INTRODUCTION

Conventional cages are the main housing system for laying hens in the United States because of high hygiene, high egg production, low level of aggression and cannibalism, and the mechanization of feed and water distribution, egg collection, and manure handling (Appleby, 1998; Rodenburg et al., 2005; Vits et al., 2005a). However, this housing system has been criticized for limiting the ability of hens to perform certain behaviors, resulting in a negative effect on their welfare (Lay et al., 2011; Freire and Cowling, 2013). These criticisms have led to gradual changes in housing system for laying hens globally. Switzerland has banned the use of conventional cages since 1992, followed by Germany in 2007 and the entire European Union in 2012. California voters passed proposition 2, which requires by the beginning of 2015 that all laying hens in California be housed in such a manner that they can stand up, lie down, turn around freely, as well as extend their limbs fully. In 2012, the United Egg Producers and the Humane Society of the United States signed an agreement requesting federal legislation that would require United States egg producers to convert hen housing from conventional cages to enriched cages over a 16-yr period (Greene and Cowan, 2012).

Furnished cage systems for laying hens, in contrast to barren conventional cages, are equipped with nests, scratching pads, nail trimmers, and perches to provide opportunities for chickens to better express their natural behaviors (Appleby et al., 2002; Vits et al., 2005b; Shimmura et al., 2010). Previous studies have shown that perching behavior is one of the ethological needs of fowl (Thorsten et al., 2010). Perches also reduce aggressive encounters by providing a way for subordinate birds to avoid dominant ones (Cordiner and Savory, 2001). Additionally, perch access during egg laying im-
proves bone strength and mineralization (Jendral et al., 2008; Nicol et al., 2009; Tactacan et al., 2009; Hester et al., 2013). In response to a stressor, the central nervous system of animals triggers physiological responses that involve the hypothalamic-pituitary-adrenal and the sympathetic-adrenomedullary axes whose ultimate purpose is to maintain homeostasis even in the most demanding circumstances (Romero, 2004; Ulrich-Lai and Herman, 2009). The neuroendocrine hormones released in response to stress include glucocorticoids [corticosterone (CORT) in birds, Matteri et al., 2000; Mostl and Palme, 2002; Carrasco and Van de Kar, 2003], catecholamines [epinephrine (EP), norepinephrine (NE), and dopamine (DA), Matteri et al., 2000; Mostl and Palme, 2002; Carrasco and Van de Kar, 2003; Brenes et al., 2008; Segovia et al., 2008], and serotonin (5-hydroxytryptamine or 5-HT, Carrasco and Van de Kar, 2003; Brenes et al., 2008). Stress in chickens often leads to immunosuppression (Siegel, 1995) causing higher heterophil to lymphocyte (H:L) ratios (Gross and Siegel, 1983). Adding enrichments to laying cages did not affect typical measurements of physiological stress such as catecholamines, CORT, H:L ratio, or antibody production compared with hens in conventional cages (Barnett et al., 1997; Shini, 2003; Guémené et al., 2004; Guesdon and Faure, 2004; Buil et al., 2006; Nicol et al., 2009; Pohle and Cheng, 2009).

Little is known about the effects of enrichments such as perches on the physiological status of pullets. Typical pullet cages used in the United States commercial industry are barren with no enrichments such as perches, scratch pads, or nail trimmers. A companion study of the current one showed that perches were beneficial to pullets by increasing leg muscle deposition and bone mineral content of some bones compared with pullets in conventional cages (Enneking et al., 2012a). The objective of this study was to examine the effect of perch access and age on physiological measurements of stress in caged White Leghorn pullets. Assuming that pullets, like laying hens, are highly motivated to perch, we hypothesize that pullets with access to perches will experience less stress than pullets that never have access to perches.

**MATERIALS AND METHODS**

**Birds and Management**

A total of 1,064 one-day-old White Leghorn chicks of the Hy-Line W36 strain were reared at the Purdue University Poultry Research Farm following management protocols similar to industry and under the guidelines approved by the Purdue University Animal Care and Use Committee (no. 10–082). Infrared beak trimming was performed at the hatchery. Birds were randomly assigned to 28 cages following hatch. Half of the cages had 2 perches/cage mounted parallel to the feeder (treatment group), whereas the remaining 14 pullet cages were without perches (control group). Perch positions within the cage, cage dimensions, number of pullets per cage, floor, perch, and feeder space per pullet, the diets fed, and lighting regimen have been reported previously (Enneking et al., 2012a).

