The genetics of three welfare indicators: tonic immobility duration, heterophil to lymphocyte ratio, and fluctuating asymmetry

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The genetics of welfare is reviewed with the research on three indicators, i.e. tonic immobility duration, heterophil to lymphocyte ratio, and fluctuating asymmetry, in different populations of chickens (Spanish breeds of layers and White Leghorn). The review includes breed and crossbreed differences, heritability estimates, and genotype by environment interaction. There were significant differences among breeds for tonic immobility duration, heterophil to lymphocyte ratio, and fluctuating asymmetry of two morphological traits (feather and spur lengths) and mean fluctuating asymmetry of three secondary sexual traits (wattle, ear-lobe, and wing-bay areas) in males. There was significant negative heterotic effects in F_1 and F_2 crosses for tonic immobility duration, heterophil to lymphocyte ratio, and fluctuating asymmetry of two morphological traits (leg and wing lengths) in males, ranging from -12% to -54%. Tonic immobility duration was a low heritable trait, with REML heritability estimate equal to 0.06 ± 0.02, although realized heritability in a selection experiment was intermediate (0.32 ± 0.10). The REML heritability for heterophil to lymphocyte ratio ranged from 0.13 ± 0.03 to 0.59 ± 0.09, that for fluctuating asymmetry of three morphological (leg, wing, and feather lengths) and two secondary sexual (wattle and ear-lobe areas) traits ranging from 0.00 ± 0.04 to 0.09 ± 0.05. There was significant genotype by environment quantitative interaction for tonic immobility duration in one (of 25) welfare-related factor, cold stress having a significant effect only on one breed, and significant qualitative interaction in two welfare-related factors, supplemental cholesterol in the diet and intermingled housing condition changing the order of merit of the breeds. There was significant qualitative interaction for heterophil to lymphocyte ratio in intermingled housing condition.

Keywords: tonic immobility; leukocyte ratio; fluctuating asymmetry; genetics; chickens

Introduction

Tonic immobility duration, heterophil to lymphocyte ratio, and fluctuating asymmetry are three frequently used indicators of welfare in chickens. Firstly, tonic immobility is a measurement for evaluating fearful behaviour in the chicken (Gallup, 1979) that represents a terminal defensive reaction and can be used as criterion for measuring the wellbeing and levels of stress of the birds. Secondly, the heterophil to lymphocyte ratio has become widely accepted as a reliable and accurate physiological indicator of the stress response in chickens (Gross and Siegel, 1983), because exposure to stressors causes it to increase progressively. This ratio is a more reliable indicator of mild to moderate stress than plasma corticosterone concentration (Maxwell, 1993). Gross and Siegel (1993) suggested that reference values for the heterophil to lymphocyte ratio of about 0.2, 0.5, and 0.8 are characteristic of low, optimal, and high degrees of stress, respectively. Finally, fluctuating asymmetry is the most recommended index of developmental instability (Palmer and Strobeck, 1992) and is considered to be a valid indicator of genetic and environmental stress during development (Parsons, 1990), especially if the analyses are based on the composite fluctuating asymmetry of multiple traits. It is characterised by a normal frequency distribution of left minus right side differences with a mean of zero (van Valen, 1962). Fluctuating asymmetry is used as a measurement of fitness and welfare following development (Jones, 1987; Moller et al., 1995).

This review consists of data from several works at our department which have analysed the genetics of these three welfare indicators in different breeds of layers, and have included breed and crossbreed differences, heritability estimates, and genotype by environment interactions.

Materials and methods

Birds of four different Spanish breeds of layers (Black Castellana, Buff Prat, Red-barred Vasca and Red Villafranquina), a synthetic breed, a White Leghorn population, and the e^y tester line (Smyth, 1976) were used. Castellana is a white shell egg layer, whereas Prat is a tinted shell egg layer, and Vasca and Villafranquina lay brown and dark brown shell eggs, respectively. Two varieties of the Prat (Buff and White) were included in the studies. All these breeds 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 (1998). The synthetic breed (Quail Castellana) originated from an F_2 cross between Black Castellana and Buff Prat (Campo and Orozco, 1986), and the White Leghorn population by crossing three strains selected for egg weight and egg number (Campo and Jurado, 1982).

Tonic immobility was induced by placing the animal 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 because of 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 latencies (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 (three 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. Logarithmic transformation was used prior to analysis.

To obtain the heterophil to lymphocyte ratio, 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 methyl alcohol fixation. One hundred leukocytes, including granular (heterophils, eosinophils, and basophils) and nongranular (lymphocytes and monocytes), were counted on one slide of each bird (the other slide was supplementary), and the heterophil to lymphocyte ratio was calculated. Square root transformation was used prior to analysis.

