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ANIMAL PRODUCTION

Methods for identifying stress caused by fasting in commercial laying hens

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ABSTRACT: Plasma corticosterone concentration (CORT), heterophil:lymphocyte ratio (H:L), catalase activity (CAT), total glutathione concentration (GSH), and thiobarbituric acid reactive substance levels (TBARS) were evaluated in 48 Hy-Line Brown laying hens, at 79 weeks of age, after being fasted for 10 consecutive days. Blood was collected on days zero, two, four, six, eight, and 10 of fasting, and a completely randomized design was adopted, with eight replicates on each day of collection, with each bird being an experimental unit. The time of maximum stress was determined for each method, using the polynomial regression analysis. The Pearson correlation analysis was also performed to determine whether the methods were interchangeable. CORT and GSH concentrations indicated that the time of maximum stress occurred at 4.3 days of fasting, whereas, the H:L and CAT activity indicated that the time of maximum stress occurred at 10 days of fasting. The malondialdehyde concentration detected by the TBARS method was highest at day zero and lowest at day 5.5 of fasting, but this method was not a reliable measure of stress. The low Pearson correlation coefficients observed among the methods made it impossible to designate only one of the tested methods as a replacement for the others, to measure the stress in laying hens during fasting.
Key words: corticosterone, fasting, heterophil:lymphocyte ratio, oxidative stress.

Metodologias para indicar estresse causado por jejum em poedeiras comerciais

RESUMO: A concentração de corticosterona plasmática - CORT, relação heterófilo:linfócito - H:L, atividade enzima catalase - CAT, concentração glutationa total - GSH e níveis de substâncias reativas ao ácido tiobarbitúrico – TBARS foram avaliadas em 48 poedeiras Hy Line Brown, 79 semanas de idade, submetidas ao jejum alimentar por 10 dias consecutivos. Coletou-se sangue no dia zero, dois, quatro, seis, oito e 10 do período de jejum alimentar, adotando-se um delineamento inteiramente casualizado, com oito repetições em cada dia de coleta, sendo cada ave uma unidade experimental. Através da análise da regressão polinomial determinou-se o momento de máximo estresse para cada metodologia. Realizou-se análise de correlação de Pearson para determinar se pode haver substituição entre os métodos. A CORT e GSH apresentaram o momento de máximo estresse aos 4,3 dias de jejum alimentar, enquanto que H:L e CAT aos 10 dias. TBARS apresentou maior concentração de malondialdeído no dia zero de jejum alimentar e a menor aos 5,5 dias, não sendo uma metodologia confiável para medir o estresse. Os baixos coeficientes de correlação de Pearson entre as metodologias impossibilitam a indicação de apenas um método em substituição aos demais para mensurar o estresse de poedeiras durante o período de jejum alimentar.
Palavras-chave: corticosterona, estresse oxidativo, jejum alimentar relação heterófilo: linfócito.

INTRODUCTION

Physiological stress is conceptualized as a sum of the defense mechanisms in the animal's body acting against any stress stimulus that is capable of unbalancing homeostasis (ROMERO, 2004); altering the hypothalamic–pituitary–adrenocortical (HPA) axis (SIEGEL, 1971); and increasing the blood concentration in the glucocorticoid hormones, particularly corticosterone, in birds (ROOS, 1960). This in turn causes heterophilia and lymphopenia (GROSS & SIEGEL, 1983) or affects the regulation of the carbohydrate, protein, and fat metabolism (EILER, 2012), which alters the behavior of birds, thereby, inducing oxidative stress (BOZKURT et al., 2016).

Stress evaluation in birds is possible when appropriate methods are used (ALM et al., 2016), however, no consensus exists on those methods that respect animal welfare (BLOKHUIS et al., 2007). However, plasma corticosterone and the heterophil/lymphocyte ratio are the most commonly used methods for stress assessment in birds (SCANES, 2016), with the plasma corticosterone concentration being the most indicated (MORMEDE et al., 2007).

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Fasting is a technique widely used in commercial laying hen production systems to induce molting (ZULKIFLI, 1999). During fasting, egg production is temporarily interrupted, allowing the reproductive system to rest and the bird to prepare for a new cycle of egg production (LEE, 1982). This procedure is considered highly stressful (HOSHINO et al., 1988; KOELKEBECK & ANDERSON, 2007), and some alternative protocols have been tested, to maintain the welfare of the birds by reducing the stress (BOZKURT et al., 2016; CERBARO et al., 2014; GONGRUTTANANUN et al., 2013). However, no studies have jointly tested the methods applied to evaluate the physiological stress arising from feed restriction in commercial laying hens.

