Animal (2010), **4:10**, pp 1709–1715 © The Animal Consortium 2010
doi:10.1017/S175173111000100X

animal

Effects of housing conditions during the rearing and laying period on adrenal reactivity, immune response and heterophil to lymphocyte (H/L) ratios in laying hens

R. O. Moe[1†], D. Guémené[2], M. Bakken[3], H. J. S. Larsen[4], S. Shini[5], S. Lervik[1], E. Skjerve[4], V. Michel[6] and R. Tauson[7]

[1]*Norwegian School of Veterinary Science, Department of Production Animal Clinical Sciences, P.O. Box 8146 dep., N-0033 Oslo, Norway;* [2]*INRA, UR83-Unité de Recherches Avicoles, 37380 Nouzilly, France;* [3]*The Norwegian University of Life Sciences, Department of Animal and Aquacultural Sciences, P.O. Box 5003, N-1432 Ås, Norway;* [4]*Norwegian School of Veterinary Science, Department of Food Safety and Infection Biology, P.O. Box 8146 dep., N-0033 Oslo, Norway;* [5]*School of Veterinary Science, Faculty of Natural Resources, Agriculture & Veterinary Science, University of Queensland, Gatton, QLD 4343, Australia;* [6]*French Food Safety Agency (AFSSA), Research Unit 'Epidemiology and Welfare of Poultry and Rabbits', BP 53, 22440, Ploufragan, France;* [7]*Swedish University of Agricultural Sciences, Department of Animal Nutrition and Management, Avian Division, Kungsängen Research Centre, Feeding and Management of Poultry, 753 23 Uppsala, Sweden*

(Received 11 January 2010; Accepted 23 April 2010; First published online 21 May 2010)

This study was conducted to evaluate the effect of early rearing conditions on physiological, haematological and immunological responses relevant to adaptation and long-term stress in white Leghorn hens with intact beaks housed in furnished cages (FC) or conventional cages (CC) during the laying period. Pullets were cage reared (CR) or litter floor reared (FR). From 16 to 76 weeks of age, hens were housed in FC (eight hens per cage) or in CC (three hens per cage). As measures of long-term stress at the end of the laying period, adrenal reactivity was quantified by assessing corticosterone responses to adrenocorticotropin challenge, and immune response was assessed by measuring antibody responses after immunization with sheep red blood cells (SRBC) and keyhole limpet haemocyanin (KLH). Heterophil to lymphocyte (H/L) ratio was employed as an indicator of stress. Rearing conditions significantly affected anti-SRBC titres ($P < 0.0001$*) and tended to affect H/L ratios (*$P = 0.07$*), with the highest values found in FR hens. Layer housing affected H/L ratio (*$P < 0.01$*); the highest ratio was found in FR birds housed in FC during the laying period. This study shows that early rearing environment affects immunological indicators that are widely used to assess stress in laying hens. However, while results on H/L ratio indicated that FR birds experienced more stress particularly when they were housed in FC during the laying period, the immune responses to SRBC in FR hens was improved, indicating the opposite. This contradiction suggests that the effects on immune response may have been associated with pathogenic load due to environmental complexity in FR and FC hens rather than stress due to rearing system or housing system* per se.

Keywords: welfare, housing, rearing, laying hens, adaptation

Implications

This study was conducted to explore effects of early pullet rearing conditions on adaptation to furnished cages (FC) during the laying period, which may be a key issue for hen welfare. Physiological, haematological and immunological variables relevant to stress and welfare were measured in birds with intact beaks housed in cages or on the litter floor during rearing, and subsequently in conventional cages or FC during the laying period. Rearing conditions affected variables relevant for hen adaptation. However, it was not evident if the effects were associated with pathogenic load or stress and environmental conditions.