The measured morphological traits to estimate fluctuating asymmetry were both right (R) and left (L) leg (metatarsus), wing (radius), second primary feather, and spur (in males) lengths, and ear-lobe, wattle, and wing-bay (in males) areas. Right and left values of an animal were taken during the same session. All lengths were measured in millimeters using a digital calliper. Trait length was the mean of the right and left traits [(R+L)/2]. The fluctuating asymmetry (FA) for a trait was defined by the absolute difference between sides [|R-L|]. The unsigned value (equivalent to the mean deviation) is more appropriate for these data than the variance of right minus left sides. A series of steps (Palmer, 1994) was followed before identifying exhibited asymmetry as FA, because there are several confounding factors that complicate the analysis of asymmetry. First, the presence of directional asymmetry (DA: normal distribution with a mean of not zero) and antisymmetry (AS: nonnormal distribution with a mean of zero) was tested by the inspection of the distribution of (R-L). The presence of DA was tested for using *t*-tests. Departures from normality (e.g., AS) were assessed using skewness and kurtosis measures; antisymmetry is characterized by bimodal (or broad-peaked) distributions, tending to be platykurtic (more intermediate values than the normal distribution). Second, FA and measurement error are normally distributed about a mean of zero. Thus, it is essential to show that the variance in asymmetry observed between individuals is greater than the variance due to measurement error. Twenty females and twenty males were randomly chosen and measured three times at three different days. Measures were analysed using a two-way ANOVA with side (fixed) and bird (random) as main factors (1 and 19 df), their interaction (19 df), and the measurement error (80 df). Significant variation between sides indicates variation in DA, whereas a significant interaction indicates significant FA (in the absence of AS). Finally, the product-moment correlation between FA and trait length was used to determine if they were independent. If a positive relationship was found between the mean value and asymmetry of a trait, this effect would be removed by dividing the absolute asymmetry score by the trait mean, defined as the relative FA: [2|R-L|/(R+L)]. Relative FA for all traits had distributions that were not normal and were transformed to arc-sin square root prior to analysis. Mean relative asymmetry was defined as the mean of the relative asymmetries of the different traits. If no size correction were needed, each |R-L| value would be standardised and averaged across traits to give a total score.

Differences among breeds were analyzed in experiments designed to study the effect of different welfare-related factors. A three-way ANOVA 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} is the analyzed measurement (tonic immobility duration, heterophil to lymphocyte ratio, and fluctuating asymmetry), μ is the overall mean, T_i is the effect of the welfare-related factor, B_j is the effect of breed, TB_{ij} is the genotype by environment interaction, R_k is the effect of replicate, TR_{ik}, BR_{jk}, and TBR_{ijk} are the replicate interactions, and ε_{ijkl} is the residual. Welfare-related factor and breed were always considered fixed effects and replicates were assumed to be a random effect. Significant differences were determined using the Student-Newman-Keuls' multiple-range test. Heritabilities were estimated using the restricted maximum likelihood (REML) procedure.

Table 1. Mean values for tonic immobility duration (s), leukocyte ratio, and fluctuating asymmetry in different populations of chickens.

Trait	Reference	Significant differences
Tonic immobility	Campo and Alvarez (1991)	White, tinted egg breeds (285 s) < brown egg breeds (448 s)
	Campo and Redondo (1997)	White Leghorn, Buff Prat, Black Castellana (319, 302, 261 s)
		Red-barred Vasca, Red Villafranquina (484, 475 s)
	Campo (2001)	Black Castellana (209 s) < Red-barred Vasca (375 s)
Leukocyte ratio	Campo and Redondo (1997)	Red-barred Vasca (0.27)
		Red Villafranquina (0.32)
		Black Castellana and Buff Prat (0.39), White Leghorn (0.42)
	Campo and Muñoz (2001)	Black Castellana (0.41) < Buff Prat (0.74)
	Campo et al. (2001)	Black Castellana (0.26)
		Red Villafranquina (0.39), Red-barred Vasca (0.44)
	Campo and Dávila (2002b)	Buff Prat and Black Castellana (0.56)
		Red-barred Vasca (0.70)
Fluctuating asymmetry	Campo et al. (2000)	Black Castellana (3.62%) < Buff Prat (6.86%)[1]
		Red-barred Vasca (6.74%) < Red Villafranquina (14.20%)[2]
	Campo et al. (2002)	e^y (15.72%) < Red Villafranquina (19.90%)[3]

[1]Feather length of males. [2]Spur length. [3]Combined.

Results and discussion

Significant differences among breeds are summarized in Table 1. White (Black Castellana, White Leghorn) and tinted (Buff Prat) shell egg layers showed significantly shorter tonic immobility duration than brown (Red-barred Vasca, Red Villafranquina) shell egg layers. Brown egg breeds had significantly lower heterophil to lymphocyte ratio than white and tinted egg breeds in some works, although the opposite was true in others, whereas white and tinted egg breeds did not differ significantly in general. There was a significant difference in relative fluctuating asymmetry among breeds for feather and spur lengths in males. The relative asymmetry for feather length was significantly lower in the Black Castellana and significantly larger in the Buff Prat, the relative asymmetry for spur length being significantly lower in the Red-barred Vasca than that in the Red Villafranuina breed. Combined relative asymmetry of wattle, ear-lobe, and wing-bay areas differed significantly in the Red Villafranquina and the e^y tester line.

