Comparison of stress biomarkers in laying hens raised under a long-term multiple stress condition

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ABSTRACT The objective of the current experiment was to compare various stress biomarkers including the heterophil to lymphocyte ratio (**H:L ratio**) in blood and the corticosterone (**CORT**) concentrations in feathers, claws, and egg yolk and to find the potential stress biomarkers in laying hens exposed to a long-term multiple stress condition. A total of 24 Hy-Line Brown laying hens at 47 wk of age were allotted to 2 distinct conditions including normal condition (**NC**) and multiple stress condition (**MC**) with 8 replicated cages. In NC treatment, 8 hens were raised individually in the cage (0.16 m^2/hen) under the temperature of 21 ± 0.6°C. In MC treatment, 16 hens were raised with 2 hens per cage to decrease space allowance (0.08 m^2/hen) and the temperature was maintained at 31 ± 1.6°C. The experiment lasted for 8 wk. The common diets and water were fed on an ad libitum basis during the experiment. Results indicated no interactions between stress conditions and duration of stress exposure for all measurements. Hens in MC treatment had a greater ($P < 0.01$) blood H:L ratio than those in NC treatment. The greater ($P < 0.05$) H:L ratio for MC treatment was observed at all weeks. Hens in MC treatment had greater ($P < 0.05$) feather CORT concentrations than those in NC treatment. Feather CORT concentrations were increased ($P < 0.05$) with duration of the experiment. However, stress conditions did not influence claw CORT concentrations. A tendency ($P = 0.081$) was observed for greater yolk CORT concentrations in MC treatment than in NC treatment. In conclusion, the H:L ratio in blood and CORT concentrations in feathers and egg yolks are considered potential stress biomarkers in laying hens exposed to stress conditions, although each measurement has its respective limitations. However, CORT concentrations in the claw appear to be insensitive to a long-term stress exposure.

Key words: blood heterophil to lymphocyte ratio, laying hen, multiple stress condition, tissue corticosterone concentration, stress biomarker

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INTRODUCTION

Poultry is continuously exposed to various stressful environments in the commercial situation, which is directly involved in the final productive performance and product quality of poultry (Lee et al., 2015). Heat stress and high stocking density have been identified the major stressors in the poultry industry (Najafi et al., 2015). However, despite a lot of efforts to ameliorate the negative effects of heat stress and high stocking density on poultry (Goo et al., 2019), no promising solutions have yet been developed. One possible reason may be the limited information regarding highly reliable and stress measurements in poultry exposed to stress conditions.

The heterophil to lymphocyte ratio (**H:L ratio**) in the blood is the most widely measured for stress responses in poultry because it can be determined easily with high reliability (Campo and Dávila, 2002; Post et al., 2003). However, the reliability of blood H:L ratio is often questionable due to possible pathogenic infection rather than stress, low sensitivity to different intensities and durations of stress, and potential diurnal variation (Davis, 2005; Cotter, 2015). As an alternative stress biomarker, corticosterone (**CORT**) concentrations in various tissues, such as feathers, excreta, and other body tissues have been suggested and increasingly used to measure stress responses in poultry (Cook, 2012; Weimer et al., 2018). Potential benefits of measuring tissue CORT concentrations include time-integrated measures of adrenocortical activity, animal-friendly measurement, and avoiding additional stress induced by investigators (Bortolotti et al., 2008; Cook, 2012; Matas et al., 2016). However, there have been limited information regarding the comparison of these stress biomarkers in poultry. Furthermore, most of previous

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poultry studies investigated the effects of a single stress condition (i.e., either heat stress or high stocking density) on stress responses during a relatively short period although poultry raised under the commercial situation is frequently exposed to multiple stress conditions with heat stress and high stocking density during a prolonged time, particularly in laying hens.

Therefore, the objectives of the current experiment were to compare various stress biomarkers including the blood H:L ratio and CORT concentrations in feathers, claws, and egg yolk, and to find the potential stress biomarkers in laying hens exposed to a long-term multiple stress condition.