The objective of the present study evaluated the plasma corticosterone concentration (CORT), heterophil:lymphocyte ratio (H:L), catalase activity (CAT), total glutathione concentration (GSH), and thiobarbituric acid reactive substance levels (TBARS), to measure stress in commercial laying hens subjected to feed restriction.

MATERIALS AND METHODS

Location, facilities, and birds

The experiment was conducted in the metabolism test room of Poultry Sector of Department of Animal Production and Food Science, Agroveterinary Science Center, Santa

Catarina State University (UDESC), southern Brazil. Twelve cages (0.5 x 0.5 m) equipped with trough feeders and nipple drinkers were placed in a climatized room. The room temperature was measured and the mean minimum and maximum temperatures observed, which were, 20.2 °C and 26.3 °C, respectively.

A total of 48 Hy-Line Brown commercial laying hens, 79 weeks of age, were housed in the room — four birds per cage. The hens were raised during the productive period on a commercial farm, according to the breeder's manual. The birds were allowed a period of adaptation to the experimental environment of seven days, and were fed a diet with the iso-nutritive ratio indicated for the laying phase and water *ad libitum*. On the first day of adaptation, the hens were labeled with a ring and randomly distributed among the cages. To determine the percentage of body weight loss, the birds were weighed individually at the beginning and end of the fasting period.

***S**tress stimulus and stress methods*

Fasting that lasted for 10 consecutive days was used as a stress stimulus, with water supplied *ad libitum*. Mortality and cannibalism were not observed. The experiment was comprised of the execution of five methods that evaluated stress: The plasma corticosterone concentration (CORT); heterophil:lymphocyte ratio (H:L); catalase enzyme activity (CAT); total glutathione concentration (GSH); and thiobarbituric acid reactive substances (TBARS) levels.

Six blood samples were taken on days zero, two, four, six, eight, and ten of fasting. On each collection day, eight birds were randomly selected, blood was collected, and the birds were returned to the cages to complete the feeding restriction period. No birds were used for a second blood collection.

For the CORT and H:L methods, the collection start time was standardized to 4 p.m., based on the CORT circadian rhythm, with the purpose of eliminating the influence of laying (BEUVING & VONDER, 1977). The collection was done through a puncture of the ulnar vein. After collection for CORT determination, the blood was prepared as smears for H:L determination.

For CAT, GSH, and TBARS, the collection start time was standardized to 8 a.m. The collection was done through a puncture of the jugular vein.

Plasma CORT method

A total of 4 ml of blood was collected from the jugular vein of each bird and stored in vials containing the anticoagulant agent heparin. The sample was then centrifuged for 10minutes at 3500 rpm, to obtain the plasma, followed by freezing at -20 °C. It was then transported to the Bet Labs laboratory in Rio de Janeiro, where the samples were analyzed using the radioimmunoassay technique, with an NP Biomedical Radioimmunoassay® kit. Manual bird containment for blood collection did not exceed 45 seconds; the collections were timed to avoid an increase in the CORT concentration due to immobilization (BEUVING & VONDER, 1978).

H:L method

Blood smears were prepared in duplicate, dried at room temperature and stained with Giemsa and May-Grunwald (Newprov®, Pinhais, Paraná, Brazil), following the manufacturer's recommendations. Microscopy was performed with an optical microscope, at 100X magnification (oil immersion) (Opticam Microscopy Technology®). A total of 200cells were counted per slide with the aid of a manual blood cell counter, for differentiation into granulocytes (heterophils, eosinophils, and basophils) and agranulocytes (monocytes and lymphocytes). For cell identification, the morphological characteristics

described by LUCAS & JAMROZ (1961) were used, and the H:L ratio was calculated by dividing the percentage of heterophils relative to lymphocytes (GROSS & SIEGEL, 1983), obtained from the mean of two slides per bird.

