull, and C. L. Campbell. 2008. Astaxanthin: a novel potential treatment for oxidative stress and inflammation in cardiovascular disease. Am. J. Cardiol. 101:S58–S68.
Quinteiro-Filho, W., A. Ribeiro, V. Ferraz-de-Paula, M. Pinheiro, M. Sakai, L. Sá, A. Ferreira, and J. Palermo-Neto. 2010. Heat stress impairs performance parameters, induces intestinal injury, and decreases macrophage activity in broiler chickens. Poult. Sci. 89:1905–1914.
Rimoldi, S., E. Lasagna, F. M. Sarti, S. P. Marelli, M. C. Cozzi, G. Bernardini, and G. Terova. 2015. Expression profile of six stress-related genes and productive performances of fast and slow growing broiler strains reared under heat stress conditions. Meta Gene 6:17–25.
Sun, T., R. Yin, A. D. Magnuson, S. A. Tolba, G. Liu, and X. G. Lei. 2018. Dose-dependent enrichments and improved redox status in tissues of broiler chicks under heat stress by dietary supplemental microalgal astaxanthin. J. Agric. Food Chem. 66:5521.
Takahashi, K., M. Watanabe, T. Takimoto, and Y. Akiba. 2004. Uptake and distribution of astaxanthin in several tissues and plasma lipoproteins in male broiler chickens fed a yeast (Phaffia rhodozyma) with a high concentration of astaxanthin. Br. Poult. Sci. 45:133–138.
Wang, X. 2000. McCullough KD, Franke TF, and Holbrook NJ. Epidermal growth factor receptor-dependent Akt activation by oxidative stress enhances cell survival. J. Biol. Chem. 275:14624–14631.
Willemsen, H., Q. Swennen, N. Everaert, P. A. Geraert, Y. Mercier, A. Stinckens, E. Decuypere, and J. Buyse. 2011. Effects of dietary supplementation of methionine and its hydroxy analog dl-2-hydroxy-4-methylthiobutanoic acid on growth performance, plasma hormone levels, and the redox status of broiler chickens exposed to high temperaturesdl-methionine analog and oxidative status. Poult. Sci. 90:2311–2320.
Yan, X., M. P. Pepper, M. Z. Vatamaniuk, C. A. Roneker, L. Li, and X. G. Lei. 2012. Dietary Selenium Deficiency Partially Rescues type 2 Diabetes–like Phenotypes of glutathione peroxidase-1–Overexpressing male mice. J. Nutr. 142:1975–1982.
Zhang, J., X. Wang, V. Vikash, Q. Ye, D. Wu, Y. Liu, and W. Dong. 2016. ROS and ROS-mediated cellular signaling. Oxidative Med. Cell Longev. 2016:4350965.
Zhang, Z., G. Jia, J. Zuo, Y. Zhang, J. Lei, L. Ren, and D. Feng. 2012. Effects of constant and cyclic heat stress on muscle metabolism and meat quality of broiler breast fillet and thigh meat. Poult. Sci. 91:2931–2937.