# Increased serum levels of advanced glycation end products due to induced molting in hen layers trigger a proinflammatory response by peripheral blood leukocytes

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**ABSTRACT** Induced molting (**IM**), a severe detriment to animal welfare, is still used in the poultry industry in some countries to increase or rejuvenate egg production and is responsible for several physiological perturbations, possibly including reactive oxidative stress, a form of metabolic stress. Because metabolic stress has been shown to induce a proinflammatory response involved in attempts to restore homeostasis, we hypothesized that similar responses followed IM. To confirm this hypothesis, we initially confirmed the establishment of oxidative stress during IM in 75-wk-old layers by demonstrating increased production of advanced glycation end products (**AGE**). Concomitant with increased oxidative metabolites, cellular stress was demonstrated in peripheral blood leukocytes (**PBL**) by increased levels of stress gene products (the glucocorticoid receptor, sirtuin-1, and heat shock protein 70 mRNA). Increased expression of stress proteins in PBL was followed by a proinflammatory response as demonstrated by increased levels of proinflammatory gene products (IL-6 and IL-1β mRNA); increased expression of these gene products was also demonstrated in direct response to AGE in vitro, thus establishing a direct link between oxidative and cellular stress. To establish a possible pathway for inducing a proinflammatory response by PBL, we showed that AGE increased a time dependent expression of galactin-3, Toll-like receptor-4, and nuclear factor - κB, all involved in the proinflammatory activation pathway. In vivo, AGE formed complexes with increased levels of circulating acute phase proteins (lysozyme and transferrin), products of a proinflammatory immune response, thereby demonstrating an effector response to cope with the consequences of oxidative stress. Thus, the harmful consequences of IM for animal welfare are extended here by demonstrating the activation of a resource-demanding proinflammatory response.

**Key words:** hen layer, proinflammatory immune response, induced molting, oxidative stress

## INTRODUCTION

Molting is a major physiological event in the annual life cycle of many avian species, and its initiation may be because of various endogenous and environmental stimuli (Dawson, 2015). In the poultry layer industry, induced molting (**IM**) is used to increase or rejuvenate egg production and is achieved by reducing the photoperiod and by either feed removal or by feeding a nutritionally deficient diet (Berry, 2003; Webster, 2003; Koelkebeck and Anderson, 2007). Induced molting is also responsible for causing significant metabolic changes, including increased metabolic rates, all cumulatively perturbing homeostasis to form metabolic stress (Mench, 2002; Webster, 2003; Najafi et al., 2016). Consequently, a series of physiological responses is initiated to repair accumulated "wear and tear" and to restore homeostasis (Blas, 2015).

The outcome of IM may increase morbidity and mortality in the affected layers and is thus an increasing issue of animal welfare (Eltringham et al., 1969; Dantzer and Mormede, 1983; Jackson and Diamond, 1996; Rauw et al., 1998; Cheng and Muir, 2005; Wein et al., 2017a). Furthermore, it was recently suggested that metabolic rate enhancement, typical of IM, impairs animal welfare as it may cause reactive oxidative stress, an imbalanced condition between oxidant formation and elimination by antioxidant mechanisms (Alonso-Alvarez et al., 2004; Balcombe et al., 2004; Lin et al., 2004). Thus,

energy-requiring metabolic processes may lead to production of free radicals or advanced glycation end products (**AGE**), both eliciting oxidative stress, whereas endogenic antioxidant mechanisms, such as glutathione-superoxide dismutase, fail to attenuate increased oxidative stress as they are already fully active. Furthermore, oxidative stress can lead to additional homeostasis deterioration by increasing the damage to cell membranes and proteins, thereby impairing their physiological activity (Cadenas and Davies, 2000; Finkel and Holbrook, 2000; Nyska and Kohen, 2002; Aschbacher et al., 2013). Thus, the metabolic imbalance resulting from IM-related stress may be compounded by a concomitant state of oxidative stress in layers subjected to intensive production regimens.

Our previous studies and those of others indicated an interaction between oxidative stress and the proinflammatory response (Lee et al., 2019; Wein et al., 2017a; Wein et al., 2017b). Thus, restoration of homeostasis in broilers and turkeys in a state of oxidative stress (induced by transport) involved activation of an innate immune response. Increased expression of proinflammatory immune-response genes (i.e., interleukin [**IL**]-6 and IL-1β) in peripheral blood leukocytes (**PBL**) as well as elevation of acute phase antioxidative proteins (i.e., lysozyme and transferrin) in serum (Wein et al., 2017a; Wein et al., 2017b). Owing to these observations and studies supporting an interaction between oxidative stress and a proinflammatory response (Li et al., 1995; Cadenas and Davies, 2000; Finkel and Holbrook, 2000; Nyska and Kohen, 2002; Gallo et al., 2014), our aims were to confirm oxidative stress due to IM in layers and to investigate whether oxidative stress is involved the activation of a proinflammatory response. Finally, we investigated the possible role of the proinflammatory response in attempts to restore homeostasis in layers subjected to IM.

## MATERIALS AND METHODS

### Animals and Husbandry

White Leghorn layers (Hyline) were reared in enclosed, environmentally controlled layer facilities (Bustan HaGalil experimental facility, Israel). In accordance with a standard rearing protocol (Poultry Section, Ministry of Agriculture, Israel), the hens were fed a complete layer ration *ad libitum* (NRC, 1994). The photoperiod was 16:8 h of light and darkness, respectively. At this time, molting was induced by altering the photoperiod and the feeding regimen as described in the following paragraphs.

### Ethics Statement

All hen studies were performed under an Institutional Animal Care and Use Committee-approved protocol of the Hebrew University of Jerusalem in compliance with Animal Welfare regulations (Approval no. IL272/10).

### Induced Molting

At the age of 73 wks, hens were randomly selected and divided into 2 groups (N = 50 per group): A control group that continued to be fed on a regular layer diet and received the same photoperiod and a group in which molting was induced by food deprivation for 75 h (Phase 1 [Webster, 2003]) and by reduction of daylight to 8 h (increased darkness to 16 h). Water was provided *ad libitum* to all birds, and temperature and rearing conditions were kept identical in both groups.

### Blood Collection

Blood was collected 72 h after initiation of IM. Birds were randomly selected, manually restrained, and 2.5 mL blood was drawn by venipuncture of the *vena cutanea ulnaris*. Collected blood was rapidly distributed into different tubes. For serum collection, 0.5 mL was placed in Vacuette Z Serum Sep Clot Activator (Greiner Bio-one, KremsmÜnster, Austria) tubes; for mRNA extraction, 0.5 mL blood was placed directly into RNAse free tubes containing TRI Reagent-BD (Molecular Research Center Inc., Cincinnati, OH); for in vitro studies, 1.5 mL blood was placed in tubes containing Alsever's solution (Sigma-Aldrich, St Louis, MO) at 1:1 v/v ratio.

