The effect of different degrees of feed restriction on heat shock protein 70, acute phase proteins, and other blood parameters in female broiler breeders

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ABSTRACT The aim of the current study was to determine the physiological response to feed restriction in female broiler breeders using a range of conventional and novel indicators. One hundred female breeders were subjected to one of five feeding regimens from d 28 to 42 as follows (i) ad libitum feeding (AL), (ii-v) 75, 60, 45, and 30% of ad libitum feed intake. Blood heterophil to lymphocyte ratio (HLR), and plasma circulating corticosterone (CORT), ghrelin (GHR), serotonin (5-HT), and dopamine (DA) and serum acute phase proteins (APP) concentrations together with brain heat shock protein (HSP) 70 level were measured. The results showed a significant effect of feed restriction on blood HLR and plasma CORT, GHR, 5-HT, DA, and brain HSP 70 levels. However, feed restriction had no effect on serum levels of APP of alpha-1 acid glycoprotein, ovotransferin, and ceruloplasmin. Serum levels of 5-HT and GHR varied curvilinearly with the feed restriction level. The relationship between brain HSP 70 and level of feed restriction was negligible. However, significant linear relationships between HLR, CORT, DA, and the level of feed restriction were noted. Thus, these 3 parameters appear to represent a straight forward relation with severity of feed restriction.

Key words: broiler breeder, feed restriction, corticosterone, heat shock protein 70, acute phase proteins

INTRODUCTION

Broiler breeders are reared under strict feed restriction programs. The restriction of 25 to 33% of ad libitum feed consumption during rearing (de Jong et al., 2002) and 50 to 90% during production is a common practice to reduce metabolic disorders and to improve productivity (Bruggeman et al., 1999; Chen et al., 2006). There is substantial evidence that restricting the intake of feed can result in physiological stress responses, boredom, stereotypies, aggression, and other abnormal behaviors in poultry (Mench, 2002; van Krimpen and de Jong, 2014). Plasma corticosterone (CORT) concentration (Zulkifli and Siegel, 1995) and heterophil to lymphocyte ratios (HLR) (Maxwell et al., 1992; Zulkifli and Siegel, 1995) have been used widely as physiological indicators of stress in avian species. There is, however, a lack of agreement among previous studies in the reliability of CORT and HLR for the assessment of chronic stress in feed restricted broiler breeders. For example, a higher CORT was noted in feed restricted breeder birds as compared to those fed ad libitum (Savory et al., 1996; Savory and Mann, 1997; Hocking et al., 2001; de Jong et al., 2002). On the contrary, Savory et al. (1993) noted otherwise in breeders fed a commercial daily restricted ration from 5 to 21 weeks of age. Working with 36-day-old White Plymouth Rocks, Zulkifli et al. (1995) reported that 60% feed restriction had negligible effect on CORT. Similarly, inconsistent results have been reported for HLR in feed restricted broiler breeders (Katanbaf et al., 1989; Savory et al., 1993; 1996; Hocking et al., 1996). D’Eath et al. (2009) concluded that relationships between various physiological parameters and feed restriction may not necessarily measure an animal’s negative subjective state resulting from hunger. For example, other than psychological stress, elevation in CORT may also be attributed to metabolic stress (de Jong et al., 2003).

In this study, we attempted to identify other physiological stress indices in feed restricted broiler breeders than CORT and HLR. It has been reported that feed restriction can elicit heat shock protein (HSP) 70 response in broiler chickens (Zulkifli et al., 2000; Zulkifli et al., 2002; Liew et al., 2003; Al-Aqil and Zulkifli, 2009). HSP plays a profound role in modifying physiological stress response and in the acquisition of stress tolerance (Craig and Gross, 1991; Kregel, 2002). Serotonin (5-HT) and dopamine (DA) are neurotransmitters with several functions in regulating the biological processes of an organism. Previous studies showed that laying hens under social stress showed elevated peripheral levels of 5-HT and DA (Cheng et al., 2001).
Ghrelin (GHR) is a 26 amino acid peptide hormone in avian species (Kaiya et al., 2002) and is thought to have a physiological function in feed intake by acting as signal of hunger (Cummins et al., 2001). Shousha et al. (2005) reported that GHR was elevated in fasted Japanese quail. Similarly, GHR was raised following feed restriction in rats (Tschöp et al., 2000; Hayashida et al., 2001).

