Tonic Immobility Reaction and Heterophil to Lymphocyte Ratio in Hens from Three Spanish Breeds Laying Pink Eggshells

J. L. CAMPO and A. REDONDO

Area de Genetica Animal, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Apartado 8111, 28080 Madrid, Spain

ABSTRACT Tonic immobility reaction and heterophil:lymphocyte ratio were studied in hens laying pink eggs from three brown egg Spanish breeds (Barred Red Vasca, Red Vasca, Red Villafranquina). There was no significant difference among breeds in terms of tonic immobility duration or heterophil:lymphocyte ratio. Tonic immobility duration was significantly shorter in the pink (220 ± 23 s) than in the normal brown shell group of hens (416 ± 23 s; \( P < 0.001 \)). Differences were consistent across the breeds, tonic immobility of hens laying pink eggs being shorter than that of control hens in all three breeds. Birds of the group with pink shells appeared to be less fearful when defined by either the duration of tonic immobility or the number of inductions to achieve it. There was a significant difference in the heterophil:lymphocyte ratio between the two groups of hens (\( P < 0.001 \)), mean value being significantly higher in the pink shell (0.73 ± 0.03) than in the control group (0.35 ± 0.03). The heterophil:lymphocyte ratio from the pink shell hens increased significantly in the three breeds. Hens that laid pink eggs had significant heterophilia and lymphopenia (\( P < 0.001 \)). Thus, hens laying pink eggs were less fearful and more stressed than control hens, as indicated by the tonic immobility reaction and the heterophil:lymphocyte ratio, respectively. A significant negative correlation was found between both measurements (\( r = -0.28; P < 0.01 \)). A similar significant negative association (\( P < 0.001 \)) was found between pink shell color and shell strength.

(Key words: pink eggshells, tonic immobility reaction, heterophil to lymphocyte ratio, fear, stress)

1996 Poultry Science 75:155-159

INTRODUCTION

Besides the greater economic losses due to shell damage occurring after the egg has been laid, an important though smaller loss is due to primary malformations of the egg. External abnormalities of the eggshell have been a serious problem to many producers because such eggs do not match consumer’s concept of the ideal brown egg. Losses may also be caused in breeding flocks because affected eggs are not usually sent to the hatchery. Steggerda and Hollander (1944) observed three types of shell variation in Barred Plymouth Rock x Rhode Island Red crosses: eggs with areas of depigmentation, eggs coated with a chalky deposit, and eggs with shell deformations. Van Ness (1949) showed that alterations in New Hampshire pullet eggs occurred in the form of an overlay of calcium carbonate on an already pigmented egg or as a roughness and lack of pigmentation. An abnormality often seen on eggs from brown layer flocks is the superficial coating of amorphous calcareous material, causing in some eggs an apparent change in color to blue-violet or pink (Lang and Wells, 1987). These abnormalities are associated with retention of eggs in the shell gland and may be due to the effect of stress on the animal (Watt and Solomon, 1988; Cranston and Solomon, 1994). Solomon et al. (1987) suggested that the abnormal eggs laid after hens have been exposed to stress are of poor external and structural quality. Hughes et al. (1986) categorized two main classes of shell abnormalities: eggs coated with a superficial layer of amorphous calcium (dusted, white banded, chalky, or pink eggs), and misshapen or bulging eggs. Translocation of hens from pens to cages, disturbances to flocks on deep litter, and exclusion from nests of birds accustomed to laying in nest boxes resulted in an increase in the proportion of abnormal eggs. These observations provided a basis for a noninvasive method of assessing stress as well as helped to account for hitherto inexplicable occurrences of declining shell quality. Hughes and Black (1976) showed that handling resulted in an increased incidence of equatorial bulges, suggesting that handling may be a stressor. Mills et al. (1987) showed that hens kept in individual cages laid fewer abnormal eggs than hens kept in groups of three or four, supporting the idea that the incidence of eggs showing abnormal calcification may provide a quick and
reliable method to measure stress in hens that lay brown eggs. Mills et al. (1991) studied two populations of ISA Brown hens with family histories of a low or a high incidence of eggshell whitening and found that hens of the population with the high incidence appeared to be more fearful than hens of the population with the low incidence, although the duration of tonic immobility did not differ significantly. Fear is an important component of stress, and tonic immobility is considered a useful index of general fearfulness in fowls (Gallup, 1979).

The heterophil:lymphocyte ratio has been proposed as an index of stress in chickens (Gross and Siegel, 1983) because exposure to stressors cause it to increase progressively. This ratio may be a more reliable indicator of mild to moderate stress than plasma corticosterone concentration (Maxwell et al., 1992; Maxwell, 1993), although its relationship with the incidence of abnormal eggshells has not been studied. Beuving et al. (1989) found that White Leghorn hens showing long tonic immobility had higher absolute heterophil:lymphocyte ratios than those showing short tonic immobility (high-fear and low-fear birds, respectively); the ratio was significantly increased after the continued denial of access to visible feed. Mauldin et al. (1979) showed that birds from the less fearful line had a significantly larger number of lymphocytes, although they were smaller in diameter.