### Blood Chemistry

***Liquid Chromatography–Mass Spectrometry Analysis of Fatty Acids Esters and Cholesterol*** Serum samples were freeze-dried, and lipids were extracted with isopropanol (1.5 ml) following sonication (for 20 min). Samples were then centrifuged at 7,000 rpm for 10 min and filtered through 0.2 μm Teflon filters before liquid chromatography–mass spectrometry (**LC-MS**) analysis. Details of the LC-MS analyses appear in Supplemental Material.

***LC-MS Analysis of Free Fatty Acids*** Samples were prepared and analyzed as described for esters and cholesterol with several differences (described in Supplemental Material).

***Total Protein*** Total protein levels were determined in serum using the Pierce BCA protein assay kit (Thermo Fischer Scientific Inc., Rockford, IL). The procedure was performed according to the manufacturer's instructions, using chicken albumin (Sigma-Aldrich) as a standard.

***Blood Glucose and Ketone Determination*** Glucose and ketone levels were determined in serum samples using FreeStyle Optium glucometer/ketonemeter and FreeStyle Optium test strips (Abbott Diabetes Care Ltd, Witney Oxon, UK) according to instructions provided by the manufacturer.

## Advanced Glycation End Products Stimulation Assay

Whole blood diluted in Alsever's solution was distributed among 6 new cell culture tubes (Nunc, Thermo Fischer Scientific Inc.); AGE protein (Abcam, Cambridge, UK) was diluted using endotoxin free PBS (25 cP; Sigma-Aldrich) and added to 4 tubes at 2 concentrations: 0.1 µg/mL and 1 µg/mL (2 tubes for each concentration). Two control tubes contained whole blood in Alsever's and endotoxin-free PBS. Tubes were then mixed gently end-to-end and incubated at 37°C for 0.5 and 1 h. Following incubation, chicken PBL were collected using methylcellulose density centrifugation as previously described (Kogut et al., 1995; Wein et al., 2017a); briefly, each of the treated and control tubes were mixed with 1% methylcellulose in endotoxin-free PBS at 1:1 ratio and centrifuged at $25 \times g$ for 7 min at room temperature. The upper layer, containing PBL, was carefully removed, transferred to a new tube, and centrifuged at $450 \times g$ for 5 min at room temperature to pellet the cells. Following centrifugation, the supernatant was discarded, and the cell pellet was treated with TRI Reagent (Molecular Research Center Inc.) according to the manufacturer's instructions and was kept at -20°C until RNA processing.

## Fluorescein Activated Cell Sorting Analysis

Peripheral blood leukocytes treated or not with AGE (1 µg/mL for 1 h) were prepared as described above. The cells were counted and divided into tubes ($1 \times 10^6$ cells/tube). Rabbit anti-human galectin-3 polyclonal antibodies (3 µg/ml; Bioss Inc., Woburn, MA) were added, and the tubes were incubated at 4°C overnight (these antibodies were cross reactive with chicken galectin-3; ELISA data not shown). Cells were then washed twice, and Alexa fluor 647-conjugated goat anti-rabbit antibodies (Jackson ImmunoReserch laboratories, West grove, PA) were added to all the tubes. Following incubation of 1.5 h at room temperature, the cells were thoroughly washed and filtered, using a 40 µm nylon Falcon cell strainer (ThermoFisher Scientific, Bohemia, NY), into Falcon polystyrene fluorescein activated cell sorting analysis (**FACS**) tubes (Becton Dickinson, Franklin Lakes, NJ). A tube without the primary anti-galectin 3 antibody, served as negative control. Samples were analyzed using BD FACSCalibur front and side scatter and FL-4 characteristics; for each sample, 10,000 cells were counted.

## Galectin-3 Immunofluorescence Assay

Peripheral blood leukocytes were obtained as described above. Cells were fixed for 10 min using 1.6% formaldehyde in PBS (Sigma-Aldrich) followed by permeabilization with 0.2% Tween 20 in PBS (Sigma-Aldrich) for 15 min. Samples were than incubated overnight at 4°C with Rabbit anti-human galectin-3 polyclonal antibody (3 µg/ml; Bioss Inc.) in 1% BSA in PBS (Sigma-Aldrich). Detection was performed using Alexa 488 Goat anti-

rabbit antibodies (1:50; Jackson Laboratories Inc., West Grove, PA). Cells were counterstained using Fluoroshield with DAPI (Sigma-Aldrich). Photos were taken using BX 51 TRF microscope (Olympus, Japan).

## Determination of Serum Lysozyme, Transferrin, AGE, and AGE Complexes by ELISA

Transferrin, lysozyme, and AGE levels were determined in serum samples by direct ELISA. Briefly, nondiluted serum samples were placed on ELISA plates. Serial dilutions in carbonate-bicarbonate buffer (pH = 9.6) of chicken transferrin (My BioSource, San Diego, CA), lysozyme (Sigma-Aldrich), or AGE (Abcam) were used as respective standards. Coated plates were incubated in a humidified chamber at 4°C overnight and were then blocked using 0.5% skim milk (BD, Difco, Sparks, MD) in PBS. Detection was performed using HRP conjugated rabbit anti-chicken transferrin (My BioSource), HRP conjugated rabbit anti-chicken hen egg lysozyme (Abcam), or rabbit anti-AGE (Abcam) and HRP conjugated goat anti-rabbit IgG (Jackson Laboratories Inc. West Grove, PA). TMB (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was used as substrate. Optical absorbance was determined at 450 nm using a Bio Tek microplate reader (Bio Tek, Winooski, VT). Serum proteins and AGE levels in serum were determined by comparing absorbance values to a standard curve formed from either purified lysozyme, transferrin, or AGE, respectively, in each plate.

AGE-lysozyme or AGE-transferrin complexes levels were determined in serum samples using sandwich ELISA. Briefly, ELISA plates (Nunc; Thermo Fischer Scientific Inc.) were coated with rabbit anti-AGE (Abcam) in carbonate–bicarbonate buffer (pH = 9.6) at a concentration of 1µ/mL. Coated plates were incubated at 4°C overnight. Plates were then washed extensively and blocked as described above. Serum samples were added, and plates were incubated overnight in a humidified chamber at 4°C. The presence of AGE-transferrin and AGE-lysozyme complexes was performed with HRP-conjugated rabbit anti-chicken transferrin (My BioSource) and HRP-conjugated rabbit anti-chicken hen egg lysozyme (Abcam), respectively. For controls, plates were coated with either purified AGE (10 µg/ml) (Abcam), lysozyme (5 µg/mL) (Sigma-Aldrich), or transferrin (5 µg/ml) (My BioSource), and the assay was performed as described above. Detection was performed using HRP-conjugated anti-AGE, anti-lysozyme, or anti-transferrin, respectively.