MATERIALS AND METHODS

Animals and Experimental Design

The protocol for the current experiment was reviewed and approved by the Institutional Animal Care and Use Committee at Chung-Ang University (IACUC No. 2020-00100). The experiment was conducted in a completely randomized design. Before the start of the experiment, all hens were raised under the common management and environment according to the Hy-Line Brown Management Guideline (Hy-Line international, 2018). A total of 24 Hy-Line Brown laying hens at 47 wk of age were allotted to 2 distinct conditions including normal condition (**NC**) and multiple stress condition (**MC**) with 8 replicated cages. In NC treatment, 8 hens were raised in the metabolic cages (35 × 45 × 55 cm^3, width × length × height) with 1 hen per cage (0.16 m^2/hen) and temperature and humidity were maintained at 21 ± 0.6°C and 74 ± 5.7%, respectively. This condition was considered a thermoneutral and comfortable environment for laying hens based on the Hy-Line Brown Management guideline (Hy-Line international, 2018). In MC treatment, however, 16 hens were raised in the metabolic cages with 2 hens per cage to decrease space allowance (0.08 m^2/hen) and temperature and humidity were maintained at 31 ± 1.6°C and 73 ± 11.3%, respectively. Therefore, hens raised in MC treatment were subjected to multiple stress conditions with both heat stress and high stocking density. The experiment lasted for 8 wk. Commercial-type diets were used for all hens. Energy and nutrient compositions in the diet were presented in Table 1. The diets and water were fed on an ad libitum basis to the birds. A 16-h lighting schedule was used during the entire experiment.

Table 1. Composition and nutrient concentrations of the diet.

Items	Inclusion levels (%)
Ingredients[1]	
Corn	61.50
Soybean meal (44.5% CP)	16.90
Corn gluten meal	1.64
DDGS	5.00
Animal fat	0.79
MDCP	1.26
Limestone	10.26
Lys H_2SO_4 (54%)	0.34
L-Thr (98.5%)	0.06
DL-Met	0.28
L-Trp	0.11
Celite	1.20
NaCl	0.27
Choline (50%)	0.10
$NaHCO_3$	0.10
Vitamin premix	0.09
Mineral premix	0.10
Total sum	100.00
Calculated energy and nutrient	
AME_n (kcal/kg)	2,740
CP (%)	14.16
Total amino acid (%)	
Lys	0.81
Met + Cys	0.73
Met	0.48
Thr	0.59
Trp	0.18
Arg	0.80
Ile	0.63
Val	0.76
Calcium (%)	4.18
Available phosphorus (%)	0.36

[1]DDGS, dried distiller's grains with solubles; MDCP, monodicalcium phosphate.

Sample Collection

Hen-day egg production and egg weight were recorded daily. However, feed intake (**FI**) and feed conversion ratio (**FCR**) were recorded weekly. Egg mass was calculated by multiplying egg weight by hen-day egg production. The data for productive performance was summarized for 8 wk of the experiment.

All hens in NC treatment (n = 8) were used for the sample collection, whereas 8 hens in MC treatment (i.e., 1 hen per replicated cage; n = 8) were selected and used for all sample collections throughout the experiment. Blood samples were drawn from the wing vein of 16 hens at 0, 2, 4, 6, and 8 wk of the experiment. The collected blood samples were immediately stored in EDTA tubes for calculating the H:L ratio. Two primary flight feather samples were also collected at 0, 2, 4, 6, and 8 wk of the experiment. Each feather was analyzed separately. In addition, claw samples were collected from digit 2 or digit 4 at 0, 4, and 8 wk of the experiment. Egg yolk samples were also collected from each replicated cage at 0, 4, and 8 wk of the experiment. The samples for feather, claw, and egg yolks were stored at −80°C and were subject to the CORT analysis.

Heterophil to Lymphocyte Ratio (H:L ratio) Analysis

The H:L ratio in the blood was analyzed according to the method of Lentfer et al., 2015 with a minor modification. The detailed procedure has been reported in the previous experiment (Yu et al., 2021).

