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A Comparison of Humoral Immune Function in Response to a Killed Newcastle's Vaccine Challenge in Caged Vs. Free-range Hy-line Brown Layers

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Abstract: The relationship between immune function and disease risk may be greatly influenced by an organism's response to chronic stressors including those that are environmentally induced. Measurements of stress-induced immune alterations have previously been made in poultry species by utilizing hematological and immunological indices. To ascertain the effects of alternative layer housing management methods on humoral immune function, Hy-line Brown hens housed on range (n = 15) or in battery style cages (n = 20) were inoculated with a killed Newcastle's vaccine. Blood serum samples were taken prior to injection and for three consecutive weeks following injection to assess antibody production. Antibody production was significantly higher in caged hens in comparison to free-range hens at pre-injection (1.69±0.70 vs. 0.069±0.069) ($p<0.0001$) and post-injection week one (2.26±0.77 vs. 0.145±0.25) ($p<0.0001$), week two (8.00±2.98 vs. 4.38±2.94) ($p<0.001$) and week 3 (9.24±2.56 vs. 6.69±3.86) ($p<0.05$). Additionally, caged hens exhibited a significantly higher level ($p<0.0001$) of total antibody production (5.30±0.23) throughout the immune challenge compared to free-range hens (2.82±0.26). Caged hens exhibited significantly higher H:L ratios (2.34±0.86 vs. 1.75±0.57) ($p<0.05$) during post-injection week 2 which correlated with the greatest difference in antibody production observed between the two groups of hens. This data indicated that environmental management methods utilized in layer hen production may influence both levels of humoral immune function and heterophil:lymphocyte ratios.

Key words: Layers, free-range, cage, humoral immune function

INTRODUCTION

The evolution of commercial egg production has been shaped by a multitude of factors including: the economy, population growth and public perception of layer hen management methods. Layer hen management methods have progressed from backyard flocks to large-scale commercial conventional cage facilities, which were designed to optimize production in an economical manner, while protecting hens from environmental extremes, predation and disease. However, due to public perception that layer hen well-being has been adversely affected by intensive conventional caged environments, the industry has been forced to implement alternative management methods such as free-range environments. Alternative management methods have also increased in popularity due to the recent passing of legislation in California entitled "Proposition 2." Proposition 2 has implemented strict regulations for the confinement of farm animals including layer hens reared in battery cages. Officially known as the Standards for Confining Farm Animals initiative or the Prevention of Farm Animal Cruelty Act, the statute states that animals must be able to turn around freely, extend their limbs and lie down comfortably within their confinement. This law will be fully implemented by January 1, 2015 and as such will have a major economical impact on both the poultry and livestock industry (Sumner *et al*., 2008).

Numerous studies have been conducted to assess the physiological and behavioral advantages for laying hens reared in caged vs. free-range environments. Cages have been shown to reduce cannibalism (Hilbrich, 1985) while promoting hygiene and decrease incidences of disease (Tauson, 1998), making them the management method of choice in most countries. The disadvantages that come with traditional caged housing is that they lack nesting facilities, restrict general freedom of movement (Appleby *et al*., 1992), limit natural behaviors such as dust bathing, prevent behaviors like wing flapping (Appleby, 1998) and increase hens' susceptibility to osteoporosis and cage-layer fatigue (Riddell *et al*., 1968).

In contrast to traditional caged housing, free-range environments allow hens to be outside while providing them with access to a veranda for shade, protection from the weather and litter for resting and dust bathing. The free-range system also provides space for exercise, nesting facilities and wing flapping. Behaviorists argue that exhibiting comfort behaviors such as wing flapping and dust bathing, is necessary and improves layer hen well-being (Rodenburg and Sonck, 2005). However, numerous aspects of the free-range environment such

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as temperature, severe weather, predation, increased incidence of cannibalism and disease cannot be regulated. Free-range production has also been found to be more labor-intensive than caged production when examined on a per-hen basis (Anderson, 2009a).