Acute phase proteins (APP) are a group of proteins that are primarily synthesized in the hepatocytes and released into the bloodstream upon conditions such as bacterial infection, inflammation, tissue injury, endotoxin exposure, and neoplasia (Murata et al., 2004; O’Reilly and Eckersall, 2014). APP function as protease inhibitors, enzymes, transport proteins, coagulation proteins, and modulators of the immune response. α1-glycoprotein (AGP) is a sialoglycoprotein produced and then secreted typically by hepatocytes. This protein is associated with homeostasis maintenance through reduction of tissue damage related to inflammatory response in extrahepatic cells (Fournier et al., 2000). Ceruloplasmin (CP) is a copper-containing ferroxidase that protects tissues from iron-mediated free radical injury by oxidation of toxic ferrous ion to nontoxic ferric form (Patel et al., 2002). Although ovotranferrin (OVT) is typically specified as a negative APP, there is some evidence that chicken serum OVT concentration is increased as an inflammation response (Toljho et al., 1995; Xie et al., 2002). OVT may participate in the innate immune system by collection of ferric ions to prevent the use of nutrients by parasites and pathogens (Law, 2002). Recent studies suggested that overcrowding (Shakeri et al., 2014) and administration of exogenous CORT (Zulkifli et al., 2014) may elevate serum levels of AGP, OVT, and CP in broiler chickens. Holt and Gast (2002) reported that the stress associated with forced molting through feed withdrawal exacerbated APP response to bacterial challenge in laying hens. Hence, the aim of the present study was to investigate HSP 70, 5-HT, DA, GHR, and APP responses to varying degrees of feed restriction in female broiler breeders. In this study, we also measured CORT and HLR which are considered to be common physiological indices of stress in avian species. Because both CORT and HLR react to a wide range of negative stimuli, there is a need to compare novel stress physiological parameters with these conventional indices.

**MATERIALS AND METHODS**

**Birds, Husbandry, and Housing**

All experimental procedures were conducted in accordance with Universiti Putra Malaysia Research Policy on animal ethics. One hundred one-day-old female broiler breeder chicks (Cobb) were obtained from a local breeder farm and were assigned randomly to 20 battery cages (5 birds/cage; 4 cages/treatment group) with wire floors in an environmentally controlled room. The length, width, and height of each cage were 90 cm, 56 cm, and 50 cm, respectively. Birds were kept in cages mainly for ease of handling during blood sampling. It has been reported that the sampling procedure itself may evoke a considerable physiological response (Beuving and Vonder, 1978). Mench et al. (1986) showed that laying hens housed in pens and cages had similar physiological stress responses. Ambient temperature was set at 32°C and gradually reduced to 23°C by d 21. The chicks were fed standard broiler breeder starter (mash form; 17% CP and 2780 kcal ME/kg) and grower (mash form; 15% CP and 2680 kcal ME/kg) from d 0 to 21 and from d 21 to 42, respectively. The birds were fed at 08:00 h and water was available ad libitum. Lights were on from 07:00 h to 15:00 h. On d 28, 20 birds (5 birds/cage) were assigned to one of five feeding regimens: (1) ad libitum feeding; (2) 75% of ad libitum feed intake; (3) 60% of ad libitum feed intake; (4) 45% of ad libitum feed intake; and (5) 30% of ad libitum feed intake. The feed restrictions were 75%, 60%, 45%, and 30% of the previous day’s feed intake of the ad libitum group.

**Experimental Treatment**

On d 42, at 08:00 h (before feeding), 12 (3 birds/cage) birds from each feeding regimen were randomly chosen and removed with minimum disturbance to flock mates. Immediately following capture, the birds were decapitated and blood samples were collected in plain and EDTA coated tubes. This procedure did not exceed 45 s and was therefore considered to have no influence on CORT (Lagadic et al., 1990; Romero and Reed, 2005). The blood sampling was accomplished at 09:10. Blood samples were centrifuged at 2000 g for 15 min at 4°C and plasma and serum were separated and stored at -80°C until further assays for determination of CORT, GHR, 5-HT, DA, and APP (AGP, OVT, and CP). To determine HLR, blood smears were prepared using Wright stain and heterophils and lymphocytes were counted to a total of 60 cells (Gross and Siegel, 1983). Immediately after blood sampling, the entire brain samples were removed, frozen quickly in liquid nitrogen and stored at -80°C until further analysis for HSP 70 density.