Corticosterone (CORT) Analysis

The CORT extraction from the feather was undertaken using a procedure described by Bortolotti et al. (2008) and Häffelin et al. (2020) with a minor modification. In short,

feathers were separated to vane and rachis with a scalpel, and then the vane was sliced with scissors into pieces of <5 mm^2. Vane pieces were placed in 50 mL tubes with 10 mL methanol (HPLC grade, Honeywell, Charlotte, NC). The tubes were sonicated in a water bath at room temperature for 30 min, and then incubated in a shaking water bath at 50°C overnight. Afterwards, the methanol was separated from the remaining feather using syringe filters and a filtration funnel (HyunDai Micro, Anseong, Republic of Korea). The feather remnant was washed twice again with 2 mL methanol and the washes were added back to the original extract. To evaporate the methanol, samples were placed in a water bath at 50°C until complete evaporation. Samples were resuspended in 500 μL of Assay Buffer 15 (Tris-buffered saline) provided in the ELISA kit (Enzo Life Sciences Inc., New York, NY). Samples were frozen at −80°C before CORT analysis.

The CORT extraction from the claw was followed by the modified method of Baxter-Gilbert et al. (2014). Briefly, claws were first washed with 1 mL distilled water and then washed again with 1 mL 100% methanol twice by vortex mixing for 10 s. Washed samples were air-dried and placed in liquid nitrogen at −196°C for more than 10 min. Frozen samples were then homogenously galvanized using the mortar and pestle. Minced claw samples were placed in 50 mL tubes containing methanol in a ratio of 0.005 g/mL. The tubes were sonicated for 30 min and then incubated in a water bath at 50°C for 24 h. Further sequential steps of methanol separation, evaporation, and re-suspension with Tri-buffered saline were conducted as described in the feather CORT extraction method.

The method described by Abobaker et al. (2017) was used for CORT extraction from the egg yolk. In brief, 0.15 g of the homogenized egg yolk was diluted in 600 μL of distilled water, vortexed for 30 s, and frozen overnight. Frozen samples were then thawed and mixed with 3 mL of 100% methanol. The mixture was shaken for 30 min and frozen again overnight. On the next day, the samples were centrifuged at 2,500 rpm for 5 min. One mL of the supernatant was collected and evaporated under a stream of nitrogen. The remaining remnants were suspended in 500 μL of Tri-buffered saline and then frozen at −80°C before CORT analysis.

The CORT analysis of the feather and egg yolk was performed on duplicates, but the CORT analysis of the claw was performed on 1 sample due to the limitation of sample collection and amount. The CORT concentrations in the feather, claw, and egg yolk were determined using the commercial Enzo Life Sciences Corticosterone ELISA Kit ADI-901-097 (Enzo Life Sciences Inc.) The calculation of CORT concentrations in the tissues was performed based on the manual of Enzo Life Sciences Corticosterone ELISA Kit ADI-901-097 (Enzo Life Sciences Inc., New York, NY), whereby the standard curve was fitted using a 4-parameter logistic curve to interpolate CORT concentrations. The detailed procedure for analyzing CORT concentrations was described previously by Häffelin et al. (2020).

Statistical Analysis

All data were analyzed as a completely randomized design. The data for productive performance were analyzed by ANOVA with the MIXED procedure of SAS (SAS Institute Inc., Cary, NC). Outliers were checked using the UNIVARIATE procedure of SAS. The LSMEANS procedure was used to calculate treatment means and the PDIFF option of SAS was used to separate the means if the difference was significant.

For stress biomarkers, including H:L ratio in the blood and CORT concentrations in the feather, claw, and egg yolk, a repeated-measure analysis was performed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC). The statistical model included the fixed effects of stress condition, time, stress condition × time interaction, and the covariate effect of initial blood H:L ratio and tissue CORT concentrations measured at the start of the experiment. Five different covariance structures were tested, and then the unstructured option was chosen for the correlation between observations within each stress condition because the unstructured option revealed the least values for Corrected Akaike Information Criterion (Wang and Goonewardene, 2004). The LSMEANS procedure was used to calculate means and the PDIFF option of SAS was used to provide the mean difference between stress conditions at each time point as well as between time points within each stress condition. A probability of $P < 0.05$ was considered significant and $0.05 \leq P \leq 0.10$ was considered a tendency.

