**Effects of moist litter, perches, and droppings pit on fluctuating asymmetry, tonic immobility duration, and heterophil-to-lymphocyte ratio of laying hens**

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**ABSTRACT** The purpose of this study was to analyze the effects of moist litter, perches, and droppings pit on the fluctuating asymmetry, the tonic immobility duration, and the heterophil:lymphocyte ratio of laying hens. In experiment 1, hens from 4 Spanish breeds and a White Leghorn population, that had been housed in moist or dry litter pens from 20 wk of age, were used. Treatment effect was significant for the fluctuating asymmetry of toe length (\(P < 0.05\)), the fluctuating asymmetry of hens housed in moist litter pens being larger. The duration of tonic immobility was significantly longer (\(P < 0.01\)) in hens housed in moist litter pens. Thus, litter moisture is associated with the well-being or fear levels of hens, as indicated by the fluctuating asymmetry and the tonic immobility duration. In experiment 2, hens from the Black Menorca breed, that had been housed in pens with or without perches from 20 wk of age, were used. The fluctuating asymmetry of wattle length and the combined fluctuating asymmetry were significantly greater (\(P < 0.001\)) in hens housed in pens without perches. Thus, the presence of perches is associated with the well-being level of birds, as indicated by the fluctuating asymmetry. In experiment 3, hens from 4 Spanish breeds and a White Leghorn population, that had been housed in pens with or without a droppings pit, were used. There was significant difference for the fluctuating asymmetry of leg length (\(P < 0.001\)) between hens housed in pens with or without a droppings pit, the fluctuating asymmetry of hens housed in pens without a droppings pit being larger. There were no significant differences for the duration of tonic immobility and the heterophil:lymphocyte ratio between both treatments. Thus, the presence of a droppings pit is associated with the well-being of hens, as indicated by the fluctuating asymmetry, and does not contribute to their fear or stress levels, as indicated by the tonic immobility duration and the heterophil:lymphocyte ratio.

**Key words:** moist litter, perch, droppings pit, well-being, stress

INTRODUCTION

Fluctuating asymmetry is considered to be a valid indicator of stress during development (Moller and Swadle, 1997), and it is used as a measure of well-being after development (Tuyttens, 2003) because it reflects the ability of the animal to cope with the sum of challenges during its growing period (Knierim et al., 2007). Housing conditions (such as temperature, humidity, litter, and air quality) are important to chicken well-being (Dawkins et al., 2004). Moist litter is responsible for a large number of well-being problems in poultry, such as increasing incidence of footpad dermatitis (Wang et al., 1998; Mayne, 2005). Excessive moisture promotes bacterial growth, which will decompose organic material producing ammonia, a highly irritating and toxic gas, in the process (Wathes, 1998; Kristensen and Wathes, 2000). The effect of moist litter on the fluctuating asymmetry has been studied only in broiler chickens by Van Poucke et al. (2007), who did not find significant difference between wet litter and control treatments for the relative fluctuating asymmetry of metatarsus (length and width), outer and middle toe lengths, metacarpal length, eye length, and wattle width. Van Poucke et al. (2007) also reported the effect of wet litter on the duration of tonic immobility (an indicator of fearful-ness; Gallup, 1979), indicating that broilers from the wet litter treatment were more fearful, with the tonic immobility duration being longer. The effect of moist litter on the heterophil:lymphocyte ratio (an indicator of chronic stress; Gross and Siegel, 1983) has not been investigated yet, although Dawkins et al. (2004) indicated that moist litter increased the production of the stress hormone corticosterone.