† E-mail: randi.moe@nvh.no

Introduction

Currently, the improvement of laying hen welfare is of great concern to the European Commission and EU egg industry (Council Directive, 1999; EFSA, 2005; Blokhuis *et al.*, 2007). In recent times, there have been concerted research efforts focusing on modifying cage designs and improving hen welfare (Appleby *et al.*, 2002; Blokhuis *et al.*, 2007). A furnished cage system (FC) attempts to provide an enriched environment to meet the behavioural needs of hens while maintaining a small group size to minimize stress (Tauson, 1998). In contrast to the conventional cages (CC), FC are equipped with nesting areas, perches and litter areas to increase opportunities for the hens to exhibit natural behaviours and thereby improve their welfare (Appleby *et al.*, 2002).

However, discrepancies in the outcome assessment regarding mortality, plumage conditions and production parameters have been reported for new housing systems (EFSA, 2005; Blokhuis *et al*., 2007), although several factors could have been responsible for the inconsistent results, including differences in cage designs, group sizes, stocking densities, application of beak trimming and the genotype of birds used (EFSA, 2005).

Access to litter at an early age stimulates ground pecking and dustbathing, and influences the propensity for injurious feather pecking in laying hens during the laying period (van de Weerd and Elson, 2006). Therefore, the early rearing environment has been presented as one of the most important factors affecting the ability of hens to adapt to the housing environment during the laying period. Early rearing conditions may be of particular importance for hens with intact beaks in order to adapt to future housing systems involving large group sizes. It has been shown that rearing pullets with intact beaks on deep litter floor resulted in a positive long-term impact on plumage conditions regardless of housing conditions (i.e. FC or CC) during the laying period (Hetland *et al*., 2004). Hens for this study had been recruited from that large-scale experiment in order to explore measures relevant to stress and adaptation that have been presented as an important approach for addressing hen welfare (Blokhuis *et al*., 2007).

The activation of the hypothalamo–pituitary–adrenal (HPA) pathways by environmental stressors plays an important role in the coordination of the physiological response to stress, including the immune response in laying hens (Siegel, 1985; Dohms and Metz, 1991). Therefore, physiological, haematological and immunological variables relevant to long-term stress may be recorded in order to understand how early rearing management practices including housing conditions influence the adaptation process of laying hens.

One approach to detect long-term stress has been using an adrenocorticotropin (ACTH) stimulation test (Thorn *et al*., 1953) to assess corticotropic axis functionality and to determine the adrenal glands capacity to respond to ACTH. This approach has previously been used to assess chronic stress in relation to housing conditions in birds (Mormède *et al*., 2007).

Studies of immune responses are another approach to assess long-term stress in birds. The T-cell dependent antibody response, using a modified ELISA with either sheep red blood cells (SRBC) or keyhole limpet haemocyanin (KLH) as antigen, has been a common protocol for pre-clinical immunotoxicity evaluations in different species (Gore, 2006). Accordingly, it has been shown that poultry exposed to various stressful environments or procedures have lower antibody production in response to a variety of antigens including SRBC (Siegel, 1985). Similarly, exposure to continuous infusions of ACTH (Mumma *et al*., 2006) or dietary corticosterone (Post *et al*., 2003), in order to mimic chronic stress, suppressed antibody responses to immunization with experimental antigens such as SRBC (Mumma *et al*., 2006) and vaccines used commercially, for example, infectious bronchitis virus vaccine (Shini *et al*., 2008). On the other hand, reducing stress during the laying period (Zulkifli *et al*., 2002) and enriching the cage environment (El-Lethey *et al*., 2000) led to increased anti-SRBC antibody titres. Under practical farm conditions, higher antibody titres in response to vaccines are reported in laying hens placed in floor pens as compared with FC (Shini, 2003). Furthermore, heterophil to lymphocyte (H/L) ratio has been widely used as a sensitive indicator of long-term stress related to immune function in laying hens (Gross and Siegel, 1983; Maxwell and Robertson, 1998), and has been employed as an index of hen welfare (Nicol *et al*., 2009). However, little is known about the long-term effects of early rearing environment on immune responses in hens housed in FC during the laying period.