A preliminary study in hens that laid pink eggs from a Spanish stock of brown layers (Campo, unpublished observations) suggested that pink color might be associated with consistent decreases in tonic immobility durations of the hens; these birds showed shorter duration of tonic immobility (149 ± 24 s) than normal hens (408 ± 36 s), and laid abnormal eggs in the absence of any apparent stressor. This paper presents the results of a study designed to test the relationships between the incidence of pink eggs, the duration of the tonic immobility reaction, and the heterophil:lymphocyte ratio, in three different brown egg Spanish breeds of chickens. In addition, an attempt was made to determine the extent to which pink color affected shell quality.

**MATERIALS AND METHODS**

Eggs showing pink shells occurred in hens of three different Spanish stocks: Barred Red Vasca, Red Vasca, and Red Villafranquina. The two varieties of the Vasca breed lay typical brown colored eggs, whereas Villafranquina is a traditional layer of very dark brown eggs. These populations are maintained at the Experimental Station of "El Encín" (Madrid), in a conservation program of genetic resources started in 1975 (Campo and Orozco, 1982), and have been described by Campo and Orozco (1980, 1983), and Campo and Alvarez (1988).

A total of 130 hens were used, equally divided into two groups: Group 1 consisted of 65 hens laying eggs with pink or chalky shells (24, 23, and 18 hens of the Barred Red Vasca, Red Vasca, and Red Villafranquina, respectively); Group 2 served as a control of 65 additional hens laying normally colored brown eggs (24, 23, and 18 hens of each breed). Hens were housed in small floor pens with trapnests to allow the eggs of each hen to be identified. Feed and water were supplied for ad libitum consumption and the lighting regimen was 14 h light:10 h darkness. Disturbance of the birds was limited to routine husbandry procedures and experimental manipulations.

Birds were tested for tonic immobility duration at 52 wk of age. Tonic immobility was induced as soon as a bird was caught by placing the hen on her back with the head hanging in a U-shaped wooden cradle (Jones and Fauré, 1981); the bird was restrained for 15 s. The observer sat in full view of the hen, about 1 m away, and fixed his eyes on the bird. If the bird remained immobile for 10 s after the experimenter removed his hands, a stopwatch was started to record latencies until the bird righted itself. If the bird righted in less than 10 s, then it was considered that tonic immobility had not been induced and the restraint procedure was repeated. 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. Testing took place between 0900 and 1400 h on five replicates at 1-wk interval (13 hens per group in each replicate).

As soon as the tonic immobility test was finished, hens were carried to a separate room and collection of blood was made immediately. Two drops of blood were taken from a small puncture in the comb of each bird, one drop being smeared on each of two glass slides. The smears were stained using May-Grünwald and Giemsa stains (Lucas and Jamroz, 1961), approximately 2 to 4 h after preparation with methyl alcohol fixation. One hundred leucocytes, including granular (heterophils, eosinophils, basophils) and nongranular (lymphocytes, monocytes) ones, were counted on one slide of each hen and the heterophil:lymphocyte ratio was calculated.

A three-way analysis of variance (Sokal and Rolf, 1981) was used, according to the model: $x_{ijkl} = m + T_i + B_j + R_k + TB_{ij} + TR_{ik} + BR_{jk} + TBR_{ijk} + e_{ijkl}$, where $x_{ijkl}$ is the tonic immobility duration, or the heterophil:lymphocyte ratio, of treatment $i$, breed $j$, and replicate $k$; $m$ is the overall mean; $T_i$ is the effect of treatment (pink vs normal shell color); $B_j$ is the effect of breed ($j = 1 \ldots 3$); $R_k$ is the effect of replicate ($k = 1 \ldots 5$); and $e_{ijkl}$ is the residual (the number of hens in the individual sub-classes was unequal, 1 being 4 or 5 in the Vasca varieties and 3 or 4 in the Villafranquina). Treatments and breeds were considered to be fixed effects and replicates were assumed to be a random effect. Logarithmic or square root transformations were used for analyses of variance, but indicated mean values are not transformed.

A total of 1,984 eggs (992 with pink shell and 992 with normal brown color) were collected to estimate shell quality. A single sodium chloride solution with specific
gravity of 1.080 (the industry standard) was used, recording if an egg floated on it or did not. Bennett (1993) indicated that it may be possible to use a single salt solution to distinguish between thick- and thin-shelled eggs. The solution was adjusted regularly with a hydrometer. To test for the independence between shell color and specific gravity a 2 x 2 chi-square was made (Snedecor and Cochran, 1980).

