PAPER

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

Michele De Marco,[1] Silvia Martinez Mirò,[2] Martina Tarantola,[1] Stefania Bergagna,[3] Elisabetta Mellia,[3] Maria Silvia Gennero,[3] Achille Schiavone[1]

[1]Dipartimento di Scienze Veterinarie, Università di Torino, Grugliasco (TO), Italy

[2]Departamento de Producción Animal, Universidad de Murcia, Spain

[3]Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Torino, Italy

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* (BSh). After a journey of 67 km (75 min) from the farmhouse 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 farmhouse 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 for AGP. An increase in the H/L ratio ($P<0.05$) was recorded at time T2 for IBh and BSh, compared to BPh ($P<0.05$). TI was significantly higher ($P<0.05$) for the local breeds, BPh and BSh, 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 rearing 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* (BSh), 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 Cavalchini *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 Cavalchini *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 (Campo and Carnicer, 1993; Campo *et al.,* 2001, 2008, 2012), and in laying hens and broiler chickens (Scott *et al.,* 1998; Zulkifli *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 immobility (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

Corresponding author: Prof. Achille Schiavone, Dipartimento di Scienze Veterinarie, Università di Torino, via Leonardo da Vinci, 44, 10095 Grugliasco (TO), Italy.
Tel. +39.011.6709208 - Fax: +39.011.2369208.
E-mail: achille.schiavone@unito.it

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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-Aqil *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 ages corresponding to the beginning of oviposition). IBh were purchased in a conventional farm where hens were ground reared (9 hens/m^2), while BSh and BPh were purchased in free-range farm (2 hens/m^2). The distance of the two farms of origin was below 2 km. All the hens were placed in transport crates (0.90x0.58x0.28 m), with 10 hens to each crate, and were then subjected to a journey of about 70 km for a total time of 75 min.

On arrival at the SDSV, the hens were divided into groups of 30 hens each, and were housed (*per* breed) in three different indoor pens (2.5×5.5 m), each with an outdoor paddock (2.5×5.5 m). The hens were free-range reared, in identical environmental conditions, at a density of 0.9 hens/m^2. Feed and water were provided *ad libitum.* All the hens were fed the same commercial diet (11.5 MJ metabolizable energy/kg feed, 166 g crude protein/kg feed). All the hens were individually identified with a shank ring. All hens were monitored during the trial (n=90). All the evaluations (body weight, blood sampling and TI) were assessed at four different sampling times: at the farmhouse of origin (T1), at 1 day (T2), 15 days (T3), and at 2 months (T4) after arrival at SDSV. The body weight of the individual hens was recorded during the four sampling times, just before the blood sampling, using a high precision scale (Signum®, Sartorius, Muggiò, MB, Italy).

Blood was sampled by means of venepuncture of the brachial vein, using a 21G needle to measure the physiological stress indicators. A sample of 3 mL of blood was collected from each hen: 1.5 mL was placed an EDTA tube and 1.5 mL in a tube without anticoagulant. A blood smear was prepared, using one glass slide for each hen, from a drop of blood without anticoagulant. The smears were stained using May-Grünwald and Giemsa stains (Campbell, 1995). The total red and white blood cell counts were determined in an improved Neubauer haemocytometer on blood samples previously treated with a 1:200 Natt-Herrick solution. One hundred leukocytes, including granular (heterophils, eosinophils, and basophils) and non-granular (lymphocytes and monocytes) leukocytes, were counted on the slide and the H/L was calculated. The tubes without anticoagulant were left to clot in a standing position at room temperature for approximately two hours to obtain serum. The serum was separated by means of centrifugation at 700 x g for 15 min and frozen at –80°C pending analysis. The serum AGP concentration (mg/mL) was assayed using a commercially available radial immunodiffusion tray (Cardiotech Services, Louisville, KY, USA).

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 TI 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 TI 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 stationery. Furthermore, in another study Zulkifli (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).

	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).

	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)^{A,Y}	0.42 (0.03)^{B}	0.39 (0.04)^{B,Y}	0.001
T2	1.69 (0.25)^{A,X}	0.44 (0.04)^{B}	0.88 (0.14)^{B,X}	0.000
T3	0.38 (0.02)^{B,Y}	0.41 (0.04)^{AB}	0.52 (0.03)^{A,Y}	0.033
T4	0.49 (0.05)^{A,Y}	0.33 (0.02)^{B}	0.47 (0.03)^{A,Y}	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; ^{X,Y}different letters on the same column 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).

	IBh	BPh	BSh	P (breed effect)
Tonic immobility duration (mean)	105.76 (14.42)^{B}	281.09 (25.66)^{A}	223.60 (21.64)^{A}	0.000
T1	57.00 (16.97)^{B}	257.44 (37.27)^{A}	191.67 (43.44)^{A}	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 heritable. 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 trend increase. IBh resulted to be the most sensitive breed to fear from crating associated with transport; the rate of increment of TI, between T1 and T2, was plus 47% for BPh, plus 71% for BSh and plus 256% for IBh. Scott *et al.* (1998), using ISA Brown laying hens and a journey time of 74 min, found a similar result for TI (statistical tendency) after crating and transport. Murphy (1978) pointed out that novelty is the stimulus characteristic that is most often associated with fear. The crating and transport of the hens was a novel stimulus, because they are not routinely treated in this manner. However for all hens, at sampling times T3 and T4, the TI duration returned to the values of sampling time T1. It can be speculated that the hens, after the fear caused by crating and transport, became accustomed to the new rearing condition and to being subject to TI. Some authors have noticed that habituation attenuates the TI response in both young chicks and adult fowls (Salzen, 1963; Gentle *et al.,* 1985; Heiblum *et al.,* 1998). Inhibition increases with the number of testing stimuli, and can last for over 2 months (Nash and Gallup, 1976). Grigor *et al.* (1995), using a medium hybrid laying strain, suggested that regular exposure to an outside environment during the rearing process increased hens' readiness to move and utilize the available outdoor area and such an experience reduced the hens' fear level.

Conclusions

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.

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