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Research Article
Sex, Genetics and Test Type Affect the Responses of Chickens to Fear Testing

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Abstract

Background and Objective: Fear tests are often used as tools to evaluate the welfare of poultry under both experimental and commercial conditions. However, responses to these tests could be affected both by the genetic makeup and the sex of the individuals tested and in addition different fear tests may vary with respect to their validity and repeatability. The objective was to determine if genetics and sex affected fear response in two different tests. **Methodology:** Males and females of six different genetic stocks of fowl were tested using two fear tests, tonic immobility (TI) and inversion (INV). The stocks were Red Junglefowl, Red Junglefowl/New Hampshire Red crosses, three different Single Comb White Leghorn (SCWL) stocks (UCD-003 and Hyline CV 20) and genetically featherless (scaleless, SL) chickens. **Results:** There were pronounced genetic effects on all TI and INV responses, with significant differences among stocks although these were not necessarily consistent across all measures. Sex differences were more consistent than genetic differences, with males of all stocks showing. Males and females also differed irrespective of genetics, with males requiring fewer induction attempts and having longer latencies to first head movement and to right than females in the TI test ($p<0.05$). Males also had less wing flapping, for less time and less intensely than females during INV ($p<0.05$). **Conclusion:** These results demonstrate that different genetic stocks of fowl react differently in different fear tests and that single fear tests should not be used to evaluate the fear response of fowl.

Key words: Fear, chicken, genetics, sex, fear test

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Competing Interest: The author has declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Fear is a major welfare concern in agricultural animal production systems. Excessive or prolonged fear in animals can result in wasted energy, injuries, behavioral inhibition, reduced ability to adapt to change, delayed maturation, decreased growth and reproduction and death[1]. For laying hens, fearfulness is also a contributor to the development and persistence of severe feather pecking behavior[2,3], which is a widespread welfare problem in commercial flocks.

Because of the importance of fear for welfare, fear testing may be a component of certification or labelling schemes that incorporate the evaluation of outcome-based welfare criteria on commercial farms. The Welfare Quality®[4] assessment protocol for laying hens, for example, includes two tests of fearfulness. One of these involves placing a novel object in the feeder or the litter area and the other involves a human approach test, but both measure fearfulness via avoidance behavior. A lack of avoidance of the novel object is considered an indicator of a positive emotional state, while a lack of human avoidance is considered an indicator of a good human-animal relationship. Indeed, many studies have shown a relationship between fearfulness and farm-specific factors such as the amount of time hens have daily visual contact with caretakers[5], experience with litter during rearing[6] and access to aerial perches[7], making fear testing a potentially valuable tool for on-farm assessment.

One concern about using fear testing for the purposes of comparing farms or farming systems is that different testing methods may measure different types or aspects of fear and with differing degrees of reliability. Miller *et al.*[8] evaluated the validity of four fear tests (emergence, novel object, novel food and predator surprise) for Japanese quail and found that all fear measures were somewhat unstable over time and were also affected by context, since responses were inconsistent across experimental situations. However, some fear responses were more stable and less likely to be context-dependent than others. Similarly, Erasmus and Swanson[9] found that only certain fear measures were stable over time in turkeys, while others were unreliable.

In addition, there can be genetic differences in fearfulness that could affect the validity of cross-farm or housing system comparisons. Fear responses in laying hens have been reported to have heritabilities ranging from 0.07-0.49[2,10,11] and breed differences in fearfulness have been reported[3,12-14]. In contrast, Anderson and Jones[15] found no differences in tonic immobility among four genetic stocks of White Leghorn hens, three random-bred control stocks from the 1950s and one commercial stock, even though these stocks differed significantly in growth parameters, livability, production characteristics and basal corticosterone levels. When differences are found they can depend upon test type. Albentosa *et al.*[16] compared different laying hen stocks and found that White Leghorns differed from the other stocks on one fear measure, but not others.

Fear-related behaviors shown by fowl include passive avoidance, freezing, tonic immobility, withdrawal (active avoidance) and vigorous escape[17]. Many fear responses are related to predation. Ratner[18] distinguished four types of fear shown across various prey species in response to predators: Freezing, fleeing, fighting and tonic immobility. Of the fear responses evaluated by Miller *et al.*[8] in Japanese quail, those related to predation (flight distance and freezing duration) had the best validity.

