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Differential Abundance of Egg White Proteins in Laying Hens Treated with Corticosterone

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ABSTRACT: Stressful environments can affect not only egg production and quality but also gene and protein abundance in the ovary and oviduct in laying hens. The oviductal magnum of laying hens is the organ responsible for the synthesis and secretion of egg white proteins. The objective of this study was to investigate the effects of dietary corticosterone as a stress model on the abundance of proteins in the egg white and of mRNA and proteins in the magnum in laying hens. After a 14-day acclimation, 40 laying hens were divided into two groups which were provided for the next 14 days with either control (Control) or corticosterone (Stress) diet containing at 30 mg/kg. Corticosterone treatment resulted in increased feed intake ($P \leq 0.05$) and decreased egg production. Two-dimensional electrophoresis (2DE) with MALDI-TOF/TOF MS/MS using eggs obtained on days 0 and 5 revealed differential abundance of egg white proteins by Stress: transiently expressed in neural precursors (TENP), hemopexin (HPX), IgY-Fcυ3-4, and extracellular fatty acid-binding protein (Ex-FABP) were decreased while ovoinhibitor and ovalbumin-related protein X (OVAX) were increased on days 5 vs 0 ($P \leq 0.05$). Expression of mRNAs and proteins was also significantly modulated in the magnum of hens in Stress on day 14 ($P \leq 0.05$). In conclusion, the current study provides the first evidence showing that dietary corticosterone modulates protein abundance in the egg white in laying hens, and it suggests that environmental stress can differentially modify expression of egg white proteins in laying hens.

KEYWORDS: *laying hens, egg white proteins, stress, corticosterone, two-dimensional gel electrophoresis, MALDI-TOF/TOF MS/MS*

INTRODUCTION

The contents of eggs in oviparous species, including chickens, are important in developing embryos. Sealed from the external environment, successful development and growth of the embryo are solely dependent upon the resources provided by the mother hen at the time of egg formation. Increasing evidence has shown that maternal resources are transferred to avian eggs, which can affect offspring growth, phenotype, and fitness.[1,2] The environmental conditions, under which the hen is reared, can therefore have a critical impact on not only laying rate and egg quality but also the quality of chicks.[1−3]

Stressful environments can cause enhanced release of hormones such as adrenocorticotrophin (ACTH) and corticosterone in hens which lay eggs with high corticosterone content.[2,4] Treatments with these hormones have been shown to cease egg production[5] or to delay the onset of egg laying with shortened duration of peak production.[3] Consistent with these findings, weights of the oviduct and ovary are profoundly decreased in these hens.[3,5] Furthermore, the number of large follicles declined in their ovaries while that of atretic follicles is remarkably increased,[5] implying stress-related changes in concentrations of ovarian hormones.[4]

Indeed, elevated corticosterone induced by either corticosterone treatment or stressful environment causes reduced plasma concentrations of testosterone, progesterone, and estrogen[1] and decreased testosterone concentrations in ovarian large follicles,[4] leading to low-quality offspring.[2] Maternal corticosterone has been shown to reduce the fertility and hatchability of eggs while increasing the numbers of early dead embryos[6] and to produce offspring that are smaller at hatching, less competitive, and less fearful, suggesting that hens being raised in stressful situations are likely to produce unhealthy chicks.[7]

Findings from both in vitro and in vivo studies have so far shown that protein synthesis in the oviduct and ovary of hens is influenced by experimental conditions, including stressful environments. Heat stress, for example, down-regulated ovarian mRNA expression of steroidogenic enzymes.[8] Stress such as molting and immune challenges has been also shown to be able to modulate gene expression in the ovary and oviduct of hens.[9,10] Furthermore, the chicken oviduct has receptors for steroid hormones[11] which have long been known to regulate the expression of mRNAs and proteins of the egg white in the oviduct.[11,12] It has been shown that dexamethasone, a synthetic glucocorticoid, causes a rapid, reversible reduction in c-myc mRNA level in the oviducts of estrogen-treated, immature chickens.[13]

Little is known, however, about whether stressful environments affect protein abundance in the egg white. In the present study, we investigated using 2DE with MALDI-TOF/TOF MS/MS the effects of dietary corticosterone as a stress model on the expression of egg white proteins in laying hens. We also determined expression of mRNAs and proteins in the oviductal magnum of these hens. Our results show that dietary corticosterone affects protein abundance in the egg white of laying hens.

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MATERIALS AND METHODS

Animals and Samples Preparation. Forty, 47 week-old Single Comb Hy-Line Brown Leghorn hens, purchased from Sangol Egg Farm (Sancheong, Korea), were housed in individual cages in a room with a light regimen of 15 h light and 9 h dark (lights on at 6:00 AM) and an ambient temperature of 20 ± 2 °C during the entire experimental period. A commercial layer diet and tap water were provided *ad libitum*. This study was approved by the Institutional Animal Use and Care Committee of Gyeongsang National University (GAR-101020-X0007).

After adaptation to the experimental environment for the first 14 days, hens were divided into two groups, each of which was provided for the next 14 days with either ethanol (Control) or corticosterone (Stress)-containing diet at 30 mg/kg.[3,14] A commercial layer diet (Nonghyup Feed Co., Ltd., Seoul, Korea), which was used as a basal diet for the adaptation, was mixed for 30 min in a mixer with ethanol (95%) with or without corticosterone (92%, Sigma) at 2.61 mg/mL to produce either Stress or Control diet, respectively. Therefore, ethanol in the diet (12.5 mL/kg) was completely evaporated at the end of the 30 min mixture.

For each hen, feed intake, egg production and egg weight were monitored daily in the morning and body weight measured weekly throughout the experimental period. Eggs, laid by the same laying hens on 0 and 5 days (days 0 vs 5) after the initiation of providing hens with the experimental diets, were selected for the ongoing proteomic analysis as egg production started to decrease around day 5 by 30% (Figure 1). Immediately after being manually separated from the egg

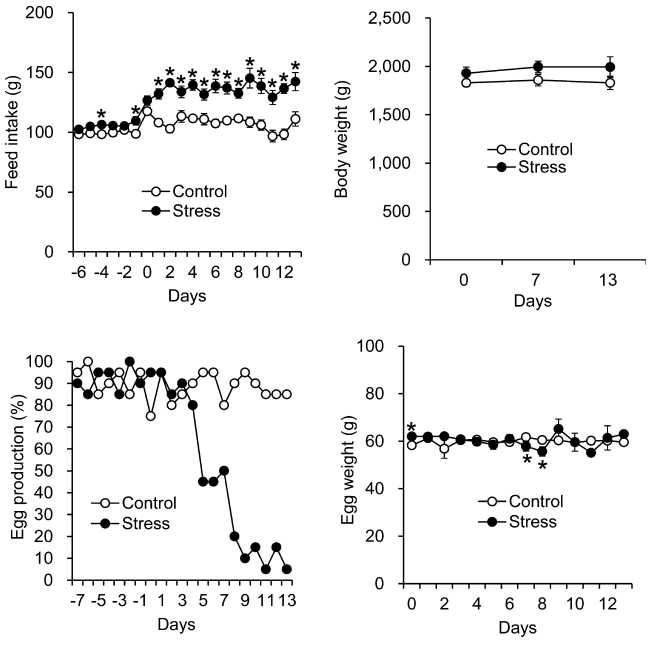

Figure 1. Feed intake (g), body weight (g), egg production (%), and egg weight (g) in laying hens. Hens were acclimatized to the experimental environment for the first 2 weeks (days −14 to 0) and then were divided into two groups to receive either ethanol- (Control) or corticosterone- (Stress) containing diet at 30 mg/kg for the next 2 weeks (days 0−14), respectively. Data are means ± SEM (n = 20 per group). *$P \leq 0.05$ vs Control.

yolk, the egg white was freeze-dried using a lyophilization freeze-dryer (#PVTFD50A, Ilshin Bio Base, Dongduchen, Korea) and then stored at −80 °C. Hens, which had a mean egg production of higher than 90% and their egg weights of being higher than 60 g during the adaptation period (63.4 ± 0.46 g in average), were selected for the study.

At the end of the entire experimental period (day 14), hens were killed using a guillotine and blood was collected from the trunk into 10 mL tubes treated with sodium heparin (BD, Franklin Lakes, NJ, USA) followed by centrifugation at 2 000*g*, 4 °C for 10 min. Plasma obtained was stored at −80 °C for further analysis. Organs including the oviduct were sectioned off, frozen in liquid nitrogen, and stored at −80 °C for later analysis. The magnum was cut into three parts of similar length before being stored at −80 °C, and the middle one was used for subsequent analysis.

Corticosterone Assay. Corticosterone concentrations in plasma were determined in duplicates each sample by ELISA using a kit (#ADI-901−097, Enzo Life Sciences, Farmingdale, NY, USA), according to the manufacture's instruction. Optical density was measured at 405 nm using a Multiskan FC Microplate Photometer (#51119000, Thermo Scientific, Waltham, MA, USA). Inter- and intra-assay coefficients of variations of this assay were 8.2% and 8.4%, respectively.