An appropriate environmental design is considered fundamental to ensure poultry welfare. Deep litter housing systems may or may not include perches for roosting and a droppings pit. The presence of perches...
may improve the welfare of chickens (Duncan et al., 1992). Olsson and Keeling (2000) indicated that laying hens kept under conditions where perching is not possible may experience reduced welfare, and Olsson and Keeling (2002) suggested that hens should be housed in systems with perches. The relationships between the use of perches and the duration of tonic immobility or heterophil:lymphocyte ratios have been analyzed in several experiments. Brake et al. (1994) reported that the age-related increase in the duration of the tonic immobility of broiler breeder pullets housed in pens was attenuated by the provision of perches during rear- ing, although the duration of the tonic immobility did not differ significantly between pullets housed with or without perches. Barnett et al. (1997) evaluated the heterophil:lymphocyte ratio of layer hens housed in cages modified with perches, and they did not find significant effects of perches. Heckert et al. (2002) did not observe significant effects on the heterophil:lymphocyte ratios between broilers housed with or without perches. Campo et al. (2005) found a significant effect of perches on the heterophil:lymphocyte ratio, the ratio of hens with perches being lower than that of hens without perches, but did not find a significant effect on the duration of tonic immobility.

The purpose of this study was to analyze the effects of moist litter, perches, and droppings pit on the fluctuating asymmetry of laying hens (experiments 1, 2, and 3, respectively). Additionally, the effects of moist litter and a droppings pit on the tonic immobility duration and the heterophil:lymphocyte ratio were also analyzed. It was hypothesized that birds housed in moist litter, without perches or without a droppings pit, would show more asymmetrical morphological traits, a longer duration of tonic immobility, and increased heterophil:lymphocyte ratios. Relative to the existing literature, the study analyzes for the first time the association between moist litter and heterophil:lymphocyte ratio and complements, with laying hens, the only previous experiment to analyze the association between moist litter and the fluctuating asymmetry or the tonic immobility duration with meat animals (Van Poucke et al., 2007). As far as the authors know, the associations between the use of perches and the fluctuating asymmetry, and the use of a droppings pit and the fluctuating asymmetry, the tonic immobility duration, or the heterophil-to-lymphocyte ratio have not been studied yet. Duration of tonic immobility and heterophil:lymphocyte ratio were not measured in the second experiment, because they had been previously analyzed by Campo et al. (2005).

**MATERIALS AND METHODS**

**General Procedure**

Different breeds of chickens, that are maintained at the Experimental Station of El Encín (Madrid, Spain) in a conservation program of genetic resources started in 1975 (Campo and Orozco, 1982), were used. Birds from all of the breed were reared together in an all-litter (pine wood shavings to approximately 7 cm over the floor) floor pen (20 m × 9 m) at a density of 10 birds/m² until 8 wk of age (mixed sexes). Equal numbers were used from each breed. Artificial light was only provided during the first week (23L:1D). Temperature was controlled with gas heaters (33 to 35°C at the chick level during the first week followed by a reduction of 3°C each week until the temperature reached 18 to 20°C at the sixth week of age). Birds were reared in another all-litter floor pen (20 × 13 m) at a density of 6 birds/m² from 8 to 20 wk of age. The lighting regimen was 8L:16D. Birds were fed standard rearing diets, containing 19% CP, 2,800 kcal of ME/kg, 1% Ca, and 0.5% available P, until 8 wk, and 15% CP, 2,700 kcal of ME/kg, 0.9% Ca, and 0.4% available P, until 20 wk. Feed and water were supplied for ad libitum consumption. From 20 to 36 wk of age, the lighting regimen was 14L:10D (light from 0700 to 2100 h) and room temperature was 16 to 20°C. Birds were fed a standard laying diet, containing 16% CP, 2,700 kcal of ME/kg, 3.5% Ca, and 0.5% available P. Feed and water were supplied for ad libitum consumption. Bird density was 4 birds/m².

