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Research Article
Evaluation of Fear and Stress in White Layers Housed in Either Conventional Cages or Enriched Colony Cage

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Abstract

Background and Objective: The US egg industry is currently moving away from conventional cage housing towards larger colony caging systems. However the most common breed in the U.S.A., the Hy-Line W-36™, has been selected based on conventional housing systems. The present study was designed to investigate the effect of housing hens in colony systems on the stress and fear response. **Methodology:** W-36 pullets were housed in either enriched colony (EC) system or conventional A-frame cages, with manure shields (CC), from 23-79 weeks of age. Plasma corticosterone (CORT, n = 60) and composite physical asymmetry (ASYM, n = 60) were used to evaluate the stress response. Tonic immobility (TI, n = 60) and inversion (INV, n = 60) were used to determine fear. Measurements were done at 23 week of age (T_1), 33 week of age (T2), 56 week of age (T3) and 79 week of age (T4). **Results:** No differences were observed ($p>0.05$) in CORT (20.8±8.6 ng mL^{-1}), ASYM (1.87±0.13 mm), latency to right during T_1 testing (259.3±16.5 sec), or intensity of flapping during INV (4.2±0.2 flaps/sec) at 23 weeks of age. The EC and CC differed in CORT at T_2 ($p = 0.02$), T_3 ($p = 0.03$) and T_4 ($p = 0.04$). Similarly, EC and CC differed in ASYM at T_2 ($p = 0.004$), T_3 ($p = 0.03$) and T_4 ($p = 0.05$). While no difference ($p>0.05$) was still observed in the latency to right during T_1 at T_2 (284.5±19.1 sec) or T_3 (284.5±19.1 sec) the EC did differ from the CC at T_4 ($p = 0.02$). The EC also flapped more intensely during INV in T_2 ($p<0.001$), T_3 ($p<0.001$) and T_4 ($p<0.001$). **Conclusion:** This indicates that the EC were more stressed and were more flighty than CC. Furthermore, it appears that housing W-36 Leghorns in enriched colony systems may not be desirable over conventional cages based on these results.

Key words: Layer, fear, stress, housing, welfare

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Competing Interest: The authors have declared that no competing interest exists.

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

INTRODUCTION

Several reviews of the literature have been conducted in an attempt to determine what the best caging system would be for optimum bird welfare. The LayWel project[1] attempted to summarize the welfare status of birds in conventional cages, furnished cages or non-cage systems. While this report has some useful information, it is often misused to make conclusions about colony cages. The furnished cages analyzed in Blokhuis *et al.*[1] were much smaller than colony cage systems. Another report by Lay *et al.*[2] compared housing systems based on available research at the time but again this did not include colony cages as research was not available. While these reports may suggest that enriched cages, which are often confused as being the same as enriched colony cages, provide better welfare[1,2] they are in fact looking at enriched systems which are much smaller than an enriched colony cage systems, therefore, these reports do not answer any questions about colony cage systems. The benefit of the conventional cage has been the ability to maintain a small group size, with a low level of social stress, resulting in low aggression and cannibalism, high egg production and increased hygiene, which may favor improved welfare of conventionally housed birds[3]. Housing birds in extremely large groups in cage-free settings can result in increased mortality[2] and social stress but allows for more natural behavioral expression. This has led to the development of colony cages as some believed they may be a middle ground between cage-free and conventional cages. The size of the cages allows for birds to be housed in groups but still in manageable numbers compared to cage-free systems.