Physiological responses to a less regulated environment may lead to a state of general stress. Stress can be described as "adaptive responses to challenges in homeostasis" (Dohms, 1991) which may include adaptations in the immune response. Studies have shown that immunomodulation, activation or suppression of the immune system, can be provoked by environmental stressors (Dohms, 1991). Environmental stressors reduce immune responses and cause immunomodulation, which is initiated by the hypothalamus-pituitary-adrenal cortical pathways. The relationship between immune function and disease risk may be greatly influenced by an individual's response to chronic stressors including those that are environmentally induced (Sapolsky, 1994). Prolonged stress responses have been observed to alter an animal's immune function (McEwen, 2003) by increasing their risk for a wide range of adverse health outcomes. Ultimately, stress heightens the risk for adverse health outcomes by suppressing the immune response thus leaving the host vulnerable to opportunistic disease (Miller and O'Callaghan, 2002).

Measurements of stress-induced immune alterations have been conducted in poultry by utilizing immunological and hematological indices. Specifically, antibody production in response to vaccine challenges and Heterophil:Lymphocyte (H:L) ratios have been used as sensitive immunological and hematological indicators of stress responses in chicken populations (Gross, 1983). Antibody production can be monitored post-inoculation as the antibody response is commonly used to assess alterations in humoral immune function (Dohms, 1983). A higher level of antibody production demonstrates increased humoral immune function, which can be utilized an indicator of the level of stress caused by an environment (Siegel, 1985). Heterophil: lymphocyte ratios can also be measured when evaluating stress response in poultry as stressors induce an elevation in plasma corticosterone concentrations (Gross, 1983) and as a result, corticosterone concentrations stimulate a rise in heterophil concentrations. Heterophils increase in circulation as a result of interactions between bone marrow and the hypothalamic-pituitary-adrenal cortical axis (Maxwell, 1998); (Shini, 2003). The H:L ratio quantifies the balance between the nonspecific, fast-acting defenses of heterophils and the antigen specific, slower-acting defenses of the lymphocytes (Shini, 2003).

A previous study examined differences in humoral immune function in caged vs. non-caged laying hens kept on the ground, indoors under identical conditions after an immunization challenge with human serum Immunoglobulin G (IgG) with or without adjuvant. The results indicated that the mean egg yield and mean egg yolk antibody titers were significantly higher in caged laying hens compared to non-caged laying hens kept on the ground regardless of the presence or absence of adjuvant (Erhard *et al*., 2000). Another study examining the physiological responses of laying hens to alternative housing systems found that hens kept in battery cages had greater heterophil levels and decreased lymphocytes in comparison to hens kept in modified cages and free-range housing systems (Shini, 2003). These findings exemplify the complexity and variation of current findings from studies examining the relationship between stress and immune function in laying hens housed in caged vs. non-caged environments. The present study aims to elucidate differences in free-range vs. caged layer hens' humoral immune function and H:L ratios as a means to assess the impact of each environment on layer hen well-being. Research has shown that production is a mechanism of well-being (Curtis, 1991). Therefore, understanding the impact of a caged vs. free-range environment on layer hen well-being will enable producers to focus on optimizing well-being of layers which will ultimately increase production.

MATERIALS AND METHODS

Pullet rearing: Pullet rearing parameters in this study were conducted in accordance with the 37th North Carolina Layer Performance and Management Test (Anderson, 2009b). Fertile eggs for the Hy-Line Brown Layers utilized in this study were received at the Piedmont Research Station, Salisbury, NC. The eggs were set and hatched concurrently at which time the chicks were sexed to remove the males using color sexing, vaccinated for Mareks disease and the chicks for the range portion were pinioned. Pinioning involved the surgical removal of a bird's metacarpals, the point on the wing where the primary flight feathers originate. The procedure was accomplished using a hot blade and a bar apparatus mounted in a Lyons trimmer. One wing (i.e. left or right), was extended and a cut was made through the joint at the Intracarpal Ligament between the Radius and Ulna and the first phalanx of the third and fourth digit. Simultaneously, the hot blade cauterized all cuts which stopped any bleeding enabling the birds to recover much faster. The pain and distress associated with this procedure at 1 day of age is similar to that of beak trimming which was done on all birds at 6-10 days. Beak trimming began at 6 days of age using a Lyons Precision beak trimmer, with a 7/64" guide hole. The trim was a block cut with an approximate blade temp of 1100^{o} F (dull red). Beak trimming was completed in less than 3 days. Pullets were not re-trimmed at any point in the rearing period.