## RNA Extraction and PCR Analysis

RNA was extracted from chicken PBL using TRI Reagent (Molecular Research Center Inc.) according to the manufacturer's instructions. Contaminating chromosomal DNA was digested with DNase I (RNAse free;

1IU/μg of RNA; Fermentas, Glen Burnie, MD) for 30 min at 37°C. RNA quality was assessed using Agilent bioanalyzer total RNA nano chip. One microgram RNA from each sample was reverse transcribed using iScript Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad, Hercules, CA) according to the manufacturers' protocol. cDNA was amplified by PCR using SsoFast Eva-Green Supermix (Bio-Rad) and specific primers for the examined genes (see Table 1 for details). Primer sequences were designed using Oligo primer analysis software (Molecular Biology Insights, Inc., Colorado Springs, CO) according to GeneBank published sequences. Each primer pair was calibrated to determine the optimal reaction temperature and cDNA concentration. Expression levels of examined genes were determined by RT-PCR. RT-PCR was performed using C1000 Thermal Cycler, and results were analyzed using Bio-Rad's CFX manager software (http://www.bio-rad.com/webroot/web/pdf/lsr/literature/10021337.pdf) (Bio-Rad). Dissociation curve analysis was performed at the end of each real-time PCR reaction to validate the presence of a single reaction product and lack of primer dimerization. Normalized expression ($\Delta\Delta C_q$) of examined genes was determined using 2 normalizing genes (ch18S and ch28S). The calculation for normalized expression is described in the following formula, which uses the calculated relative quantity calculation:

$$\text{Normalized Expression}(GOI)_{sample} = \frac{RQ \text{ sample } (GOI)}{\left(RQ_{\text{sample (Ref 1)}} \times RQ_{\text{sample (Ref 2)}} \times \ldots \times RQ_{\text{sample (Ref } n)}\right)^{1/n}}$$

Where:

RQ = Relative Quantity of a sample
Ref = Reference gene in a run that includes one or more reference genes in each sample
GOI = Gene of interest (one target)

### Statistical Analysis

The FACS analysis data were analyzed by FlowJo software (FlowJo, LLC data analysis software, Ashland, OR), and statistical analysis was perform using Kolmogorov–Smirnov test (**K**–**S** test): a nonparametric test of the equality of continuous, one-dimensional probability distributions, used to compare 2 samples.

Results of all other experiments are mean averages of 3 separate experiments ± SEM; in individual experiments, no less than 10 biological samples were evaluated (different representative individuals were randomly sampled); thus, the total displayed results are from no less than 30 biological samples. Statistical analyses were performed using JMP software (SAS Institute Inc., Cary, NC). Data were analyzed using Welch $t$ test (significance of differences was at least $P = 0.05$).

Wilcoxon each paired test was used for multiple comparisons. Interactions between experiments were found to be insignificant ($P > 0.05$).

## RESULTS

Induced molting is expected to alter metabolic homeostasis and induce nutrition-related stress. To confirm this expectation, we initially assessed the physiological status of the layers as determined by total protein, glucose, ketones (β-hydroxybutyrate), cholesterol, and cholesterol derivative levels in serum. Results presented in Table 2 show that during IM, the total protein levels in sera of treated animals were not significantly altered relative to those of control layers. However, serum glucose levels decreased during IM ($\sim$20% reduction; $P < 0.05$), as were the levels of cholesterol ($\sim$80%; $P < 0.05$). In contrast, levels of cholesterol derivatives in serum increased (oleic ester $\sim$ 257% and linoleic ester $\sim$ 233%; $P < 0.05$), suggesting a transition from glucose to lipids as a metabolizable energy source. Supporting this conclusion, a parallel significant decrease in levels of monounsaturated and polyunsaturated fatty acids was also observed (Supplemental Material).

To test whether the transfer to lipid metabolism was accompanied by production of reactive oxidative metabolites, we initially determined by quantitative ELISA the levels of advanced glycation end products AGE in serum of layers undergoing IM. Advanced glycation end product levels in control hens (n = 10 in each of 3 experiments) were on average 5.2 ± 1.5 ng/ml, whereas levels in IM hens were 14.5 ± 2.3 ng/ml; this increase in AGE levels ($\sim$3-fold) was significant ($P < 0.05$). Next, we determined cellular stress responses in circulating PBL concomitant with the elevation of AGE levels.

As we had previously demonstrated that the elevation of sirtuin 1 (**SIRT1**), glucocorticoid receptor (**GCR**), and heat shock protein 70 (**HSP70**) mRNA was indicative of a cellular stress response in PBL, we determined mRNA levels of these genes. Peripheral blood leukocyte mRNA was prepared, as described in methods, 72 h after the initiation of IM and then probed for expression of SIRT1, GCR, and HSP70. Results presented in Figure 1 show that mRNA for all 3 genes was increased in PBL following IM ($P < 0.05$), and the level of increase was two-fold to three-fold. These results confirm cellular stress in PBL of IM hens.

We then determined the expression of proinflammatory-related genes in hen PBL as expressed by mRNA levels of IL-1ß and IL-6 cytokines (Figure 2). We found a $\sim$ 2-fold increase in mRNA expression of both IL-1ß and IL-6 cytokines in IM hens when

**Table 1.** Genes and primer sequences.

| Gene | Forward/Reverse | Sequence | Gene Bank Ref |
|------|-----------------|----------|---------------|
| 18s | F | 5′-CGGCGTCCAACTTCTTAGAGG-3′ | AF173612.1 |
| | R | 5′-CTGCCGGCGTAGGGTAGACAC-3′ | |
| 28s | F | 5′-ACGGTACTTGTTGGCTATCGG-3′ | DQ018756.1 |
| | R | 5′-GCCCAAGTCCTTCTGATCGAG-3′ | |
| HSP70 | F | 5′-GGGCTCCACTCGTATTCC-3′ | EU747335.1 |
| | R | 5′-TGGTGGTGTTACGCTTGA-3′ | |
| Transferrin | F | 5′-TTTCAAAGACTCTGCCATAATGC-3′ | NM_205304 |
| | R | 5′- TTGCTCTTCTCATCCTTGCCTAC- 3′ | |
| Lysozyme C | F | 5′-ACATAACAGCGAGCGTGAAC-3′ | NM_205281 |
| | R | 5′-CTCCTCACAGCCGGCAGCCT-3′ | |
| IL-1β | F | 5′-CTTCATCTTCTACCGCCTGGACA-3′ | NM_204524.1 |
| | R | 5′-TCGGGTTGGTTGGTGATG-3′ | |
| IL-6 | F | 5′-AGAAATCCCTCCTCGCCAAT-3′ | HM179640.1 |
| | R | 5′-AAATAGCGAACGGCCCTCA-3′ | |
| SIRT1 | F | 5′-CCAGACCTTCCAGATCCTCA-3′ | NM_001004767.1 |
| | R | 5′-TGCAACATGTTCAATGTGT-3′ | |
| GCR | F | 5′- GCAAACTTCTCCGGGTCT-3′ | DQ227738.1 |
| | R | 5′- CCCTGATCCCTGGCACCTATTCC-3′ | |
| TLR4 | F | 5′- TCAAGGTGCCACATCCATAC -3′ | NM_001030693.1 |
| | R | 5′- CTCCTGCAGGGTATTCAAGT -3′ | |
| NFκβ1 | F | 5′-GCCCAAGTATCGAGATGTCAACA-3′ | AF000241.1 |
| | R | 5′-CCGCTGCCTCCACCGTAG-3′ | |