More recently, the Coalition for Sustainable Egg Supply[4] has reported that hen mortality is much higher in aviary systems compared to conventional or colony cage systems. They also found that hens in the aviary and colony cages had higher keel bone deviations and fractures than hens in conventional cages[5]. Hens in conventional cages had the highest incidence of foot problems but hens in aviaries had more severe problems when they occurred. Birds in either cage system had worse feather coverage due to cage wear[5]. The CSES also found that aviaries and enriched cages allowed for improved behavioral expression and overall bone strength[4]. While this coalition's work is useful, they used a Lohmann LSL white layer that is less commonly used in industry so the results may not have been representative of the more typical Hy-Line White Leghorn (W-36). Dikmen *et al.*[6] found no differences between conventional and enriched cages in tonic immobility, blood glucose, total cholesterol, triglyceride or calcium values but did observe higher heterophil/lymphocyte ratios in conventionally caged birds. Matur *et al.*[7] found that conventionally caged birds exhibited higher heterophil/lymphocyte ratios and lower antibody titers than furnished caged housed birds. Meng *et al.*[8] also saw decreased stress in furnished caged birds when compared to conventionally caged birds. Li *et al.*[9] also concluded that furnished caged housed birds had better welfare than conventionally caged birds over a variety of parameters in including tonic immobility response. It is important to note that all of these studies[6-9] used brown layers and the Coalition For Sustainable Egg Supply project used a less commonly used Lohmann LSL white layer. This becomes important as it has been demonstrated that genetics can greatly impact how birds respond to stressors[10,11] and producers are more likely to want to continue to utilize the W-36 as they have high egg production and efficiency. However, the W-36 Leghorn has been bred to be suited for conventional cage systems and may not fare well in alternative systems.

Two important factors that will be important when birds are shifted from more conventional housing to alternative housing systems will be how the birds fear and stress levels are altered compared to current systems that the birds were bred to be grown within. While stress is not inherently negative[12], it occurs when an animal experiences changes in the environment that stimulate responses aimed at reestablishing homeostasis[13]. While the animal tries to reestablish homeostasis, it diverts energy away from reproduction, immune function and development[14]. The most common way to measure stress is via the hormones released by the hypothalamic pituitary axis[15]. In chickens, this hormone is corticosterone. Another way to measure stress is via physical asymmetry which is a simple comparison of bilateral structures on a bird, structures on the left and right side of the bird are measured and a larger difference indicates greater asymmetry[16]. Physical asymmetry has been strongly correlated to stress in many studies, with greater asymmetry indicating a stronger physiological response to stressors[17-19] and is a useful tool for determining the current stress level[20].

As fear is an unpleasant and aversive state and can be tied to stress levels and performance it is also an important animal welfare measure. Since poultry are prey animals, predator avoidance is the major component of their fear response. Ratner[21] defines 4 such behaviors as a progression from freezing, to fleeing, fighting and finally tonic immobility. Once a chicken is captured it will attempt to struggle and break free[21]. This response can be measured via an inversion. Newberry and Blair[22] stated that inversion testing (INV) is a practical measure of fear for birds used in commercial production. The most used fear test in poultry is tonic

immobility (TI). This response is characterized by a sustained period of non-responsiveness brought about by physical restraint[23,24] and is considered to be the final stage of fear response in wild animals[21] when they cannot escape a predator.

The objective of this study was to evaluate the stress susceptibility and fear response of the more commonly used W-36 strain of the White Leghorn housed in conventional or enriched colony cage. To accomplish this equal numbers of laying hens were housed in conventional or enriched colony systems from 18 until 79 weeks of age. The hens were periodically subjected to two fear tests (inversion and tonic immobility) and had their stress susceptibility determined by plasma corticosterone concentrations and composite physical asymmetry. It is hypothesized that the W-36 laying hens will exhibit more stress and fear when housed in the enriched colony cages when compared to conventional cages due to the known behavioral characteristics of this strain such as increased fearfulness when compared to brown strains[10].