**Blood Sampling**

Two birds per cage were randomly selected for sampling at 4, 6, and 12 wk of age. For each age, blood samples were collected at the same time of day starting at 0800 h in an alternating order of treatment and control until all cages were sampled. Pullets were sedated with sodium pentobarbital (30 mg/kg of BW) via intracardiac injection at 4 and 6 wk of age and by intravenous (brachial vein) injection at 12 wk of age. A 3- to 5-mL blood sample was collected from each pullet via cardiac puncture within 2 min of removing the chicken from its cage. The blood sample was placed into an EDTA-coated test tube. An aliquot of 500 μL of whole blood was retained for 5-HT and Trp analysis; the remaining blood sample was centrifuged at 700 × g for 15 min to obtain plasma for CORT, EP, NE, and DA analyses. Whole blood and plasma were kept at −80°C until assayed.

**H:L Ratio**

Duplicate blood smears on glass slides were made per sampled pullet using the blood in the EDTA-coated test tubes. Blood smears were stained with Camco 3 Step Staining Reagents (Cambridge Diagnostic Inc., Fort Lauderdale, FL). The leukocytes were counted at 1,000 × (oil immersion lens) until a total of 100 cells per slide was reached. The leukocyte counts included heterophils, lymphocytes, monocytes, basophils, and eosinophils. The H:L ratio was calculated by dividing the number of heterophils by the number of lymphocytes (Cheng et al., 2001). The ratios from the 2 slides for each pullet were averaged, and the mean was used for the statistical analysis.

**HPLC Assay**

Plasma EP, NE, and DA concentrations were measured using a commercial catecholamine analysis kit (Themo Scientific, Sunnyvale, CA) following the protocol described previously (Cheng et al., 2001). Briefly, plasma samples were acidified and deproteinized with 4 M perchloric acid, followed by centrifugation. The acid supernatants and internal standard dihydroxybenzylamine were added and absorbed into an alumina minicolumn to bind the catecholamines. The column was rinsed and eluted with the washing solutions supplied by the company. After addition of eluents into the reverse-phase column, catechols were detected with Coulochem II electrochemical detectors by liquid chro-
matography. The concentrations of EP, NE, and DA were determined using a standard curve developed by using commercially supplied standards (Sigma-Aldrich Corporation, St. Louis, MO). Ratios of EP:NE were calculated.

For detecting blood 5-HT and Trp levels, whole blood was acidified with freshly prepared 3% ascorbic acid, then deproteinized with 4 M perchloric acid. After centrifugation, the supernatants were filtered through a syringe filter and injected onto the column (Cheng et al., 2001). Samples were analyzed using HPLC. The concentrations of 5-HT and Trp were calculated from a reference curve made using standards (Sigma-Aldrich Corporation).

**RIA**

Plasma concentrations of CORT were measured in duplicate using a commercial \(^{125}\)I CORT RIA kit (MP Biomedicals, Orangeburg, NY) following the method described previously (Cheng et al., 2001).

**Statistical Analysis**

All of the measurements were done in duplicate; data were not used when the CV was greater than 10% between duplicates. A 2-way ANOVA was used in the data analysis (Steel et al., 1997). Perch treatment and age of the birds were considered to be fixed effects. Appropriate transformations were performed for normality when it was needed. Because statistical trends were similar for both transformed and untransformed data, the untransformed least squares means and SEM were presented. The MIXED model procedure of the SAS Institute (2008) was used on all traits. The Tukey-Kramer test was used to partition differences among means due to age effects (Oehlert, 2000). Age-adjusted Pearson correlation coefficients were used to assess the relationship between hormones (Rodgers and Nicewander, 1988).

**RESULTS**

The perch treatment (Table 1) or its interaction with age (data not presented in tables) did not affect any parameter measured in the study. Concentrations of circulating EP, NE, EP:NE ratio, and Trp were lower at 12 wk of age compared with the levels at both 4 and 6 wk of age (\(P < 0.0001\)). Levels of 5-HT were also lower in 12-wk-old pullets compared with 6-wk-old pullets, but not 4-wk-old pullets (\(P = 0.0007\)). In contrast, concentrations of DA were less at 4 wk compared with the levels at both 6 and 12 wk of age (\(P < 0.0001\)).