Abbreviations: GCR, glucocorticoid receptor; HSP, heat shock protein 70; SIRT1, sirtuin 1; TLR, Toll-like receptor.

compared with control untreated hens ($P < 0.05$). To investigate whether the proinflammatory response observed in PBL of IM hens could have been directly induced by AGE, PBL from nutritionally balanced hens (endogenic AGE $\sim$ 5 ng/ml serum) were stimulated, in vitro, with AGE (0.1 or 1 μg/mL). mRNA of HSP70 (indicator of stress response), IL-1ß and IL-6 was determined 0.5- and 1 h post stimulation (Figure 3). mRNA levels in control cells (no exogenous AGE) remained stable throughout the experiment period. Advanced glycation end product (1 μg/m) induced increasing expression of HSP70 mRNA with time (Figure 3, upper panel: $\sim$50% and $\sim$100% after 0.5 and 1 h culture, respectively–$P < 0.05$ at both time points). A strong proinflammatory response, as expressed by increased of IL-1ß and IL-6 cytokine mRNA levels, was determined for both AGE dosages after 1 h culture: AGE stimulated cells displayed $\sim$40 fold increase in IL-1ß mRNA levels and a $\sim$4-6 fold increase

IL-6 mRNA levels (all increases–$P < 0.05$) (Figure 3 – middle and lower panels). Thus, AGE was directly linked to stress and proinflammatory responses in hen PBL, with the proinflammatory response appearing later than the immediate stress response.

As PBL directly responded to AGE, we then determined the whether expression of galactin 3 (a putative AGE receptor) was responsive to AGE stimulation in vitro. Hen PBL incubated, or not, with 1 μg/ml AGE for 1 h were stained with polyclonal antigalectin antibodies and subjected to both FACS analysis and immunocytochemistry (Figure 4). Incubation with AGE caused a marked right shift in galectin 3 expression (Figure 4A): The left curve represents galectin 3 distribution on PBL in the absence of AGE and the right distribution–galectin cells following incubation with AGE. Data analysis of the distribution change (see Methods) revealed a K–S difference of 80.7% and a K–S probability of >99% (both relative to the control cells,

**Table 2.** Effects of induced molting (IM) on serum metabolites in laying hens.

| | Group | |
|---|---|---|
| Parameter | Control | IM |
| Total Protein (mg/ml) | $31.78 \pm 2.09$ | $33.15 \pm 1.84$ |
| Glucose (mg/dl) | $203.90 \pm 1.15^{a}$ | $166.44 \pm 7.32^{b}$ |
| Ketones (β-HBA; mmol) | $0.27 \pm 0.03^{b}$ | $5.35 \pm 0.36^{a}$ |
| Cholesterol (LC-MS units)[1] | $5.30 \pm 0.30^{a}$ | $1.00 \pm 0.06^{b}$ |
| Cholesterol derivative: Oleic ester $C_{45}H_{79}O_2$ (LC-MS units)[1] | $0.42 \pm 0.03^{b}$ | $1.21 \pm 0.07^{a}$ |
| Cholesterol derivative: Linoleic ester $C_{45}H_{77}O_2$ (LC-MS units)[1] | $0.32 \pm 0.02^{b}$ | $1.13 \pm 0.07^{a}$ |

The listed metabolites were determined by LC-MS in serum of control or IM layers 72 h after food withdrawal. Welch $t$ test was used to determine significance of differences between group mean values ($\pm$SEM, n = 10 per group; the measurement was repeated 3 times with different serum samples and with similar results). Values with different superscripts are significantly different ($P < 0.05$).

Abbreviations: LC-MS, liquid chromatography–mass spectrometry.

[1]LC-MS units: relative amount of cholesterol and its derivates are expressed in LC-MS arbitrary units.

respectively), indicative of a significant cell responses to AGE. Immunocytochemistry was used to identify the cells expressing galectin 3 (Figures 4B and 4D). Several cell types stained positive for galectin-3 with differing densities as implied by Figures 4C and 4D: Galectin 3 positive cells were polymorphonuclear cells (yellow arrows), monocytes (red arrows), and lymphocytes (white arrows; small mononuclear cells); erythrocytes and thrombocytes did not stain positively.

The involvement of galectin 3 could lead to activation of multiple proinflammatory pathways culminating in the activation of nuclear factor - κB (**NF-κB**); one pathway involving Toll-like receptor (TLR)-4 was investigated. Hen PBL were incubated, or not, with 1 µg/ml AGE and TLR-4, and NF-κB mRNA expression was determined 0.5-h and 1-h poststimulation. Results in Figure 5 show that TLR-4 mRNA levels increased as early as 0.5 h after AGE stimulation ($P < 0.05$) and then returned to base levels 1h after stimulation. Interestingly, NF-κB mRNA levels remained at base levels after 0.5 h and then increased at 1 h poststimulation ($P < 0.05$). This temporal difference suggests that TLR-4 activation preceded that of NF-κB, thus providing additional evidence for AGE's role in activation of proinflammatory pathways.

After establishing a proinflammatory response to AGE in PBL of IM hens, we investigated responses of acute phase proinflammatory proteins. We initially determined serum levels of lysozyme and transferrin and found increased serum levels of both proteins in IM hens (Table 3; $P < 0.05$). We then analyzed lysozyme and transferrin mRNA levels in PBL of IM and control birds and found these to be elevated as well (Table 4—IM in vivo; $P < 0.05$). To link this elevation to increased AGE, we cultured PBL from nutritionally balanced hens (endogenic AGE $\sim$ 5 ng/mL serum) in the presence of AGE (1 µg/ml) for 1 h and then processed PBL mRNA to detect lysozyme and transferrin expression. We found that mRNA levels of both transferrin and lysozyme was increased following culture with AGE (Table 4—PBL cultured with AGE; $P < 0.05$). Thus, metabolic stress induced by IM was linked to increased AGE that, in turn, was linked to induction of a proinflammatory response in PBL and to the increase levels of effector proinflammatory plasma proteins (lysozyme and transferrin).

