ABSTRACT
Measurements of the heterophil:lymphocyte (H/L) ratio (invasive technique) and corticosterone in yolk and albumen (noninvasive techniques) were used to measure stress in 3 commercial laying strains, Lohmann White (LW), H&N White (HN), Lohmann Brown (LB), and a noncommercial cross (CR) between Rhode Island Red (male) and Barred Plymouth Rock (female), kept in conventional cages or floor pens. All chicks were reared in their respective environments, and 450 and 432 pullets were placed at 18 and 7 wk of age in cages and floor pens, respectively. Blood from 12 hens per strain was taken at 19, 35, and 45 wk of age in each housing system. A total of 100 heterophils and lymphocytes were counted and their ratio (H/L ratio) was calculated. Corticosterone was measured in yolk and albumen from 12 hens per strain in each housing system at 22 and 45 wk of age. The H/L ratio was within the normal range. The interaction between environment and strain for the H/L ratio showed that in both environments, LB and CR hens had a higher H/L ratio than LW and HN layers. In cages, there were significant differences in H/L ratios between LW and HN hens that were likely due to genetic differences. The LW hens had significantly lower corticosterone concentrations in yolk than LB hens. In cages but not floor pens, yolk corticosterone concentrations at wk 22 were significantly higher than at wk 45. In floor pens but not cages, albumen corticosterone at wk 22 was higher than at wk 45. The H/L ratios suggest that none of the hens were unduly stressed, and corticosterone levels in yolk and albumen support the suggestion that hens adapted to their environments with age. Although measurement of yolk corticosterone and the H/L ratio may be comparable, the measurement of corticosterone level in the albumen may differ because it is secreted over a short time.

Key words: conventional cage, floor pen, laying hen, heterophil:lymphocyte ratio, egg corticosterone

INTRODUCTION
According to McBride (1980), an animal at first tries to avoid an unpleasant stimulus and if unsuccessful, it undergoes a psychophysiological process of habituation. These animals deal with stressors at a physiological level by adrenocortical activation (acute stress response), followed by a period of adaptation. If an animal fails to adapt, a stage of exhaustion (general adaptation syndrome; Selye, 1976) can occur depending on the degree of exposure to the stressor.

Corticosterone, also known as the stress hormone, is the major glucocorticoid in birds (Harvey et al., 1980). In commercial poultry, stressors such as heat, food and water deprivation (Beuving, 1980), and transportation (Broom and Knowles, 1989) increase glucocorticoid production, which is mediated by the release of adrenocorticotropic hormone from the pituitary gland (Munck et al., 1984). The heterophil:lymphocyte (H/L) ratio is a hematological measure that has been shown to be a reliable indicator of long-term stress (Gross and Siegel, 1983; Maxwell and Robertson, 1998; Elston et al., 2000; Mahboub et al., 2004; Campo et al., 2005). However, handling and blood sampling are invasive techniques and are stressors in themselves (Harvey et al., 1980). Therefore, a noninvasive means of measuring corticosterone could be helpful in identifying stressful conditions.

Initial studies conducted by Fraser and Emtage (1976) found degenerative metabolites of vitamin D in albumen, which raises the possibility of finding other plasma solutes in egg albumen. Corticosterone assays in eggs are becoming popular, noninvasive techniques for measuring stress in hens (Downing and Bryden, 2002; Hayward and Wingfield, 2004; Rubolini et al.,...
The gradual accumulation of yolk and albumen during egg formation over 10 d (Johnson, 1986) and 6 h (Downing and Bryden, 2002), respectively, could provide an integrated reflection of circulating hormones over these periods, thereby providing noninvasive measures of long-term and short-term stress, respectively, in laying hens.

The environment contributes to the well-being of an animal, but the genotype is also important (Wall, 2003). Hormone levels are affected by the genotype and in turn affect growth and egg production (Bayyari et al., 1997; Nestor et al., 2000). The genotype and phenotype of an animal activate the neuroendocrine system that helps the animal in its behavioral adaptability (Lamont, 1994; Mench and Duncan, 1998). The objective of this study was to determine the effects of conventional cages and floor pens on the H/L ratio in blood and albumen and yolk corticosterone in eggs of 4 strains of laying hens to assess their levels of stress. A secondary goal was to determine whether the corticosterone level in the egg can be used to measure stress.