CAT, GSH, and TBARS methods

A total of 3 ml of blood/bird was collected in EDTA tubes and used for each method. The labeled samples were stored in a freezer at -80 °C and sent to the laboratory for processing. To assess the CAT activity, the blood was first homogenized in a 150-mM saline solution, and the protein content was measured using the Coomassie stain method (BRADFORD, 1976). The CAT activity was determined by following the decrease in hydrogen peroxide (H2O2) absorption, at 240 nm, and expressed in µmol/mg protein/min (BOVERIS & CHANCE, 1973). To evaluate the GSH concentration, the indirect non-protein thiols (NPSH) content method was used and evaluated at 412 nm, after reaction with DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)]. The proteins were removed by the addition of 0.5 M perchloric acid (ELLMAN, 1959), and the NPSH content results were presented as µmol SH/g blood. Lipid peroxidation was measured by the amount of malondialdehyde (MDA) obtained by the TBARS method, as described by BUEGE & AUST (1978). The results were reported in nmol MDA/g blood. All analyses were performed on whole blood samples.

Statistical analysis

The analysis was performed by a completely randomized design, with five stress-indicating methods (treatments), at six sampling times, with eight replications. The data were analyzed using the Shapiro-Wilk test for Normality, and the Bartlett's Test for homogeneity of variance. Polynomial regression analysis was performed to determine the day of maximum stress identified by each method. As all methods provide quantitative results, correlations were possible with the Pearson correlation. All statistical analyses were performed using SAS (2011).

RESULTS AND DISCUSSION

The birds weighed 2.072 g ± 137 g at the beginning and 1.501 g ± 126 g at the end of the fasting period, resulting in a mean weight reduction of 27.7%. This reduction indicated that the fasting to which the birds were subjected was successful, as recommended by CERBARO et al. (2014).

The plasma CORT levels showed a quadratic behavior determined by polynomial regression, with the peak stress being observed at 4.3 days of fasting (Figure 1a). The birds had an initial mean CORT level of 1.76 ng/ml, with a subsequent increase in the hormone till it reached the peak stress (4.3 days), at a level of 3.69 ng/ml.

It is important to highlight that the CORT levels increased in the first movement, followed by a decrease after 4.3 days of fasting. The response to fasting in the initial adaptation phase is short and involves metabolic transition to a stable fasting physiological state. The basal metabolic rate declines and the plasma corticosterone concentration increases (WEBSTER, 2003; CHEREL et al., 1988), as observed in this study. After that, there is a phase of long-term economy that may last weeks or even months. During this time, the specific basal metabolic rate is almost constant, the locomotor activity is reduced to conserve energy, and the plasma corticosterone concentrations are low (WEBSTER, 2003; CHEREL et al., 1988), as observed in this study.

This result indicated that feeding restriction is a stress stimulus capable of altering the HPA axis, thereby increasing the CORT plasma levels. The assumption is that, in the face of stress, the neural stimuli act on the hypothalamus and increase the secretion of the hormone that releases corticotropin (CRH), which in turn stimulates the adenohypophysis to release the adrenocorticotropic hormone (ACTH). At high blood concentrations, the latter stimulates the adrenal cortex to release steroid hormones into the systemic circulation (SIEGEL, 1971), and the first endocrine change observed in laying hens subjected to feeding restriction is a higher plasma CORT concentration (BERRY, 2003). CORT is delivered to the bloodstream according to the body's needs, to mobilize energy, as this hormone promotes gluconeogenesis, which helps to maintain the plasma glucose levels in the early phase of food deprivation (WEBSTER, 2003).

The blood H:L ratio of the birds presented a linear behavior, determined by the polynomial regression analysis (Figure 1b). The H:L ratio increased with the days of fasting, with the time of greatest stress being 10 days, at the end of fasting. This method has been shown to be effective as an indicator of stress in laying hens, where an increase in the H:L ratio during feed restriction is interpreted as an attempt by the body to combat the physiological stress that results from lack of food and consequent loss of body weight (DAVIS et al., 2000). The increase in the H:L ratio is also due to the increase in CORT (WEBSTER, 2003). However, a direct relation cannot be established, as the regression equations of both are