AGE-lysozyme and AGE-transferrin complexes neutralize oxidative effects of AGE. To locate the presence of either AGE-transferrin or AGE-lysozyme complexes in IM hen sera, we developed a sandwich ELISA for AGE complexes (described in materials and methods). Interestingly, results in Figure 6 reveal that AGE-lysozyme and AGE-transferrin complexes are present in normal untreated chicken serum; this indicates a possible steady state of oxidant neutralization by lysozyme and transferrin. However, during IM, the relative quantity of these complexes increased ($P < 0.05$) and particularly so in the case of lysozyme-AGE complexes; this indicates an elevation of antioxidative measures during oxidative stress due to IM.
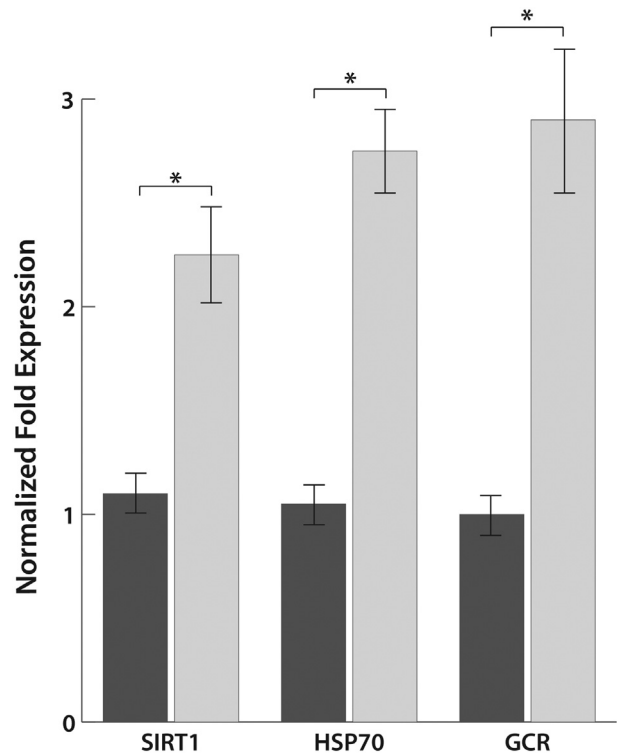


**Figure 1.** Induced molting (IM) causes elevation of sirtuin 1 (SIRT1), heat shock protein 70 (HSP70), and the glucocorticoid receptor (GCR) mRNA in layer PBL. PBL were isolated 72 h after initiation of IM, and mRNA was purified as described in Methods. Specific primers (Table 1) were used to determine normalized expression levels of SIRT1, HSP70, and GCR by RT-PCR in both the control (dark grey columns) and IM (light grey columns) and groups. Welch $t$ test was used to determine significance of differences between group mean values ($\pm$SEM, n = 10 per group; the measurement was repeated 4 times with different mRNA samples and with similar results). For each gene, the asterisks indicate that expression in the IM group was significantly higher than that of the control group ($P < 0.05$). Abbreviation: PBL, peripheral blood leukocyte.
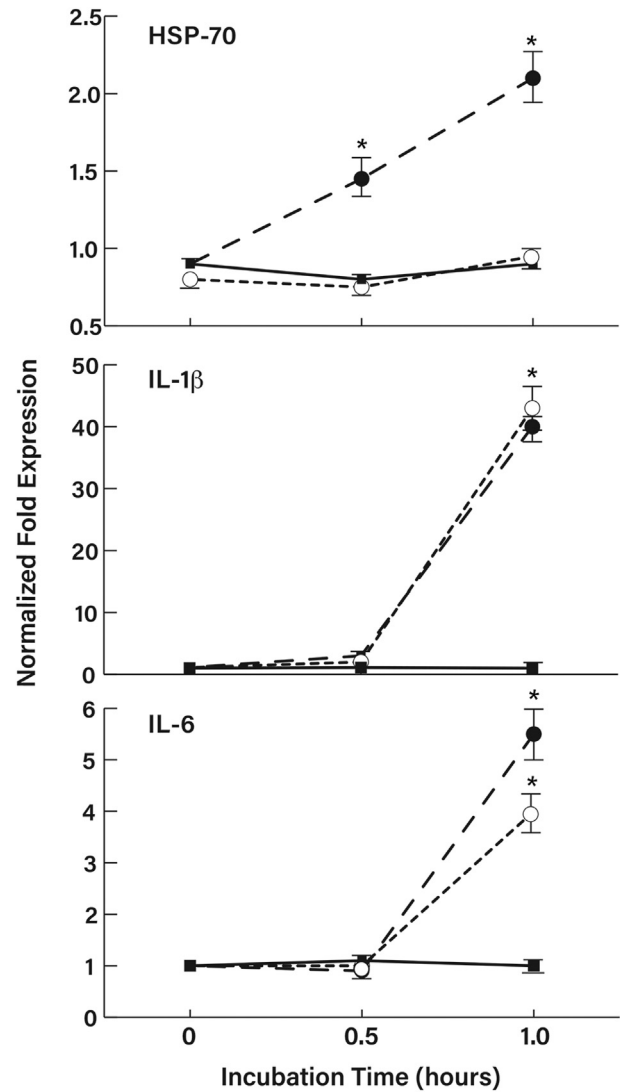
## DISCUSSION