Reagents and Apparatus. Sodium dodecyl sulfate (SDS), phenyl methanesulfonyl fluoride (PMSF), tris(hydroxymethyl)aminomethane (Tris), urea, thiourea, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS), dithiothreitol (DTT), glycerol, bromophenol blue, isopropanol, silver nitrate, sodium thiosulfate pentahydrate and iodoacetamide were purchased from Amresco (Cochran, GA, USA); protease inhibitor cocktail from Roche (Indianapolis, IN, USA); agarose from GeorgiaChem (Suwanee, GA, USA); trypsin from Promega (Fitchburg, WI, USA); potassium ferricyanide, ammonium bicarbonate (ABC), ∂-cyano-4-hydroxycinnamic acid (CHCA), trifluoroacetic acid (TFA), formaldehyde and ethidium bromide (EtBr) from Sigma (St. Louis, MO, USA); 2-D Quant kit and immobilized pH gradient (IPG) buffer from GE Healthcare Life Sciences (Little Chalfont, UK); PrimeScript first strand cDNA synthesis kit from Takara (Ootsu, Japan); SYBR Green PCR Master Mix and Sequazyme Peptide Mass Standard Kit (Calibration 1) from Applied Biosystems (ABI, Foster City, CA, USA); IPG strip (pH 4−10 Nonlinear (NL), 180 × 3 × 0.5 mm) from Genomine (Pohang, Korea); Gradi-Gel II gradient PAGE analysis kit from Elpis (Daejeon, Korea); methanol, acetic acid and acetonitrile (ACN) from DaeJung (Siheumg, Korea); and Easy-spin total RNA extraction kit from Intron (Sungnam, Korea).

Extraction of Solubilized Proteins from Egg White for Proteomics. Lysis buffer A consisted of 1% SDS, 1 mM PMSF, protease inhibitor cocktail, and 100 mM Tris-HCl (pH 6.8), and lysis buffer B of 7 M urea, 2 M thiourea, 4% CHAPS, 0.1 M DTT, and 40 mM Tris-HCl (pH 6.8). One gram of dried egg white was ground into fine powder using a mortar and a pestle. Twenty mg of powdered egg white was dissolved in 25-fold volume lysis buffer A of the weight of egg white (v/w) and then dissolved in the same volume of lysis buffer B as lysis buffer A. The solution was shaken gently at room-temperature (RT) for 1 h, followed by sonication with a 2 s-on and 9 s-off cycle for five times. After incubation at RT for 1 h, this solution was centrifuged at 15 000*g*, 4 °C for 20 min to remove debris. The supernatant was incubated at 4 °C for 1 h and then the process was repeated twice to remove debris. The solubilized protein extracts were quantified using a 2-D Quant kit (#80-6483-56, GE).

SDS-PAGE. Egg white protein (10 μg) was diluted in a buffer (2% SDS, 10% glycerol, and 125 mM Tris-HCl (pH 6.8)), which was loaded on 4−20% premade SDS polyacrylamide gels (#84713, Thermo Scientific) at 20 mA, RT for 3 h until the bromophenol blue dye, containing a standard protein marker (#161-0374, Bio-Rad, Hercules, CA, USA) of 10−250 kDa, disappeared from the bottom of the gel.

Two-Dimensional Electrophoresis (2DE). Egg white protein (200 μg) was mixed in rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2.5% DTT, 10% isopropanol, 5% glycerol, and 2% IPG buffer) to a total volume of 450 μL. The sample was loaded onto a swelling tray in which IPG strips (pH 4−10 NL) were allowed to be rehydrated overnight. The first dimensional isoelectric focusing (IEF) was then performed using an IEF unit (#28926930, Ettan IPGphor 3, GE) at 20 °C, 50 V for 30 min, 100 V for 30 min, 250 V for 1 h, 8 000 V gradient for 9 h and 8 000 V for 6 h. The IPG strips were placed for 15 min in an equilibration solution (6 M urea, 30% glycerol, and 2% SDS, and 50 mM Tris-HCl (pH 8.8)) containing 1% DTT, and then

Table 1. Primer Information Used in the Present Study

Full name (protein and gene names)	Gene symbols	Accession no.	Forward (5′ → 3′) Reverse (5′ → 3′)	Size
Ovalbumin	SERPINB14	NM_205152.1	TTCCTGGGTAGAAAGTCAGACAAAT	100
			GACAATGGCATTAACCAGAACCA	
Ovalbumin-related protein Y (OVAY)	SERPINB14B	NM_001031001.1	TGGCCTTGGGAATGACAGA	100
			GAACACCCCATGGACAGCAT	
Ovotransferrin	Ovotransferrin	NM_205304.1	CAGTGATGGCAGAACGATATGAC	112
			TTCCAGTTGACGTTGCTGTCTT	
Extracellular fatty acid -binding protein (Ex-FABP)	EX-FABP	NM_205422.1	GGAGGACCTTGCACATGATGA	100
			GTGTAGTTCCGCTCCCTAGCA	
Transiently expressed in neural precursors (TENP)	TENP	NM_205026.1	CCAAACGAGCAGCTGATGTC	103
			TGCCCTGCTTGGAGAACAC	
Ovoinhibitor	OIH	NM_001030612.1	ACCAGCGTTGCCAAAAAGC	111
			GCAGGCCACCACCTGTCT	
Hemopexin (HPX)	Hemopexin	M37319.1	AGCGCGTGGACTTGACAAA	107
			GCAGCAGGTAGATGCCATCA	

treated with the same equilibration solution containing 2.5% iodoacetamide for 15 min. The second dimensional separation was performed on 7.5% SDS polyacrylamide gels using a Gradi-Gel II gradient PAGE analysis kit (#EBA-1056, Elpis). The gels were placed into a Protean II XL for 2DE (#165−3188, Bio-Rad) at 20 mA, RT for 18 h until the bromophenol blue dye, with the standard protein marker of 10−250 kDa (Bio-Rad), disappeared from the bottom of the gel. After the electrophoresis, the gels were fixed in a fixation solution (38% water, 50% methanol, and 12% acetic acid) overnight and stained in a silver nitrate solution as previously described.[15] Gel images were scanned using an imaging densitometer (#GS-800, Bio-Rad), and the intensity of protein spots from 3 gel images each group was processed using an image master (#28−9380−55, Image Master 2D Plantinum, v.7.0, GE), followed by the determination of molecular weights of the spots.

Matrix-Assisted Laser Desorption−Ionization Time-of-Flight/Time-of-Flight Mass Spectrometry/Mass Spectrometry (MALDI-TOF/TOF MS/MS). Selected spots were excised out from 2DE gels and subjected to in-gel digestion with trypsin as previously described.[15] Shortly, each of the spots predetermined by the image analysis was repeatedly picked up using a pipet tip into a 1.5 mL tube, and a destaining process was performed to remove the silver dye. Individual protein spots, 50 μL of 30 mM potassium ferricyanide, and 50 μL of 0.1 M sodium thiosulfate pentahydrate were vortexed for 1 min and spun down to remove the supernatant. To break the disulfide bonds, the samples were incubated in 0.1 mL of 10 mM DTT in 0.1 M ABC at 56 °C for 45 min and then the supernatant was removed after centrifugation. For alkylation, they were incubated again in 0.1 mL of 55 mM iodoacetamide in 0.1 M ABC at RT for 30 min, and then spun down to remove the supernatant. Individual precipitates were incubated with 5 μL of 50 ng trypsin in order to cleave peptide chain mainly at the carboxyl side of the amino acids lysine or arginine in 25 mM ABC at 37 °C overnight for in-gel digestion and spun down to collect the supernatant. The digested products were then dried using a vacuum concentrator (Ecospin 3180C, Hanil Science Industrial, Incheon, Korea) and stored at −20 °C. The peptides, dissolved in 1 μL of matrix solution (0.05 g CHCA in 0.1% TFA/50% ACN), were eluted onto a MALDI plate (#1016629, ABI), and was air-dried in a clean bench. MS and MS/MS analyses were performed on an ABI 4800 Plus TOF-TOF Mass spectrometer (ABI) using a mass standard kit (calibration mixture 1, ABI), according to the method described by Kwon et al.[15] The spectrometer used a 200 Hz ND:YAG laser operating at 355 nm and air as a collision gas. At least, ten most intensive ionized peptides per MALDI spot, with signal/noise ratios >25, were selected for subsequent MS/MS analysis in 1 kV mode and 1 000−1 500 consecutive laser shots. MS/MS spectrum data were searched against the UniProt Knowledgebase (UniProtKB) by ProteinPilot (v.3.0) in combination with MASCOT as the database search engine and with peptide and fragment ion mass tolerance of 50 ppm. Both carbamidomethylation of cysteine and oxidation of methionine were taken into account during the search of the peptides. High confidence identifications had statistically significant search scores which were equivalent to the expected values on the MASCOT at $P \leq 0.05$ and were consistent with the pI and MW of the individual proteins, which accounted for the majority of ions present in the mass spectra.

mRNA and Protein Expression Analysis of the Magnum. Total RNA was isolated from homogenized magnum tissue using an Easy-spin total RNA extraction kit (#17221, Intron), according to the manufacturer's protocol. One hundred mg of frozen magnum tissue was homogenized in 1 mL of lysis buffer (Intron). Each of the homogenized samples was mixed with 200 μL of chloroform and then centrifuged at 13 000 rpm, 4 °C for 10 min to harvest the supernatant. Four hundred μL of the supernatant was mixed with 400 μL of a binding buffer (Intron) by inverting and spun down, which was incubated at RT for 1 min and loaded into a spin column (Intron). The spin column was centrifuged at 13 000 rpm, RT for 30 s, and the eluted buffer was discarded. Seven hundred μL of washing buffer (Intron) was loaded into each column and centrifuged again in the same way as before. After washing, the column membrane was dried by centrifugation at 13 000 rpm, RT for 1 min, soaked with 50 μL of the elution buffer (Intron) and then centrifuged at 13 000 rpm to extract RNA. The total amount of RNA was determined by measuring optical density at 260 nm using a Nanodrop2000C spectrophotometer (Thermo Scientific). RNA quality was assessed by loading 2.5 μg of each RNA sample on a 1% agarose gel with formaldehyde and EtBr to visualize the bands of 28S and 18S rRNA.