**DISCUSSION**

Stress activates the hypothalamic-pituitary-adrenal and sympatho-adrenomedullary systems causing the adrenals to enlarge and release glucocorticoid and catecholamines, respectively (Siegel, 1995; Goldstein and Kopin, 2008). Increases in circulating levels of CORT (the major glucocorticoid in chickens), catecholamines (mainly EP and NE) as well as the EP:NE ratio, an expression of sympatho-medulloadrenal balance between epinephrine and norepinephrine output (Rouex et al., 2006), are indicative of chickens experiencing stress (Siegel, 1995; Cheng and Fahey, 2009; Lay et al., 2011). Similar to plasma CORT and the catecholamines, brain
levels of 5-HT also increased in response to various stressors such as restraint or immobilization (Shimizu et al., 1992), forced swimming (Kirby et al., 1995), and tail pinch (Kalén et al., 1989; Vahabzadeh and Fillenz, 1994) in rats. However, the biological role of peripheral 5-HT in response to stress is still unclear and has not been studied in pullets.

Little research has been conducted on the effect of rearing environment of pullets on physiological measurements of stress. In a study conducted by Buil et al. (2006), pullets reared on the floor compared with cages had higher fecal CORT levels when placed in furnished cages during egg laying, suggesting that the housing system for rearing should be similar to the housing system for egg laying to minimize stress. In the current study, plasma concentrations of CORT and catecholamines, EP:NE ratio, and blood levels of 5-HT and Trp in caged pullets up to 12 wk of age were similar between pullets with and without access to perches, suggesting that a stress response was not induced. In a companion study by Enneking et al. (2012a), the right adrenal weight was similar between pullets housed in conventional cages with or without perches, again offering evidence that pullets of this study showed no physiological indication of being stressed.

In addition to hormones, the response of leukocytes to stressors is another indicator of physiological stress. Increasing circulating levels of glucocorticoids leads to an increase in the H:L ratio (Maxwell, 1993). However, similar to the stress hormones, there has been little reported in the scientific literature on the effect of pullet housing systems on the H:L ratio. No difference in the H:L ratio was found in broilers housed in floor pens with and without perches at various density from 32 to 42 d of age (Heckert et al., 2002). Hens housed in floor pens with perches had a lower H:L ratio than hens without access to perches (Campo et al., 2005), suggesting that the welfare of hens housed in floor pens during egg laying could be improved by providing perches (Olsson and Keeling, 2000). The H:L ratios of the current study confirm the results of the hormonal profile and the adrenal weights (Enneking et al., 2012a), indicating that the absence of perches in conventional pullet cages compared with perch access does not elicit a stress response.

Laying hens are highly motivated to perch (Lambe and Scott, 1998; Olsson and Keeling, 2002). If perching is thwarted, hens display frustration and unrest (Olsson and Keeling, 2000) which could lead to a stress response (Campo et al., 2005). Perching behavior in caged pullets has been less studied, and little is known about a pullet’s motivation to perch. However, in a companion paper of the current study, we have demonstrated that pullets use perches if given access to them. Specifically, caged pullets, as early as 2 wk of age, began to use the 2 metal perches provided at 1 d of age, although the perching incidence was low at this age (Enneking et al., 2012b). As the pullets aged, perching frequency increased reaching a peak at 12 wk of age; this level of perching activity (31 to 37% of the pullets were using 1 of the 2 perches at night) was sustained until the end of observations at 16 wk of age (Enneking et al., 2012b). Naive pullets of the current study that were never exposed to perches showed no evidence of eliciting a stress response, suggesting that prior perch experience with subsequent denial may be needed to induce stress.

The reasons for the age-related decrease in circulating EP and NE concentrations in the current study are unclear, but might be related to a pullet’s response to handling. Activation of the sympatho-adrenomedullary system results in the rapid secretion of EP and NE into the peripheral circulation (Spasojevic et al., 2009; Strahler et al., 2013), leading to an increase in cardiovascular activity and a boost of energy (Lundberg, 2005; Shan et al., 2010). Because both EP and NE are released by adrenal medullary cells in response to acute stress, the positive correlation between EP and NE of the current study was not unexpected and agrees with a previously reported correlation (Linsell et al., 1985). Younger pullets, aged 4 and 6 wk, may have reacted more vigorously to the handling experience compared with 12-wk-old pullets, causing the release of EP and NE. Sedation with pentobarbital was more difficult in the younger pullets than the 12-wk-old pullets, which could have contributed to the higher levels of circulating EP and NE.

