Effect of genotype and transport on tonic immobility and heterophil/lymphocyte ratio in two local Italian breeds and Isa Brown hens kept under free-range conditions

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Abstract

This study was undertaken to investigate the effect of transport and genotype on the welfare and fear response of laying hens through a comparison of three breeds reared in free-range conditions: a commercial strain, the Isa Brown (IBh), and two local chicken breeds, the Bionda Piemontese (BPh) and the Bianca di Saluzzo (BSB). After 67 days of transport from the farm of origin to the experimental station, ninety hens, divided according to breed, were free-range reared for two months. Body weight (BW), tonic immobility (TI), red and white blood cells, heterophil to lymphocyte (H/L) ratio and □1-acid glycoprotein (AGP) were assessed at four different sampling times: at the farm of origin (T1), at 1 day (T2), 15 days (T3) and at 2 months (T4) after arrival at the experimental station. No statistical differences were found between the four sampling times for BW, total red and white blood cells or AGP. An increase in the H/L ratio (P<0.05) was recorded at time T2 for IBh and BSB, compared to BPh (P<0.05). TI was significantly higher (P<0.05) for the local breeds, BPh and BSB, than for the commercial strain IBh. The results of this study suggest that genetic and adaptive differences can affect both, physiological and ethological parameters.

Introduction

The increased demand for traditional agriculture products, linked to the territory and to the natural cycles of the seasons, is opening new prospects concerning the poultry production chain. In Italy, over the past decade, rural farming using local breeds has increased. This may not be competitive for industrial farming but it is suitable for extensive rearing, such as in free-range and organic systems. Local breeds are used in these farms and great attention should be paid to animal welfare issues (stoking density, social behavior, quality of feed) (Lazzaroni et al., 2001).

In the countryside of the Piemonte region (North-West Italy), there are two local chicken breeds, Bionda Piemontese (BPh) and Bianca di Saluzzo (BSB), that are well suited to these farming practices, because of their grazing ability (Asproavic, 2002). Furthermore, both breeds are extremely hardy with good disease resistance (Guidobono Cavachlina et al., 2007). In addition, both are good producers of eggs, with an average annual production of 200 and 180 eggs, respectively (Asproavic, 2002). The Bionda Piemontese breed is characterized by a buff colored coat with a black tail, while the Bianca di Saluzzo breed is completely white (Asproavic, 2002). For all these reasons, these two local chicken breeds are both suggested for traditional recipes and are included in the Slow Food Presidia. Slow Food is a global, grassroots organization with supporters in 150 countries around the world who link the pleasure of good food with a commitment to their community and the environment (Slow Food, 2013).

Studies regarding these two breeds are limited (Lazzaroni et al., 2001; Guidobono Cavachlina et al., 2007; Schiavone et al., 2009), and no literature is available concerning their response to stress and fear. Some studies have been performed to characterize other Italian breeds (Castellini et al., 2002; Cerolini et al., 2010; Dal Bosco et al., 2012; Madeddu et al., 2013; Mugnai et al., 2009, 2011; Zanetti et al., 2010, 2011). Nevertheless, some experimental trials have been carried out concerning poultry response (in terms of ethological and physiological welfare indicators) to several factors, such as the type of rearing system (from cages to free-range), crating, and transport, in Spanish chicken breeds (Campos and Càrnicer, 1993; Campos et al., 2001, 2008, 2012), and in laying hens and broiler chickens (Scott et al., 1998; Zulifi et al., 2000, 2001; Salamano et al., 2010).

Some blood parameters have been used as physiological indicators of the stress response of chickens. The heterophil-to-lymphocyte (H/L) ratio is affected by stress factors and could be used as an indicator of stress in hens. The H/L ratio is correlated to a hen’s health status and responds to stimuli associated with diet, chronic bacterial infections, stress, light and trauma and it varies according to the change in the percentage of heterophils and lymphocytes in the blood (Gross and Siegel, 1983; Maxwell, 1993; Maxwell and Robertson, 1998). The □1-acid glycoprotein (AGP) is a positive acute phase protein (APP) whose particular feature is that its serum concentration increases in response to an inflammatory and non-inflammatory challenge. Some recent studies on chickens have highlighted a relationship between the AGP response and psychophysical stress, suggesting that this response may be induced by stressful events to which hens could be exposed during daily management (Schiavone et al., 2009; Salamano et al., 2010). For these reasons, AGP can be considered a stress indicator in chickens. Fear is certainly considered an important stress factor that can seriously damage poultry welfare and performance, and fear reduction is of major importance (Jones et al., 2005). Fear is an emotional state that is derived from an aversive stimulus which leads to ethological and physiological changes that arise in animals to engage this stimulus (Moberg, 1985). Tonic immobilization (TI) is an unlearned catatonic state that is used as a method to estimate a state of fear because it is considered to be positively connected to fear and its duration could