For magnum tissues, proteomics was performed based on the methods similar to those for egg white with some modification: magnum tissues (100−200 mg) were homogenized in lysis buffers A and B of 10-fold magnum volume, followed by incubation, sonication and centrifugation to obtain solubilized protein. Protein (150 ug) was loaded onto IPG strip (pH 4−7 L) and 12% SDS polyacrylamide gels, followed by MALDI-TOF/TOF MS/MS analysis.

Reverse Transcription (RT) and Real-Time Quantitative PCR (qPCR). Relative expressions of each gene encoding the proteins differentially expressed in the egg white were determined in the magnum of the oviduct. Primer sequences, shown in Table 1, were designed with software (Primer Express, v. 3.0.1, ABI), in which glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a housekeeping gene. Two μg of RNA was reverse-transcripted using a PrimeScript first strand cDNA synthesis kit (#6110A, Takara) in a final volume of 20 μL according to the manufacturer's protocol. qPCR was then performed using a Step One Plus Real-Time PCR System (ABI) with Power SYBR Green PCR Master Mix (#4368702, ABI). Thermal cycling had 3 stages which were 1) activation of DNA polymerase at 95 °C for 10 min, 2) fluorescent detection of amplification by 40 cycles at 95 °C for 15 s and at 60 °C for 1 min, and 3) fluorescent

detection of melting curve at 95 °C for 15 s, 60 °C for 1 min, with a heating rate of 0.5 °C/s between 60 °C ~ 95 °C, and 95 °C for 15 s.

Statistical Analysis. All experimental data were represented as mean ± SEM. Student's *t*-test was performed using Microsoft Excel program to assess differences between Control and Stress at $P \leq 0.05$. Feed intake, body weight and egg production were measured in 20 hens each group to estimate mean values of each parameter. For corticosterone concentrations, mean values were calculated from 8 hens each group. For 2DE analysis, mean values of each spot were calculated from triplicated data in egg white samples and magnum tissues. Protein spots, which were differentially expressed at least 1.2 fold between days 0 and 5 in each group, were selected from 2DE gels, and statistically analyzed by a *t*-test ($P \leq 0.05$). Relative mRNA expression in magnum samples collected from 8 hens each group was measured in duplicates based on the $2^{-\Delta\Delta CT}$ method, and differences between both groups were analyzed by *t*-test.

RESULTS

Animal Study. No hens were found dead from any of the dietary treatments during the whole period of the experiment. During the adaption period (days −7−0), feed intake, egg production, and egg weight were relatively constant. Stress resulted in significant increase in mean-daily feed intake compared with Control (days 1−7: 109 ± 1 g for Control vs 136 ± 2 g for Stress, $P \leq 0.0001$; days 8−13: 105 ± 2 g for Control vs 137 ± 2 g for Stress, $P \leq 0.0001$). No differences were, however, detected in body weight between the two groups although it was slightly higher in the Stress group throughout the entire experimental period (Figure 1). Egg production was drastically decreased to approximately 30% in Stress on day 5 while Control had about 90% (days 1−7: 89 ± 3% for Control vs 70 ± 8% for Stress, $P \leq 0.05$; days 8−13: 88 ± 2% for Control vs 12 ± 2% for Stress, $P \leq 0.0001$). Dietary corticosterone resulted in a significant decrease in the number of follicles equal to or greater than 10 mm in diameter and in increased corticosterone concentrations in plasma ($P \leq 0.05$) (Figure 2).

Differential Protein Abundance in Egg White of Treated and Untreated Hens (Day 5 after Treatment). Because egg production was sharply reduced around 4 days (45%) after hens started to consume dietary corticosterone while no changes were detected between the two groups during the adaptation period (Control: 89 ± 3% vs Stress: 92 ± 2%, $P \leq 0.47$, for the second week of the adaptation period), eggs produced on day 5 were used for subsequent proteomic analysis. Protein profiles of the egg white were significantly changed in the eggs laid between days 0 and 5 in Stress whereas they remained unchanged in Control (Figures 3−5). SDS-PAGE analysis showed a drastic reduction in protein bands between 37 and 50 kDa and between 50 and 75 kDa on day 5 compared with day 0 in Stress, whereas such changes were not seen in Control (Figure 5). These findings were confirmed using 2DE analysis (Figure 4).

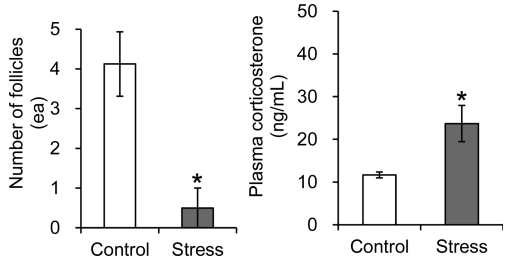

Figure 2. Number of follicles (≥10 mm in diameter) and plasma concentrations of corticosterone (ng/mL) in laying hens. Laying hens were acclimatized to the experimental environment for the first 2 weeks (days −14 to 0) and then were divided into two groups to receive either ethanol- (Control) or corticosterone- (Stress) containing diet at 30 mg/kg for the next 2 weeks (days 0−14), respectively. The number of follicles equal to or greater than 10 mm in diameter was counted at the end of the experiment (n = 8 per group), and blood obtained from the trunk of the hens was determined for plasma corticosterone concentrations by ELISA (n = 8 per group). Data are means ± SEM. *$P \leq 0.05$ vs Control.

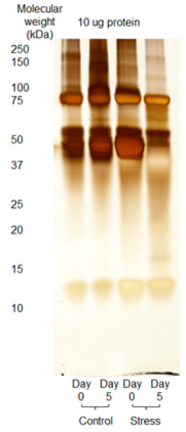

Figure 3. SDS-PAGE analysis of egg white proteins of hens. Laying hens were acclimatized to the experimental environment for the first 2 weeks (days −14 to 0) and then were divided into two groups to receive either ethanol- (Control) or corticosterone- (Stress) containing diet at 30 mg/kg for the next 2 weeks (days 0−14), respectively. Eggs laid by the same hens on days 0 and 5 in each group were used for proteomic study. Egg white protein samples (10 μg) were loaded on 4−20% SDS polyacrylamide premade gel and stained with silver nitrate staining.

Thirty-four protein spots in Stress were up- or down-expressed at least 1.2-fold compared with Control (Figure 4). Eight protein spots (#6−12 and 18) were significantly up-expressed on day 5 ($P \leq 0.05$) but six (#2, 15, 22, 27, 28, and 29) were down-expressed ($P \leq 0.05$) in Stress. By contrast, such change was detected in the only one protein spot (#19) from Control, which was up-expressed on day 5 compared with day 0 ($P \leq 0.05$).

From 34 protein spots, 9 proteins were identified through MALDI-TOF/TOF MS/MS analysis and the data mining based on subsequent bioinformatics: ovoinhibitor, ovalbumin-related protein X (OVAX), transiently expressed in neural precursors (TENP), hemopexin (HPX), ovotransferrin, IgY-Fcυ3-4, extracellular fatty acid-binding protein (Ex-FABP), ovalbumin-related protein Y (OVAY), and ovalbumin. Of nine spots (#3−11) that were estimated as ovoinhibitor, 6 spots (#4−8 and 11) were determined as ovoinhibitor. While expression of this protein was significantly increased over 5 days in egg white in Stress ($P \leq 0.05$), no such significant changes were detected in Control (Tables 2 and 3). Three spots (#12, 18, and 24) were determined as OVAX, and corticosterone-treatment resulted in significant increase in 2 spots ($P \leq 0.05$) whereas no significant changes were detected in Control. Eight spots were considered to be TENP: among them, 4 spots (#17, 18, 27, and 28) were confirmed as TENP. Corticosterone treatment up-regulated 1 spot (#18) and down-regulated the other three spots (#17, 27, and 28) but no