In studies on hen welfare in FC, most attention has concentrated on mortality, health and production parameters (Tauson, 1998; Rodenburg *et al*., 2009) and few have focused on the effects of FC on hen's physiological and haematological measures, while employing beak-trimmed hens (Shini, 2003; Pohle and Cheng, 2009; Tactacan *et al*., 2009). Nevertheless, these investigators did not examine the effects of early rearing conditions on measures of long-term stress during the laying period. Studies of stress involving rearing conditions and effects in subsequent housing systems are of particular importance to assess welfare in hens that are not beak trimmed.

In this study, the effects of early rearing conditions in cages (CR) or floor pens (FR) and housing systems during the laying period (CC or FC) on long-term stress measures (i.e. adrenal reactivity, humoral immune response and H/L ratios) were investigated in laying hens with intact beaks. In addition, a possible interaction between the variables during rearing and laying conditions was examined.

Material and methods

Birds and housing

White Lohmann Selected Leghorn chicks were hatched at the Research Station of the Norwegian Poultry Association, Norway. The day after hatch, 1600 chicks were transferred to an adjacent building and kept on a deep litter floor (FR) or in conventional wire mesh rearing cages (CR) throughout the rearing period from 0 to 16 weeks of age. For FR, 800 pullets were divided into eight pens at a stocking density of 13 birds/m^2. For CR, 800 pullets were housed in conventional wire mesh rearing cages (Big Dutchman) with 12 pullets per cage at a stocking density of 448 cm^2/bird. None of the rearing alternatives included a perch. Chicks were vaccinated against Marek's disease (TAD – Marek – vac vet., LAH Cuxhaven, Lohmann Animal Health GmbH & Co. KG, Cuxhaven, Germany), coccidiosis (Paracox vet., Schering – Plough, Intervet/Schering-Plough Animal Health, Boxmeer, The Netherlands) and avian encephalomyelitis (TAD – AE – VAC vet., LAH Cuxhaven) on days 1 and 7, and week 12 after hatch, respectively.

At 16 weeks of age, pullets were transferred to an adjacent building and housed in 36 CC, housing three hens/cage, at a density of 730 cm^2/hen; or in 12 Victorsson FC, housing eight hens/cage, with a minimum space allowance of 750 cm^2/hen including a litter bath and nest box and perch

with 12 cm allowance per bird. This experimental design allowed for four rearing and housing combinations: CR/CC; CR/FC; FR/CC; and FR/FC. This study was part of another large-scale study (Hetland *et al*., 2004), and the results reported here are from a total of 160 individuals randomly selected from these rearing and housing alternatives, that is, 80 birds (20 per rearing and housing alternative) for the ACTH and immunization challenge, and a further 80 birds (20 per rearing and housing alternative) for the H/L trial. Individual hens were not marked according to rearing pen or cage number when they were transferred to layers cage systems, and it was therefore not possible to include pen or rearing cage number as a factor. The cages were placed in the same room and hens were kept there during the entire laying period, that is, from 16 to 76 weeks of age. Birds were not beak trimmed.

Measurements

Indicators of hen physiology (i.e. adrenal responsiveness to a challenge with ACTH), haematology (i.e. differential white blood cell counts including H/L ratio) and immune function (i.e. humoral immune response to immunization with SRBC and KLH) were used to assess the long-term effects of rearing and housing. In short, 20 hens per rearing alternative (CR or FR) and cage model (CC or FC) (i.e. a total of 80 hens) were randomly selected and individually marked with coloured foot rings and then used for the adrenal responsiveness (50 weeks of age) and immune function assessment beginning at 62 weeks of age, with weekly blood sampling for 6 weeks. Another 80 birds (20 hens per cage model and rearing alternative) were used for the H/L ratio assessment at 70 weeks of age.