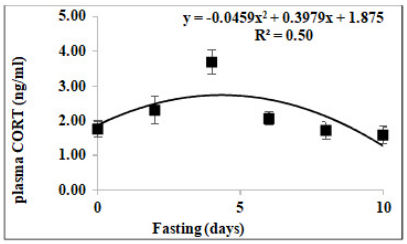

Figure 1 (a) – CORT

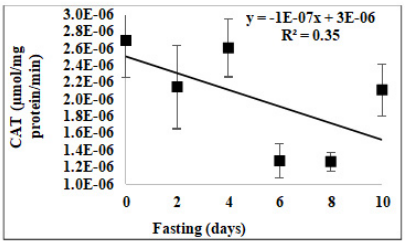

Figure 1 (c) – CAT

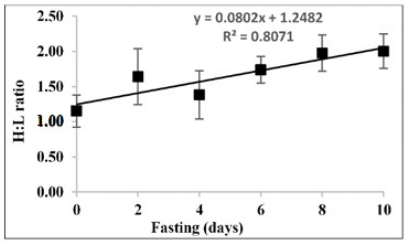

Figure 1 (b) – H:L

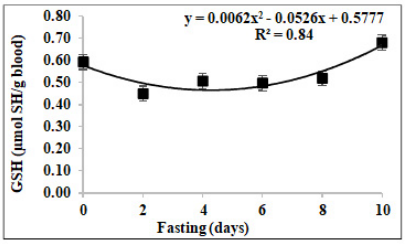

Figure 1 (d) – GSH

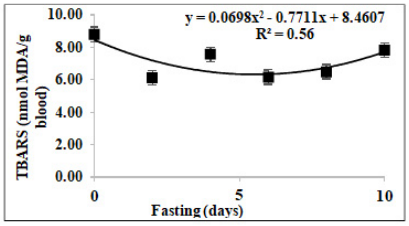

Figure 1 (e) – TBARS

Figure 1 - Methods that evaluate stress in Hy-Line Brown laying hens subjected to feeding restriction for 10 days. The points in the figures represent the mean plasma corticosterone concentration (CORT), catalase activity (CAT), heterophil/lymphocyte ratio (H:L), total glutathione concentration (GSH), and thiobarbituric acid reactive substances (TBARS), measured every two days, along with the respective standard error of the mean.

different. In the present study, the birds have shown greater stress at the end of feeding restriction; the higher the H:L ratio, the greater the stress indication in the bird (GROSS & SIEGEL, 1983).

No appropriate reference values exist for the H:L ratio in birds (COTTER, 2015). In our study, the ratio ranged from 1.15 to 2.00 during the fasting period. However, DAVIS et al. (2000) obtained an H:L ratio of 0.71 in laying hens during the peak of feeding restriction, and when these birds were 20 weeks of age, the ratio was significantly lower (0.082).

The polynomial regression analysis of the CAT activity showed a linear behavior. As the fasting days advanced, the enzymatic activity decreased (Figure 1c), with the time of maximum stress that occurred at 10 days of restriction. The method was able to detect stress and was considered effective for this purpose. In this case, the practice of restricting the feed in commercial laying hens could induce oxidative stress by suppressing the antioxidant capacity of the body.

Studies involving stress induction in birds show that a decrease occurs in the enzyme activity. Stress induction in broilers, through the administration of lipopolysaccharides from *Escherichia coli,* leads to a decrease in the CAT activity and an increase in the MDA concentration (ZHENG et al., 2016). Cyclophosphamide administration in the broilers results in a decrease in CAT activity, an increase in MDA concentration, and a lower GSH concentration (YU et al., 2015). However, both studies cited had the objective of evaluating the body's response after incorporation of ingredients with antioxidant capacity into the ration. No studies have been found, which demonstrated that feeding restriction is a stress agent, with subsequent CAT evaluation.

The GSH concentration showed a quadratic behavior according to the polynomial regression analysis (Figure 1d). After day zero, the birds showed a decrease in GSH till they reached the time of maximum stress, that is, at 4.3 days of fasting, when the lowest GSH concentration was observed.

Stress leads to an increase in the GSH released from the liver and other tissues into the bloodstream for detoxification of the body. Norepinephrine stimulates this process on the basis of the availability of circulating glutathione, for absorption by the extrahepatic tissues. This helps to reduce oxidative stress (SONG et al., 2000). Based on the glutathione redox system, dismutation of H2O2 in H2O + O2 can be performed by the action of the GSH-Px enzyme (YU et al., 2015), which is obtained by the transformation of the reduced form of glutathione to its oxidized form (HUBER et al., 2008). However, when the antioxidant defense system becomes deficient, or, the production of the reactive oxygen species (ROS) prevails, oxidative stress is established (SANI et al., 2015), and the GSH concentration tends to decrease (ZAREI & SHIVANANDAPPA, 2013). This oxidative stress can be observed for up to 4.3 days of fasting, when the GSH concentration decreases continuously, indicating that the bird's body has not been able to control the stress generated by the feeding restriction until this time.