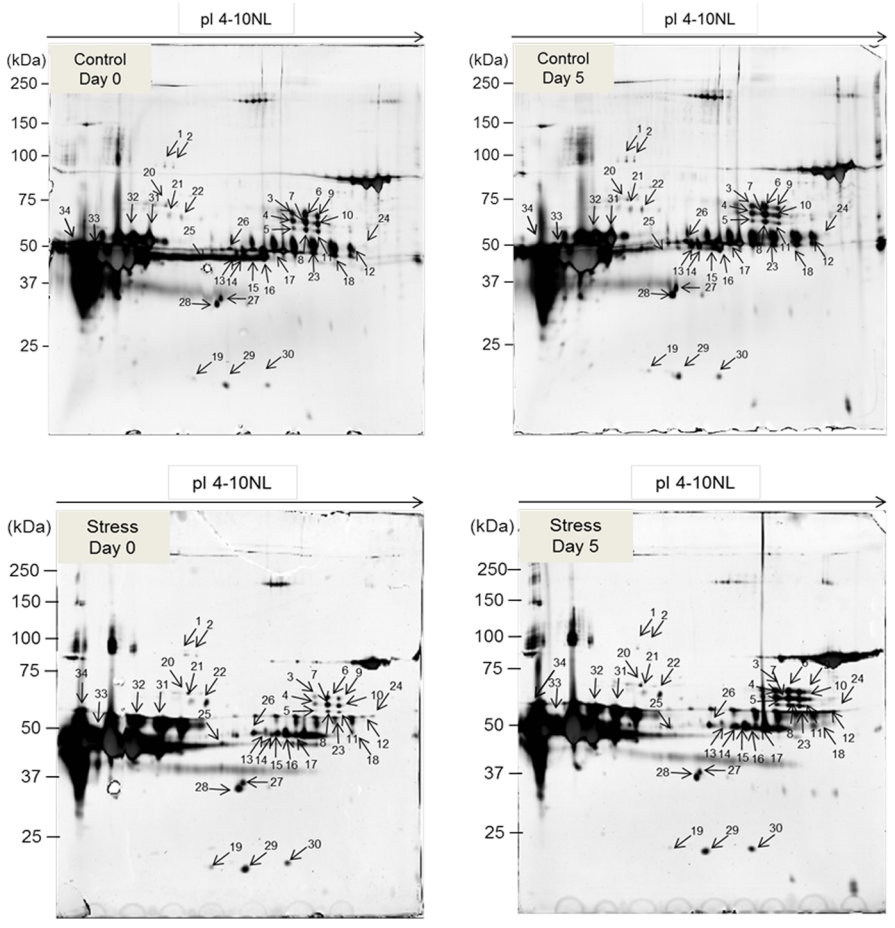

Figure 4. Representative two-dimensional electrophoresis (2DE) of hen's egg white proteins. Laying hens were acclimatized to the experimental environment for the first 2 weeks (days −14 to 0) and then were divided into two groups to receive either ethanol- (Control) or corticosterone- (Stress) containing diet at 30 mg/kg for the next 2 weeks (days 0−14), respectively. Eggs laid by the same hens on days 0 and 5 in each group were used for proteomic study. The 2DE gel images of egg white were obtained from eggs laid on days 0 and 5 by the same laying hens treated with Control or Stress. Egg white protein samples (200 μg) were loaded on 7.5% SDS polyacrylamide gels using a Gradi-Gel II gradient PAGE analysis kit and stained with silver nitrate staining. Protein spots determined are ovoinhibitor (#4−8 and 11), OVAX, ovalbumin-related protein X (#12, 18, and 24), TENP, transiently expressed in neural precursors (#17, 18, 27, and 28), HPX, hemopexin (#21 and 22), ovotransferrin (#24), IgY-Fcυ3-4 (#27), Ex-FABP, extracellular fatty acid-binding protein (#29), OVAY, ovalbumin related protein Y (#31 and 32) and ovalbumin (#33 and 34), whereas spots are estimated for ovoinhibitor (#3, 9, and 10), TENP (#13, 14, 15, and 16), OVAY (#23 and 26), CAL, chondrogenesis-associated lipocalin (#19 and 30), and HPX (#20) based on recent publications. However, estimation for proteins was not possible for spots #1, 2, and 25, due to either lack of reference data or ambiguity of reference.

significant change was found in Control. Of three spots estimated as HPX, 2 spots (#21 and 22) were confirmed. HPX expression was significantly reduced by Stress on days 5 over 0 ($P \leq 0.05$), but no such changes were seen in Control. One spot (#27) was identified as IgY-Fcυ3-4, expression of which was significantly reduced in Stress ($P \leq 0.05$) but not in Control. Similarly, another spot (#29) corresponding with Ex-FABP was significantly down-expressed by corticosterone-treatment ($P \leq 0.05$) but not by control. Among 4 spots which were estimated as OVAY, two spots (#31 and 32) were verified as OVAY, and one (#32) spot was significantly increased in Control ($P = 0.06$), but not in Stress. Although 2 spots (#33 and 34) were identified to be ovalbumin protein, no significant changes were found in both groups over 5 days. Two spots (#19 and 30), which were estimated to be chondrogenesis-associated lipocalin (CAL), were significantly increased in Control ($P \leq 0.05$) but not in Stress. One spot (#24), which was determined as ovotransfferin, was detected in Stress but not in Control.

Proteins "determined" were identified based on proteomic analysis in the present study (Table 3) whereas proteins "estimated" were found to have similar molecular weight (MW) and pH in the 2DE gels to those reported in recent papers.[16,17] Spots #18, #24, and #27 were determined to contain each of two different proteins (OVAX and TENP; OVAX and ovotransferrin; and TENP and IgY-Fcυ3-4, respectively) (Tables 2 and 3).

Some of the proteins show differences between the theoretical (MWt) and observed (MWo) molecular weights (Table 2). The latter was based on the analysis of 2D gels using an image analyzer. The MWo values for some proteins were nearly the same as (#4, 5, 7, 8, 11, 17, 18, and 29), much smaller than (#24, 27, 28, and 34), or much larger than (#6, 12, 18, 21, 22, 24, 27, 31, 32, and 33) those of MWt.

Differential Protein Abundance in Magnum of Treated and Untreated Hens (Day 14). Of the proteins differentially abundant in the egg white on day 5 there were 6 proteins (ovalbumin, ovoinhibitor, ovotransferrin, hemopexin, IgY-Fcυ3-4, and OVAY) which were also differently abundant

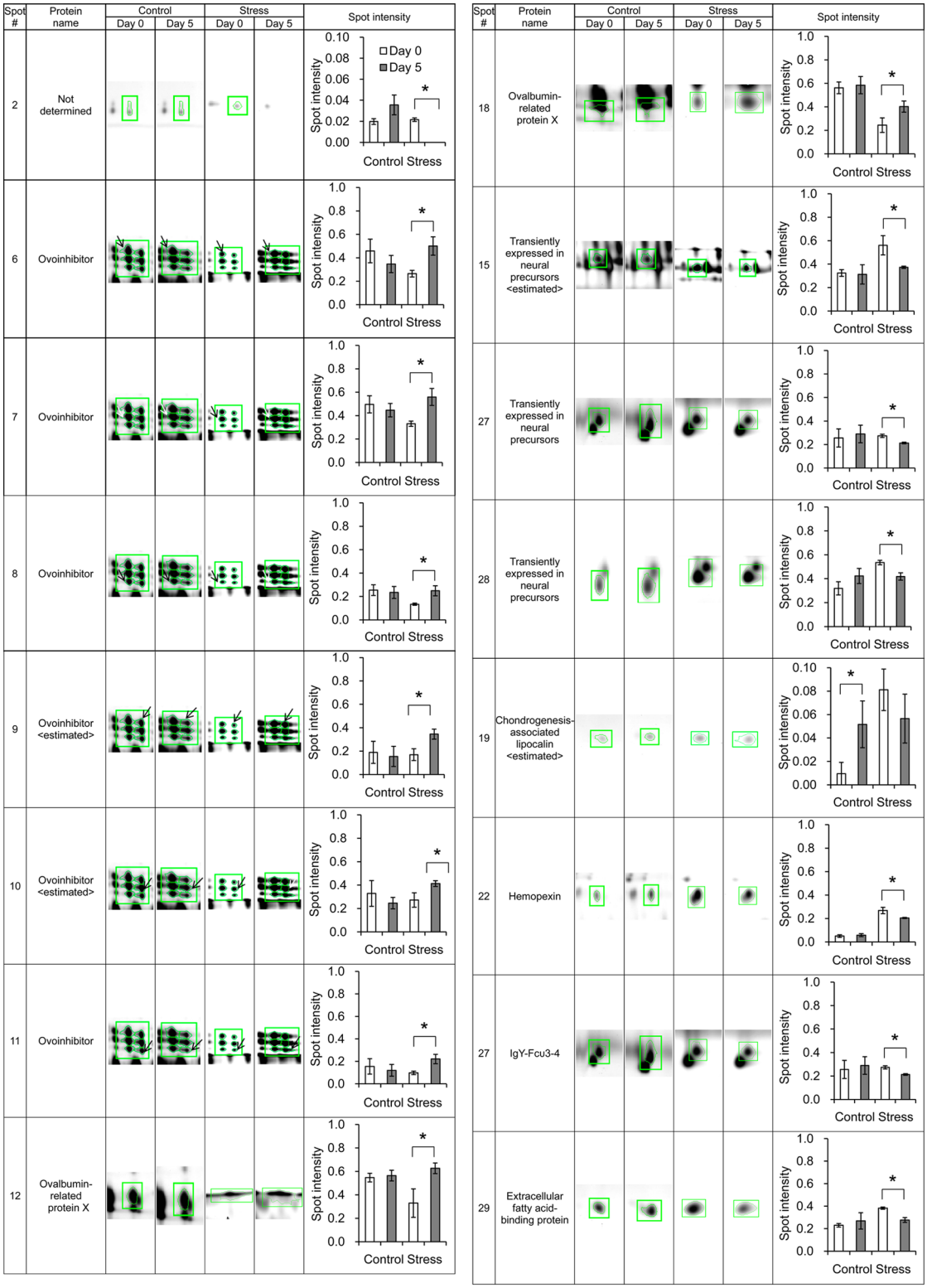

Figure 5. Differential expression of egg white proteins in spots derived from two-dimensional electrophoresis (2DE). Laying hens were acclimatized to the experimental environment for the first 2 weeks (days −14 to 0) and then were divided into two groups to receive either ethanol- (Control) or corticosterone- (Stress) containing diet at 30 mg/kg for the next 2 weeks (days 0−14), respectively. Eggs laid by the same hens on days 0 and 5 in each group were used for proteomic study. The 2DE gel images of egg white were obtained from eggs laid on days 0 and 5 by the same laying hens treated with Control or Stress. Individual spots on the gels were matched between the 2 days and between two treatments, and their intensities equal to or greater than 1.2-fold in expression were compared between days 0 and 5 in each treatment and were shown in graphs. The spot numbers are the same as those specified in Figure 4. Data are means ± SEM (n = 3 per day per group). *All spots identified show statistically significant differences at $P \leq 0.05$ by t-test.