ACTH stimulation

Initial corticosterone concentrations and maximum reactivity of the adrenal glands using a challenge with 1 to 24 ACTH were assessed at 50 weeks of age. Each bird was injected i.m. in the *pectoralis major* muscle with 10 μg/kg BW 1 to 24 ACTH (Immediate Synacthen 0.25 mg, Novartis, Oslo, Norway). This dose has previously been used to induce maximal HPA axis response in laying hens (Kjaer and Guémené, 2009). Blood samples (1 ml) were drawn from the right wing vein immediately before, and then from the left wing vein 15 min after injection. Blood was then immediately transferred to a Lithium–Heparin-coated centrifuge tube. Hens were placed in individual boxes immediately after the zero-blood sample and ACTH injection, removed after 15 min, a blood sample taken and then returned to their home cage. Blood was stored on ice and centrifuged within 6 h; plasma was separated and stored at −20°C for corticosterone assay. Plasma corticosterone concentrations were measured in duplicate using a specific radioimmunoassay (Etches, 1976).

Antibody titres

The same hens were used for adrenal and immune challenges. At 62 weeks of age, blood samples (1 ml) were obtained from the right wing vein to determine pre-immunization antibody titres. Subsequently, 1 ml of a 2% (v/v) of SRBC (SIGMA) in phosphate buffer saline (PBS) and 1 ml of a 250 μg/ml KLH (No H7017, Sigma-Aldrich Inc., St. Louis, MO, USA) diluted in PBS (31 mM PBS containing 0.46 M NaCl and 41 mM sucrose) were injected i.m. in the right and left front parts of the *pectoralis major* muscle, respectively. Blood samples were collected at 7, 14, 21, 28, 35 and 42 days post-immunization. Serum from blood was stored frozen until analysed for the presence of anti-KLH IgG-specific antibodies and antibodies to SRBC. Antibodies against KLH were measured by an enzyme-linked immunosorbent assay (ELISA), as described by Korver *et al*. (1984), and slightly modified in that we used skim milk powder (Merck, Germany) in PBS-Tween 20 as blocking solution, and by the 2-fold dilution of serum samples. The lowest dilution tested was 1 : 100. The serum antibody titre was measured by using a horseradish peroxidase linked goat anti chicken IgG-Po (Fc) (AA129P, Serotec, Oxford, UK) and the enzyme substrate, ABTS (2,2′-azinobis (3-ethylbenzthiazolinesulphonic acid)) (Sigma-Aldrich Inc.). The enzyme reaction was stopped by adding sodium azide (NaN_3, 1 mg/ml) and measured immediately with a Titertek Multiskan® Plus ELISA reader (Labsystems, Titertek Multiskan, Eflab. Oy, Helsinki, Finland; wavelength 405 nm). The antibody titre values were $\log_2$ – transformed ($\log_{10}$ of the titre divided with $\log_{10}$ 2) to normalize the distribution. The final titre was defined as the reciprocal ($\log_2$) of the highest dilution of serum showing positive reaction, that is, at least 0.1 OD_{405}, which exceeded by more than a factor of two of the average OD_{405} of the mean normal control samples in the same test plate.

Anti-SRBC titres were quantified using a haemagglutination assay (Ling and Catty, 1988). In short, whole blood (heparinized) from sheep was mixed 1 : 2 with Alsevers solution and kept overnight at 4°C. The SRBC was washed three times in PBS and a 0.5% (v/v) SRBC suspension was made for the assay. Serum (25 μl) was diluted with PBS (25 μl) in titration plates. The lowest serum dilution tested was 1 : 2. To all wells 50 μl 0.5% SRBC was added and the suspension was gently mixed. The plates were kept for 2 h at room temperature before the anti-SRBC antibody endpoint titre of each animal was estimated as the reciprocal ($\log_2$) of the highest dilution of serum showing haemagglutination.