Different regimens of food deprivation/limitation together with altered photoperiod induce molting in layers, as well as in other birds (Berry, 2003; Webster, 2003; Dawson, 2015). Induced molting is used in the layer industry to rejuvenate the laying capacity in hens following its gradual decline with age (Webster, 2003). Induced molting is only one of several recorded physiological responses following diet manipulation. All these result in a shift in homeostasis, such as the metabolic shift from carbohydrates to other energy resources like lipids and/or proteins (Webster, 2003). As shifts in homeostasis have been shown to induce metabolic and oxidative stress (Celi and Chauhan, 2013) and as metabolic signaling was shown in the human to be tightly linked to proinflammatory signaling (Osborn and Olefsky, 2012), the objectives of the present study were to demonstrate an oxidative stress response in hens undergoing IM and to investigate whether the proinflammatory immune response, as expressed by PBL, was activated and involved in measures to restore homeostasis.
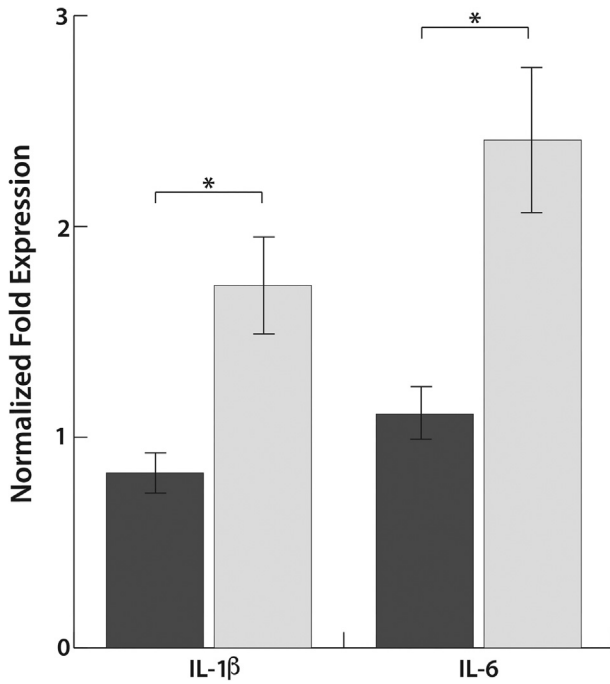
**Figure 2.** Induced molting (IM) causes elevation of IL-1β and IL-6 in layer PBL. PBL were isolated 72 h after initiation of IM, and mRNA was purified as described in Methods. Specific primers (Table 1) were used to determine normalized expression levels of IL-1β and IL-6 by RT-PCR in both the control (dark grey columns) and IM (light grey columns) and groups. Welch $t$ test was used to determine significance of differences between group mean values (±SEM, n = 10 per group; the measurement was repeated 3 times with different mRNA samples and with similar results). For each gene, the asterisks indicate that expression in the IM group was significantly higher than that of the control group ($P < 0.05$). Abbreviation: PBL, peripheral blood leukocyte.



**Figure 3.** AGE in vitro induces expression of HSP70, IL-1ß, and IL-6 in PBL derived from nutritionally balanced hens. Purified PBL were stimulated, in vitro, with AGE (0 μg/ml–full squares, 0.1 μg/ml–blank circles, 1 μg/ml–full circles), and mRNA expression was determined at 0, 0.5 and 1 h poststimulation. Each data point is the average ± SEM of 5 identical cultures from a single bird. The experiment was repeated 10 times with PBL from 10 different layers with similar results. Welch $t$ test was used to determine significance of differences between the different time points relative to 0 h; for each gene, the asterisks indicate that expression was significantly higher than at time 0 h ($P < 0.05$). Abbreviations: AGE, advanced glycation end products; HSP70, heat shock protein 70; PBL, peripheral blood leukocyte.

Initially, we concentrated our efforts to define the metabolic status of hens subjected to IM by monitoring the changes in glucose, protein, lipids, or lipid derivative levels in the serum and to demonstrate homeostatic imbalances (Rajman et al., 2006; Ishikawa et al., 2014). Our observations indicated that during IM, glucose levels decreased as expected (Viscarra and Ortiz, 2013). The IM food regimen caused a fasting response typical of phase 2 (Webster, 2003): This was demonstrated by a decrease in serum cholesterol levels, increased levels of cholesterol derivatives in serum as well as by changes in serum fatty acid composition. Similar findings have been reported in mice and ducks (Lien et al., 1999; Bergman et al., 2006). These results suggest that in IM layers, there is a shift in energy sources from glucose to lipids (Eltringham et al., 1969; Sheets et al., 2006; Wang et al., 2006). Utilization of fatty acids and lipids as an alternative energy source during food limitation is not unique to chickens and occurs in ruminants as well (Drackley and Andersen, 2006).

Our observations that SIRT1 levels increased significantly in PBL obtained from IM hens suggest the involvement of SIRT1 in energy homeostasis of chicken PBL and the initiation of a proinflammatory response. The involvement of SIRT1, a $NAD^{+}$-dependent protein, as an energy senor in cellular energy homeostasis has
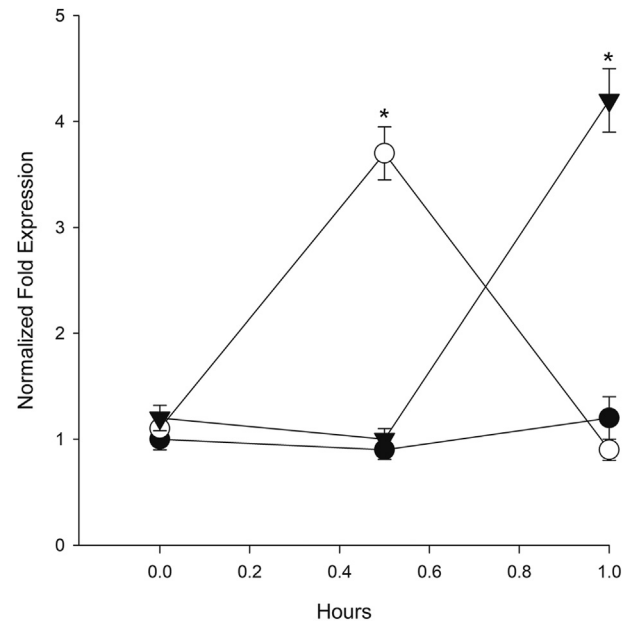
been previously demonstrated (Cantó and Auwerx, 2012; Nogueiras et al., 2012). SIRT1 has also been shown to be an effector protein, having both cytosolic and nuclear activity (Hipkiss, 2008; Hayashida et al., 2010; Cantó and Auwerx, 2012). Upregulation of SIRT1 enhances the affinity of HSF-1 to the HSP70 gene promotor, a product indicative of stress (Wein et al., 2017a; Wein et al., 2017b) and prolongs their binding duration (Johnson and Fleshner, 2006). Thus, the increase of both SIRT1 and HSP70 mRNA during IM indicates that the metabolic energy shift induced a stress response in hen PBL. This conclusion is supported by the increase of GCR mRNA in PBL; GCR regulation
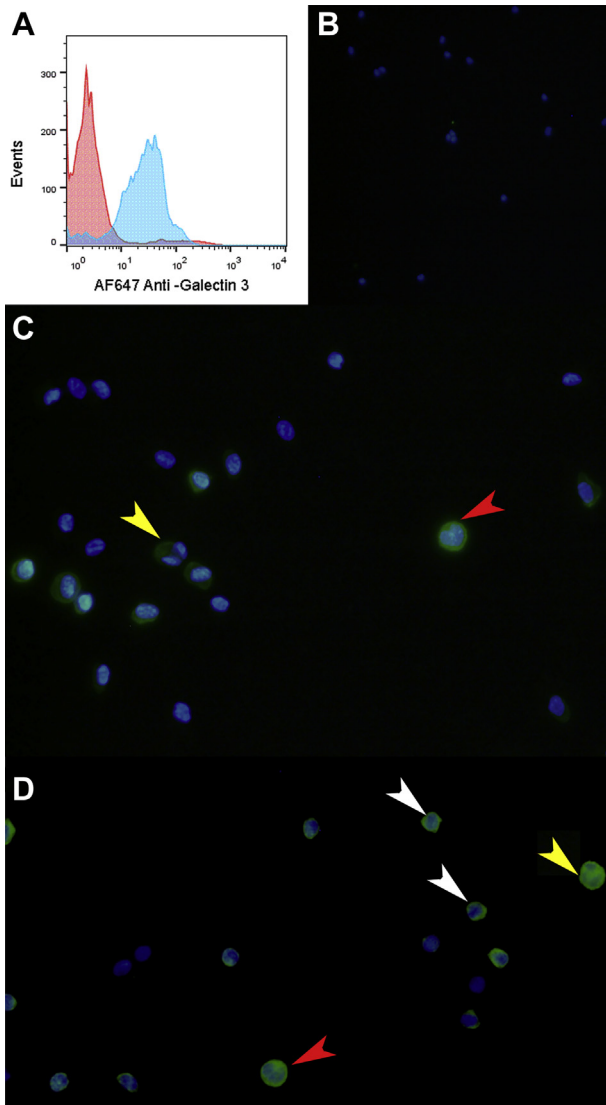
**Figure 4.** AGE in vitro increases expression of galactin-3 in layer PBL. Hen PBL incubated, or not, with 1 µg/ml AGE for 1 h and were stained with rabbit anti-human galectin antibodies and then with fluorescein-labeled goat anti-rabbit antibodies. Labeled cells were subjected to both FACS analysis (A) and immunocytochemistry (B–D). Panel B, a negative control, indicates cells that were labeled directly with fluorescein-labeled goat anti-rabbit antibodies. The results are from the PBL of a single hen; similar results were obtained from 10 different layers. Cells were identified by size and nuclear morphology as stained by DAPI. Arrow key: yellow–polymorphonuclear cells (segmented nuclei), red–monocytes (large mononuclear cells with kidney shaped nuclei), white–lymphocytes (small mononuclear cells). Abbreviations: AGE, advanced glycation end products; FACS, fluorescein activated cell sorting analysis; PBL, peripheral blood leukocyte.