**ELISA Assays**

Plasma CORT was determined using a double antibody radio-immunoassay kit (IDS Ltd., Bolton, UK). The intra- and inter-assay variations for CORT measurements were <7% and <9%, respectively, and the sensitivity was 0.17 ng/mL according to the manufacturer’s information. An enzyme immunoassay kit (Cussabio Ltd., Wuhan, China) was used for plasma GHR measurement. The intra- and inter-assay variations for GHR measurements were <15% for both, respectively, and the sensitivity was less than 12.5 pg/mL according...
to the manufacturer’s information. An enzyme immunoassay kit (Cusabio Ltd., Wuhan, China) was also used to measure plasma 5-HT. The intra- and inter-assay variations for 5-HT measurements were <8% and <10%, respectively, and the sensitivity was less than 0.47 ng/mL according to the manufacturer’s information. Plasma DA was determined using an enzyme immunoassay kit (LDN Labor Diagnostika Nord., Nordhorn, Germany). The intra- and inter-assay variations for DA measurements were <10.8% and <12.1% respectively, and the sensitivity was less than 3.3 pg/mL according to the manufacturer’s information.

**SDS – PAGE and Immunoblot Analysis for HSP 70 Density**

The level of HSP 70 protein expression were determined as previously described (Soleimani et al., 2012) with some modification. Briefly, brain samples (0.3 g, whole cerebrum) were homogenised with 1.5 mL of protein extraction buffer (20 Mm Tris, pH 7.5; 0.75 M sodium chloride) and 10 μL/mL protease inhibitor cocktail (Precision Plus Protein, Bio-Rad, Hercules, CA), and centrifuged at 20,000 g for 30 min at 4°C. The supernatant were separated and the total protein and HSP 70 quantity were measured using a bicinchoninic acid protein assay kit (Sigma Chemical Co., St. Louis, MO) and SDS-PAGE. The final brain HSP 70 concentration was calculated as an arbitrary unit of band density relative to total protein concentration of each sample.

**APP Assay**

The serum level of AGP concentration was determined using a commercial ELISA kit specific to chickens (Cat. No.: NB-E60049, Life Diagnostics Inc., West Chester, PA). The OVT and CP levels were determined as previously described by Zulkifli et al. (2014).

**Statistical Analysis**

Data were subjected to ANOVA using the GLM procedure of SAS (SAS Institute Inc., Cary, NC) to determine the significance of any variations among the 5 treatments. For better assessment of hunger, the data collected were regressed against the 5 treatments. Prior to regression analysis, orthogonal polynomial contrast was used to explore the shape of the response of the variables measured and the 5 equally spaced feed restriction levels. Relationships between measured parameters and feed restriction levels were generated using the correlation coefficient and regression polynomial options of SAS. Statistical significance is considered as $P \leq 0.05$.

**RESULTS**

Feed restriction affected CORT ($P < 0.0001$), HLR ($P = 0.0092$), 5-HT ($P < 0.0001$), DA ($P = 0.0003$), GHR ($P < 0.0001$), and brain HSP 70 density ($P = 0.0008$). Significant linear relationships were noted for CORT ($P = 0.006; r^2 = 0.943$) (Figure 1), HLR ($P = 0.003; r^2 = 0.959$) (Figure 2), and DA ($P = 0.009; r^2 = 0.922$) (Figure 3). There were curvilinear relationships between serum levels of 5-HT ($P = 0.003; r^2 = 0.999$) (Figure 4), GHR ($P = 0.014; r^2 = 0.996$) (Figure 5), and the level of feed restriction. The linear or curvilinear relationship between brain HSP 70 density and the level of feed restriction was negligible ($P = 0.097; r^2 = 0.655$ and $P = 0.293; r^2 = 0.707$, respectively) (Figure 6). Feed restriction had no effect on AGP, OVT, and CP (Table 1).
Figure 3. Plasma levels of dopamine (DA) of female broiler breeders and the estimated relationship between the restriction level and DA (n = 8).

Figure 4. Plasma levels of serotonin (5-HT) of female broiler breeders and the estimated relationship between the restriction level and 5-HT (n = 11).

Figure 5. Plasma levels of ghrelin (GHR) of female broiler breeders and the estimated relationship between the restriction level and GHR (n = 8).

Figure 6. Brain levels of heat shock protein (HSP) 70 of female broiler breeders and the estimated relationship between the restriction level and HSP 70 (n = 7).

Table 1. The pooled effect of various levels of feed restriction on means ± SEM of serum α1 acid glycoprotein (AGP), ovotransferrin (OVT), and ceruloplasmin (CP) levels in female broiler breeders.