Two tests that utilize anti-predator responses for measuring fear in fowl are tonic immobility and inversion. Tonic immobility occurs during the final stage of a predator-prey interaction when the animal is unable to escape the predator[18] and consists of the temporary suppression of the righting response, reduced vocalization, intermittent eye closure, rigidity, Parkinsonian-like muscle tremors in the extremities, altered electroencephalographic patterns and change in heart rate, respiration and core temperature[19]. It is a widely used test of fearfulness that has been found to be repeatable and reliable[9,20]. The inversion test[21] evaluates an earlier stage of the predator response, fight or flight, by measuring the intensity of the attempt to break free of the grasp of a simulated predator. This response has been shown to be correlated with tonic immobility in broiler chickens[22].

The objective of this study was to determine how different genetic stocks of fowl respond during tonic immobility and inversion tests. Birds of both sexes of five different genetic stocks of fowl were tested: The ancestor of the domestic fowl (Red Junglefowl), a Junglefowl/domestic fowl hybrid (Red Junglefowl X New Hampshire Red), two different stocks of Single-comb White Leghorns (one commercial and one highly inbred) and a genetically featherless line of New Hampshire Red crosses. In addition, birds were tested twice to evaluate response stability and conducted factor analyses to assess the validity of the two tests across differed among the different genetic stocks.

MATERIALS AND METHODS

Animals and husbandry: Five different genetic stocks of chickens available through the Poultry Genetic Resources Conservation Program in the Department of Animal Science at the University of California, Davis, were used in this study: Red Junglefowl (RJF, N = 18, males = 5, females = 13), a Red Junglefowl/New Hampshire Red cross (RJFNH, N = 20, males = 5, females = 15), commercial Single Comb White Leghorn (SCWL, Hyline CV-20, N = 20, males = 5, females = 15), an inbred SCWL (UCD-003, N = 20, males = 5, females = 15), and a featherless (Scaleless) New Hampshire Red line (SL, N = 20, males = 10, females = 10). All birds were adults ranging from 1-3 years of age. They were housed singly in wire cages measuring either 0.46 m×0.46 m (SCWL and RJFNH, as well as male SL) or 0.3 m×0.3 m (RJF and female SL) and managed according to the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching[23]. Feed (Purina Mills Layena® SunFresh® Recipe, St. Louis, MI, 16% protein) and water were provided *ad libitum* throughout the study and the photo period was 16L:8D.

Fear testing: Two fear tests were conducted on each bird, as described below. The tests were conducted 3 days apart and were counter-balanced to eliminate potential carry-over effects such that half of the birds were first tested on the inversion test and then on the tonic immobility test, while test order was reversed for the remaining birds. These tests were then repeated on the same birds 30 days after the first testing period.

Inversion Test (INV): This test involved holding the bird upside down by its legs with one hand until it ceased wing flapping, or for a maximum of 30 sec[21]. The testing was video recorded for later analysis (Cannon, ZR900, Melville, NY, USA, 24 fps). The duration of flapping and number of wing flaps were recorded and the wing flapping intensity was then calculated by dividing the number of wing flaps by the duration of flapping.

Tonic immobility (TI): Tonic immobility testing was conducted as previously described[21]. In brief, birds were removed from their cage and placed on their backs in a wooden cradle and held there for 15 sec. If a bird righted before 10 sec it was re-induced up to three times, if it could not be induced in three tries it was scored as 0. Latency to first head movement and latency to right were recorded. If a bird failed to right within 120 sec it was given the maximum time score and the test was terminated. All TI testing was carried out in the room in which the birds were housed.

Statistics: A GLM with genetics, sex, testing period, genetics x sex, testing period x sex, genetics x testing period and genetics x sex x testing period was used to determine treatment effects on latency to first head movement, latency to right, number of wing flaps, flapping duration and flapping intensity. The least significant difference *post hoc* test was to test all possible comparisons ($p<0.05$). All of the assumptions of GLMs were tested using the Shapiro-Wilk test for normality and Levene's test for homogeneity of variance, no transformations were needed. Since the number of inductions for the TI data did not meet the assumption of equal variance, they were compared using the non-parametric Kruskal-Wallis test on the equality of the medians for treatment differences, testing period effects on the number of inductions were compared using the Wilcoxon signed ranks test. When significant differences were found for these two measures, the Dwass Steele Critchlow-Fligner method[24] was then used to test all possible comparisons. All analyses were performed using SAS 9.3 for Windows (SAS Institute Inc.). A p value of<0.05 was considered to indicate significance for the GLM, Kruskal-Wallis and *post-hoc* tests.

RESULTS

There was no effect of testing period on any measure for either test, nor were there any significant two or three-way interactions in the GLM models. However, there were significant main effects of genetic stock and sex for both TI and INV measures (Fig. 1).