The measured morphological traits were both right (R) and left (L) middle toe length, leg (metatarsus) length, wing (radius) length, wattle length, and leg width at 36 wk of age. Right and left values of a bird were taken during the same session. All 4 lengths and leg width were measured in millimeters using a digital caliper. Trait size was the mean of the right and left traits [(R + L)/2]. All traits showed normal frequency distributions. The fluctuating asymmetry for a trait was defined by the absolute difference between sides [|R − L|], equivalent to the mean deviation. A series of steps (Palmer, 1994; Knierim et al., 2007) were followed before identifying exhibited asymmetry as fluctuating asymmetry (normal distribution of signed right minus left differences with a mean of zero) because there are several confounding factors that complicate the analysis of asymmetry: different types of bilateral asymmetry (fluctuating asymmetry, directional asymmetry, and antisymmetry), measurement error, and relation between fluctuating asymmetry and trait size (Campo et al., 2008). Thus, the fluctuating asymmetry was estimated confidently, and it was not confounded with measurement error. Relative fluctuating asymmetry was used for all traits (|R − L|/(R + L)); it had distributions that were not normal and were transformed to arcsin square root before analysis. Combined relative asymmetry was defined as the mean of the relative asymmetries of the different traits.

To obtain the heterophil:lymphocyte ratio (on the same day as morphological traits), birds were carried to a separate room, and blood was collected immediately. Two drops of blood were taken from a small puncture in the comb of each bird, 1 drop being smeared on each of 2 glass slides. The smears were stained using...
May-Grünwald and Giemsa stains (Lucas and Jamroz, 1961), approximately 2 to 4 h after methyl alcohol fixation. One hundred leukocytes, including granular (heterophils, eosinophils, and basophils) and nongranular (lymphocytes and monocytes), were counted on 1 slide of each bird (the other slide was supplementary), and the heterophil:lymphocyte ratio was calculated. Ratios were transformed to square root before analysis.

Birds were tested for tonic immobility on the day after the blood sampling. They were caught and carried in an upright position to a separate neighboring room. A few seconds after the bird was caught, tonic immobility was induced by placing the bird on its back with the head hanging in a U-shaped wooden cradle (Jones and Faure, 1981). The bird was restrained for 10 s. The observer sat in full view of the bird, about 1 m away, and fixed his eyes on the bird to give the fear-inducing properties of eye contact. If the bird remained immobile for 10 s after the experimenter removed his hands, a stopwatch was started to record latency (s) until the bird righted itself. If the bird righted itself in less than 10 s, then it was considered that tonic immobility had not been induced, and the restraint procedure was repeated (3 times maximum). If the bird did not show a righting response over the 10-min test period, a maximum score of 600 s was given for righting time. Thus, tonic immobility duration ranged from 0 to 600 s. Durations were logarithmically transformed before analysis.

**Experiment 1 (Moist Litter)**

Hens from 3 different Spanish breeds (Blue Andaluza, Black Castellana, Buff Prat), 1 synthetic breed (Quail Silver Castellana), and a White Leghorn population were used. Blue Andaluza and Black Castellana are white shell egg layers, whereas Buff Prat is a tinted shell egg layer. The synthetic breed originated from an F2 cross between Black Castellana and Buff Prat (Campo, 1996). A total of 120 hens (24 per breed) from 2 different replicates (hatches) separated by 14 d were used to study the effect of moist litter on fluctuating asymmetry, duration of tonic immobility, and heterophil:lymphocyte ratio at 36 wk of age. There were 20 all-litter (pine wood shavings) floor pens (6 birds per pen) in the experiment (10 per replicate: 1 for each breed and treatment). The pens were 1.5 m × 1.0 m. The moist litter treatment consisted of 60 hens (12 hens of each breed in 2 replicates of 6 hens) housed at 20 wk of age in pens in which water (3 L) was applied daily and distributed evenly across the litter. The control treatment consisted of 60 additional hens (12 hens of each breed in 2 replicates of 6 hens) housed at 20 wk of age in partially littered pens with a raised (30 cm from the ground) slatted floor (without perches) covered with droppings pit and pine wood shavings litter on the rest of the floor; the slatted area occupied approximately one-third of the floor. Perches were in the slatted floor area.