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Table 2. Proteins Identified by Two-Dimensional Electrophoresis (2DE), and Matrix-Assisted Laser Desorption-Ionization Time-of-Flight/Time-of-Flight Mass Spectrometry/Mass Spectrometry (MALDI-TOF/TOF MS/MS) Followed by Bioinformatics

Spot no.[a]	Protein name[b]	Accession no.[c]	MWt[d] /MWo[e] (kDa)	pI	Score[f]	Coverage (%)[g]	Matched peptides[h]	Matched peptide sequences[i]
4	Ovoinhibitor	P10184	52/59	6.2	58	32	14	K.DGTSWVACPR.N/R.NLKPVCGTDGSTYSNECGICLYNR.E/R.EHGANVEK.E/K.HVTIDCSPYLQVVR.D/R.DGNTMVACPR.I/K.LHDGECK.L/R.TLVACPR.I/R.ILSPVCGTDGFTYDNECGICAHNAEQR.T/R.QEIPEIDCDQYPTR.K/K.VSPICTMEYVPHCGSDGVTYSNR.C/R.CFFCNAYVQSNR.T
5	Ovoinhibitor	P10184	52/54	6.2	47	23	14	K.DGTSWVACPR.N/R.EHGANVEK.E/K.HVTIDCSPYLQVVR.D/R.DGNTMVACPR.I/K.LHDGECK.L/R.TLVACPR.I/R.QEIPEIDCDQYPTR.K/R.CREEVPELDCSK.Y/K.VSPICTMEYVPHCGSDGVTYSNR.C/R.CFFCNAYVQSNR.T
6	Ovoinhibitor	P10184	52/63	6.2	46	22	13	K.DGTSWVACPR.N/R.NLKPVCGTDGSTYSNECGICLYNR.E/R.EHGANVEK.E/K.HVTIDCSPYLQVVR.D/R.DGNTMVACPR.I/K.LHDGECK.L/R.TLVACPR.I/R.QEIPEIDCDQYPTR.K/R.CREEVPELDCSK.Y/R.CEEDITK.E
7	Ovoinhibitor	P10184	52/58	6.2	362[d]	33	19	K.DGTSWVACPR.N/R.NLKPVCGTDGSTYSNECGICLYNR.E/K.HVMIDCSPYLQVVR.D/R.DGNTMVACPR.I/R.TLVACPR.I/R.ILSPVCGTDGFTYDNECGICAHNAEQR.T/K.CRQEIPEIDCDQYPTR.K/R.QEIPEIDCDQYPTRK.T/R.CREEVPELDCSK.Y/K.VSPICTMEYVPHCGSDGVTYSNR.C/R.CFFCNAYVQSNR.T
8	Ovoinhibitor	P10184	52/54	6.2	46	17	10	K.DGTSWVACPR.N/R.EHGANVEK.E/K.HVTIDCSPYLQVVR.D/R.DGNTMVACPR.I/K.LHDGECK.L/R.TLVACPR.I/K.SHDGRCK.E/K.VSPICTMEYVPHCGSDGVTYSNR.C/R.CFFCNAYVQSNR.T
11	Ovoinhibitor	P10184	52/53	6.2	37	20	11	K.DGTSWVACPR.N/R.NLKPVCGTDGSTYSNECGICLYNR.E/R.EHGANVEK.E/K.HVTIDCSPYLQVVR.D/R.DGNTMVACPR.I/K.LHDGECK.L/R.TLVACPR.I/R.QEIPEIDCDQYPTR.K/R.CFFCNAYVQSNR.T /
12	OVAX	P01013	26/52	5.1	79	30	7	K.TAFNAEDTREMPFHVTK.Q/K.ILELPFASGDLSMLVLLPDEVSDLER.I/K.VYLPQMK.I/K.HSPESEQFR.A/K.HNPTNTIVYFGR.Y
17	TENP	O42273	47/51	5.5	39	13	6	K.VADLWLSVVPEAGLR.L/R.LGIEVELR.V/R.VAPLHAVPMPVR.I
18	TENP	O42273	47/50	5.5	147[d]	23	10	R.APDCGGILTPLGLSYLAEVSKPHAEVVLR.Q/K.VADLWLSVIPEAGLR.L/R.LGIEVELR.I/K.ITQVGSLYHEDLPITLSAALR.S/R.VVLEEGR.A/R.MMISTAVIEDAELSLAASNVGLVR.A /
18	OVAX	P01013	26/50	5.1	103[d]	27	8	K.TAFNAEDTREMPFHVTK.Q/K.ILELPFASGDLSMLVLLPDEVSDLER.I/K.VYLPQMK.I/K.HSPESEQFR.A/K.HNPTNTIVYFGR.Y
21	HPX	Q90WR3	30/60	5.9	35	24	9	R.ERPWLDAGPCDAALR.W/R.YYCLQGTQFYR.F/R.FRPHSWEVLPGYPR.D/R.DLRDYFIPCPGR.G/R.GGHQLVEGYPR.A
22	HPX	Q90WR3	30/61	5.9	340[d]	48	18	R.ERPWLDAGPCDAALR.W/R.YYCLQGTQFYR.F/R.FRPHSWEVLPGYPR.D/R.DLRDYFIPCPGR.G/R.DYFIPCPGR.G/R.MYLIQGSQVSIYVSGR.G/R.GGHQLVEGYPR.A/K.ADAAFTCPGSAELYVITGDR.M/R.HADEPQPLPYDGVDGAMCTADGIYLLR.G
24	OVAX	P01013	26/52	5.1	108[d]	27	8	K.TAFNAEDTREMPFHVTK.Q/K.ILELPFASGDLSMLVLLPDEVSDLER.I/K.HSPESEQFR.A/K.HNPTNTIVYFGR.Y
24	Ovotransferrin	P02789	78/52	6.7	63	21	10	R.SAGWNIPIGTLLHR.G/K.AQSDFGVDTKSDFHLFGPPGK.K/K.GEADAVALDGGLVYTAGVCGLVPVMAER.Y/K.TDERPASYFAVAVAR.K/R.TAGWVIPMGLIHNR.T/R.TGTCNFDEYFSEGCAPGSPPNSR.L/K.YFGYTGALR.C/K.NLQMDDFELLCTDGR.R/K.DLTKCLFK.V
27	TENP	O42273	47/38	5.6	246[d]	24	10	M.GALLALLDPVQPTR.A/K.VADLWLSVIPEAGLR.L/K.ITQVGSLYHEDLPITLSAALR.S/R.VVLEEGR.A/R.MMISTAVIEDAELSLAASNVGLVR.A/R.AALLEELFLAPVCQQVPAWMDDVLR.E
27	IgY-Fcυ3-4	UPI000 18BF9C1	25/38	5.6	112[d]	30	4	R.NTGPTTPPLIYPFAPHPEELSLSR.V/R.AVPATEFVTTAVLPEER.T
28	TENP	O42273	47/36	5.6	232[d]	20	13	K.VADLWLSVIPEAGLR.L/K.ITQVGSLYHEDLPITLSAALR.S/R.VVLEEGR.A/R.MMISTAVIEDAELSLAASNVGLVR.A/R.AALLEELFLAPVCQQVPAWMDDVLR.E
29	Ex-FABP	P21760	20/24	5.6	351[d]	52	17	R.SEVAGKWYIVALASNTDFFLR.E/K.WYIVALASNTDFFLREK.G/R.ISFLGEDELEVSYAAPSPK.G/K.SYAVIFATR.V/R.TLHMMR.L/R.SREVSPTAMAIFR.K/R.EVSPTAMAIFR.K/R.ERNYTDEMVAVLPSQEECSVDEV.-
31	OVAY	P01014	44/52	5.2	638[d]	40	16	K.FCFDVFNEMK.V/K.VHHVNENILYCPLSILTALAMVYLGAR.G/K.VLHFDSITGAGSTTDSQCGSSEYVHNLFK.E/K.TFSVLPEYLSCAR.K/R.KFYTGGVEEVNFK.T/K.IAFNTEDTR.E/K.ILELPYASGDLSMLVLLPDEVSGLER.I/K.HSLELEEFRADHPFLFFIR.Y/R.ADHPFLFFIR.Y/R.YNPTNAILFFGR.Y
32	OVAY	P01014	44/52	5.2	645[d]	44	20	K.FCFDVFNEMK.V/K.VHHVNENILYCPLSILTALAMVYLGAR.G/K.VLHFDSITGAGSTTDSQCGSSEYVHNLFK.E/K.LYVDKTFSVLPEYLSCAR.K/R.KFYTGGVEEVNFK.T/R.QLINSWVEK.E/K.IAFNTEDTR.E/K.ILELPYASGDLSMLVLLPDEVSGLER.I/K.HSLELEEFRADHPFLFFIR.Y/R.ADHPFLFFIR.Y/R.YNPTNAILFFGR.Y
33	Ovalbumin	P01012	43/49	5.2	645[d]	38	19	R.DILNQITKPNDVYSFSLASR.L/R.YPILPEYLQCVK.E/R.GGLEPINFQTAADQAR.E/R.ELINSWVESQTNGIIR.N/K.AFKDEDTQAMPFR.V/R.VTEQESKPVQMMYQIGLFR.V/K.LTEWTSSNVMEER.K/K.ISQAVHAAHAEINEAGR.E/R.ADHPFLFCIK.H/K.HIATNAVLFFGR.C
34	Ovalbumin	P01012	43/21	5.2	488[d]	38	19	R.DILNQITKPNDVYSFSLASR.L/R.LYAEERYPILPEYLQCVK.E/R.GGLEPINFQTAADQAR.E/R.ELINSWVESQTNGIIR.N/K.AFKDEDTQAMPFR.V/R.VTEQESKPVQMMYQIGLFR.V/K.LTEWTSSNVMEERK.I/K.ISQAVHAAHAEINEAGR.E/R.ADHPFLFCIK.H/K.HIATNAVLFFGR.C