**Figure 5.** AGE in vitro induces expression of TLR-4 and NF-κB. Hen PBL were incubated, or not, with 1 µg/ml AGE and TLR-4 and NF-κB mRNA expression was determined 0.5- and 1-h poststimulation (Full circles: PBL without AGE for both genes; blank circles–TLR-4; full triangles—NF-κB). Each data point is the average ± SEM of 5 identical cultures from a single bird. The experiment was repeated 5 times with PBL from 10 different layers with similar results. Welch $t$ test was used to determine significance of differences between the different time points relative to 0 h; for each gene, the asterisks indicate that expression was significantly higher than that at time 0 h ($P < 0.05$). Abbreviations: AGE, advanced glycation end products; PBL, peripheral blood leukocyte; TLR, Toll-like receptor.

is compound and is influenced by several factors including GCR ligands, glucocorticoids, and HSP proteins (Okret et al., 1991; Kirschke et al., 2014). Hence, our results demonstrating the concurrent increase in GCR mRNA in chicken PBL together with increased mRNA expression of SIRT1 and HSP70 provide evidence for induction of cellular stress following IM.

Intensified lipid metabolism and accumulation of free fatty acids (particularly polyunsaturated free fatty acids), increase the risk of lipid peroxidation and free radical assaults leading to formation of reactive

metabolites such as AGE (Balcombe et al., 2004; Negre-Salvayre et al., 2008). Indeed, we demonstrate that IM led to increased AGE levels in serum. The production of AGE is hazardous, as they might cause loss of function to major serum proteins such as albumin or increase spontaneous reactivity against serum proteins and cell membrane proteins forming irreversible bonds impairing normal function (Lapolla et al., 2005; Negre-Salvayre et al., 2008; Jaisson and Gillery, 2010). Advanced glycation end product metabolites are conceived to be endogenous danger signaling molecules (danger associated molecular patterns [**DAMPS**]) that cause cell stress and activate innate immune cells as well as the proinflammatory cascade, characterized by elevated proinflammatory cytokines (IL-6, IL-1ß) (Lapolla et al., 2005; Rosin and Okusa, 2011). Accordingly, our observations showed increased mRNA levels of TLR-4, NF-κB as well as increased mRNA levels of IL-6 and IL-1ß in PBL from IM hens. Collectively, these results suggest that in the chicken AGE may be recognized as a DAMP inducing cellular stress (as expressed by elevated levels of HSP70 mRNA) and that leads to activation a TLR-4 dependent pro-inflammatory cascade.

To confirm AGE as a DAMP, we exposed normal PBL to AGE in vitro. We found that stimulation of PBL with AGE in vitro, led to increased HSP70 mRNA and TLR-4 mRNA levels, that were followed by increased NF-κB,

**Table 3.** Lysozyme and transferrin serum levels following induced molting (IM) in layers.

| Serum protein | Control | IM |
|---|---|---|
| Lysozyme (μg/ml) | 6.5 ± 0.8[b] | 16.5 ± 2.0[a] |
| Transferrin (mg/ml) | 2.5 ± 0.5[b] | 29.5 ± 4.5[a] |

Lysozyme and transferrin levels were determined in serum of control or IM layers 72 h after food withdrawal by direct ELISA as described in Methods. Welch $t$ test was used to determine significance of differences between group mean values (±SEM, n = 10 per group; the measurement was repeated 3 times with different serum samples and with similar results). Values with different superscripts are significantly different ($P < 0.05$).

IL-6, and IL-1ß mRNA levels. This suggested that AGE binding induced a TLR-4–NF-κB proinflammatory signaling cascade in these cells, thus supporting the in vivo results described above. Further support for this is found in studies in mammals showing that activation of AGE receptors induced leukocyte sensitization via a TLR-4–NF-κB pathway (Dasu et al., 2010; Freeman, 2006; Hodgkinson et al., 2008; Jaisson and Gillery, 2010; Lapolla et al., 2005; Moore and Stewart et al., 2010; Ohgami et al., 2001; Yang et al., 2005).