**MATERIALS AND METHODS**

**Birds, Housing, and Management**

Commercial chicks [Lohmann White (LW), H&N White (HN), and Lohmann Brown (LB)] were obtained from Pacific Pride Chicks (Abbotsford, British Columbia, Canada), and chicks from a cross of Rhode Island Red males and Barred Plymouth Rock females (CR; Silversides et al., 2007) were produced at the Agassiz Research Centre. Birds were provided with 9 h of light per day until 18 wk and 14 h afterward. A standard layer ration was provided to allow ad libitum consumption in both housing systems. Temperature was between 21 and 23°C and humidity was approximately 70%. Care of hens was in accordance with the guidelines described by the Canadian Council on Animal Care (1993), and all procedures were approved by the Animal Care Committee of the Agassiz Research Centre.

Caged birds were reared in pullet cages, and 450 birds (120 birds each of LW, LB, and HN strains and 90 birds of the CR strain) were housed randomly with 3 birds per cage (688 cm²/bird) at wk 18 (Singh et al., 2009). Cages were arranged on 2 tiers in double-sided rows. Each cage was provided with a feeder in front and 2 nipple drinkers at the back. Birds in adjacent cages shared access to the drinkers. Birds in floor pens were reared from d 1, and 432 birds (120 birds each of LW and HN, 105 of LB, and 87 for CR strain) were housed randomly at 7 wk in 4 or 5 pens per strain with 21 to 24 birds per pen (6,115 to 6,990 cm²/bird). In floor pens, water was provided via nipple drinkers and feed was provided in tube feeders. Pens included perches and nest boxes from the second week of age. In addition to the standard rations, birds in each floor pen were fed 200 g/pen of whole wheat 3 d a week on alternate days. In both housing systems, birds started laying at approximately wk 20, and the laying trial was continued until the birds were 50 wk of age (Singh et al., 2009).

**H/L Ratio**

Blood was sampled for H/L ratio when the birds were 19, 35, and 45 wk of age. A total of 12 hens per strain were randomly selected from each cage or floor pen. Two drops of blood were taken from the right brachial vein within a minute of catching the hen to avoid stress due to handling (Broom and Knowles, 1989) and were dropped separately onto the slide. Blood smears were made on each slide, air-dried, fixed with methyl alcohol, and stained with Giemsa stain (Humason, 1979). On each slide, heterophils and lymphocytes were counted until a total of 100 cells was reached. After averaging the cells of 2 slides, the ratio of heterophils to lymphocytes was calculated.

**Corticosterone Assay**

Eggs were collected for measuring corticosterone in yolk and albumen from 12 hens per strain in each housing system at 22 and 45 wk of age and were stored at −20°C. The albumen was separated from the yolk and both were freeze-dried, homogenized, and stored at −20°C for further analysis. Exraction procedures for yolk and albumen were carried out according to Cook et al. (2009), and corticosterone was assayed using Assay Design Corticosterone Enzyme Immunoassay kits (901-097, Assay Designs Inc., Ann Arbor, MI) as per the instructions provided with the kit.

Briefly, approximately 100 mg of yolk was weighed and vortexed for 30 s with 2 glass beads in 500 µL of water, after which 5 mL of petroleum ether:diethyl ether (30:70 vol/vol) was added, and the mixture was vortexed for 1 min. After centrifugation at 2,800 × g for 5 min, the mixture was frozen at −80°C, and the supernatant was transferred to a 10-mL glass tube and dried under N₂ at 40°C. The sample was reconstituted in 1 mL of 90% ethanol, vortexed until dissolved, and frozen at −80°C. The ethanol sample was allowed to thaw and centrifuged at 2,500 × g at 4°C for 5 min. The supernatant was decanted into a new 10-mL tube. Two milliliters of hexane was added, and the mixture was vortexed for 30 s, centrifuged at 2,500 × g at 4°C for 1 min, and frozen at −80°C. The hexane layer was removed and discarded and the ethanol extract was dried under N₂ at 40°C. The dried extract was reconstituted immediately in 500 µL of assay buffer, and the sample was transferred to a microtube and centrifuged at 14,000 × g at 4°C for 15 min.