<table>
<thead>
<tr>
<th>Feeding regimen</th>
<th>AGP (ng/ml)</th>
<th>OVT (mg/ml)</th>
<th>CP (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL</td>
<td>7.44 ± 0.85</td>
<td>0.24 ± 0.04</td>
<td>5.95 ± 0.41</td>
</tr>
<tr>
<td>75AL</td>
<td>6.93 ± 0.98</td>
<td>0.21 ± 0.01</td>
<td>5.46 ± 0.69</td>
</tr>
<tr>
<td>60AL</td>
<td>8.04 ± 0.48</td>
<td>0.19 ± 0.02</td>
<td>4.99 ± 0.49</td>
</tr>
<tr>
<td>45AL</td>
<td>6.32 ± 0.21</td>
<td>0.19 ± 0.01</td>
<td>6.90 ± 0.43</td>
</tr>
<tr>
<td>30AL</td>
<td>8.12 ± 0.98</td>
<td>0.21 ± 0.02</td>
<td>5.14 ± 0.46</td>
</tr>
</tbody>
</table>

Analysis of variance: Probability
Feeding regimen 0.3483 0.6315 0.0619

1 AL = ad libitum; 75, 60, 45 and 30AL = 75%, 60%, 45% and 30% of ad libitum feed intake.
n = 8 for AGP and CP and 9 for OVT.

DISCUSSION

The noted significant linear relationships between CORT, HLR, and DA and the level of feed restriction suggest that these physiological indices are suitable to measure stress attributed to feed restriction in broiler breeders. Corticosterone is the main hormone associated with stress in avian species and has been frequently used to monitor physiological response to stressors (Siegel, 1980; Gross and Siegel, 1985; Zulkifli and Siegel, 1995; Post et al., 2003). de Jong et al. (2003) reported a third-grade hyperbolic relationship between CORT and level of feed restriction in breeders. The authors noted a low CORT in birds feed restricted 50 to 90% of ad libitum. In our study, however, CORT elevated significantly with increasing level of feed restriction. The elevated CORT in feed restricted breeders could be attributed to both physiological and psychological stress (Zulkifli et al., 1995, 2006). On a cautionary note, however, D’Eath et al. (2009) highlighted several possible problems with the use of CORT as a measure of chronic hunger. According to the authors, CORT may also be influenced by positive stimuli, and the body weight of an animal. They also suggested that
Elicitation of adrenocortical activity is known to precede heterophilia and lymphopenia (Gross and Siegel, 1993; Maxwell, 1993). Corticosterone can alter the circulating leucocytes counts by suppressing activation and proliferation of lymphocytes (Munck et al., 1984). Gross and Siegel (1983) compared leucocytic and hormonal responses to environmental challenges and exogenous CORT and concluded that HLR was a more reliable indicator of the perceived magnitude of stressors than CORT values in avian species. The present findings concur with those reported previously (Katanbaf et al., 1989; Maxwell et al., 1992; Savory et al., 1993) that chronic feed restriction increases HLR. We noted a significant linear relationship between level of feed restriction and HLR. Maxwell (1993) indicated that even though HLR may be a reliable measure of stress response in avian species, its value as a criterion during periods of extreme stress where basophilia and heteropenia may develop is limited. Inconsistent HLR reactions to chronic quantitative feed restriction have been reported in poultry (D’Eath et al., 2009).

Neurotransmitters such as 5-HT and DA are known to play critical adaptive roles in stress response regulation (Chauoloff, 2000). It has been reported that 5-HT is part of the response to stressors such as handling and separation from cage mates in chickens (Gruss and Braum, 1997). However, work in rats (Chik et al., 1986) suggested that feed restriction reduced circulating 5-HT. Chik et al. (1986) reported that serum level of 5-HT of fasted rats did not increase until 10 to 13 h after refeeding. These differences could be due to physiological and behavioral differences existing between avian and mammalian species. Cheng et al. (2003) reported that social stress elevated plasma DA concentration in laying hens. The present findings showed that feed restriction elevated both DA and 5-HT in broiler breeders. However, DA and 5-HT varied linearly and curvilinearly with level of feed restriction, respectively. Hence, DA may be considered as a reliable measure of feed restriction stress.