MATERIALS AND METHODS

Animals and husbandry: A total of 120 Hy-Line W-36 laying hens were housed for 56 weeks starting at 21 weeks of age. Hens were obtained from a commercial farm at 19 weeks of age and placed into each system at time of arrival to the research farm. The hens were randomly divided over two housing systems: (1) conventional cages (CC, n = 60 hens) and (2) an enriched cage (EC, n = 60 hens). The hens in the conventional cages were housed in groups of 4 in a total of 15 adjacent A-frame cages that were 610×508 mm (774.7 cm^2 per bird). The enriched caged hens were housed in a group of 60 in a fully enriched colony cage (Versa Colony System, Chore-Time, Milford, In, USA, 1321×3658 mm, 805.4 cm^2 per bird) that included perches, nesting area and scratch area. All hens were housed in the same barn and within 15 m of each other on the same side of the barn. Feed and water were provided *ad libitum*. All hens were wing-banded for individual identification. The hens were managed according to the guidelines set forth in the Guide for the Care and Use of Agricultural Animals in Research and Teaching[25]. Data was collected when the hens were 23, 33, 56 and 79 weeks of age. The order in which sampling was done was the following, blood collection, asymmetry, tonic immobility and inversion.

Fear response

Inversion: The inversion test (INV) was conducted in the building in which the hens were housed, using methods described by Newberry and Blair[22] and Archer and Mench[26]. Each treatment had 60 hens tested. Hens were caught and placed in transport crates in groups of 15 birds at a time and time within the crate was consistent across treatments. Each hen was taken individually from their crate, held upright in front of a camera (Panasonic PV-DV2030, Kadoma, Osaka, Japan) with a hand supporting the breast and the other firmly grasping both legs and then inverted by removing the hand from the back of the hen and allowing the hen to hang freely upside down. Once the hen ceased flapping for several seconds it was placed back in its cage. After all the hens were inverted and recorded, the video file was transferred to a computer. Using PowerDirector 11 (CyberLink, Taipei, Taiwan) to analyze the video file, the time was found for each hen's duration of flapping (measured from time the hand was removed from the back to time of last wingbeat) and the number of wingbeats in the time was counted. Longer and more intense flapping was considered to indicate more fearfulness[22].

Tonic immobility: Tonic Immobility (TI) was conducted on 60 hens per treatment. Methods were modified from previous research by Jones[24] and Archer and Mench[26]. Hens were caught and placed in transport crates in groups of 15 birds at a time and time within the crate was consistent across treatments. Hens in the colony cage were caught with a leg hook to make catching as quick as possible to limit stress. Briefly, each hen was individually taken and placed on its back in a cradle. The head of the hen was covered with one hand while the breast was held with the other for approximately 15 sec to induce tonic immobility, after which time contact was removed and a timer was started. If the hen righted itself in under 15 sec, the timer was reset and the above procedure was performed again. If again the hen righted in under 15 sec, it was recorded as a time of 0. Otherwise the time of righting (or attempting to right) was recorded, with a maximum of 10 min. Longer times to first head movement and righting were considered to indicate more fearfulness[24].

Both fear tests were performed by the same person and same order at each time point. The T_1 test was conducted over two days and was performed at the same time each day, with equal numbers of hens from each treatment insure that there was no difference in diurnal testing. All birds were caught each testing day and on the second day if a bird had been noted to have been already tested the previous day it was returned to its housing.

Stress susceptibility

Composite asymmetry: Composite physical asymmetry of 60 hens per treatment was measured at each time point

following the protocol outlined in Archer and Mench[19]. Using a calibrated Craftsman IP54 Digital Caliper (Sears Holdings, Hoffman Estates, IL), the middle toe length, metatarsal length and metatarsal width were measured for both the right and left legs. The composite asymmetry score was calculated by taking the sum of the absolute value of left minus right of each trait, then dividing by the total number of traits. Thus, the formula for this trial would be:

$$\text{Trial} = \frac{(|\text{L-R}|\text{MTL}+|\text{L-R}|\text{ML}+|\text{L-R}|\text{MW})}{3} = \text{Composite asymmetry score}$$

Higher composite asymmetry score indicates increased stress susceptibility.