The TBARS analysis showed a quadratic behavior, as indicated by the polynomial regression, and the time of maximum stress occurred at 5.5 days of the feeding restriction (Figure 1e). On day zero, the birds had a higher amount of MDA, and with the progression of fasting, a reduction occurred up to the minimum amount determined by the regression, at 5.5 days. The results of the present study are different from those reported in the literature, which indicated that birds subjected to feeding restriction have increased MDA levels (BOZKURT et al., 2016).

No correlation was observed between the methods on day zero of the feeding restriction (Table 1), indicating that the methods cannot be replaced by one another in a situation where the birds are not under stress. However, some methods showed a high positive correlation on certain days of fasting, allowing the replacement of indicators. This can be observed between CORT, H:L, and GSH on the eighth day; between CORT and CAT on the sixth day, and between CORT and TBARS on the second day of collection. The H:L ratio showed a high positive correlation with the GSH on the fourth day and with TBARS on the second day. On the eighth day of collection, the GSH concentration had a high positive correlation with TBARS. Notably, the above results indicated that one method can be replaced by another, according to the feeding restriction day. However, when the total period of 10 days of fasting is considered, no indicator can be completely replaced by another method tested in the study, given the inconsistent values of the coefficients indicated by the Pearson correlation.

CONCLUSION

The plasma corticosterone concentration, heterophil:lymphocyte ratio, total glutathione concentration, and catalase activity methods are

Table 1 - Pearson correlation coefficient (r) of methods for evaluating stress in commercial laying hens subjected to 0, 2, 4, 6, 8, and 10 days of feeding restriction.

Methods		----------0---------		--------2--------		--------4--------		--------6--------		----------8---------		-------10--------	
		r	P *value*	r	P *value*	r	P *value*	r	P *value*	r	P *value*	r	P *value*
CORT	H:L	0.009	0.986	0.501	0.390	0.159	0.762	0.021	0.969	0.716	0.110	-0.394	0.382
	CAT	0.035	0.947	-0.310	0.690	-0.042	0.937	0.672	0.143	-0.105	0.822	-0.423	0.344
	GSH	-0.787	0.115	-0.238	0.700	-0.001	0.999	0.102	0.848	0.700	0.121	-0.278	0.594
	TBARS	-0.328	0.590	0.835	0.078	0.107	0.840	-0.529	0.280	0.555	0.331	0.374	0.465
H:L	CAT	-0.696	0.125	-0.138	0.862	-0.741	0.057	0.014	0.978	0.126	0.788	-0.264	0.568
	GSH	-0.559	0.327	-0.825	0.043	0.735	0.060	-0.516	0.294	0.212	0.687	0.380	0.400
	TBARS	-0.659	0.155	0.636	0.249	0.012	0.982	-0.605	0.203	0.256	0.621	-0.471	0.287
CAT	GSH	0.280	0.648	0.027	0.966	-0.400	0.373	-0.071	0.909	-0.122	0.794	0.266	0.610
	TBARS	0.117	0.825	-0.006	0.991	-0.852	0.031	-0.268	0.663	-0.256	0.624	-0.545	0.263
GSH	TBARS	0.557	0.443	-0.772	0.072	-0.238	0.608	0.351	0.440	0.987	0.002	-0.504	0.307

CORT = plasma corticosterone concentration (ng/ml); H:L = heterophil:lymphocyte ratio; CAT = catalase (µmol/mg protein/min); GSH = total glutathione (µmol SH/g blood); TBARS = thiobarbituric acid reactive substances (nmol MDA g blood); P value = probability.

indicated for measuring stress in fasting commercial laying hens. The plasma corticosterone and total glutathione concentration methods indicated that the time of maximum stress was at 4.3 days of fasting, whereas, the heterophil:lymphocyte ratio and catalase activity methods determined it to be at 10 days of fasting.

On analysing, the TBARS level was not found to be an efficient method, as this method detected the highest amount of malondialdehyde at day zero of fasting and the lowest at day 5.5. The Pearson correlation coefficients were low and inconsistent among the methods, which made it impossible to use only one method in place of the others, to measure the stress in laying hens during fasting.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTIONS

The authors contributed equally to the design and writing of the manuscript. All authors critically reviewed the manuscript and approved the final version.

BIOETHICS AND BIOSECURITY COMMITTEE APPROVAL

The project was submitted and approved by the Ethics Committee on Animal Experimentation of UDESC under protocol number 5377250116 and is therefore in accordance with the obligations of Law 11.794/2008 (BRASIL, 2008), with Decree 6.899/2009, and with the standards established by the Conselho Nacional de Controle da Experimentação Animal (National Council for the Control of Animal Experimentation).

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