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be related to a high or low level of fearfulness (Jones, 1986). TI is one of the most frequently used tests, and it is regarded as a relatively robust measure of underlying fearfulness, particularly in poultry (Campo and Carnicer, 1993; Scott et al., 1998; Campo et al., 2001, 2008, 2012; Riedstra and Groothuis, 2002; Al-Aql et al., 2013).

Several studies have examined how transport can affect the welfare status of hens. Hens are not routinely subjected to the stimuli associated with transport. The acts of being crated and transported are likely to be novel and, potentially, frightening or disturbing to hens (Murphy, 1978). Manual handling is, in itself, frightening for chickens (Scott and Moran, 1992; Zulkifli et al., 2000). Zulkifli (2003) observed that the transportation of poultry induced an augmentation of the H/L ratio, according to the duration of the journey. Mills and Nicol (1990) observed that fear levels, evaluated by means of TI, in laying hens, following crating and transport, was more frightening than after catching alone. Scott et al. (1998) pointed out that there was no reason to expect that hens from free-range environments are less frightened during transport than hens from cages.

The purpose of this study was to investigate how some indicators of fearfulness and stress vary in relation to a stressful stimuli like transport and a change of environment in respect of genotype using three different breeds of laying hens reared in a free-range system.

### Materials and methods

The study was conducted between March and May 2011 at the Struttura Didattica Speciale Veterinaria (SDSV), University of Torino (Italy). The trial was carried out by comparing two local Italian chicken breeds, Bianca di Saluzzo (BSh) and Bionda Piemontese (BPh), and a commercial strain Isa Brown (IBh). Thirty hens for each strain were purchased, at the age of 26 weeks for BSh and BPh, and at the age of 16 weeks for IBh (both breeds used in this study (P<0.05). A marked statistical trend was considered for P<0.1.

All hens (n=90) were tested for TI in a separate room on the day after the blood sampling. Each individual was carefully and gently caught with both hands, held in an inverted manner, and carried to a separate room (no visual contact with other hens) for the TI measurements. TI was induced by placing the hen on its back with its head hanging in a U-shaped plastic cradle and gently restraining the hen on the breastbone for 10 s. The observer then retreated approximately 1 m and fixed his eyes on the hen because of the fear-inducing properties of eye contact, but made no unnecessary noise or movement (Jones and Faure, 1981). A stopwatch was started to record latency until the hen righted itself. If the hen righted itself in less than 10 s, the restraining procedure was repeated. If TI was not induced after 3 attempts, the duration of TI was considered 0 s. If the hen did not show a righting response over the 10-min test period, a maximum score of 600 s was given for the righting time. Thus, tonic immobility duration ranged from 0 to 600 s (Zulkifli et al., 2000). After measuring the TI, the hen was spray-painted on the back, for identification purposes and returned to its home pen.

Normality of data distribution was assessed using the Shapiro-Wilk test, while the homogeneity of variances was tested using the Levene test. Data were analyzed using an analysis of variance (ANOVA) for repeated measures, followed by Tukey’s post-hoc test. The main factors were the breed of the hens, for each sampling time, and the time of sampling, within the breed. Square root (heterophil-to-lymphocyte ratio) or logarithmic (tonic immobility duration) transformations were used for the analyses of variance, but the original mean values have been presented. All the statistical analyses were performed using the SPSS statistics package, ver. 17.0 (SPSS, 2008) and the data are presented as the mean and standard error (SE). Differences were considered statistically significant for P<0.05. A statistical trend was considered for P<0.1.