Plasma corticosterone: At each time point, 60 hens per treatment had blood collected. The area around the jugular vein was sanitized with 70% alcohol and in preparation, between 1-2 mL of blood were collected from each bird. Hens were caught and placed in transport crates in groups of 15 birds at a time and time within the crate was consistent across treatments. Blood collection took less than one minute per bird once removed from the crate. The blood was allowed to clot for 24 h at 4°C, the vacutainers were spun down in a Beckman GS-6R centrifuge (Beckman Coulter, Brea, CA) for 15 min at 4000 rpm to separate the clot. The serum was poured off into 2 mL microcentrifuge tubes and stored at -20°C until further analysis. Plasma corticosterone concentrations were measured using a commercially available ELISA kit (Enzo Life Sciences, ADI-901-097, Farmingdale, NY). The inter and intra-assay %CV were both under 5%. Higher plasma corticosterone concentrations indicate higher stress susceptibility. The blood was collected the day prior to behavioral testing. Each bird was handled a total of 5 times per time point across the fear and stress measure testing.

All methods were approved by the Mississippi State University IACUC committee (AUP# 14-097).

Statistical analysis: To investigate treatment effects on inversion, tonic immobility, composite asymmetry and corticosterone the GLM procedure was used with treatment, testing day and treatment×testing day as factors. The least significant difference test was used to test all planned comparisons. All of the assumptions were tested (Shapiro-Wilk test for normality, Levene's test for homogeneity of variance). No transformations were needed to meet assumptions. All analyses were performed using SAS 9.3 for Windows (SAS Institute Inc.). Significant differences were at $p<0.05$.

RESULTS

Fear response: The results of the fear testing are presented in Table 1. The main effect of treatment ($F_{1,480} = 6.75$, $p = 0.01$) and testing day ($F_{3,480} = 13.4$, $p<0.001$) as well as the interaction of the two ($F_{3,480} = 3.37$, $p = 0.02$) was significant for latency to right during tonic immobility testing. No differences between housing treatments was observed in latency to right during tonic immobility testing at 23 (pooled mean = 259.4±16.5 sec), 33 (pooled mean = 284.5±19.1 sec), or 56 (pooled mean = 360.6±22.7 sec) weeks of age ($p>0.05$), however, at 79 weeks of age the conventional cage birds (414.8±27.1 sec) had a longer latency to right than the enriched colony cage birds (325.9±25.6 sec, $p = 0.02$). The main effect of treatment ($F_{1,480} = 36.8$, $p<0.001$) and testing day ($F_{3,480} = 104.6$, $p<0.001$) as well as the interaction of the two ($F_{3,480} = 125.5$, $p<0.001$) was significant for flapping intensity during inversion. Initially at 23 weeks of age there was no difference between treatments in flapping intensity during inversion (pooled mean = 4.22±0.19 flaps/sec, $p>0.05$), however, differences between treatments were observed at all other time points (Table 1). The enriched colony cage birds flapped more intensely than the conventional caged birds during inversion at 33 (EC, 5.22±0.10 flaps/sec vs CC, 4.25±0.36 flaps/sec, $p<0.001$), 56 (EC, 5.75±0.14 flaps/sec vs CC, 3.88±0.22 flaps/sec,

Table 1: Fear response of laying hens housed in either conventional or enriched colony cages after 0, 10, 33 and 56 weeks of being housed in each system

Measure	Number of birds tested	Weeks of age	Weeks housed	Conventional cages	Enriched colony cages	p-value
Tonic Immobility	60	23	4	244.50±14.3	274.20±18.7	>0.05
(latency to right, sec)		33	10	310.20±17.7	258.80±20.4	>0.05
		56	33	375.70±21.3	345.40±24.0	>0.05
		79	56	414.80±27.1	325.90±25.6	0.02
Inversion (flapping	60	23	4	3.97±0.19	4.47±0.18	>0.05
intensity, flaps/sec)		33	10	4.25±0.36	5.22±0.10	<0.001
		56	33	3.88±0.22	5.75±0.14	<0.001
		79	56	3.04±0.23	4.11±0.18	<0.001

Values are expressed as Mean±SE

Table 2: Stress susceptibility of laying hens housed in either conventional or enriched colony cages after 0, 10, 33 and 56 weeks of being housed in each system