The chicks were equally divided between two pullet rearing facilities. The cage brooding and rearing system consisted of 6 replicates. Each replicate was comprised of 4 cages filled with 13 brown-egg (13 per 24" x 26" cage) pullets on the day of hatch totaling 52 chicks per Quad-deck cage system in a light tight house. All chicks were brooded in the same cage during the entire 17 wk rearing period with a floor space allowance of 48 in^2, 4.7 cm (1.8 in) of feeder space/bird and 1:6.5 nipple drinkers to bird ratio. Paper was placed on the cage floor for the first 7 days within each of the replicate cages and was removed at the time of beak trimming. This represented 312 birds started in cages.

The second group of chicks was reared in accordance with free-range standards as practiced by specialty egg producers. They were brooded in an environmentally controlled floor brood-grow facility consisting of a single room divided into individual pens that were 32" x 72" with 34" of linear feeder space, 6 nipple drinkers and linear roosting space of 32". Each of the 17 pens (replicate) were filled with 15 brown-egg pullets each on the day of hatch for a rearing allowance of approximately 929 cm^2/pullet, 5.7 cm (2.3 in) of feeder space, 1:2.5 nipple drinkers to bird ratio and bird roost space of 2.1 cm (0.8 in). This represented a total of 255 pullets which were moved to the range units.

The pullets for the range facilities were moved to the range house and paddocks at 12 weeks of age. The range environment included a range hut for roosting and protection and paddocks that were separated into four pens housing up to 75 hens each. Pullets had access to feed, nipple waterers and roosts in order to gain familiarity with their environment and to facilitate nest box usage. All other rearing procedures and vaccinations were the same as their cage-reared flock mates.

The range pullets were placed in a range hut that provided 929 cm^2/pullet, 13 cm of roosting space/pullet and 1 nest/8 hens. The range hut had a timer and light powered via battery and solar cell, supplemental propane heater for winter conditions to maintain an interior temperature above 7.2°C (45°F) which is the lower level of the chickens Thermal Neutral Zone (TNZ) where body temperature will be maintained via a feed intake increase. The pullets had access to the outdoors throughout the day and appeared to return to the range hut during the dark for roosting and protection. Husbandry, lighting and supplemental feed were allocated on the same basis as flock mates in cages in order to minimize the variables between flock mates. Range density was based upon a 500 hen/acre static equivalency 8.04 m^2/hen. The range pens were 21.3 m x 21.3 m (70' x 70') and were enclosed by a fence 1.8 m (6 ft) with the lower chain link section being 1.2 m (4 ft). Pullets were fed *ad libitum* by hand daily with Starter feed containing Amprol during the initial brooding period to achieve the breeder recommended body weights at each weigh interval. This was followed by Grower and Developer diets (Anderson, 2009b). Pullets were moved on to the next rearing diet at the point of achieving target body weight goals or after a prescribed time interval. Expected feed transition intervals were; starter 0 to 6 wks; grower 6 to 12 wks; developer 12 to 15 wks; pre-lay diet 15 to 16 wks. The pre-lay diet was provided no earlier than the last week in the rearing facility through the interim prior to reaching the threshold day length of 14 h. All mortality was recorded daily, but mortality attributed to the removal of males (sex slips) and accidental deaths from a replicate were excluded. Pullet vaccination schedules were identical between the rearing treatments. Pullet vaccination schedules and the lighting schedule for the pullet controlled environment facility and range rearing are outlined in the Single Production Cycle Report of the Thirty Seventh North Carolina Layer Performance and Management Test 37 (Anderson, 2009b).