Leucocyte counts

At 70 weeks of age, a total of 80 other birds (two hens randomly selected per cage) were individually gently removed from their cage. Immediately after the capture, a drop of blood was taken from a small puncture of the wing vein. The blood was immediately smeared onto a glass slide and dried. Later, the smears were fixed and stained using May–Grünewald–Giemsa stain according to standard procedures, and one hundred leucocytes were counted once on each slide using a light microscope and 1000× magnification. The H/L ratios were determined by dividing the number of heterophils by the number of lymphocytes.

Statistical analysis

After establishing the database in Excel® (2003) for Windows, the data were transferred to the statistical programs JMP

(8.0 for Windows, SAS Institute Inc.) for further analysis. The data were examined using graphical and tabular methods before a full model was established using the GLM platform. Initial corticosterone concentrations were described using median and range, as the data were not normally distributed. The increase in corticosterone was however normally distributed, allowing a linear regression model to be constructed. The linear regression model was built using the outcomes of increases of plasma corticosterone concentrations, leucocyte differential counts and immune response, and housing and rearing as fixed explanatory factors. Models with and without interactions between housing and rearing were tested. Model fit was assessed using standard procedures including check for normality of residuals using quantile normal plots. For the immune response, the model was established in Stata (10/SE for Windows, StataCorp, College Station, TX, USA) with the hen as a random factor in the GLM regression model. Models were built and assessed as in JMP. Owing to the skewed distribution of data for differential counts and the H/L ratio, the data were presented as median and range using the Wilcoxon rank sum test for group comparison.

Results

ACTH stimulation

Median initial base concentrations of plasma corticosterone were 0.88 ng/l (range 0.11 to 6.7), while the response concentrations were 16.9 ng/l (range 1.5 to 38.0) after ACTH challenge. The median increase was 16.03 ng/l (range 0.96 to 36.6) and clearly significant ($F = 150.3$, $df = 1$, $P < 0.0001$). No effect of rearing ($F = 0.44$, $df = 1$, $P = 0.51$) or housing ($F = 0.52$, $df = 1$, $P = 0.47$) was found.

Antibody titres

Hens responded to immunizations with a clear increase in antibody titres to SRBC and anti-KLH IgG, respectively, as illustrated in Figure 1 (SRBC) and Figure 2 (KLH). Anti-SRBC titre was significantly affected by rearing conditions ($F = 68.1$, $df = 1$, $P < 0.0001$) where highest titre was found for FR hens. No effects of housing conditions were found. However, interactions between layer housing and rearing were found for KLH titre ($F = 29.5$, $df = 1$, $P < 0.001$), where the highest titre was found in FR hens that had been housed in FC.

Leucocyte counts

Data on differential leucocyte counts and H/L ratio are presented in Table 1. Layer housing conditions significantly affected H/L ratios ($F = 7.9$, $df = 1$, $P < 0.01$). Hens housed in FC showed the highest values of H/L compared with hens housed in CC. When adjusted for a housing effect, rearing conditions tended to affect H/L ratio ($F = 3.45$, $df = 1$, $P = 0.07$), where FR resulted in the highest H/L ratio.

Discussion

Initial plasma corticosterone concentrations were low, indicating that hens were not hypersensitive, resulting from chronic stressful housing conditions (Guémené *et al.*, 2006). Furthermore, 1 to 24 ACTH injection led to significant increases of corticosterone concentrations in all groups. Thus, no exhaustion of the adrenal glands was found, indicating that hens had not experienced severe chronic stress related to any of the present experimental rearing or housing conditions. On the other hand, it has to be emphasized that high doses of ACTH stimulation can only detect maximum