Approximately 50 mg of albumen was vortexed for 10 s with 2 glass beads in 1 mL of water. Five milliliters of petroleum ether and diethyl ether (30:70 vol/vol) mix-
ture was added and vortexed for 10 s. After centrifuga-
tion at 3,500 × g for 5 min, the mixture was frozen 
at −80°C and the supernatant transferred to a 10-mL 
glass tube and dried under N$_2$ at 40°C. The sample was 
reconstituted in 500 µL of water, vortexed for 10 s, 2 
mL of petroleum ether was added, and the mixture was 
vortexed for 10 s, centrifuged at 3,500 × g, and frozen 
at −80°C. The supernatant was decanted into a new 
10-mL tube and dried under N$_2$ at 40°C. The dried 
extract was reconstituted in 500 µL of assay buffer and 
centrifuged at 14,000 × g at 4°C for 15 min.

Statistical Analysis

The data on H/L ratio and corticosterone measure-
ment in yolk and albumen were subjected to an ANOVA 
using PROC GLM of SAS (Version 9.1, SAS Institute 
Inc., Cary, NC). Blood and eggs were collected from 
different birds at each age. The experimental units varied 
from 30 to 40 in cages and from 4 to 5 in floor 
pens. The model included the effects of environment, 
strain, age, and interactions between them. The 3-way 
interaction was not significant for any measure and was 
dropped from the model. Significant 2-way interactions 
were investigated by separation of the data according 
to one of the main effects and reanalyzed including only 
significant interactions. When effects were significant, 
means were separated using least squares means. A 
P-value <0.05 was considered to be significant for all 
analyses.

Table 1. Ratio (±SEM) of heterophils to lymphocytes (H/L) in blood and corticosterone concentra-
tion in the yolk and albumen of eggs from 4 strains of laying hens kept in conventional cages and 
floor pens1

<table>
<thead>
<tr>
<th>Attribute</th>
<th>H/L ratio</th>
<th>Yolk</th>
<th>Albumen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cages</td>
<td>Floor pens</td>
</tr>
<tr>
<td>Environment</td>
<td>0.24 ± 0.003</td>
<td>24.7 ± 1.38</td>
<td>26.9 ± 1.74</td>
</tr>
<tr>
<td></td>
<td>0.23 ± 0.003</td>
<td>25.2 ± 1.38</td>
<td>26.0 ± 1.74</td>
</tr>
<tr>
<td>Strain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LW</td>
<td>0.19 ± 0.005</td>
<td>19.8$^a$ ± 1.96</td>
<td>28.5 ± 2.46</td>
</tr>
<tr>
<td>LB</td>
<td>0.27 ± 0.005</td>
<td>27.3$^a$ ± 1.96</td>
<td>26.0 ± 2.46</td>
</tr>
<tr>
<td>HN</td>
<td>0.16 ± 0.005</td>
<td>26.8$^{ab}$ ± 1.96</td>
<td>26.8 ± 2.46</td>
</tr>
<tr>
<td>CR</td>
<td>0.33 ± 0.005</td>
<td>26.1$^{ab}$ ± 1.96</td>
<td>24.6 ± 2.46</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wk 22</td>
<td>0.26 ± 0.004</td>
<td>29.2 ± 1.38</td>
<td>28.1 ± 1.74</td>
</tr>
<tr>
<td>wk 35</td>
<td>0.23 ± 0.004</td>
<td>29.2 ± 1.38</td>
<td>28.1 ± 1.74</td>
</tr>
<tr>
<td>wk 45</td>
<td>0.22 ± 0.004</td>
<td>20.7 ± 1.38</td>
<td>24.8 ± 1.74</td>
</tr>
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<td>ANOVA</td>
<td></td>
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<tr>
<td>Environment</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Strain</td>
<td>&lt;0.01</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Age</td>
<td>&lt;0.01</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Environment × strain</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Environment × age</td>
<td>&lt;0.01</td>
<td>NS</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Strain × age</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

$^a,b$Means of yolk corticosterone for strains with different superscripts are different at $P < 0.05$.

$^1$Total number of observations was 288 for H/L ratio and 192 for measures of corticosterone.