RESULTS

Productive Performance

The BW, BW gain, hen-day egg production, egg weight, egg mass, FI, and FCR were influenced by multiple stress conditions (Table 2). Hens in MC treatment had less ($P < 0.05$) BW, BW gain, hen-day egg production, egg weight, egg mass, FI, and FCR than hens in NC treatment. This result indicates that our MC treatment designed to induce both heat stress and high stocking density was sufficient to produce stressful conditions for laying hens.

Table 2. Effect of stress conditions on productive performance of laying hens during 8 wk of the experiment.[1]

	Treatments[2]			
Items	NC	MC	SEM	*P*-value
BW, g	1,906	1,693	34.5	<0.01
BW gain, g	181	−3	31.0	<0.01
Hen-day egg production, %	98	90	1.0	<0.01
Egg weight, g	60	57	0.8	<0.05
Egg mass, g	59	52	1.1	<0.01
Feed intake, g/hen/d	117	94	1.4	<0.01
Feed conversion ratio, g/g	2.00	1.84	0.030	<0.01

[1]Data are least squares means of 8 replicates per treatment.
[2]NC, normal condition; MC, multiple stress condition.

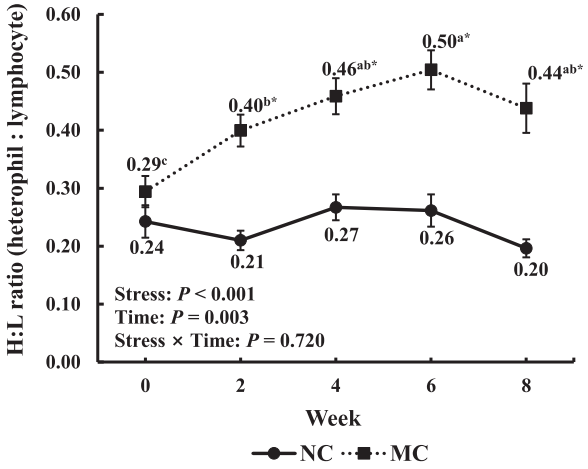

Figure 1. Heterophil to lymphocyte ratio (H:L ratio) in the blood of laying hens raised at either normal condition (NC) or multiple stress condition (MC) during 8 wk of the experiment. Data are shown as means ± SEM (n = 8). [a,b,c]Means with different superscripts are different within MC treatment ($P < 0.05$). Asterisk mark (*) indicates that means at each time point between NC and MC treatments are different ($P < 0.05$).

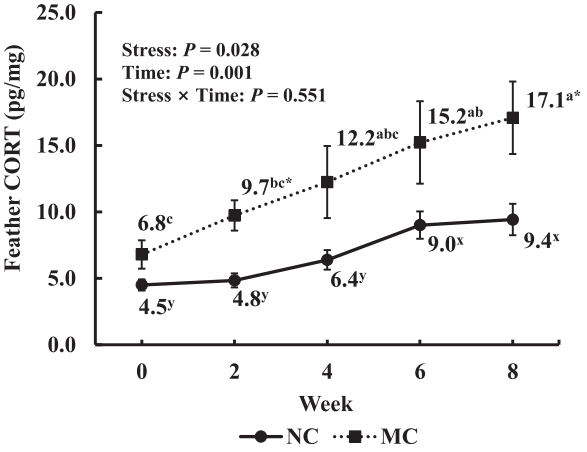

Figure 2. Feather corticosterone (CORT) concentrations of laying hens raised at either normal condition (NC) or multiple stress condition (MC) during 8 wk of the experiment. Data are shown as means ± SEM (n = 8). [a,b,c]Means with different superscripts are different within MC treatment ($P < 0.05$). [x,y]Means with different superscripts are different within NC treatment ($P < 0.05$). Asterisk mark (*) indicates that means at each time point between NC and MC treatments are different ($P < 0.05$).