Killed newcastle's vaccine challenge and heterophil: lymphocyte ratios: In conjunction with the 37th North Carolina Layer Performance and Management Test (Anderson, 2009b), 15 Hy-line Brown hens randomly selected from three different range pens and 20 Hy-line Brown hens randomly selected from four different cages located within a high rise, environmentally controlled facility with three banks of Quad-deck (4-tier) high cages were utilized in this study. At 71 weeks of age, both groups of hens were inoculated with 0.1 mL of Newcastle Disease Vaccine Killed Virus (AVIPRO 105 ND) subcutaneously just inferior to the nape of the neck. Immediately prior to inoculation and for three consecutive weeks post-inoculation, 2-3 cc of blood were collected from alternating left and right brachial veins of each hen into serum collection tubes. All blood samples were allowed to clot for 2 h and serum was decanted into microcentrifuge tubes and frozen at -20°C for later analysis. Serum was analyzed with the APMV-1-Avian Paramyxovirus-1 ELISA to assess Optical Densities (OD) of antibodies produced that were specific to the killed Newcastle's vaccine (North Carolina Veterinary Diagnostic Laboratory System, Rollins Laboratory, Raleigh, NC).

Individual hens' Heterophil:Lymphocyte (H:L) ratios were also assessed throughout the duration of the vaccine challenge by evaluating blood smears made just prior to inoculation and for three consecutive weeks following inoculation. For each time period, one blood smear was made per hen immediately after drawing blood from the brachial vein using the 2-slide wedge method (Houwen, 2000). After air-drying, the slides were immediately stained using 100% Wright's stain and rinsed with distilled water. Slides were allowed to air dry and one hundred granular heterophils and non-granular lymphocytes were counted once on each slide using oil immersion microscopy at 100x magnification. H:L ratios for each bird were then determined by dividing the total number of heterophils by the total number of lymphocytes for each slide.

Statistical analysis: All data were subjected to ANOVA utilizing the GLM procedure (SAS, 2009). Mean differences were separated via the PDIFF option of the GLM procedure of SAS.

RESULTS AND DISCUSSION

Humoral immune response: Antibody production was significantly higher in caged hens in comparison to free-range hens (Table 1) at pre-injection (1.69±0.70 vs. 0.069±0.069) ($p<0.0001$) and post-injection week 1 (2.26±0.77 vs. 0.145±0.25) ($p<0.0001$), week 2 (8.00±2.98 vs. 4.38±2.94) ($p<0.001$) and week 3 (9.24±2.56 vs. 6.69±3.86) ($p<0.05$). Differences in average antibody production between caged and free-range hens were greatest at week 2 post-injection, with differences of 1.62 at pre-injection, 2.11 at week 1 post-injection, 3.62 at week 2 post-injection and 2.55 at week 3 post-injection. Caged hens also exhibited a significantly higher level ($p<0.0001$) of total antibody production (5.30±0.23) throughout the immune challenge compared to free-range hens (2.82±0.26). This data demonstrates a more robust humoral immune function in caged hens in response to a killed Newcastle's virus compared to free-range hens.

Heterophil:Lymphoctye ratios: H:L ratios (Table 2) did not differ significantly between caged hens and free-range hens at pre-injection (1.07±0.80 vs. 1.31±1.32) and post-injection week 1 (2.15±0.93 vs. 2.12±0.98) and week 3 (1.28±0.43 vs. 1.34±0.38). However, caged hens did exhibit a significantly higher H:L ratio compared to free-range hens at post-injection week 2 (2.34±0.86 vs. 1.75±0.57) ($p<0.05$) which is also the time period where the greatest difference in antibody production between caged and free-range hens was observed. No significant differences in average H:L ratios during the vaccine challenge were observed between caged and free-range hens (1.69±0.14 vs. 1.62±0.17).