**Statistical Analysis**

To test the differences in fluctuating asymmetry, tonic immobility duration, and heterophil:lymphocyte ratio between treatments in experiments 1 and 3, a 3-way ANOVA (Sokal and Rohlf, 1981) was used with the statistical model:

\[ x_{ijkl} = \mu + T_i + B_j + TB_{ij} + r_k + Tr_{ik} + Br_{jk} + TBr_{ijk} + \varepsilon_{ijkl}, \]

where \( x_{ijkl} \) = the analyzed measurement; \( \mu \) = the overall mean; \( T_i \) = the effect of treatment (moist vs. dry...
RESULTS

Moist Litter (Experiment 1)

Mean litter moisture on both treatments averaged 71 and 12% in moist and dry litter treatments, respectively. Mean values indicating the effect of moist litter on fluctuating asymmetry are summarized in Table 1. Hens housed in moist litter had significantly ($P < 0.05$) greater relative asymmetry for toe length than hens housed in dry litter. Treatment effect was not significant for the relative asymmetry of leg, wing, and wattle lengths; leg width; and the combined relative asymmetry of the 5 traits. Breed effect was not significant and there was no significant treatment × breed interaction for any measure of fluctuating asymmetry. Similar results were obtained for the absolute fluctuating asymmetry. The effect of moist litter was significant ($P < 0.01$) on the tonic immobility duration. Tonic immobility of hens housed in moist litter was longer than that of hens housed in dry litter (Table 1). There were significant differences among breeds in terms of tonic immobility duration ($P < 0.05$). The tonic immobility duration was significantly longer for Blue Andaluzas (292 s) than for Quail Silver Castellanas (105 s). There was no significant treatment × breed interaction for the tonic immobility duration. Treatment, breed, and treatment × breed interaction were not significant sources of variation for the heterophil:lymphocyte ratio.

Perches (Experiment 2)

Mean values indicating the effect of treatment on fluctuating asymmetry are summarized in Table 2. There was a significant effect of perches ($P < 0.001$) on the relative fluctuating asymmetry of wattle length and the combined relative asymmetry of the 5 traits. The relative fluctuating asymmetry for hens housed with perches was lower than that of hens housed without perches. Treatment effect was not significant for the relative asymmetry of toe, leg, and wing lengths and leg width, although all asymmetry measurements, whether or not significant, pointed in the same direction, hens housed without perches having the most asymmetrical toes, legs, wings, and wattles. Hens housed with perches tended to have lower relative fluctuating asymmetry of wing length ($P < 0.10$).

Droppings Pit (Experiment 3)

There was a significant effect of the droppings pit on the relative fluctuating asymmetry of leg length ($P < 0.001$). Hens housed without a droppings pit had significantly greater relative asymmetry than hens housed with a droppings pit (Table 3). Treatment effect was not significant for the relative asymmetry of toe, wing, and wattle lengths; leg width; and the combined relative asymmetry of the 5 traits. Breed effect was not significant, and there was not a significant treatment × breed interaction for any measure of relative asymmetry. Similar results were obtained for the absolute

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### Table 1. Mean relative asymmetry ($\times 100$) of various morphological traits, tonic immobility duration (s), and heterophil:lymphocyte ratio in hens from 5 different breeds housed in moist or dry litter (experiment 1; n = 120)

<table>
<thead>
<tr>
<th>Litter</th>
<th>Toe length</th>
<th>Leg length</th>
<th>Wing length</th>
<th>Wattle length</th>
<th>Leg width</th>
<th>Combined</th>
<th>Tonic immobility</th>
<th>Heterophil:lymphocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moist</td>
<td>2.47$^a$</td>
<td>0.73</td>
<td>0.95</td>
<td>1.53</td>
<td>2.64</td>
<td>1.66</td>
<td>263$^a$</td>
<td>0.85</td>
</tr>
<tr>
<td>Dry</td>
<td>1.98$^b$</td>
<td>0.75</td>
<td>0.82</td>
<td>1.59</td>
<td>2.54</td>
<td>1.53</td>
<td>184$^b$</td>
<td>0.80</td>
</tr>
<tr>
<td>SEM</td>
<td>0.15</td>
<td>0.07</td>
<td>0.07</td>
<td>0.11</td>
<td>0.24</td>
<td>0.07</td>
<td>23</td>
<td>0.04</td>
</tr>
</tbody>
</table>

$^{a,b}$Means within the same column with no common superscript differ ($P < 0.05$).
fluctuating asymmetry, that for leg length being significantly \((P < 0.001)\) greater in hens housed without a droppings pit \((0.95 \text{ mm})\) than in hens housed with a droppings pit \((0.62 \text{ mm})\).