Stress Biomarkers

Repeated-measures analysis was conducted to investigate the interactive effects of stress condition and time on stress biomarkers, including blood H:L ratio and CORT concentrations in the feather, claw, and egg yolk.

No interactive effects between stress conditions and time were observed for blood H:L ratio (Figure 1). However, the main effects of stress conditions and time were significant ($P < 0.05$) for blood H:L ratio. Hens in MC treatment had greater ($P < 0.01$) blood H:L ratio than those in NC treatment. Blood H:L ratio differed ($P < 0.01$) with the duration of experiment. In MC treatment, the greatest ($P < 0.05$) H:L ratio was observed at 6 wk, whereas no differences were identified at all time points in NC treatment. The greater ($P < 0.05$) H:L ratio for hens in MC treatment than in NC treatment was observed at all time points.

There were no interactive effects between stress conditions and time for feather CORT concentrations (Figure 2). However, the main effects of stress conditions and time were significant ($P < 0.05$) for feather CORT concentrations. Hens in MC treatment had greater ($P < 0.05$) feather CORT concentrations than those in NC treatment. Feather CORT concentrations were increased ($P < 0.05$) with the duration of experiment. In both MC and NC treatments, feather CORT concentrations were the greatest ($P < 0.05$) at the end of the experiment (i.e., 8 wk). However, differences in feather CORT concentrations between stress conditions were only detected ($P < 0.05$) at 2 and 8 wk of the experiment.

A tendency ($P = 0.078$) for interaction between stress conditions and time was observed for claw CORT concentrations (Figure 3). One of the main effects, stress conditions, was not significant, but claw CORT concentrations were decreased ($P < 0.05$) with the duration of experiment. In both MC and NC treatments, claw CORT concentrations were the least ($P < 0.05$) at the end of the experiment (i.e., 8 wk). No differences in claw CORT concentrations between stress conditions were observed at each time point.

No interactive effects between stress conditions and time were observed for CORT concentrations in egg yolk (Figure 4). Hens in MC treatment tended ($P = 0.081$) to have a greater CORT concentration in egg yolk than hens in NC treatment, but no significant time effects were observed. In MC treatment, CORT concentrations in egg yolk at 4 and 8 wk were greater than those at the start of the experiment, whereas no differences were observed among time points in NC treatment.

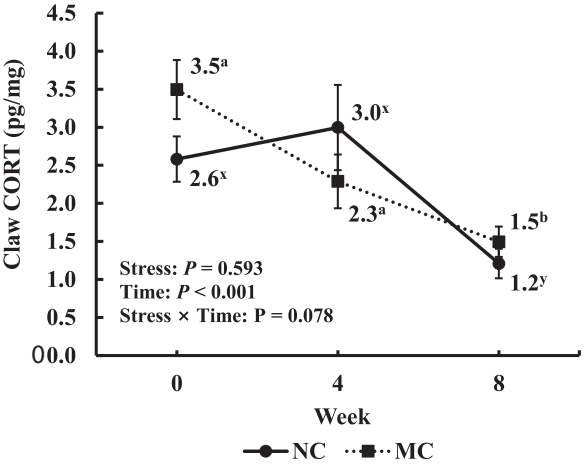

Figure 3. Claw corticosterone (CORT) concentrations of laying hens raised at either normal condition (NC) or multiple stress condition (MC) during 8 wk of the experiment. Data are shown as means ± SEM (n = 8). [a,b]Means with different superscripts are different within MC treatment ($P < 0.05$). [x,y]Means with different superscripts are different within NC treatment ($P < 0.05$).