Estimates of heritability are included in Table 2. The intermediate realized heritability of tonic immobility using the White Leghorn population indicates that this trait should respond to selection, although the heritability estimated by REML in the Quail Castellana breed was low. Heritabilities of heterophil to lymphocyte ratio estimated by REML in the Quail Castellana breed differed substantially, with estimates being high or low after two or four generations, respectively. The heritability of the relative fluctuating asymmetry estimated by REML in the Quail Castellana breed was not significantly different from zero for all five traits. The heritability of the combined relative asymmetry was still very low (0.01 ± 0.03), indicating that fluctuating asymmetry is determined mainly by environmental sources of variation.

Table 2. Heritability estimates for tonic immobility duration , leukocyte ratio, and relative fluctuating asymmetry.

Trait		Reference	Heritability	Procedure
Tonic immobility		Campo and Carnicer (1993)	0.32 ± 0.10	Realized
		Campo et al. (2006)	0.06 ± 0.02	REML
Leukocyte ratio		Campo and Dávila (2002a)	0.59 ± 0.09	REML
		Campo et al. (2006)	0.13 ± 0.03	REML
Fluctuating asymmetry	of leg length	Campo et al. (2005a)	0.02 ± 0.03	REML
“ “	of wing length		0.09 ± 0.05	
“ “	of feather length		0.00 ± 0.04	
“ “	of ear-lobe area		0.00 ± 0.07	
“ “	of wattle area		0.02 ± 0.03	

Significant heterotic effects are summarized in Table 3. The significantly shorter tonic immobility duration showed by the F_2 cross and two different F_1 crosses suggests important negative heterotic effects for this trait. Significant negative heterotic effects were also found for heterophil to lymphocyte ratio in F_1 and F_2 crosses, and for relative fluctuating asymmetry of leg and wing length in males from the F_2 cross between the Black Castellana and Buff Prat breeds.

Table 3. Heterotic effects for tonic immobility duration, leukocyte ratio, and fluctuating asymmetry in different crossbreeds of chickens.

Trait	Reference	Crossbreed	Heterosis (%)
Tonic immobility	Campo and Alvarez (1991)	(Black Castellana x Buff Prat) F_2	-22
	Campo and Carnicer (1993)	(White Leghorn x Black Castellana) F_1	-31
		(Buff Prat x White Prat) F_1	-54
	Campo and Redondo (1997)	(Black Castellana x Buff Prat) F_2	-33
Leukocyte ratio	Campo and Redondo (1997)	(Black Castellana x Buff Prat) F_2	-43
	Campo and Dávila (2002a)	(Quail Castellana x White Leghorn) F_1	-26
		(Quail Castellana x White Leghorn) F_2	-36
Fluctuating asymmetry	Campo et al. (2000)	(Black Castellana x Buff Prat) F_2	-38[1]
		(Black Castellana x Buff Prat) F_2	-12[2]

[1]Leg length of males. [2]Wing length of males.

Significant genotype by environment interactions are included in Table 4. There was significant genotype by environment quantitative interaction for tonic immobility duration in one of 25 welfare-related factor (cold stress), cold stress having a significant decreasing effect only on the Red-barred Vasca breed. There was significant qualitative interaction for tonic immobility duration in two welfare-related factors (supplemental cholesterol in the diet and intermingled housing condition), changing the order of merit of the breeds. Cholesterol in the diet increased tonic immobility in the Red-barred Vasca breed and decreased it in the Buff Prat breed. Intermingling reduced significantly tonic immobility of Quail Castellana whereas had no significant increasing effect on the tonic immobility of Black Castellana. There was also significant qualitative interaction for the heterophil to lymphocyte ratio in intermingled housing condition, the ratio being significantly lower in the intermingled Black Castellana, whereas intermingling had no significant increasing effect on the ratio of birds from the Quail Castellana breed.

Table 4. Genotype by environment interactions for tonic immobility duration and leukocyte ratio in different welfare-related factors.

Trait	Reference	Welfare-related factor	Breed
Tonic immobility	Campo and Carnicer (1994)	Cold	Red-barred Vasca (173 s < 349 s)
	Campo and Carnicer (1995)	Cholesterol	Red-barred Vasca (446 s > 271 s) Buff Prat (215 s < 413 s)
	Campo et al. (2005b)	Intermingling	Quail Castellana (164 s < 317 s)
Leukocyte ratio	Campo et al. (2005b)	Intermingling	Black Castellana (0.32 < 0.59)

In conclusion, between breeds variation was common for tonic immobility duration and heterophil to lymphocyte ratio, but rare for relative fluctuating asymmetry, whereas within breeds additive genetic variation was generally low for the three indicators of welfare and specially for the fluctuating asymmetry, and significant heterosis was found in all three indicators of welfare, as it should be expected for traits with low heritability. Besides, there was generally no significant genotype by environment interaction for tonic immobility and leukocyte ratio, indicating that a specific difference of environment had the same effect on different genotypes; only three welfare-related factors had inconsistent differences across breeds.

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