Shousha et al. (2005) and Kaiya et al. (2007) reported that fasting increases GHR in quail and layer chicks, respectively. On the contrary, Richards et al. (2006) indicated that GHR did not change significantly following feed restriction and refeeding in broiler chickens. Studies in rats, cows, and humans suggested that fasting may elevate plasma GHR levels and that following refeeding, the concentration will return to initial values (Tschöp et al., 2000). In the present study, we noted a curvilinear relationship between GHR and level of feed restriction. There is the question of whether GHR is an indicator of nutritional status or stress attributed to feed restriction. Plasma GHR may act as a short-term signal of hunger and satiety. Kaiya et al. (2008) indicated that GHR represents a key link between nutritional status in the stomach and the neuroendocrine response from the brain. It is known that GHR and 5-HT act on the hypothalamus and inhibit feed intake by stimulation of the 5-HTergic system (Zendehdel et al., 2013) or release of corticotrophin releasing factor (Saito et al., 2005) in chickens. The preceding activation of hypothalamic–pituitary–adrenal axis results in corticosterone release from the adrenal glands and thus metabolic manifestations of feed restriction stress. Furthermore, lack of GHR interaction with hypothalamic neuropeptide Y in chickens would also further contributes to anorexia in feed restricted birds. Neuropeptide Y is known to stimulate feed intake in broiler chickens (Ando et al., 2001; Saito et al., 2005) and is considered to be a homeostasis measure against feed restriction (Boswell et al., 1999). Given the close relationship between 5-HTergic system, neuropeptide Y, and stress, we may consider GHR as a measure of stress attributed to feed restriction. However, the noted curvilinear relationship between GHR and level of feed restriction suggests that it is not a straightforward indicator of stress severity.

Expression of HSP is considered an essential mechanism for cells to cope with both thermal and non-thermal stressors (Craig and Gross, 1991; Kregel, 2002). It has been suggested that HSP 70 plays a profound cytoprotective role in the gastrointestinal tract after injury or stress (Ehrenfried et al., 1996). HSP expression may be required for cells to recover from metabolic insults (Kregel, 2002). Work in broiler chickens has consistently showed that feed restriction increased HSP 70 density in the brain (Zulkifli et al., 2002; Liew et al., 2003; Al-Aqil and Zulkifli, 2009; Soleimani et al., 2011). In the present study, although HSP 70 was elevated significantly by feed restriction, there was neither linear nor curvilinear relationship between brain HSP 70 level and feed restriction level. Zulkifli et al. (2002) subjected broiler chicks to 40% and 60% feed restriction at 4, 5, and 6 days of age and observed a similar brain HSP 70 level in both groups. It seems that although the expression of HSP 70 is closely related to stress tolerance (Kreb and Feder, 1997), it may not be able to reflect the magnitude of feed restriction.

It is well documented that inflammatory response to different type of infections may trigger APP response and their concentrations in avian species (Holt and Gast, 2002; Buyse et al., 2007; Rath et al., 2009; Georgieva et al., 2010). There is, however, a dearth of information regarding APP response to non-infl ammatory challenges in poultry. Shakeri et al. (2014) showed that overcrowding elevated AGP, CP, and OVT. The present findings suggest that APP may not be affected by feed restriction in broiler breeders. Although APP have been proposed to be markers of stress in mammalian species (O’Reilly and Eckersall, 2014), other researchers failed to note haptoglobin response to physical stressors in cattle (Asemgeest et al., 1995; Hickey et al., 2003). Elevation in glucocorticoids during stressful events has been suggested as a possible mechanism behind the stress-induced APP response.
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(Castell et al., 1990). In the present study, feed restriction resulted in a significant increase in CORT. It thus appears that the link between stress and APP is rather complex and needs further elaboration. It is noteworthy to mention that recent work in our laboratory (unpublished) indicated that APP response may only triggered after 30 h from onset of feed deprivation in broiler chickens.

In conclusion, our results indicate that the conventional physiological indices such as CORT and HLR are indeed reliable to assess stress caused by feed restriction in broiler breeders. However, the metabolic role of CORT may complicate its interpretation as a stress indicator (D’Eath et al., 2009). The linear relationship between DA and level of feed restriction suggests that this neurotransmitter is a potential marker of stress magnetite in feed restricted breeders. Serum levels of 5-HT and GHR are not clear indicators of feed restriction level. Although feed restriction altered brain HSP 70 level, it may not be considered as a reliable indicator. Acute phase proteins were not affected by different levels of feed restriction. It should be noticed that in the present work, assessment of the physiological response to feed restriction was for 2 weeks only. In a commercial setting, broiler breeders will be subjected to feed restriction during the growing and laying periods. Evidence is accumulating to show that chickens readily habituate to feed restrictions of moderate durations (Rees et al., 1985; Zulkifli et al., 1994; Bruggeman et al., 1999). Thus, an important point of discussion is whether the studied physiological parameters will remain reliable to evaluate long term feed restriction in broiler breeders.

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