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Table 2. continued

[a]Spot numbers correspond to the labels in Figure 4. Spots #1, 2, 3, 9, 10, 13, 14, 15, 16, 19, 20, 23, 25, 26, and 30 were not determined due to the limit of either database availability or instrumental detection. [b]Abbreviations: OVAX, ovalbumin-related protein X; TENP, transiently expressed in neural precursors; HPX, hemopexin; Ex-FABP, extracellular fatty acid-binding protein; and OVAY, ovalbumin related protein Y. [c]Accession numbers are from the UniProt Knowledgebase (UniProtKB) databases. [d]Molecular weights in theory. [e]Calculated molecular weights from 2D gels using by ImageMaster 2D Platinum 7.0. [f]Score (MOWSE), used in MASCOT PMF (peptide mass fingerprint), represents MS data from the UniProtKB database. Scores greater than 84 are significant ($P \leq 0.05$). [g]Sequence coverage. [h]The number of both ionized and nonionized peptides identified using MALDI-TOF/TOF MS/MS were matched with the whole peptides. [i]The sequences of peptides identified using MALDI-TOF/TOF MS/MS were matched with the whole sequences.

in the magnum of Stress ($P \leq 0.05$) on day 14, whereas no significant changes were detected for Ex-FABP and TENP (Figure 6). Ovoinhibitor, ovalbumin, and IgY-Fcυ3-4 had at least two different spots which were significantly increased or decreased in the magnum whereas the expression of ovotransferrin and hemopexin was up-regulated. OVAY was decreased ($P \leq 0.05$), and no significant changes of both Ex-FABP and TENP were detected (Figure 6).

Differential Gene Expression in Magnum of Treated and Untreated Hens (Day 14). The genes, whose proteins were significantly up- or down-expressed in the egg white proteomic study ($P \leq 0.05$), were analyzed using qPCR in protein extracts of the magnum of the oviduct on day 14 (Figure 7). The seven genes encoding the proteins ovalbumin, OVAY, ovotransferrin, Ex-FABP, TENP, ovoinhibitor, and HPX were significantly down-expressed ($P \leq 0.0001$) in the magnum of laying hens treated with corticosterone compared with Control. One twenty-ninths for ovalbumin, 1/46 for OVAY, 1/12 for ovotransferrin, 1/23 for Ex-FABP, 1/33 for TENP, 1/16 for ovoinhibitor, and 1/49 for HPX genes were down-expressed in Stress against Control. The expressions of both the genes and proteins for HPX, TENP, Ex-FABP, OVAY, and ovalbumin were equally decreased. The gene for ovoinhibitor was down-regulated but its protein up-regulated.

DISCUSSION

Corticosterone Decreases Egg Production. Corticosterone, the primary stress hormone in avian species including chickens, stimulates gluconeogenesis for survival to provide the body with energy when chickens are under stress.[2,4] Stress can affect performance in quantitative and qualitative manners as well as health by causing endocrine, metabolic, physiological, and behavioral changes in chickens.[5,7] The results of the present study show that although laying hens treated with corticosterone consumed more feed and had higher plasma corticosterone concentrations than did control hens, body weight was not significantly different from Control ($P = 0.11$). In a sharp contrast to the current results, corticosterone treatments show different effects in laying hens: corticosterone injected subcutaneously at 2 mg/kg body weight twice a day caused significant reduction in feed intake and body weight,[18] whereas dietary corticosterone at 1.5 mg/bird/day enhanced feed intake while reducing body weight gain.[19] Administration of corticosterone via drinking water at 20 mg/L for 1 week at 7, 11, and 15 weeks of age resulted in reduced body weight in laying hens.[3] ACTH, which stimulates the secretion of corticosterone from the adrenal and was delivered via osmotic pumps, increased feed intake and body weight in laying hens.[5] The controversy among these results may have resulted from different methods such as age of birds and delivery and dosage of corticosterone.

In the present study Stress caused significant reduction in egg production with relatively constant egg weight while increasing plasma corticosterone concentrations. The reduced egg production can be accounted for in part by decreased numbers of ovarian follicles (≥10 mm in diameter) in this study and by increased numbers of atretic follicles and reduced weights of the oviduct and ovary.[3,5] A strong negative relationship has been reported between elevated corticosterone concentrations and reduced egg production in studies in which hens were treated with ACTH via osmotic pumps,[5] subcutaneous corticosterone injections,[18] or corticosterone administration by way of either feed[19] or drinking water.[3] Indeed, stressful

Table 3. Relative protein abundances that were increased (+) or decreased (−) in the egg white produced on day 5 by the same laying hens treated with Control or Stress, compared with those on day 0

			Control		Stress	
Protein name[a]	Spot no.	Determined or estimated[b]	Relative change[c]	*P*-value	Relative change	*P*-value
Ovoinhibitor	3	Estimated	+1.1	0.21	+2.3	0.17
	4	Determined	−1.1	0.30	+2.6	0.14
	5	Determined	−1.1	0.28	+2.0	0.18
	6	Determined	−1.3	0.20	+1.9	0.02
	7	Determined	−1.1	0.30	+1.7	0.02
	8	Determined	−1.1	0.39	+1.9	0.03
	9	Estimated	−1.2	0.40	+2.0	0.03
	10	Estimated	−1.3	0.26	+1.5	0.05
	11	Determined	−1.3	0.35	+2.3	0.02
OVAX	12	Determined	+1.1	0.39	+1.9	0.04
	18[d]	Determined	+1.1	0.40	+1.6	0.05
	24[d]	Determined	-	-	+1.9	0.16
TENP	13	Estimated	+1.4	0.10	−1.5	0.13
	14	Estimated	-	-	−1.6	0.25
	15	Estimated	−1.1	0.45	−1.5	0.04
	16	Estimated	+1.3	0.14	−1.5	0.08
	17	Determined	−1.1	0.39	−1.3	0.12
	18[d]	Determined	+1.1	0.40	+1.6	0.05
	27[d]	Determined	+1.1	0.38	−1.3	0.01
	28	Determined	+1.3	0.13	−1.3	0.02
HPX	20	Estimated	+1.1	0.38	−1.2	0.37
	21	Determined	+1.3	0.22	−1.2	0.30
	22	Determined	+1.2	0.33	−1.3	0.04
IgY-Fcυ3-4	27[d]	Determined	+1.1	0.38	−1.3	0.01
Ex-FABP	29	Determined	+1.2	0.31	−1.4	0.01
OVAY	23	Estimated	+1.1	0.14	+1.3	0.17
	26	Estimated	+1.2	0.26	−1.3	0.10
	31	Determined	+1.1	0.33	−1.2	0.10
	32	Determined	+1.1	0.06	−1.2	0.11
Ovalbumin	33	Determined	−1.1	0.40	−1.4	0.28
	34	Determined	+1.1	0.30	−1.3	0.19
Ovotransferrin	24[d]	Determined	-	-	+1.9	0.16
CAL	19	Estimated	+5.4	0.05	−1.4	0.21
	30	Estimated	+1.9	0.13	−1.3	0.22

[a]Abbreviations: OVAX, ovalbumin-related protein X; TENP, transiently expressed in neural precursors; HPX, hemopexin; Ex-FABP, extracellular fatty acid-binding protein; OVAY, ovalbumin related protein Y; and CAL, chondrogenesis-associated lipocalin. [b]Proteins "Determined" were those identified based on MALDI TOF/TOF MS/MS analysis followed by bioinformatics in the present study. In contrast, proteins "Estimated" have not been identified due to the detection limit of the mass spectrometry. Proteins of these spots were estimated based on both those reported in recent papers[16,17] and the observational molecular weight (MWo) from the 2D gels of the current study using an image master. Ovoinhibitor (#3, 9, and 10): 64, 61, and 56 kDa; TENP (#13−16): 51 kDa; HPX (#20): 66 kDa; OVAY (#23 and 26): 52 and 51 kDa; CAL (#19 and 24): 24 and 25 kDa, respectively. [c]Relative changes of proteins show the spot intensity of any given protein on day 5 against those on day 0 under the same treatment. [d]Spots #18 (OVAX and TENP), #24 (OVAX and ovotransferrin,) and #27 (TENP and IgY-Fcu3-4) were determined as two different proteins.

environment caused reduced plasma concentrations of testosterone, progesterone, and estrogen,[1] which resulted in decrease ovarian function[1] and low-quality offspring.[2] This proclivity suggests that various environmental stimuli enhancing the release of corticosterone in plasma can contribute to decreased egg production and quality.

Corticosterone Modulates Egg White Protein. The present study is the first to present evidence suggesting that dietary corticosterone can not only contribute to decreased egg production and quality but also modify the expression of proteins in the egg white. We found several proteins that differentially expressed in the egg white in response to dietary corticosterone: ovoinhibitor and OVAX were significantly increased while TENP, Ex-FABP, HPX, and IgY-Fcυ3-4 were decreased. With the exception of OVAY, however, such changes were not seen in Control, suggesting that the changes are treatment-specific.