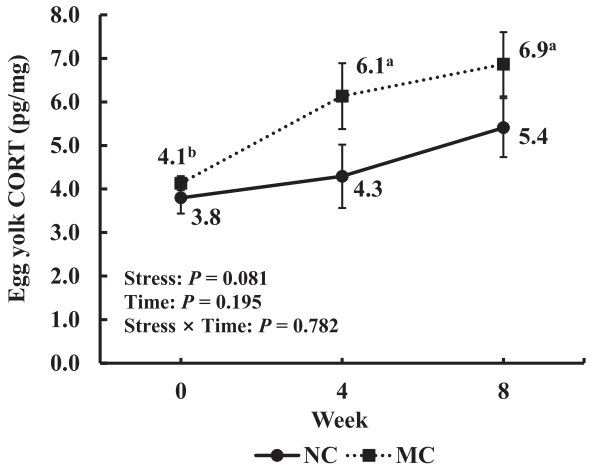

Figure 4. Egg yolk corticosterone (CORT) concentrations of laying hens raised at either normal condition (NC) or multiple stress condition (MC) during 8 wk of the experiment. Data are shown as means ± SEM (n = 8). [a,b]Means with different superscripts are different within MC treatment ($P < 0.05$).

DISCUSSION

Blood H:L ratio is the most widely used as a stress biomarker in poultry (Zulkifli et al., 2009; Al-Aqil et al., 2013; Shakeri et al., 2014). Increasing CORT levels in the body by various stressors are known to facilitate the transmission of lymphocytes from the blood circulation into body tissues, but it concomitantly promotes the transmission of heterophils from the bone marrow into the blood circulation; therefore, poultry exposed to various stress conditions generally show an increase in blood H:L ratio (Bishop et al., 1968; Fauci, 1975; Dhabhar, 2002). It has been reported that blood H:L ratio is increased in poultry exposed to heat stress (McFarlane and Curtis, 1989; Mashaly et al., 2004; Felver-Gant et al., 2012) or high stocking density (Shakeri et al., 2014; Kang et al., 2016; Lee et al., 2018). In the current experiment, a greater blood H:L ratio was observed in MC treatment than in NC treatment.

It appeared, however, that blood H:L ratio was not linearly responsive to increasing duration of stress exposure because the greatest H:L ratio was observed at 6 wk and then tended to be maintained or rather decreased slightly at 8 wk. This result may indicate that poultry may have a limited capacity to increase blood H:L ratio when exposed to a long-term and continuous stress condition possibly with high intensities. Previous experiments reported that birds treated with CORT via drinking water showed a linear increase in blood H:L ratio during the first day, but a decrease by the continuous CORT treatments during 7 to 10 d (Shini et al., 2008). The reason may be decreased responsiveness of blood H:L ratio to increasing CORT levels with a long-term stress exposure, but the direct evidence for this speculation is currently lacking.

It has been demonstrated that circulating CORT can be readily deposited in the feather and the amount of its CORT deposition is positively correlated with both duration and intensity of stress conditions (Bortolotti et al., 2008, 2009). Moreover, feather collection is non-invasive, and therefore, feather CORT concentrations are recognized as an animal-friendly stress biomarker in poultry (Häffelin et al., 2021). Previous experiments reported that heat stress (Kim et al., 2021) and high stocking density (Robertson et al., 2017; von Eugen et al., 2019; Yu et al., 2021) increased the feather CORT concentrations of poultry, which was confirmed by our results.

Interestingly, however, we found that feather CORT concentrations in NC treatment were also increased with duration of the experiment, which led to a significant main effect of time. A similar increase in feather CORT concentrations during the experiment was also observed in broiler chickens raised under normal conditions (Weimer et al., 2018). These results may indicate that the feather is very responsive and accumulative to circulating CORT levels because birds raised under normal conditions also have a certain level of circulating CORT. Moreover, our observation is also likely related to the general physiology of laying hens. Leishman et al. (2021) reported that a female-line turkey had greater feather CORT concentrations than a male-line turkey. Bortolotti et al. (2008) also reported that the feather CORT concentrations were highly and positively correlated to the number of laying eggs.