A recent review (Pinkas and Aschner, 2016) divides the receptors for AGE in to 2 groups: One group mediates AGE internalization and degradation and includes CD36 and macrophage scavenger receptors I and II. The second group initiates signal transduction that leads to proinflammatory responses and includes RAGE and galectin 3 (Vlassara et al., 1995; Menini et al., 2016). Galactin 3, as a receptor for AGE, has been shown to induce a proinflammatory cascade in numerous innate immunity cell types (Sato and Nieminen, 2002; Pugliese et al., 2014). Furthermore, galectin 3 has been implicated in the development of metabolic disorders because it favors glucose homeostasis and prevents the deleterious activation of adaptive and innate immune response to obesogenic/diabetogenic stimuli (Pugliese et al., 2014). To date, the receptor for AGE in the chicken is unknown. Herein, the increase in galactin 3 membrane expression on several PBL cell types in response to AGE in vitro provides indirect support to

**Table 4.** Lysozyme and transferrin mRNA in PBL following induced molting (IM) in layers.

| Treatment | mRNA | Control | IM |
|---|---|---|---|
| IM—in vivo | Lysozyme | 1.2 ± 0.1 | 3.8 ± 0.3* |
| | Transferrin | 1.5 ± 0.5 | 3.5 ± 0.5* |
| PBL cultured with AGE in vitro | Lysozyme | 1.1 ± 0.3 | 3.7 ± 0.4* |
| | Transferrin | 1.5 ± 0.5 | 3.6 ± 0.5* |

Lysozyme and transferrin mRNA levels were determined in PBL of control or IM layers. In the in vivo treatment, mRNA was prepared from separated PBL 72 h after food withdrawal. In the in vitro treatment control, PBL were cultured in the presence of AGE (1 μg/ml) for 1 h and then processed to detect lysozyme and transferrin-specific mRNA. Values are mRNA normalized fold expression units as described in methods. Welch $t$ test was used to determine significance of differences between group mean values (±SEM, n = 10 per group; the measurement was repeated 3 times with different mRNA samples and with similar results). Values with asterisks are significantly higher than their respective controls ($P < 0.05$).
Abbreviations: AGE, advanced glycation end products; PBL, peripheral blood leukocyte.
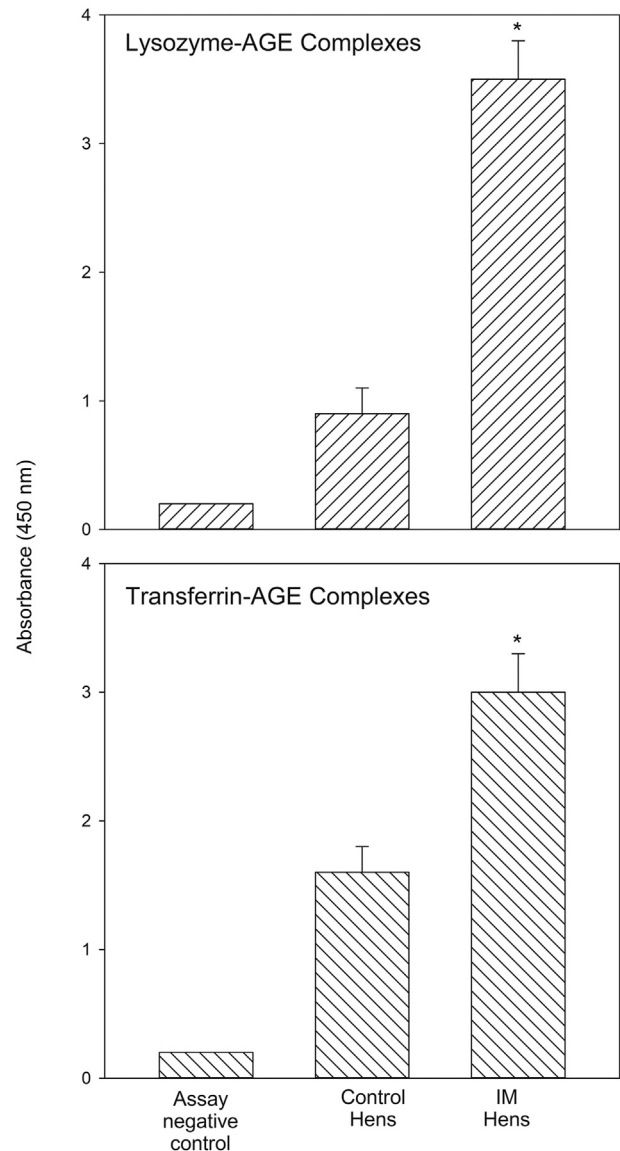


**Figure 6.** IM increases levels of lysozyme-AGE and transferrin-AGE complexes in serum. Age complexes were determined by ELISA (detailed in Methods). Welch $t$ test was used to determine significance of differences between column (group) mean values (±SEM, n = 10 per group; the measurement was repeated 5 times with different serum samples and with similar results). Welch $t$ test was used to determine significance of differences between the complex levels in serum of control and IM hens; for each complex, the asterisks indicate that the level of complexes was higher in the IM group ($P < 0.05$). Abbreviations: AGE, advanced glycation end products; IM, induced molting.

propose galactin 3 as a possible receptor for AGE in chicken PBL.

Previously published observations by our group and others showed that stress induces upregulation of serum acute phase proteins, including lysozyme and transferrin and suggested that these acute phase proteins act as a means to restore homeostasis (Cray et al., 2009; Kim et al., 2011; Yildiz and Altunay, 2011; Najafi et al., 2016; Wein et al., 2017a; Wein et al., 2017b). Induced molting led to increased levels and mRNA of lysozyme and transferrin and thus support the previous observations. Furthermore, our current findings provide a possible mode of action by which lysozyme

and transferrin reduce the hazardous effects of AGE. We demonstrated that both proteins form complexes with AGE and that these complexes increased significantly in IM hens. Thus, in agreement with previous studies, transferrin-AGE and lysozyme-AGE indicate capture and neutralization of AGE, thereby reducing and removing the oxidative effects following lipid metabolism and contributing toward the restoration of homeostasis (Kogut et al., 1995; Mitsuhashi et al., 1997; Gallo et al., 2014).

To conclude, we have demonstrated that IM induced oxidative and cellular stress in laying hens. The oxidative stress was due to the generation of AGE metabolites. In parallel, a proinflammatory response was initiated in PBL that possibly served to reduce the effects of the oxidative stress as well as to neutralize AGE by means of serum acute phase proteins (transferrin and lysozyme). Importantly, the harmful consequences of IM practice for animal welfare are extended by demonstrating the activation of a resource-demanding proinflammatory response.

## ACKNOWLEDGMENTS

## SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at http://doi.org/10.1016/j.psj.2020.04.009.

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