In the current study, there were marked differences in antibody production and minor differences in H:L ratios in response to a killed Newcastle's Disease Virus between free-range and caged flock-mates reared under identical conditions, including vaccination schedules (Anderson, 2009b). Specifically, caged hens exhibited a much more robust humoral immune response directed towards the production of antibodies against the killed Newcastle's Disease Virus vaccine challenge. Because antibody responses to commonly used vaccines allow assessment of alterations in humoral immune function (Dohms and Saif, 1983) and due to the fact that in poultry, decreased immune antibody responses have been observed in hens placed in stressful environments (Siegel, 1985), it is possible that free-range hens in this study endured environmental stressors specific to the range environment that suppressed their humoral immune response to the vaccine challenge performed during this trial. Environmental stressors in the range environment include: extreme fluctuations in environmental temperature, severe weather, predation and increased incidences of cannibalism and disease that cannot be regulated in an uncontrolled environment. Caged hens do not experience the aforementioned stressors due to the highly regulated, protected and controlled physical and social environment provided by battery cages.

In contrast to our observation of reduced humoral immune function in free-range hens, Shini (2003) did not observe any alterations in humoral immune function based on antibody titres of brown laying hens reared in conventional battery cages, modified cages and an intensive free-range housing system to commercially used Newcastle Disease (ND) and Infection Bronchitis (IB) at 35 weeks of age. Similarly, Tactacan *et al.* (2009) examination of ND antibody titres in laying hens at 61 wks of age housed in traditional vs. enriched cages did not reveal any affect of cage design on antibody production. However, Erhard *et al.* (2000) detected significantly higher antibody titres and mean immunologbulin Y concentrations in the egg yolk of caged laying hens immunized with human serum immunoglobulin G compared to hens kept on the ground with straw and a nest for laying. It is quite apparent from these findings that a large degree of variation exists in humoral immune function of laying hens reared in different housing environments. This variation is most likely due to the fact that humoral immune function is subject to the influence and interaction of numerous physiological, environmental, genetic and nutritional factors.

Previous studies in chickens have suggested that an increased H:L ratio is associated with increased

Table 1: Free-range vs. caged Newcastle's Disease (ND) antibody titres (log_{10}).

Source	Pre-injection	One week post-injection	Two weeks post-injection	Three weeks post-injection
Cage	1.690±0.70****	2.260±0.77****	8.00±2.98***	9.24±2.56*
Range	0.069±0.069	0.145±0.25	4.38±2.94	6.69±3.86

Data are expressed as the mean ± SEM. *($p<0.05$), **($p<0.01$), ***($p<0.001$), ****($p<0.0001$)

Table 2: Free-range vs. caged H:L ratios in response to a killed newcastle's vaccination challenge.

Source	Pre-injection	One week post-injection	Two weeks post-injection	Three weeks post-injection
Cage	1.07±0.80	2.15±0.93	2.34±0.86*	1.28±0.43
Range	1.31±1.32	2.12±0.98	1.75±0.57	1.34±0.38

Data are expressed as the mean ± SEM. *($p<0.05$), **($p<0.01$), ***($p<0.001$), ****($p<0.0001$)

environmental stress (McFarlane and Curtis, 1989) based on the supposition that environmental stressors induce an elevation in plasma corticosterone concentrations which, in turn, stimulates a rise in heterophil concentration. However, in the present study, no significant differences in average H:L ratios during the vaccine challenge were observed between caged and free-range hens. Similarly, Tactacan *et al*. (2009) did not observe significant differences in H:L ratios between hens housed in conventional vs. enriched cages. Shini (2003), on the other hand, observed elevated H:L ratios of hens in conventional cages compared to hens in modified cages or intensive free-range systems.

In the present study, caged hens exhibited significantly higher H:L ratios compared to free-range hens two weeks post-injection which correlated with the time period where the greatest difference in average antibody production between caged and free-range hens occurred. It is possible that the significantly higher H:L ratio and elevated antibody production observed in caged hens two weeks post-injection represents peak activation of caged hens' humoral and cell-mediated immune responses.

In summary, the results of the present study provide evidence that free-range hens may experience significant environmental stressors that suppress their humoral immune function but do not alter hematological indices such as H:L ratios in comparison to caged hens. Differences in humoral immune function between free-range and caged hens may indicate differing levels of existing stress conditions between the two housing designs.

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