$^2$LW = Lohmann White; LB = Lohmann Brown; HN = H&N White; CR = noncommercial cross between Rhode Island Red (male) and Barred Plymouth Rock (female).
different. Hens in floor pens had significant higher albumen corticosterone concentration at wk 22 compared with wk 45, and in cages, egg albumen corticosterone was not significantly different between different ages (Table 3). At wk 22, albumen corticosterone was higher in floor pens, and at wk 45, it was higher in cages. Another 2-way interaction was found between strain and age for egg albumen corticosterone and is shown in Table 4. Although the interaction was significant, none of the means were separated by the procedure used.

**DISCUSSION**

Differential responses among leukocyte populations can be used to measure stress. Heterophils and lymphocytes are responsive to stress, are easy to identify (Maxwell and Robertson, 1998), and are commonly used to indicate long-term stress. Corticosterone is the major adrenal glucocorticoid hormone that increases in birds under conditions of stress. Corticosterone has short-term effects on the physiology and behavior of laying birds and also on their long-term performance.

Our 4 strains responded differently for the H/L ratios. Zekarias et al. (2000) found that commercial brown-egg layers have higher H/L ratios than white-egg strains, supporting our findings of higher H/L ratio values for brown-egg layers in both housing systems and suggesting that the H/L ratio is affected by the genetic origin of the hen. The H/L ratios that we found for commercial white-egg layers (LW and HN) agree with those found by Gross and Siegel (1983) for a commercial White Leghorn strain housed in cages and by Sturkie and Griminger (1986) for adult White Leghorn females. Cheng and Muir (2005) found strain differences among randomly bred population of White Leghorns, and our finding of differences in the H/L ratio between LW and HN hens in cages similarly demonstrates strain differences.

Among brown-egg layers, the higher H/L ratios for CR hens than LB hens may relate to the lower intensity of selection of the parent lines compared with that of commercial lines (Silversides et al., 2007). Over the past few decades, commercial breeding programs have likely ignored the ability of the animal to cope with modern production environments in favor of production traits, and the CR hens may be more capable of mounting a stress response. Onbasilar and Aksoy (2005) found high H/L ratios when hens were given very little space (394 cm²) but no difference in H/L ratios between hens kept with 656 or 1,968 cm². We found that one of the White Leghorn strains (LW) had higher ratios in cages, which suggests a possible difference due to floor space, but the other strains did not. For these strains, the space may have been above a threshold for a response in the H/L ratio in both cages (688 cm²/bird) or floor pens (7,000 cm²/bird). The higher H/L ratios in cages at the start of the laying cycle (19 wk) compared with those at mid cycle (45 wk), which was not seen for birds in floor pens, could indicate that the hens in cages are stressed at the start of lay, but adapt to their environment.

Various studies have shown the presence of corticosterone in yolk and albumen of eggs (Downing and Bryden, 2002; Hayward and Wingfield, 2004; Cook et al., 2009) and that the concentration increases in stressful environments (Downing and Bryden, 2002; Eriksen et al., 2003). In this study, higher yolk corticosterone concentrations in eggs from cages at 22 wk are similar to our findings for the H/L ratio and suggest that hens in cages were under stress for the 10-d period of yolk formation (long-term stress) at the beginning of the laying period. For hens in cages, both the yolk corticosterone concentrations and the H/L ratio decreased with age, which may be a demonstration of the adaptation of the hens to their environment by the end of the laying period. Lower egg yolk corticosterone concentrations in LW hens than LB, HN, and CR hens found in

**Table 2.** Heterophil:lymphocyte ratio (±SEM) in blood of 4 strains of laying hens kept in conventional cages and floor pens

<table>
<thead>
<tr>
<th>Main effect</th>
<th>Conventional cages</th>
<th>Floor pens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain²</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LW</td>
<td>0.21 ± 0.007</td>
<td>0.18 ± 0.007</td>
</tr>
<tr>
<td>LB</td>
<td>0.28 ± 0.007</td>
<td>0.26 ± 0.007</td>
</tr>
<tr>
<td>HN</td>
<td>0.16 ± 0.007</td>
<td>0.17 ± 0.007</td>
</tr>
<tr>
<td>CR</td>
<td>0.33 ± 0.007</td>
<td>0.34 ± 0.007</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wk 22</td>
<td>0.28 ± 0.006</td>
<td>0.23 ± 0.006</td>
</tr>
<tr>
<td>wk 35</td>
<td>0.24 ± 0.006</td>
<td>0.22 ± 0.006</td>
</tr>
<tr>
<td>wk 45</td>
<td>0.20 ± 0.006</td>
<td>0.25 ± 0.006</td>
</tr>
</tbody>
</table>

¹²Means within strain and age with different superscripts are different at P < 0.05.