Genetic effects: There was a significant effect of genetics on all measures for both the TI (induction attempts $H_5 = 40.35$, $p<0.0001$, latency to first head movement $F_{4,192} = 10.86$, $p<0.001$, latency to right, $F_{4,192} = 7.26$, $p<0.001$) and INV (number of wing flaps $F_{4,192} = 12.03$, $p<0.001$, duration of wing flapping $F_{4,192} = 11.05$, $p<0.001$, wing flapping intensity $F_{4,192} = 16.04$, $p<0.001$) tests.

Post-hoc tests for TI comparing all genetic stocks to one another (all $0.05<p\geq0.02$) revealed that the major differences were for the RJF, RJFNH and CV20 stocks. The RJF and RJFNH required more induction attempts than CV20, in addition, RJF had a shorter latency to first head movement than all other stocks. The CV20 stock had the longest latency to first head movement of any genetic stock.

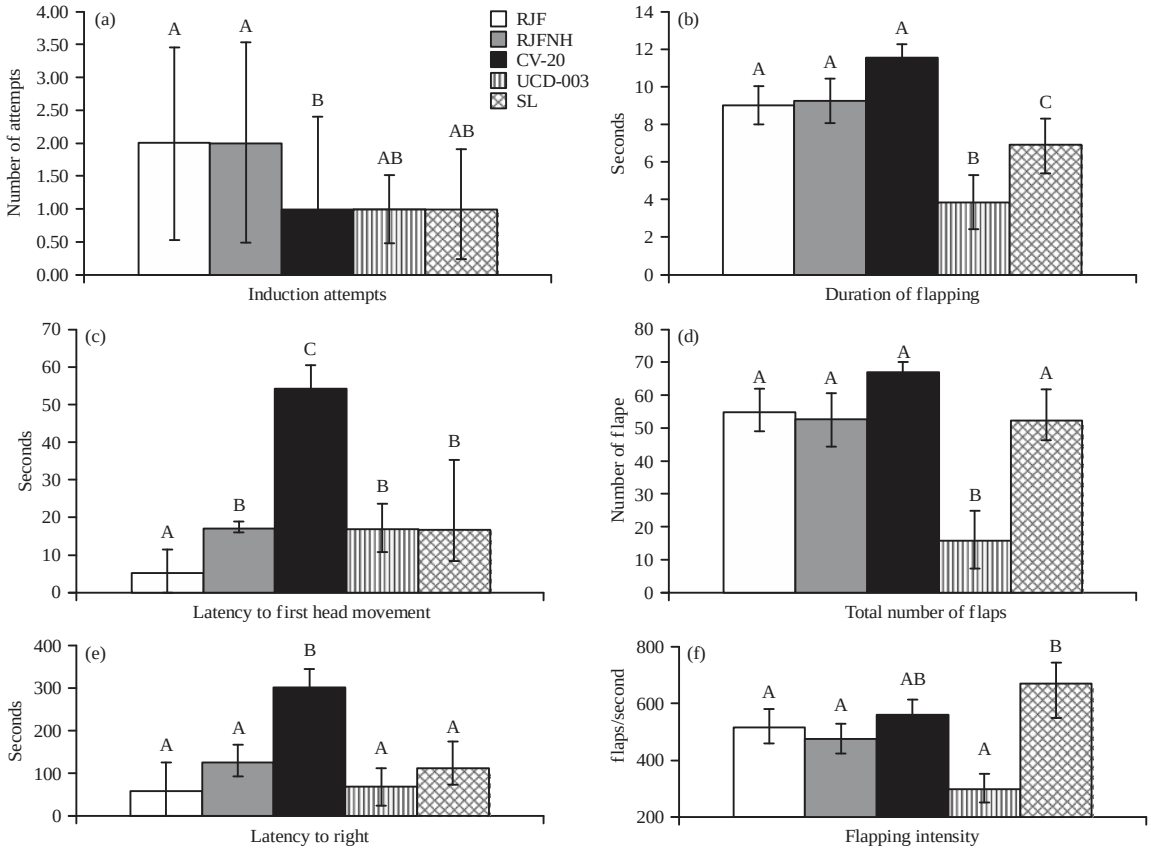

Fig. 1(a-f): (a-c) Results of tonic immobility and (d-f) inversion fear tests conducted on six genetic stocks of chicken. (a) Induction attempts (Medians, number attempts±95% C.I.), (b) Latency to first head movement (means, s±S.E.), (c) Latency to right (Medians, s±95% C.I.), (d) Duration of flapping (means, s±S.E.), (e) Total number of wing flaps (means, number of flaps±S.E.) and (f) Intensity of flapping (means, flaps/second±S.E.)
Bars with different letters indicate significant differences (p<0.05)