Measure	Number of birds tested	Weeks of age	Weeks housed	Conventional cages	Enriched colony cages	p-value
Plasma corticosterone	60	23	4	20.90±10.80	20.60±6.40	>0.05
(ng dL^{-1})		33	10	11.10±3.3	26.70±4.90	0.02
		56	33	7.50±3.1	12.80±2.50	0.03
		79	56	6.60±1.4	20.00±4.70	0.04
Composite asymmetry	60	23	4	1.82±0.11	1.92±0.15	>0.05
score (mm)		33	10	1.58±0.11	2.08±0.13	0.004
		56	33	1.65±0.10	1.83±0.10	0.03
		79	56	1.55±0.09	2.00±0.15	0.05

Values are expressed as Mean±SE

p<0.001) and 79 weeks of age (EC, 4.11±0.18 flaps/sec vs CC, 3.04±0.23 flaps/sec, p<0.001).

Stress susceptibility: The results of the stress susceptibility measures are presented in Table 2. The main effect of treatment ($F_{1,480}$ = 3.9, p = 0.049) and testing day ($F_{3,480}$ = 0.8, p>0.05) as well as the interaction of the two ($F_{3,480}$ = 1.1, p>0.05) was significant for plasma corticosterone concentrations. At 23 weeks of age no differences were observed between housing systems in plasma corticosterone (pooled mean = 20.8±8.6 ng dL^{-1}, p>0.05), however, at all other time points differences were observed between treatments. The enriched colony cage birds had higher plasma corticosterone concentrations than the conventional cage birds at 33 (EC, 26.7±4.9 ng dL^{-1} vs CC, 11.1±3.3 ng dL^{-1}, p = 0.02), 56 (EC, 12.8±2.5 ng dL^{-1} vs CC, 7.5±3.1 ng dL^{-1}, p = 0.03) and 79 weeks of age (EC, 20.0±0.4.7 ng dL^{-1} vs CC, 6.6±1.4 ng dL^{-1}, p = 0.04). The main effect of treatment ($F_{1,480}$ = 13.1, p<0.001) was significant but testing day ($F_{3,480}$ = 1.0, p>0.05) and the interaction of the two ($F_{3,480}$ = 1.1, p>0.05) were not significant for composite asymmetry score. Similarly, the enriched colony birds had higher composite asymmetry scores at 33 (EC, 2.08±0.13 mm vs CC, 1.58±0.11 mm, p = 0.004), 56 (EC, 1.83±0.10 mm vs CC, 1.62±0.10 mm, p = 0.03) and 79 weeks of age (EC, 2.00±0.15mm vs CC, 1.55±0.09 mm, p = 0.05) while starting with similar composite asymmetry scores at 23 weeks of age (pooled mean = 1.87±0.13 mm, p>0.05).

DISCUSSION

All birds began this current study with similar fear responses and stress levels based on the measure collected. However, as time passed and the birds were housed longer in the two systems it became clear that the Hy-line W36 White Leghorn was more flighty and more stressed in the enriched colony cage when compared to the conventional cage system. Two fear measures used in this current study were tonic immobility and inversion. No differences in latency to right during tonic immobility were observed between housing systems until the last time point. Tonic immobility response is the most commonly used method of fear assessment in poultry[24] and is the bird's last resort to escape predation[21]. This current study observed that by 79 weeks of age the layers housed in the conventional cage exhibited longer latencies to right than those housed in the enriched colony cage. This indicates that the birds were less fearful in the enriched colony cage as measured in this test. This contradicts what Dikmen *et al.*[6] observed when they found no differences in tonic immobility between layers housed in conventional or enriched cages, however, they did not test birds older than 66 weeks of age and used a brown strain of layer. It is possible that they may have observed similar effects as this current study if they had tested older birds. Li *et al.*[9] did conclude that birds housed in furnished cages birds had shorter latencies to right than housed in conventional cages which are similar to the results observed in this current study at 79 weeks of age. Li *et al.*[9], however, used a different style of cage, a different strain of bird and younger birds at the time of testing making a direct comparison impossible. The difference observed between housing types in this current study and Li *et al.*[9] seem to contradict Kujiat *et al.*[27] which concluded that hens housed in larger groups had longer latency to right during tonic immobility when compared those housed in smaller groups. One possible explanation for this is the hens housed in the conventional cages were better able to work out a pecking order than in the enriched caged hens, however, this was not measured in the current study and merits future investigation. Results of several studies on laying hens suggest that as group sizes increase and hens are no longer able to discriminate between all individuals in the group, they switch from a social strategy of maintaining pecking order to a strategy of social tolerance[28]. Adding to that Jones and Faure[29] found that dominant birds had longer latencies to right than subordinates making it possible that the results of this current study might have been affected by the number of "dominant" hens tested from the conventional cages vs the enriched colony cage.