### Results

The average bodyweight (SE) of the hens was 1651 (31.84) g, 1801 (39.50) g and 1830 (41.61) g for IBh, BPh and BSh, respectively. No statistical differences were found between the genotypes and different sampling times. The data obtained for the total count of red and white blood cells and AGP are reported in Table 1. These data are presented for each group as the mean value of the four sampling times (T1, T2, T3 and T4), because no significant effect of sampling time both was found within the groups. The total leucocytes was affected by breed and showed lower values for BPh and BSh than IBh (P<0.05). The values obtained for the H/L ratio are reported in Table 2. Statistical differences were recorded between the three genotypes and between the four sampling times. The mean H/L ratio values were lower for the BPh breed than the other two breeds used in this study (P<0.05). A marked
change in the H/L ratio was recorded for the four sampling times with a significant increase in IBh and BSh at time T2, 1 day after transport (P<0.05). IBh exhibited a higher H/L ratio than BPh or BSh (P<0.05) at sampling times T1 and T2. Furthermore, BSh was higher than IBh at T3, and BPh was the lowest at T4. The data obtained for the T1 test are presented in Table 3. Significant differences between breeds were observed, with the two local breeds, BPh and BSh, having longer tonic immobility duration than IBh (P<0.05). No statistical differences for T1 were recorded for the four sampling times. However, a tendency towards significance for IBh and BSh (P=0.055 and P=0.090, respectively) was observed at the T2 sampling time.

**Discussion**

The blood leucocyte concentration was different for the three breeds and this could probably be due to genetic differences (Maxwell, 1993; Schiavone et al., 2009). Nevertheless, the values obtained in this study reflect those found in previous studies performed using the same breeds, where the hens in both studies were reared in free-range conditions (Schiavone et al., 2009; Salamano et al., 2010). The mean AGP concentration was not affected by crating or transport, and no difference between genotypes was found. The AGP values recorded in this study for IBh and BSh were slightly higher than those recorded in previous studies (Schiavone et al., 2009; Salamano et al., 2010). The H/L ratio values showed some differences for the four sampling times. The highest value of the H/L ratio in IBh and BSh was found at the T2 sampling time, thus showing how transport has led to a stress increase in these two genotypes. This can be corroborated by the fact that, at the successive sampling times (T3 and T4), the H/L ratio of both IBh and BSh returned to mean values that were similar to those observed at the sampling time, T1. Zulkifli et al. (2001) reported that broiler chickens subjected to crating and transportation had greater H/L ratio than those that were crated and left stationary. Furthermore, in another study Zulkifi (2003) pointed out that there was an increase in the H/L ratio following a 60 min or a 120 min journey. The differences in the H/L ratio between the three breeds are probably due to genetic factors. Al-Murrani et al., (1997) underlined that the H/L

### Table 1. Erythrocyte and leucocyte counts and □1 acid glycoprotein according to the hen genotype (mean and SE) (n=120 for the total mean).

| Genotype | IBh | BPh | BSh | P (effect breed) | P (sampling time effect) |
|----------|-----|-----|-----|------------------|-------------------------|
| Erythrocyte, × 10^12/L | 2.40 (0.05) | 2.58 (0.05) | 2.53 (0.05) | ns | ns |
| Leucocyte, × 10^9/L | 18.73 (0.95)^a | 16.09 (0.51)^b | 13.97 (0.61)^b | 0.000 | ns |
| AGP, mg/mL | 0.52 (0.03) | 0.49 (0.02) | 0.46 (0.03) | ns | ns |

IBh, Isa Brown hen; BPh, Bionda Piemontese hen; BSh, Bianca di Saluzzo hen; AGP, □1 acid glycoprotein. ^a,b Different letters on the same row values are statistically significant at P<0.05; ns, not significant.

### Table 2. Heterophil to lymphocyte (H/L) ratio according to the hen genotype and the sampling time (mean and SE) (n=120 for total mean; n=30 for T1, T2, T3 and T4, respectively).

| Genotype | IBh | BPh | BSh | P (breed effect) |
|----------|-----|-----|-----|------------------|
| Mean | 0.64 (0.08)^a | 0.40 (0.02)^b | 0.55 (0.04)^a | 0.001 |
| T1 | 0.63 (0.06)^ab | 0.42 (0.03)^b | 0.39 (0.04)^b | 0.001 |
| T2 | 1.69 (0.25)^ab | 0.44 (0.04)^b | 0.88 (0.14)^ab | 0.000 |
| T3 | 0.38 (0.02)^ab | 0.41 (0.04)^b | 0.52 (0.03)^b | 0.033 |
| T4 | 0.49 (0.05)^ab | 0.33 (0.02)^b | 0.47 (0.03)^b | 0.005 |
| P (sampling time effect) | 0.000 | ns | 0.000 |