Post-hoc tests for INV comparing all genetic stocks to one another revealed that the major differences were for the UCD-003 and SL (all p<0.05). The number of wing flaps and the duration and intensity of wing-flapping were all numerically lowest in the UDC-04 stock. The UCD-003 stock was significantly different on these measures from all other genetic stocks (all p<0.05) except SL stock. The duration of wing flapping in UCD-003 did not differ statistically from SL, with SL having a shorter duration than all of the other stocks. Wing-flapping intensity was greater in SL than in all other stocks except CV20.

Sex effects: For the TI test, there was effect of sex on latency to right ($F_{1, 192}$ = 4.27, p = 0.04), number of induction attempts (H_1 = 5.53, p = 0.02) and latency to first head movement ($F_{1, 192}$ = 13.42, p<0.001). Overall males had shorter latency to right (pooled means±SE for the genetic stocks: Males = 228.9±26.2 sec, females = 178.6±16.0 sec), required fewer (1.5±0.09 overall) induction attempts than females (1.8±0.06) and also had a longer latency to first head movement (pooled means±SE for the genetic stocks: Males = 35.9±8.4 sec, females = 16.7±2.6 sec). Table 1 summarizes the data for all genetic strains. There was a sex x strain interaction for latency to right ($F_{4,192}$ = 3.94, p = 0.004) and latency to first head movement ($F_{4,192}$ = 2.37, p = 0.05).

For the INV test, there were sex differences for all measures (number of flaps $F_{1, 192}$ = 38.24, p<0.001, duration of flapping $F_{1, 192}$ = 28.29, p<0.001, intensity of flapping $F_{1, 192}$ = 38.60, p<0.001). Males overall flapped less (33.8±4.4 flaps), for less time (5.8±0.6 sec) and less intensely (4.0±0.4 flaps/sec) than females (55.5±3.0 flaps, 9.2±0.5 sec, 5.6±0.2 flaps/sec, p<0.05). Table 1 summarizes the data for all genetic strains. There was a sex x strain interaction for

Table 1: Sex differences within genetic stocks in tonic immobility and inversion measures

		Tonic immobility				Inversion	
Genetic stock	Sex	Induction attempts (Number±95% CI)	Latency first head movement (sec.±SE)	Latency to right (sec.±SE)	Duration of flapping (sec.±SE)	# of flaps (Number±SE)	Flapping intensity (flaps/sec.±SE)
RJFNH	male	2.0±1.4	32.2±14.2	98.8±25.5	32.2±14.4	32.2±14.5	32.2±14.6
	female	2.0±1.4	12.4±3.1	252.0±41.1	12.4±3.3	12.4±3.4	12.4±3.5
	p-value	0.98	0.17	0.01	0.01	0.01	0.01
RJF	male	1.5±1.8	7.3±2.3	151.1±34.6	7.3±2.5	7.3±2.6	7.3±2.7
	female	2.0±1.3	4.4±0.8	90.5±21.8	4.4±0.1	4.4±0.1	4.4±0.1
	p-value	0.26	0.84	0.32	0.01	0.01	0.01
SL	male	1.0±1.5	23.6±7.5	251.6±54.8	23.6±7.7	23.6±7.8	23.6±7.9
	female	2.0±1.4	10.7±3.9	198.5±49.3	10.7±3.1	10.7±3.1	10.7±3.1
	p-value	0.14	0.30	0.32	0.12	0.22	0.39
CV-20	male	1.0±0.0	100.7±41.1	407.9±62.0	100.7±41.3	100.7±41.4	100.7±41.5
	female	1.0±1.0	38.9±9.1	265.8±28.7	38.9±9.3	38.9±9.4	38.9±9.5
	p-value	0.27	0.01	0.02	0.01	0.01	0.01
UCD-003	male	1.0±0.6	33.9±14.5	227.8±59.7	33.9±14.7	33.9±14.8	33.9±14.9
	female	2.0±1.4	11.5±2.8	65.4±9.9	11.5±2.10	11.5±2.1	11.5±2.1
	p-value	0.01	0.12	0.01	0.16	0.59	0.18
Pooled	male	1.0±1.4	35.5±7.4	273.3±26.4	5.3±0.6	29.7±3.9	3.6±0.4
	female	1.0±1.4	17.8±2.3	239.6±17.2	9.7±0.4	55.5±2.7	5.4±1.8
	Overall	$H_1 = 5.53$	$F_{1, 233} = 4.56$	$F_{1, 232} = 4.33$	$F_{1, 232} = 51.66$	$F_{1, 232} = 56.21$	$F_{1, 232} = 59.18$
	p-value	p = 0.02	p = 0.04	p = 0.04	p<0.0001	p<0.0001	p<0.0001

number of flaps ($F_{4,192} = 8.55$, $p<0.001$), duration of flapping ($F_{4,192} = 7.08$, $p<0.001$) and intensity of flapping ($F_{4,192} = 3.62$, $p = 0.007$).