In a similar manner to circulating NE and EP, plasma CORT also was higher in 4- and 6-wk-old pullets compared with 12-wk-old pullets, but the effect only approached significance ($P < 0.07$, Table 1). The catecholamine response to stress is quicker (Lundberg, 2005) than the CORT and the H:L responses (Siegel, 1995). Because handling would be an acute stressor, the CORT and H:L responses to handling perhaps did not

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**Table 2. Age-adjusted correlation coefficients for peripheral concentrations of corticosterone, dopamine, epinephrine, norepinephrine, serotonin, and Trp of White Leghorn pullets**

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Dopamine</th>
<th>Epinephrine</th>
<th>Norepinephrine</th>
<th>Serotonin</th>
<th>Trp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corticosterone</td>
<td>0.05</td>
<td>0.00</td>
<td>−0.19</td>
<td>0.15</td>
<td>0.07</td>
</tr>
<tr>
<td>Dopamine</td>
<td></td>
<td>0.03</td>
<td>0.00</td>
<td>−0.04</td>
<td>−0.06</td>
</tr>
<tr>
<td>Epinephrine</td>
<td></td>
<td></td>
<td>0.54**</td>
<td>0.03</td>
<td>−0.06</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td></td>
<td></td>
<td></td>
<td>−0.04</td>
<td>0.15</td>
</tr>
<tr>
<td>Serotonin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.54**</td>
</tr>
</tbody>
</table>

1 Values for hormones averaged over the ages of the pullets (4, 6, and 12 wk of age).

**The r value is significant at $P < 0.01$.**
have enough time to develop before the pentobarbital took effect. Campo and Dávila (2002) also did not find differences in H:L ratios of a Spanish breed of chickens (Quail Castellana) between 8 and 12 wk of age.

Unlike EP and NE, pullets demonstrated a decrease in circulating DA at 4 wk of age. Specifically, plasma DA concentrations were similar between 6- and 12-wk-old pullets with concentrations that more than doubled when compared with levels of 4-wk-old pullets. Because DA is the precursor of EP and NE (Kuchel, 1991) and cannot cross the blood-brain barrier (Rios Romenets et al., 2013), one could postulate that the DA concentrations in pullets at 12 wk of age accumulated when plasma EP and NE were at their lowest level because DA was not needed for the synthesis of EP and NE. However, because this inverse relationship between plasma concentrations of DA compared with NE or EP was not apparent at 6 wk of age and the correlation of DA with EP and NE was not different from 0, it is difficult to explain the age-related changes in circulating DA based on this postulation. Stress-related studies of the functions of DA as a neurotransmitter are usually focused on the brain (Cabib and Puglisi-Allegra, 2012; Lataster et al., 2013).

As EP and NE decreased in the plasma of 12-wk-old pullets, a similar trend occurred with blood concentrations of 5-HT and Trp at 12 wk of age when compared with 4 (numerical trend for 5-HT) and 6 wk of age. Because Trp is the precursor of 5-HT (Gallup et al., 1977) and Trp can cross the blood-brain barrier (Zlokovic, 2001), it is conceivable that the circulating levels of these 2 metabolites positively correlated with one another. When circulating levels of Trp are not needed for peripheral 5-HT synthesis, it, unlike DA, does not accumulate in the blood. The Trp crosses the blood-brain barrier to be used by the brain to synthesize 5-HT. Similar results of concentrations of Trp correlating with 5-HT (r = 0.54; P < 0.0001) have been reported in the brain and blood of rats (Biggio et al., 1974; Weinberger et al., 1978). Because it has been shown that brain levels of 5-HT increased in response to stressors in rats (Kalen et al., 1989; Shimizu et al., 1992; Vahabzadeh and Fillenz, 1994; Kirby et al., 1995), peripheral levels of 5-HT may respond in a similar manner. The lower levels of blood 5-HT in 12-wk-old pullets compared with younger pullets (specifically, the 6-wk-old pullets) may be related to these older pullets experiencing less handling stress as described for EP and NE.

In conclusion, pullets in the current study that were never exposed to perches showed no evidence of eliciting a stress response. Specifically, perch access or denial from hatch to 12 wk of age in caged White Leghorns resulted in similar levels of circulating catecholamines, CORT, 5-HT, Trp, EP:NE ratio, and H:L ratio. The increase in circulating EP, NE, 5-HT (numerical increase at 4 wk), and Trp in 4- and 6-wk-old pullets compared with 12-wk-old pullets is unclear, but may have been due to acute handling stress at younger ages. Plasma CORT levels and the H:L ratio, indicators of long-term stress, were unaffected by age.

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**REFERENCES**