The present results of proteomic analysis show that some spots were identified as two or more different proteins. There is one spot (#18) that was identified as both OVAX and TENP. As shown in Table 3, expression of the spots that were determined as OVAX protein was consistently increased in the egg white associated with stress. With the exception of spot #18, which was positively expressed, those that were determined as TENP protein were consistently negatively regulated. Therefore, it is plausible to consider, based on these data and the literature,[16] that spot #18 is OVAX protein rather than TENP. However, the spot (#24), which was determined as both OVAX and ovotransferrin, was positively expressed in the egg white by Stress whereas the spot (#27), which was

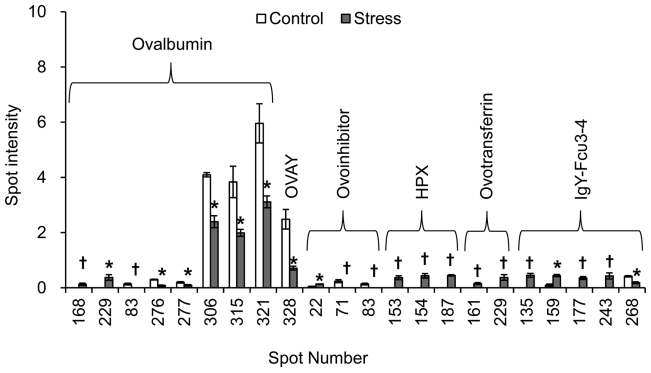

Figure 6. Differential expression of proteins in the magnum of the oviduct in hens. Laying hens were acclimatized to the experimental environment for the first 2 weeks (days −14 to 0) and then were divided into two groups to receive either ethanol- (Control) or corticosterone- (Stress) containing diet at 30 mg/kg for the next 2 weeks (days 0−14), respectively. The magnum obtained on day 14 was used for proteomic analysis. After individual protein spots on the gels derived from two-dimensional electrophoresis (2DE) were matched between two groups, their intensity was quantified and compared to identify protein spots that expressed equal to or greater than 1.2-fold, and is shown in graphs. Data are means ± SEM (n = 3 per group). Spots identified by ∗ show significant differences at $P \leq 0.05$ by t-test, and those identified by † were detected in only one group. Abbreviations: OVAY, ovalbumin related protein Y; and HPX, hemopexin.

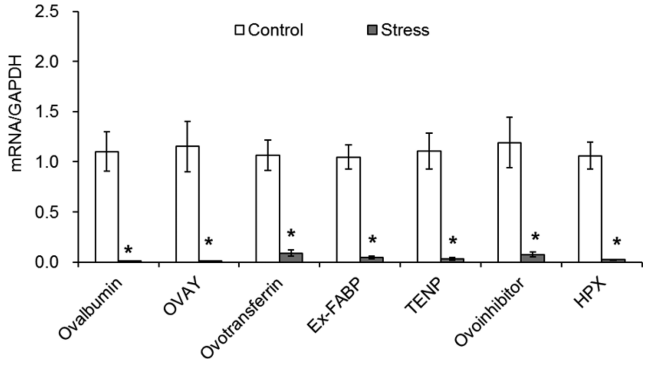

Figure 7. mRNA expression of ovalbumin, OVAY, ovotransferrin, Ex-FABP, TENP, ovoinhibitor, and HPX genes in the magnum of the oviduct in laying hens. Laying hens were acclimatized to the experimental environment for the first 2 weeks (days −14 to 0) and then were divided into two groups (n = 20 each) to receive either ethanol- (Control) or corticosterone- (Stress) containing diet at 30 mg/kg for the next 2 weeks (days 0−14), respectively. Hens were killed using a guillotine, and the magnum was cut off at the end of the entire experimental period. Relative mRNA expression of individual genes against GAPDH was determined in duplicate using qPCR. Data are means ± SEM (n = 8 birds each group). *$P \leq 0.0001$ vs Control. Abbreviations: OVAY, ovalbumin related protein Y; Ex-FABP, extracellular fatty acid-binding protein; and HPX, hemopexin.

determined as both TENP and IgY-Fcυ3-4, was negatively expressed. Due to the limit of data available, whether or not each of these spots has a single protein or includes two different proteins remains to be determined.

In the present study, MWo values for some spots were similar to, lower than, or higher than those for MWt, suggesting their possible degradation or polymerization during the preparation of proteins for the 2DE analysis, which is consistent with those reported by Guerin-Dubiard et al.[16] and Wang et al.[17]

Corticosterone Modulates Protein and Gene Expression in Magnum. Several differential proteins, with the exception of Ex-FABP, TENP, and OVAX, were common between the egg white and oviductal magnum of hens administered with dietary corticosterone. Furthermore, we also found that mRNA expression of the genes encoding these proteins was significantly modified in the magnum.

There are different patterns of expression among proteins in the egg white and genes and proteins in the magnum in the current study. This could be attributable to the differences in collection time between eggs and the oviduct: eggs laid on days 0 and 5 were used for proteomic analysis whereas mRNA and protein expression was determined using the oviduct obtained at the conclusion of the whole experiment (day 14), thus being highly likely to reflect different physiological and cellular events from those on day 5. The oviduct was visually regressed in these hens treated with dietary corticosterone for 14 days in this study, and differential expression of genes[9,10] and proteins[20] has been reported in the oviduct of hens subject to molting. Another explanation is that the time and process of transcription may be different among individual genes, after which translation for protein biosynthesis would occur.[21]

Ovoinhibitor. Ovoinhibitor is one of the 4 proteinase inhibitors found in the egg white that are ovostatin, ovomucoid, ovoinhibitor, and cystatin.[11] Distinct expression of ovoinhibitor mRNA was detected in the liver, magnum, and uterus of chickens, and the protein was up-regulated in the liver of prelaying hens (14−16 weeks of age) compared with laying hens (41 weeks).[22] Ovoinhibitor has MW of 48.6−49 kDa,[11] consists of 1.5% of total egg white protein,[11] and inhibits serine proteinase such as trypsin, chymotrypsin, and elastase.[23] Synthesis and secretion of ovoinhibitor are controlled by estrogen and progesterone,[11] as seen in other enzymes in the oviduct and ovary.[11]

In the current study, ovoinhibitor was significantly up-expressed ($P \leq 0.05$) in the egg white produced from hens treated with corticosterone, but its gene expression was down-regulated in the magnum by Stress ($P \leq 0.001$). Significant changes of this protein were also seen in the oviduct. Estrogen increases the transcription rate of ovoinhibitor in the liver,[11] although concentrations of estrogen were not measured in this study. A recent study shows that expression of 6 ovoinhibitor precursor spots was strongly increased in fertilized egg white on 7 days after hatching.[24] Discrepancy in expression patterns between the gene and protein of ovoinhibitor remains to be solved.

Ovalbumin, Ovalbumin-Related Protein X (OVAX), and Ovalbumin-Related Y (OVAY). Ovalbumin is the major protein in the egg white, consisting of 54% egg white protein.[25] Ovalbumin, OVAX, and OVAY proteins are encoded by three distinct genes, the ovalbumin, ovalbumin X, and ovalbumin Y genes, respectively, belonging to the ovalbumin gene family located within a 40 kDa region.[26] Ovalbumin is composed of 386 amino acids, alternatively named as allergen Gal d II, egg albumin, plakalbumin, or allergen=Gal d 2 (UniProtKB ID: P01012); OVAX of 232 amino acids, alternatively named as gene X protein (UniProtKB ID: P01013); and OVAY of 388 amino acids, alternatively named as gene Y protein (UniProtKB ID: P01014). According to the nomenclature guidelines of serpin, ovalbumin, gene X, and gene Y are named as serpinb14, serpinb14c, and serpinb14b, respectively.[26] OVAX has been

shown to be similar to OVAY in the amino acid sequence and structure.[8,27] OVAY is present in the yolk,[28] and OVAX is approximately 2.4 mg/mL in average in egg white and seems to show antimicrobial activity.[29]

Glucocorticoids (e.g., cortisol and corticosterone) increase the expression of ovalbumin mRNA and protein in the chicken oviductal magnum.[30,31] Study showed that ovalbumin gene expression and protein synthesis in the magnum were increased in chicks administered with glucocorticoid at 2 mg/chick every 6 h, but decreased in those treated with 10 mg of glucocorticoid.[31] Synthesis and secretion of ovalbumin are also controlled by estrogen.[32] Corticosterone treatment reduces plasma concentrations of estrogen[1] and expression of its gene and protein.[30,31] Stress such as molting can also decrease plasma concentrations of estrogen[33] and reduce ovalbumin protein expression in egg shell.[34]

In the present study, OVAX protein abundance was significantly increased in the egg white of hens treated with dietary corticosterone at 30 mg/kg diet but not in Control. Expression of ovalbumin and OVAY proteins remained unchanged in both groups. On the other hand, gene expression of ovalbumin and OVAY was significantly decreased in the magnum of Stress. Together, these findings suggest that expression of these genes and proteins is differentially regulated by glucocorticoids depending on hormone concentrations and severity of stress.

Transiently Expressed In Neural Precursors (TENP). Protein TENP is a member of BPIFB subfamilies which belong to the bactericidal permeability-increasing protein (BPI) fold-containing (BPIF) superfamily,[35] and it consists of 440 amino acids.[36] The full name of the gene encoding this protein, known as TENP, is BPI fold containing family B, member 7, the official symbol of which is BPIFB7 (NCBI gene ID: 395882). As indicated in its name "transiently expressed in neural precursors", TENP was originally reported to be temporarily expressed only in the retina and brain of embryonic chicks being hatched but not in the other organs such as the heart, liver, and kidney.[36] mRNA expression diminished over time in the brain and retina of the chicken embryo but up-regulated in adult birds.[37] TENP has been now well-known to be present in the extraneural organs/tissues such as the oviductal magnum and appears to be avian specific, expressing almost exclusively in the magnum of adult birds.[37] In fact, protein TENP has been shown to be detected in the egg: the egg white[16,27] and yolk[28] of unfertilized chicken eggs and in emu egg white,[38] suggesting that expression of this protein is independent of fertilization and avian species.