RESULTS

Replicates, replicate by treatment, replicate by breed, or replicate by treatment by breed interactions were not significant for either analyzed measurement (tonic immobility duration, heterophil:lymphocyte ratio, and its numerator or denominator), and they were pooled with the residual to give a two-way factorial model of the treatment and breed effects. Mean values indicating pink shell color effects on tonic immobility duration are summarized in Table 1. Hens laying pink eggs showed significantly (P < 0.001) shorter tonic immobility reaction (220 ± 23 s) than hens with normal colored brown eggs (416 ± 23 s), when treatments mean square was tested against residual mean square. There were no significant differences among breeds in terms of tonic immobility duration and treatment by breed interaction was not significant, indicating consistent differences between pink and normal shells. Tonic immobility of hens laying pink eggs was shorter than that of control hens in all three breeds: 280 ± 39 vs 450 ± 39 s (Red Vasca), 197 ± 38 vs 365 ± 38 s (Barred Red Vasca), and 176 ± 44 vs 442 ± 44 s (Villafranquina).

The heterophil:lymphocyte ratio was significantly higher within the pink shell group than within the control group of hens (Table 1; P < 0.001); mean values being 0.73 ± 0.03 and 0.35 ± 0.03, respectively. The ratio did not differ significantly among breeds and the interaction treatment by breed was not significant, indicating consistent differences between pink and normal shells. Tonic immobility of hens laying pink eggs was shorter than that of control hens in all three breeds: 280 ± 39 vs 450 ± 39 s (Red Vasca), 197 ± 38 vs 365 ± 38 s (Barred Red Vasca), and 176 ± 44 vs 442 ± 44 s (Villafranquina).

The heterophil:lymphocyte ratio was significantly higher within the pink shell group than within the control group of hens (Table 1; P < 0.001); mean values being 0.73 ± 0.03 and 0.35 ± 0.03, respectively. The ratio did not differ significantly among breeds and the interaction treatment by breed was not significant, the heterophil:lymphocyte ratio increasing in the three breeds (0.77 ± 0.05 vs 0.35 ± 0.05, 0.67 ± 0.05 vs 0.37 ± 0.05, and 0.75 ± 0.06 vs 0.33 ± 0.06 in the Red Vasca, Barred Vasca, and Villafranquina, respectively). In hens laying pink eggs, there was a significant increase in heterophil number (39 ± 1 vs 24 ± 1), together with a corresponding decrease in lymphocytes (58 ± 1 and 73 ± 1, respectively). Neither breed nor treatment by breed effects were significant, the changes in leucocyte numbers being similar in the three breeds. In the Red Vasca, hens with pink eggs showed 41 ± 2 heterophils and 57 ± 2 lymphocytes, whereas mean numbers for the control hens were 24 ± 2 and 72 ± 2, respectively. Hens laying pink eggs from the Barred Red Vasca had 37 ± 2 heterophils and 60 ± 2 lymphocytes, compared with 25 ± 2 heterophils and 72 ± 2 lymphocytes showed by the control group. Finally, the pink egg group of the Villafranquina had 41 ± 2 heterophils and 57 ± 2 lymphocytes, and the control group for this breed had 23 ± 2 and 74 ± 2 heterophils and lymphocytes, respectively.

Correlations between the tonic immobility reaction and the heterophil:lymphocyte ratio in each hen was calculated (Sokal and Rholf, 1981). Both traits were significantly and negatively correlated; the correlation coefficient was -0.28 (P < 0.01), indicating that hens with shorter tonic immobility tended to have a higher heterophil:lymphocyte ratio. Correlations between tonic immobility duration and heterophil or lymphocyte number were equal in absolute value, intermediate, and significantly different from zero (-0.32 and 0.32, respectively; P < 0.01).

The chi-square test for the independence between shell coloration (pink or normal brown) and specific gravity (> 1.080 or < 1.080) showed significant association between the two classifications (Table 2). As the observed number of pink eggs floating in the salt solution with 1.080 specific gravity exceeded that expected (+36.5), we concluded that pink eggs tend to be floating eggs (positive association).

TABLE 1. Mean tonic immobility duration, heterophil:lymphocyte ratio, number of heterophils, and number of lymphocytes for pink and normal shells from brown egg laying stocks

<table>
<thead>
<tr>
<th>Eggshell color</th>
<th>Tonic immobility (s)</th>
<th>Heterophil:lymphocyte</th>
<th>Heterophil number</th>
<th>Lymphocyte number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pink</td>
<td>220 ± 23</td>
<td>0.73 ± 0.03</td>
<td>39 ± 1</td>
<td>58 ± 1</td>
</tr>
<tr>
<td>Normal</td>
<td>416 ± 23</td>
<td>0.35 ± 0.03</td>
<td>24 ± 1</td>
<td>73 ± 1</td>
</tr>
<tr>
<td>SEM</td>
<td>23</td>
<td>0.03</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

A,B Means within the same column with no common superscript differ significantly (P < 0.001).