There was no significant effect of droppings pit on the duration of tonic immobility and the heterophil:lymphocyte ratio, mean values being similar in both treatments. Breed effect and treatment \(\times\) breed interaction were not significant for any analyzed measurement.

**DISCUSSION**

The main finding of experiment 1 was that moist litter affected fluctuating asymmetry of middle toe length, but not fluctuating asymmetry of leg, wing, and wattle, confirming that it is trait-specific. Hens housed in moist litter had the relative fluctuating asymmetry of middle toe length almost 25\% larger than hens housed in dry litter, suggesting that middle toe length asymmetry was a sensitive indicator of stress. These results agree with those reported by Van Poucke et al. (2007). Although they found that the effect of moist litter on the absolute fluctuating asymmetry of middle toe length approached significance \((P = 0.06)\), they did not find significant effects of moist litter on the absolute fluctuating asymmetry of leg length, wattle length, and leg width \((less than 0.2 \text{ mm for all traits})\), suggesting that fluctuating asymmetry might not be a suitable indicator to detect variations of welfare status due to the moisture of the litter in broiler chickens. In the current study, different breeds of layers (2 white shell eggs, a tinted shell egg, a synthetic population, and a White Leghorn population) housed under semi-intensive conditions were used. As would be expected, there were significant differences in the duration of tonic immobility between the hens housed in moist or dry litter, with the mean value being significantly longer within the hens housed in moist litter. The duration of tonic immobility was approximately 43\% longer in the hens housed in moist litter than that shown by the hens housed in dry litter, suggesting that moist litter is aversive for chickens. This fact agrees with the results reported by Van Poucke et al. (2007), who indicated that broiler chickens from the wet litter treatment experienced elevated fearfulness.

There were no significant differences in the heterophil:lymphocyte ratio between both treatments, with the effects of moist litter not being manifested in changes in the heterophil:lymphocyte ratio, in disagreement with the result indicated by Dawkins et al. (2004) for the significant effect of moist litter on the production of corticosterone. Although the heterophil:lymphocyte ratio is a more reliable indicator of stress than corticosterone levels in plasma, this rule applies only when moderate stress exists (Maxwell, 1993). During extreme stress, as in a moist litter situation, the heterophil:lymphocyte ratio may not be an accurate measurement of stress.

There was significant association between perches and the fluctuating asymmetry of wattle length and the combined fluctuating asymmetry of all 5 traits (experiment 2). Hens housed without perches had relative fluctuating asymmetry of wattle length twice as large as hens housed with perches, and the absence of perches significantly increased the combined fluctuating asymmetry almost 50\%. The fluctuating asymmetry results suggest that hens were less stressed when given perches, confirming that the welfare of chickens housed on the floor could be improved with perches (Duncan et al., 1992; Olsson and Keeling, 2000, 2002). Combining asymmetries from different traits increases the probability of detecting stress effects (Leung et al., 2000), especially if there is no significant kurtosis or significant platykurtosis; there was no evidence of kurtosis for leg length, wattle length, and leg width, whereas middle toe and leg lengths showed significant leptokurtosis (Campos et al., 2008). Although tonic immobility duration or heterophil:lymphocyte ratio were not measured in the current study, results agree with that of Campos et al. (2005) in the Black Menorca breed, who reported that heterophil:lymphocyte ratio was significantly affected by the presence of perches. However, they disagree with those of Barnett et al. (1997) and

**Table 2.** Mean relative asymmetry \((\times100)\) of various morphological traits in hens from the Black Menorca breed housed with or without perches \((\text{experiment} 2; n = 120)\)