Protein TENP consists of 0.1−0.5% of dried egg white protein[27] with MW of 47−50 kDa.[16] In the present study, TENP in the egg white was down-regulated by Stress while no such changes were seen in Control, and we even failed to detect TENP expression in the magnum. A recent study has shown that TENP mRNA was largely increased in the magnum of the oviduct in laying hens compared with nonlaying hens and in juvenile chicks administrated with either estrogen or progesterone, and was site-specifically expressed in the magnum of adult chickens and ducks,[37] suggesting gonadal regulation of TENP expression. The roles of TENP are not clear in chickens but appear to be associated with natural defense of avian eggs and embryonic development.[36]

Hemopexin (HPX). HPX is a plasma glycoprotein which is mainly expressed in the liver and less abundantly in the central and peripheral nervous systems, and it has a high binding affinity to heme in an equimolar ratio to protect the body from oxidative damage.[39] HPX increases the resistance of proteolytic digestion when it binds to heme.[40] HPX belongs to the acute phase protein and is increased by inflammation and stress.[39] Avian serum HPX is a 52 kDa α_1-glycoprotein and can be detected from a few days before the egg hatches.[40] HPX concentrations in 4-day-old chicks were half those of adults (3.5 months old), and its concentrations in plasma were gradually increased after hatching.[41] HPX protein concentrations in plasma are significantly increased by immune challenge such as injection of lipopolysaccharide.[42] Both full length (IgY) and truncated isoforms (Fc) of IgY were isolated from ducks vaccinated against *Escherichia coli* (*E. coli*) and used to make *E. coli*-Ig immune complexes. When IgY and IgY (Fc) were injected intravenously into naive Mallard ducks, plasma HPX concentrations were higher in ducks given either 0:100 ratio or 100:0 ratio than in those given 50:50 or *E. coli* alone.[43] HPX has been recently shown in a variety of eggs produced from different raising conditions in nutrients or hens' strains, which suggests that its abundance can be affected by hens' strain, nutrients, or housing conditions.[17]

In the present study we found that HPX protein in the egg white and its gene in the magnum were significantly down-expressed in laying hens treated with corticosterone whereas HPX protein was increased in the magnum. Considering the findings that HPX concentrations are increased by acute stress such as inflammation,[39] it is notable in the current study that both HPX protein and gene expressions were different depending on what was investigated, which is worthy of further study.

IgY-Fcυ3-4. Yolk proteins occupy approximately 16% of the total weight of egg yolk in fresh eggs.[23] Livetin fraction is a type of egg yolk proteins including α-, β-, and γ-livetin. Both yolk α-livetin and chicken serum albumin are the same protein.[23] β-Livetin is a D-2-glycoprotein with a MW of 45 kDa. A third protein, γ-livetin, is IgY with a MW of approximately 167 kDa.[23] Avian IgY is closely related to the ancestors of mammalian IgG and IgE.[44] The predominant serum immunoglobulin (Ig) in chickens has been proposed to be called IgY rather than IgG.[45] IgY consists of two heavy and two light chains,[46] and IgY-Fcυ3-4 is an IgY-Fc fragment containing heavy chain constant domains 3 (Cυ3) and 4 (Cυ4).[44,46]

As IgY in the egg has passive immunity granted from the mother, it is transported into the yolk by receptors from blood, and its amount in the yolk is related to plasma IgY concentrations.[47] IgY was detected in 3.26−6.02 mg per 1 mL of hen's plasma and in 1.15−2.26 mg per 1 mL of the egg yolk.[47] Human (h) IgG was increased to maximum in the yolk on 5 days after laying hens were administered with hIgG.[48] Although hIgA, but not hIgG, was detected from egg albumen,[48] the current results, showing the presence of IgY-Fcυ3-4 in the egg white of hens treated with corticosterone, suggest the presence of IgY in the egg white. Indeed, IgY has been shown to be detected in the egg white (3.57−9.58 μg/mL), at a much lower concentration than that in the yolk (1.15−2.26 mg/mL).[47] When some of the hens were infected with pathogen, IgY concentrations were increased in the circulation, which in turn resulted in an elevation of IgY in the egg.[49] In the present study, the abundance of IgY-Fcυ3-4 was rather decreased in the egg white, which cannot be accounted for by possible infection of the hens. Furthermore, these hens had an increased feed intake during the entire treatment period (Figure 1). Thus, down-expression of IgY-Fcυ3-4 in the egg

white means that the eggs of hens treated with corticosterone or raised under stressful environment are highly likely to have less amount of antibody than those in normal environment. In other words, if breeder hens are being raised under stress, their offspring produced are likely to be susceptible to diseases.[7] Concentrations of IgY in egg yolk and white were not determined in this study.

Extracellular Fatty Acid-Binding Protein (Ex-FABP). Fatty acids are present in complex with binding proteins rather than free form in order to be dissolved and transported in biological fluids for metabolism because fatty acid is hydrophobic. Ex-FABP is identified for the first time from the bone and cartilage of the developing chicken embryo[50] and then was also detected in muscle fibers and blood granulocytes in chickens.[50] It is also called protein Ch21, quiescence-specific protein, p20K (UniProtKB ID: P21760), or chondrogenesis-associated lipocalin, and it belongs to the chondrogenesis-associated lipocalin superfamily.[50] Ex-FABP is transported in the extracellular fluid and serum following binding to long-chain unsaturated fatty acids.[50] Ex-FABP is involved in heart development, fatty acid transport, and lipid metabolism, and it supposedly acts as a cell survival protein that protects cells from toxic lipid accumulation of fat.[51] Expression of Ex-FABP is enhanced in the liver of embryonic chickens by inflammatory agents such as lipopolysaccharide,[52] and it plays a role as an acute phase protein that is reduced by treatment with anti-inflammatory agents.[52,53] Ex-FABP gene expression is significantly increased in the lung, liver, and spleen of 3-week-old White Leghorn chicks infected with *E. coli*.[52,53] Growth inhibition of *E. coli* was observed in the presence of Ex-FABP,[54] suggesting that this protein possesses an antibacterial activity. On the other hand, suppressing gene expression of Ex-FABP resulted in increased apoptosis, decreased differentiation, and proliferation of cells.[52]

The results of the present study show that both Ex-FABP in the egg white and its gene in the magnum were significantly down-regulated in hens treated with dietary corticosterone. We failed however to detect Ex-FABP expression in the magnum using 2DE plus MALDI-TOF/TOF MS/MS. Although the relationship between Ex-FABP expression in the egg white and gene expression of Ex-FABP in the lung or liver has not been investigated in the present study, the present findings in the egg and magnum show an inverse relationship with protein and gene expressions in other organs (e.g., embryo, lung, liver, and spleen) of chickens infected.[52−54] Together, these results suggest that the reduced expression of Ex-FABP in the egg white may have resulted from the reduced expression of its gene, specifically in the magnum, but not the lung, liver, or spleen. From this perspective, a recent proteomic study,[55] which shows that the amount of an Ex-FABP precursor was gradually decreased in the egg white as the storage temperature of eggs was increased, implies that Ex-FABP can be used as an indicator for the evaluation of egg quality.

Ovotransferrin. Ovotransferrin is a 78 kDa, disulfide-rich (15 disulfide bonds) single-chain glycoprotein consisting of 686 amino acids and accounts for 13% of total egg white proteins.[25] Ovotransferrin is a member of the transferrin family that has high ability to bind two Fe^{3+} ions (iron-binding capacity) and can be transferred into the egg white through the oviduct of hens.[56] Ovotransferrin and its hydrolysates have been shown to exert antimicrobial, antiviral, and antioxidant activities and to play as markers for stress and metabolic diseases in chickens infected with various pathogens.[56] Concentrations of ovotransferrin are increased in serum of chickens infected with pathogens or treated with inflammatory agents.[56] As ovotransferrin is regulated by inflammatory or microbial stress,[56] its concentrations in blood could be used as a diagnostic marker of chickens subject to infection or inflammation. Glucocorticoids (e.g., cortisol and corticosterone) stimulate enhanced synthesis of ovotransferrin mRNA and protein in the chicken magnum in vivo.[31] We found in the current study that ovotransferrin was slightly but not significantly up-regulated in the egg white by corticosterone and was significantly increased in the magnum while its gene expression was significantly reduced in the magnum. Others showed that gene and protein expression was increased in the magnum of chicks administrated with glucocorticoid at 2 mg/chick in every 6 h but decreased in chicks at 10 mg,[31] suggesting that their expression depends upon its concentrations used.

In summary, the results of the present study show that dietary corticosterone modulates the expression of proteins in the egg white. Similar findings were observed in the expression of proteins and genes of the magnum. Recent studies have shown, by proteomics, that expression of egg white proteins are affected by egg storage conditions, time, or fertilization.[55,57] In other words, the quantity of individual proteins consisting of the egg white can be changed in response to environmental conditions, which can have a direct impact on the quality of eggs. Egg quality can be affected by various factors, such as nutrients, ambient temperature, housing conditions, and stressful conditions, under which hens are raised,[58−60] and by the age and strain of hens.[58−60] Thus, egg quality has been evaluated based on external and internal aspects of the egg as well as on a food safety perspective that has long been a subject of public concern, having been linked to hens' welfare. Therefore, egg white proteins could be used as markers which help estimate the state of animal welfare or housing environment at the time of laying the egg. In conclusion, the findings from the current study provide evidence for the first time that dietary corticosterone modulates protein abundance in the egg white in laying hens, and suggest that environmental stress can differentially modify expression of egg white proteins in laying hens.

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Notes

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