It was hypothesized that the extent of increasing CORT concentrations in the feather of hens in MC treatment would be greater than hens in NC treatment in this experiment; however, we failed to find any significant interaction. The reason may be related to an inherently large variation in feather CORT concentrations of poultry (Weimer et al., 2018). In particular, the variation in MC treatment was greater than in NC treatment, which appears to be reasonable because of the greater individual variation in feather CORT accumulation rate of hens exposed to stress conditions than normal conditions (Leishman et al., 2021). In addition, it should be noted that feather CORT concentrations in laying hens may also vary with stage and extent of feather growth because growing feathers are more responsive to changes in blood CORT levels than full-grown and inert feathers (Bortolotti et al., 2008; Lattin et al., 2011; Jenni-Eiermann et al., 2015). Therefore, there may be a possibility that stress conditions may influence the growth rate of feathers; however, the clear evidence has not been reported previously in poultry.

As observed for feather CORT concentrations, circulating CORT can diffuse into claws because both feathers and claws are composed largely of the keratin (Warnock et al., 2010). It was reported that CORT concentrations were measurable in the claw of turtles and chameleons (Baxter-Gilbert et al., 2014; Matas et al., 2016). Therefore, it can be speculated that the claw of birds may also be used for CORT status in response to stress conditions. In the current experiment, a considerable amount of CORT was detected in the claw of laying hens, regardless of stress conditions. To our knowledge, this study is the first to determine CORT concentrations in the claw of poultry as affected by stress conditions. However, CORT concentrations in the claw were not

affected by stress conditions. Moreover, the claw CORT concentrations were decreased with duration of the experiment, suggesting that CORT concentrations in the claw are unlikely a candidate stress biomarker in poultry. These results were unexpected because a considerable amount of CORT was presented in the claw of poultry. The possible reason for this unexpected result may be very slow growth rate of claw, resulting in limited accumulation time of CORT in the claw. Based on the previous experiment measuring the growth rate of claw in birds (i.e., 0.034 ± 001 mm/d; Hahn et al., 2014), we speculated that approximately 2 mm of claw grew during the current 8-wk experiment. Therefore, it was suspicious that 8 wk of stress conditions may be insufficient to affect the CORT concentrations of claw. Interestingly, CORT concentrations were decreased with duration of the experiment. The observation is not easily explained because circulating CORT is expected to be continuously retained in the claw. Therefore, this result may be caused by other factors such as sampling position and different digits of the claw in the feet. Further studies are required to disclose the reason for decreased CORT concentrations in the claw of poultry with increasing duration of stress exposure.

A relationship between circulating CORT levels and CORT concentrations in egg yolk has been reported (Rettenbacher et al., 2005; Downing and Bryden, 2008; Babacanoğlu et al., 2013), indicating that CORT concentrations in egg yolk may be a possible stress biomarker in laying hens (Singh et al., 2009; Pu et al., 2019). Our results also revealed increased CORT concentrations in egg yolk of hens in MC treatment with increasing duration of experiment, whereas it was not the case for hens in NC treatment. However, the fact that CORT concentrations in egg yolk did not differ between 4 and 8 wk of experiment may indicate that this measure is not a good reflection of the time-stress response under prolonged stress conditions. Similar results were also observed by Hayward and Wingfield (2004) who reported that the continuous infusion of CORT in the blood of Japanese quails increased CORT concentrations in egg yolk during the initial 7 d, but did not change after 7 d. The reason for this observation may be related to the fact that hens produce eggs almost daily, leading to a limitation of CORT accumulation in egg yolk as responded linearly to increasing duration of stress exposure. Thus, CORT concentrations in egg yolk may not be a suitable stress biomarker in laying hens exposed to a long-term stress.

CONCLUSIONS

The H:L ratio in the blood and CORT concentrations in feathers and egg yolks are considered potential stress biomarkers in laying hens exposed to multiple stress conditions despite their respective limitations. Blood H:L ratio and CORT concentrations in egg yolk show a limited ability to reflect a long-term and time-course change in the stress response. Feather CORT concentrations are likely to increase with duration of stress exposure due to the continuous production of CORT in poultry, irrespective of stress exposure. The claw CORT concentrations appear to be insensitive to a long-term stress exposure.

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DISCLOSURES

The authors declare no conflicts of interest.

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