<table>
<thead>
<tr>
<th>Housing</th>
<th>Toe length</th>
<th>Leg length</th>
<th>Wing length</th>
<th>Wattle length</th>
<th>Leg width</th>
<th>Combined</th>
</tr>
</thead>
<tbody>
<tr>
<td>With perches</td>
<td>1.99</td>
<td>0.75</td>
<td>0.93</td>
<td>4.47</td>
<td>3.33</td>
<td>2.30b</td>
</tr>
<tr>
<td>Without perches</td>
<td>2.39</td>
<td>0.77</td>
<td>1.15</td>
<td>8.90</td>
<td>3.87</td>
<td>3.42*</td>
</tr>
<tr>
<td>SEM</td>
<td>0.20</td>
<td>0.07</td>
<td>0.09</td>
<td>0.66</td>
<td>0.32</td>
<td>0.15</td>
</tr>
</tbody>
</table>

\(^*\) Means within the same column with no common superscript differ \((P < 0.05)\).

**Table 3.** Mean relative asymmetry \((\times100)\) of various morphological traits in hens from 5 different breeds housed with or without a droppings pit \((\text{experiment} 3; n = 120)\)

<table>
<thead>
<tr>
<th>Housing</th>
<th>Toe length</th>
<th>Leg length</th>
<th>Wing length</th>
<th>Wattle length</th>
<th>Leg width</th>
<th>Combined</th>
</tr>
</thead>
<tbody>
<tr>
<td>With droppings pit</td>
<td>2.93</td>
<td>0.64b</td>
<td>1.29</td>
<td>6.53</td>
<td>2.96</td>
<td>2.87</td>
</tr>
<tr>
<td>Without droppings pit</td>
<td>2.53</td>
<td>1.01*</td>
<td>1.43</td>
<td>5.35</td>
<td>3.39</td>
<td>2.74</td>
</tr>
<tr>
<td>SEM</td>
<td>0.21</td>
<td>0.07</td>
<td>0.10</td>
<td>0.54</td>
<td>0.30</td>
<td>0.14</td>
</tr>
</tbody>
</table>

\(^*\) Means within the same column with no common superscript differ \((P < 0.05)\).
Heckert et al. (2002) for heterophil:lymphocyte ratio and Brake et al. (1994) and Campo et al. (2005) for the duration of tonic immobility, who indicated that these indicators of stress levels and fearfulness were not influenced by the presence of perches in broilers and layers. Campo et al. (2007) indicated that fluctuating asymmetry was not associated with heterophil:lymphocyte ratio and the duration of tonic immobility, suggesting that these measurements are influenced by different biological mechanisms.

There was significant difference in fluctuating asymmetry of leg length between hens housed with or without a droppings pit (experiment 3). Hens housed without a droppings pit had a relative fluctuating asymmetry of leg length almost 58% larger than hens housed with a droppings pit. However, fluctuating asymmetries of toe length, wing length, wattle length and leg width, tonic immobility duration, and heterophil:lymphocyte ratio were not associated with the presence of a droppings pit, suggesting that a partially slatted floor system to collect most of the manure beneath the slats is not critical to hen welfare, fear, and stress.

In conclusion, results of the current study indicate that moist litter resulted in greater relative asymmetry of toe length and duration of tonic immobility in hens from 4 different Spanish breeds and the White Leghorn, suggesting that this physical stress is associated with some measures of well-being and fear. Hens from the Black Menorca breed housed without perches had greater relative fluctuating asymmetry of wattle length and the combined relative fluctuating asymmetry of the 5 traits, suggesting that the well-being of birds can be improved with perches. The absence of a droppings pit resulted in greater fluctuating asymmetry of leg length in hens from 4 different Spanish breeds and the White Leghorn, suggesting that it negatively affects this measure of well-being. Results should be confirmed in the future by applying the stress conditions during the rearing period, measuring the fluctuating asymmetry in 20-wk-old birds.

REFERENCES