The chi-square value was 10.85, with one degree of freedom, giving P < 0.001.

TABLE 2. Observed and expected (in brackets) frequencies of pink and normal eggs floating or not floating in a salt solution with 1.080 specific gravity

<table>
<thead>
<tr>
<th>Shell color</th>
<th>Floating</th>
<th>Not floating</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pink</td>
<td>548</td>
<td>444</td>
<td>992</td>
</tr>
<tr>
<td></td>
<td>(511.5)</td>
<td>(480.5)</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>475</td>
<td>517</td>
<td>992</td>
</tr>
<tr>
<td></td>
<td>(511.5)</td>
<td>(480.5)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1,023</td>
<td>961</td>
<td>1,984</td>
</tr>
</tbody>
</table>

1 The chi-square value was 10.85, with one degree of freedom, giving P < 0.001.
DISCUSSION

The difference in duration of tonic immobility between hens laying pink shell eggs and those laying normal brown shell eggs was significant. Hens that laid pink eggs showed a shorter tonic immobility reaction than those that laid normal colored brown eggs in the three breeds; the pink shell group of hens having only 53% of the tonic immobility duration of the group with normal shells. This finding disagrees with that of Mills et al. (1991), who reported a longer duration of tonic immobility in birds from a commercial stock with normal brown shell eggs. This finding disagrees with that of Mills et al. (1991), who reported a longer duration of tonic immobility in birds from a commercial stock with normal brown eggs. This was in agreement with the findings of Gross and Siegel (1983), but differs from those of Campo and Alvarez (1991), who reported that feed restriction significantly diminished the tonic immobility duration in six different breeds.

However, this ratio is an accurate indicator of stress, and it has been demonstrated that stress results in an increase in the proportion of eggs laid with abnormal shells (Mills et al., 1987; Watt and Solomon, 1988). Hughes et al. (1986) showed that transfer from pens to cages, disturbance by catching and wing-banding, and closing the nest box, resulted in retention of eggs in the shell gland and an increased proportion of abnormal eggs, pink eggs following moderate retention. Steggerda and Hollander (1944), and van Ness (1949) had already suggested that eggs coated with a chalky deposit, and eggs overlaid with calcium carbonate were due to retention in the oviduct.

In our study, the birds had not been deliberately disturbed in any way and those laying pink or brown eggs were housed similarly. It might be possible that failures in the usual husbandry operations, such as shortages in water or lighting, resulted in the birds laying abnormal eggs; however, the production of pink eggs from a hen remained fairly constant from day to day, suggesting an apparent genetic component for the trait. The accuracy of the heterophil:lymphocyte ratio as a measurement of stress is limited to moderate stress; Maxwell et al. (1992) showed that a heterophilia may be the response to mild or moderate stress, but a basophilia may result after severely stressing birds.

Hens laying pink eggs were less fearful and more stressful than control hens, as indicated by the tonic immobility reaction and the heterophil:lymphocyte ratio, respectively. A significant negative correlation between the two measurements was found, supporting a consistent intra-individual association between tonic immobility and leucocyte ratio. This fact does not agree with the results reported by Beuving et al. (1989) in White Leghorn hens differentiated according to their short or long tonic immobility durations (77 ± 5 and 818 ± 46 s, respectively). In that study, the heterophil:lymphocyte ratio was higher in high-fear (0.54 ± 0.07) than in low-fear (0.34 ± 0.04) hens, although both groups showed similar precannulation ratios (0.23 ± 0.03 and 0.16 ± 0.02, respectively). Although fear and stress are not synonymous, they are closely linked and the interpretation of the above findings is equivocal; it is interesting that Beuving et al. (1989) and Maxwell et al. (1992) found significant increases in heterophil:lymphocyte ratio accompanying feed restriction. This agrees with the results of Gross and Siegel (1983), but differs from those of Campo and Alvarez (1991), who reported that feed deprivation significantly diminished the tonic immobility duration in six different breeds.

It is not clear whether the relationship between fearfulness and blood leucocyte response to pink egg formation is a stress response, a physiological stage, or both. Pink shell seems to be associated with retention of egg in the shell gland, and may or may not be due to the effect of stress. It might be possible that if one egg were retained in the shell gland, the egg would be laid on a day following one on which no egg had been laid, and its shell would have a pink coloration.

The negative association between pink color and shell thickness found by using a specific gravity test should be confirmed in the future by measuring the individual shell strength. The estimated incidence of pink eggs (about 5%), should not be a cause of important economic loss in the three stocks studied.
REFERENCES