²Total number of observations was 288.

²LW = Lohmann White; LB = Lohmann Brown; HN = H&N White; CR = noncommercial cross between Rhode Island Red (male) and Barred Plymouth Rock (female).

**Table 3.** Corticosterone (pg/ng ± SEM) in yolk and albumen of eggs from laying hens at wk 22 and 45 kept in conventional cages and floor pens

<table>
<thead>
<tr>
<th>Age</th>
<th>Yolk corticosterone</th>
<th>Albumen corticosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cage</td>
<td>Floor pens</td>
</tr>
<tr>
<td>wk 22</td>
<td>31.2 ± 1.96</td>
<td>27.9ab ± 1.96</td>
</tr>
<tr>
<td>wk 45</td>
<td>18.1 ± 1.96</td>
<td>23.4a ± 1.96</td>
</tr>
</tbody>
</table>

¹Means for each measure with different superscripts are different at P < 0.05.

¹Total number of observations was 192.
this study may be explained by differences between the strains similar to those found in the H/L ratio.

In eggs from floor pens, albumen corticosterone concentrations were also higher at the start of the laying period, indicating stress over the 6-h period of albumen deposition. The birds in floor pens were already accustomed to their environment when they began to lay because they were reared in the pens from d 1. This short-term stress could be associated with the initiation of egg laying because it decreased as the hens grew older. The data on the H/L ratio suggest that none of the laying hens were unduly stressed and that on corticosterone in yolk and albumen support the suggestion that hens adapted to their environments with age.

A secondary goal of our study was to determine whether the corticosterone level in the egg could be used to measure stress. Although we could not directly associate the H/L ratio of a hen with specific eggs, we can make a general comparison. Both the H/L ratio and yolk corticosterone concentration are measurements of stress over a relatively long time period, and our results were generally in agreement, suggesting that egg yolk corticosterone level can be used to measure stress in a fashion similar to the H/L ratio. In contrast, our measurements of the H/L ratio and the albumen corticosterone concentration in the cage environment disagree, possibly because the albumen is secreted over a short time. The albumen corticosterone level may indicate short-term stress in contrast to the yolk corticosterone level and the H/L ratio, which infer long-term stress (Gross and Siegel, 1983). Our results indicate that although the measurement of yolk corticosterone and the H/L ratio may be comparable, the corticosterone level in the albumen may differ because it is secreted over a short time period.

ACKNOWLEDGMENTS

Help with the corticosterone assay provided by Denise Frohlich, Pierre Lepage, and Sigrid Marchand of the Lacombe Research Centre is deeply appreciated. Thanks to Lisa Hederson and the poultry staff of the Agassiz Research Centre for providing animal care to the birds. We also thank an anonymous reviewer and Laki Goonewardene of Alberta Agriculture and Rural Development (Edmonton, Alberta, Canada) for their statistical advice. We would also like to thank the Specialty Birds Research Committee of the University of British Columbia, the British Columbia Egg Marketing Board (Abbotsford, British Columbia, Canada), and Agriculture and Agri-Food Canada (Ottawa, Ontario) for financial support.

REFERENCES


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<tr>
<th>Age</th>
<th>LW (pg/mg ± SEM)</th>
<th>LB (pg/mg ± SEM)</th>
<th>HN (pg/mg ± SEM)</th>
<th>CR (pg/mg ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wk 22</td>
<td>24.5 ± 3.48</td>
<td>31.8 ± 3.48</td>
<td>31.3 ± 3.48</td>
<td>24.3 ± 3.48</td>
</tr>
<tr>
<td>wk 45</td>
<td>32.4 ± 3.48</td>
<td>20.1 ± 3.48</td>
<td>22.3 ± 3.48</td>
<td>24.3 ± 3.48</td>
</tr>
</tbody>
</table>

1 Total number of observations was 192.
2 LW = Lohmann White; LB = Lohmann Brown; HN = H&N White; CR = noncommercial cross between Rhode Island Red (male) and Barred Plymouth Rock (female).