IBh, Isa Brown hen; BPh, Bionda Piemontese hen; BSh, Bianca di Saluzzo hen; T1, at the farmhouse of origin; T2, 1 day; T3, 15 days; T4, 2 months after arrival at the experimental station. ^a,b Different letters on the same row values are statistically significant at P<0.05; ns, not significant.

### Table 3. Tonic immobility duration according to the hen genotype and the sampling time (mean and SE) (n=120 for total mean; n=30 for T1, T2, T3 and T4, respectively).

| Genotype | IBh | BPh | BSh | P (breed effect) |
|----------|-----|-----|-----|------------------|
| Tonic immobility duration (mean) | 105.76 (14.42)^b | 281.09 (25.66)^b | 223.60 (21.64)^b | 0.000 |
| T1 | 57.00 (19.37)^b | 257.44 (37.27)^b | 191.67 (43.44)^b | 0.000 |
| T2 | 203.13 (33.95)^b | 379.00 (60.90)^a | 327.53 (49.07)^ab | 0.033 |
| T3 | 83.40 (27.90)^b | 243.44 (52.30)^a | 189.80 (33.75)^a | 0.008 |
| T4 | 76.27 (16.66)^b | 255.42 (50.39)^a | 185.40 (37.80)^a | 0.008 |
| P (sampling time effect) | 0.055 | ns | 0.090 |

IBh, Isa Brown hen; BPh, Bionda Piemontese hen; BSh, Bianca di Saluzzo hen; T1, at the farmhouse of origin; T2, 1 day; T3, 15 days; T4, 2 months after arrival at the experimental station. ^a,b Different letters on the same row values are statistically significant at P<0.05; ns, not significant.
ratio is under the control of a few genes and is inheritable. Campo et al. (2008) found that the effect of the housing system (litter vs free-range) on the H/L ratio varied from chicken breed to chicken breed, and it was not significant in three of the five studied breeds. Another point is that the free-range rearing system did not affect the IBh H/L ratio. Mugnai et al. (2011) found that H/L was affected by housing system (cage vs organic) for both Brown Hy-Line hen and Ancona hen (another Italian breed), with higher value for caged birds. Similar results were observed in another study carried out by our research group (Salamano et al., 2010).

In our study, BPh and BSh reacted more fearfully in the TI test than IBh. Tonic immobility has a genetic component, with certain genetic strains showing longer TI durations than others (Jones et al., 1991). Mugnai et al. (2011) found that TI was affected by both genotype and rearing system (standard cage system and organic production) being the TI longer in Brown Hy-Line than in Ancona hen. Campo and Carnicer (1993) demonstrated that the age-related increase in TI may be associated with the approach of sexual maturity and the hens’ changed endocrine state. No significant differences were found for the four sampling times and the lack of significance can be attributed to the high variability in the TI results. Nevertheless, there was a tendency towards significance at sampling time T2 for IBh and BSh, which would seem to point out how crating and transport, which were novel stimuli for the hens, led to a fear reaction and unpleasant human contact. Poultry Sci. 85:621-626.

The present findings suggest genetic and adaptive differences to stressful stimuli that affect both physiological and ethological parameters. Genotype and transport play important roles highlighting differences in response to stress and fear among different breeds. The H/L ratio has been confirmed to be a reliable parameter to evaluate the stress status of chickens derived from crating and transport, and a similar trend was observed when TI was performed. The commercial strain IBh resulted to be suitable for free-range rearing systems.

The two local chicken breeds used in this study were more susceptible to fearful than the commercial strain probably due to genetic factors. Nevertheless, in presence of stressful and fearful situations, these two local breed have proved to be more resistant, demonstrating to suffer less than IBh. Due to the increased demand for traditional agriculture products, several conservation programs on local chicken populations are ongoing, thus detailed studies on aspects related to poultry behavior and stress response in order to ensure increasing animal welfare levels are recommendable.

Conclusions

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De Marco et al.

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