DISCUSSION

Results of the present study demonstrate that both genetics and sex can affect the fear responses shown by chickens to two tests of fear. While the RJF stock was consistently less fearful than the SCWL genetic stocks during TI, there were SCWL which exhibited statistically similar fear levels during the INV. This is contradictory to previous research which said that RJF were more fearful than SCWL[25]. Furthermore, there were differences even among the SCWL genetic stocks illustrating that alterations within genetic stock genetics can change the fear response of fowl. This second point is especially important. As demonstrated by Gallup[26] genetic selection can alter the fear response, so while selecting for things such as improved production and disease resistance the fear response could be affected if one does not consider it.

It is also clear based on this study that not only do genetic stocks of fowl differ in their fear response but they also react differently during different types of fear tests. Some genetic stocks such as the RJF had short durations to right during TI and long durations of flapping and high intensity of flapping during INV while other genetic stocks had short durations to right during TI and short durations of flapping and low intensity of flapping during INV (i.e., UCD-003). The RJF's results indicate a bird that has a strong fight/flight response and low TI response while the UCD-003 demonstrate a low fight/flight response and a low TI response. This one example illustrates why more than one test should be used to test fear responses as suggested by Jones and Mills[27]. The TI response was similar between the RJF and the UCD-003 variety indicating similar fear levels but the INV responses were opposite. Similarly, Schutz *et al.*[28] saw no difference between RJF and SCWL in their TI responses but Campler *et al.*[25] saw differences between RJF and SCWL in other fear tests. But if one just looked at TI results both genetic stocks fear responses looked similar and one would not know this unless other fear tests were carried out.

This is not a new idea as Jones[29] concluded that generalization of results and interpretations from one variety of fowl should be approached with caution. Not a single variety in this study exhibited similar responses in all the fear measures. This is astounding as there were only two tests conducted with just three measures each and these only focusing on just two of the four types of anti-predator far responses described by Ratner[18]. Ranter[18] defined freezing, fleeing, fighting and tonic immobility as the four types of anti-predator fear responses. While tonic immobility and fighting are the most reliable and relevant for commercial production likely these genetic stocks would also differ in their responses in freezing and fleeing behavior as well as other types of fear tests that are not anti-predator directed.

It is also of note that males tended to be much more passive in their response during INV but took less time to

move their heads and righted similar to females in the TI test. Jones[29] observed that male chicks in an open field test had differing fear responses when compared to females. This makes it important to balance or designate only one sex to be tested when evaluating fear response and further demonstrates that all fowls are not created equal. When evaluating the housing, management, or any other factor related to poultry production for its effect on fear responses one must first consider the variety of fowl being used as well as the sex. This study clearly demonstrates that the variety and sex of fowl being test can result indifferent types of fear responses predominate depending on the sex, variety and test. If one only considers external factors (housing system) and not the internal factor of the bird (i.e., genetics, gene expression, epigenetics) itself, erroneous conclusions can be reached and invalid recommendations made about the suitableness of housing systems or genetic stocks of birds for those housing systems.

CONCLUSION

It is concluded that not a single variety exhibited similar responses in all the fear measures. Different genetic stocks of fowl react differently in different fear tests. Therefore, single fear tests should not be used to evaluate the fear response of fowl.

SIGNIFICANCE STATEMENT

Fear tests are often used as part of welfare assessments. These welfare assessments are often used to determine the appropriateness of differing housing conditions for optimal welfare. It is likely, though, that genetic differences between breeds as well as within breeds may affect the fear response of poultry. Sex differences also are likely to exist in fear response. These differences in fear response make generalizing the acceptableness of differing housing systems based on one breed or strain an unacceptable practice. This study found that genetic differences among fowl may result in differing fear responsiveness. Furthermore, different genetic lines may behave differently in different fear tests. Therefore, using only one fear test to compare different genetic stocks of fowl is undesirable and a variety of tests should be used to get the best assessment of fear.

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