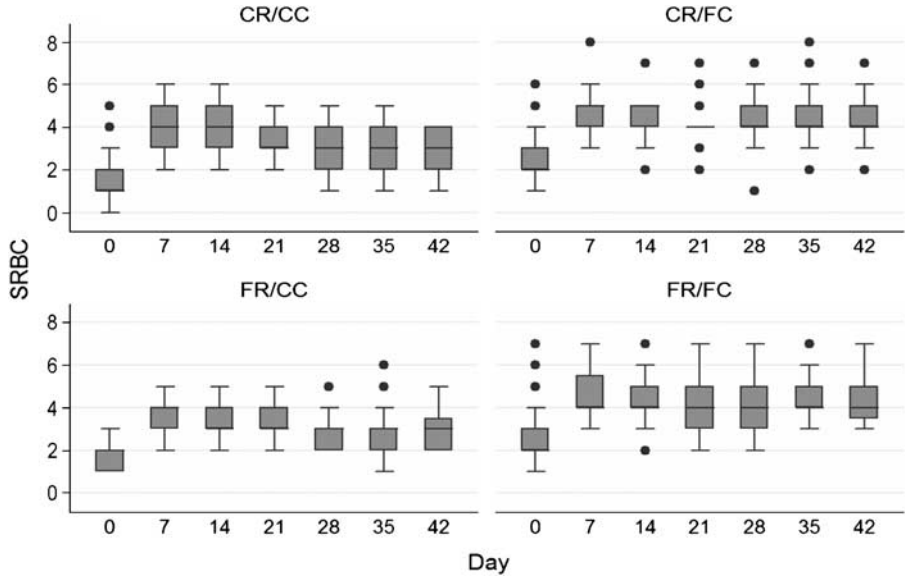

Figure 1 Anti-SRBC titre (log 2) in laying hens before (0) and after (days 7 to 42) immunization with Sheep Red Blood Cells (SRBC). Results are presented as the reciprocal (log2) of the highest dilution of serum showing haemagglutination. Hens had been exposed to four rearing and housing combinations: CR/CC, CR/FC, FR/CC or FR/FC (CR: Cage rearing, FR: Floor rearing, CC: Conventional cages and FC: Furnished cages). Results are presented as a box plot, where the box (including a median line) represents the 25 to 75 percentile, the range line represents upper adjacent values based on inter-quartal range and dots represent outside values.

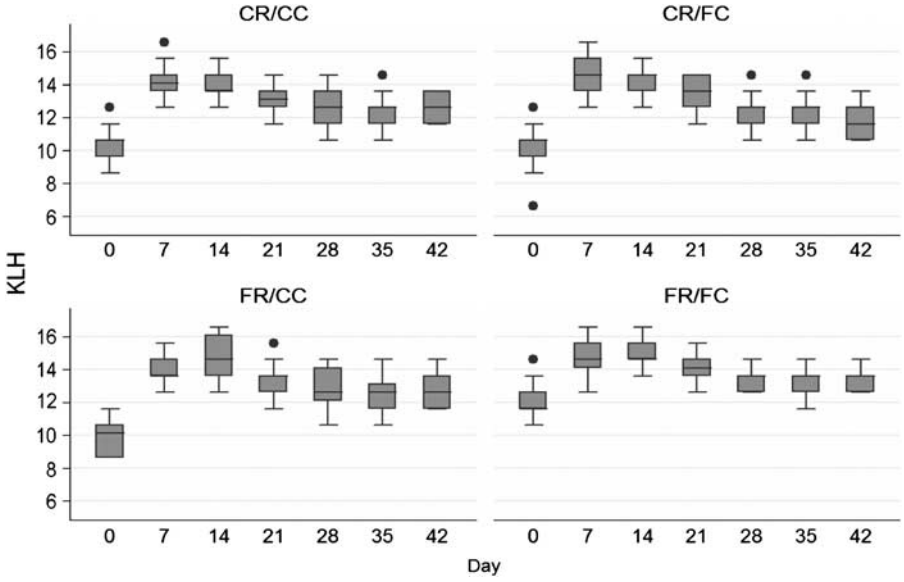

Figure 2 Anti-keyhole limpet haemocyanin (KLH) IgG titre (log 2) in laying hens before (day 0) and after (days 7 to 42) immunization with KLH. Results are presented as the reciprocal (log2) of the highest dilution of serum showing positive reaction. Hens had been exposed to four rearing and housing combinations: CR/CC, CR/FC, FR/CC or FR/FC (CR: Cage rearing, FR: Floor rearing, CC: Conventional cages and FC: Furnished cages). Results are presented as a box plot, where the box (including a median line) represents the 25 to 75 percentile, the range line represents upper adjacent values based on inter-quartal range and dots represent outside values.