The inversion test also showed differences in fear behavior between the housing systems. It was, however, opposite from what was observed at 79 weeks in the tonic immobility test. Hens housed in the enriched colony system exhibited higher intensity of flapping during inversion testing from 33-79 weeks of age indicating more fearfulness. With no other research using this fear testing technique to investigate differences in housing systems there isn't the ability to compare to this current study's result. The results of this study do clearly indicate that W-36 hens housed in the enriched colony system will be more flighty and struggle more vigorously during catching and restraint.

In both plasma corticosterone and composite asymmetry scores the hens in the enriched colony system were more stress susceptible from 33-79 weeks of age. This increased stress susceptibility in the enriched colony birds could be due to the fact that there are different underlying levels of sociality in hens. Sociality among hens has profound effects on all aspects of social interaction, including affiliation, aggression and social structure[30-33]. Therefore, birds not suited for larger group housing may exhibit chronic social distress[33-35] making birds that have low-sociality ill-suited for being housed in large social groups. Barnett *et al.*[36] found evidence of increased stress in hens in furnished cages kept in groups of 16 compared to groups of eight hens illustrating that group size plays a role in response to housing design.

Previous researchers comparing laying hen housing systems for animal welfare did not use the W-36 White Leghorn. This is important as it has been demonstrated that genetics can greatly impact how birds respond to stressors[10,11] as well as their fear response[32]. White Leghorns have even been shown to have lower blood serotonin levels when compared to brown strains[37]. Lower serotonin levels have been associated with high fearfulness[38]. As egg producers are more likely to want to continue to utilize the W-36 as they have high egg production and efficiency proper housing of them is imperative for good animal welfare. If white strains and eggs are desired to be raised in alternative systems it is clear they need to be bred selectively to be able to appropriately cope with those systems. In fact, Jones and Hocking[35] concluded that selective breeding should be used to reduce fearfulness to improve animal welfare.

Blokhuis *et al.*[1] and Lay *et al.*[2] attempted to summarize the literature but the enriched cages in their reviews are not those that are commercially available today. Large projects such as the one undertaken by the Coalition for Sustainable Egg Supply are good steps but they only give a picture of the welfare of one strain of bird. It is evident from the results of this current study that the Hy-line W36 White Leghorn will have poorer welfare in the enriched colony cage systems than if they are housed in conventional cages. They will be flightier and have higher stress susceptibility in the enriched cage systems making those systems and this strain of bird non-compatible. Genetic selection for a White Leghorn with high egg production and feed efficiency as well as the ability to cope with alternative housing systems that will force it to be in larger groups with more area to move should be done. Until that point housing this strain in those systems is not advisable for optimum welfare of the birds.

SIGNIFICANCE STATEMENT

- Currently over 90% of laying hens are housed in conventional cages in the United States, however, there is a push to shift from this type of housing to alternative housing designs
- Traditional alternatives have consisted of cage-free or free range but in the last decade the colony cage systems have been seen as a viable alternative
- These colony cages allow birds to be housed in larger groups (30-60 birds) and provide them with enrichment (perches, nest boxes and dust bathing/foraging areas) while minimizing the pitfalls of cage-free or free range systems such as disease spread and cannibalism
- It is not known how factors such as stocking density, genetics, lighting and temperature will affect the production and welfare of the birds in colony cages

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