Table 1 *Effects of early rearing conditions and housing conditions during the laying period on differential leucocyte counts in laying hens with intact beaks. Four rearing and housing combinations were investigated: CR/CC: Cage rearing, housed in conventional cages; FR/CC: Floor rearing, housed in conventional cages; CR/FC: Cage rearing, housed in furnished cages; FR/FC: Floor rearing, housed in furnished cages. Results are presented as median (range)*

Rearing and housing combination	H/L ratio	Lymphocytes	Heterophils	Eosinophils	Basophils	Monocytes
CR/CC	0.20 (0.05 to 0.6)	70.8 (56.5 to 87.0)	13.8 (4.0 to 34.0)	1.8 (0 to 4.0)	0.8 (0 to 3.0)	8.8 (4.0 to 14.5)
FR/CC	0.26 (0.07 to 0.60)	68.5 (53.5 to 82.5)	18.0 (6.0 to 33.5)	1.5 (0 to 4.5)	0.5 (0 to 4.0)	11.5 (3.5 to 20.0)
CR/FC	0.28 (0.05 to 0.82)	69.0 (47.0 to 87.0)	19.0 (4.5 to 38.5)	2.0 (0 to 5.0)	0 (0 to 2.5)	8.0 (2.5 to 14.5)
FR/FC	0.35 (0.08 to 1.10)	65.5 (39.0 to 84.0)	24.0 (7.0 to 43.0)	2.0 (0.5 to 7.5)	1.0 (0 to 3.0)	7.5 (2.0 to 14.0)

reactivity of the adrenal glands. Therefore, it cannot be ruled out that minor differences in stress levels could have been detected by exploring adrenal sensitivity using lower doses of ACTH (Guémené *et al*., 2006). Our present finding is thus not in contradiction with those of other investigators (e.g. Hester *et al*., 1996) who have shown an improvement in the bird's capacity to adapt to stress when exposed to similar social conditions during the rearing and laying period.

Housing conditions did not influence immune response to immunization, which suggests that hens are capable of building the same level of immune protection in different housing conditions during the laying period. However, layer housing affected H/L ratio. Larger group size in FC (eight hens) compared with CC (three hens) showed increased H/L ratios presumably caused by social ranking and social stress (Gross and Siegel, 1980; Craig and Muir, 1996; Hester *et al*., 1996). Our results are supported by Barnett *et al*. (2009) who found that group size in FC was probably more influential on hen welfare than the type of housing facilities provided. Our results contrast with findings by Shini (2003) who found higher H/L ratio in hens housed in CC when compared with modified cages and free-range conditions. It should be noted that birds used in that study were beak trimmed. However, Scholz *et al*. (2008) also reported lower H/L ratios in hens with intact beaks kept in groups of 40 layers in compartments with perches (Eurovent). It appears that beak trimming alone may not explain the discrepancy in results. There are many other factors that influence the H/L ratio in hens, including age, infectious agents and hen–human relationships (Gross and Siegel, 1983). In this study, all birds were non-beak trimmed and the bird management was similar. Therefore, we suggest that housing condition (group size and access to litter in FC) may have induced stress and influenced H/L ratios in hens.

Rearing conditions tended to affect H/L ratio ($P = 0.07$). A small sample size and large variation in the results can explain why these differences did not reach significance. In agreement with Wall and Tauson (2005), FR and later housing in FC

resulted in the highest H/L ratio, and accordingly represented the most stressful rearing and layer housing combination. In contrast, CR hens that were housed in CC had the lowest H/L ratio, indicating less stress. Faure (1991) showed that chicks reared on large floor pens worked intensively in order to gain access to more space in an operant conditioning cage during the laying period. Therefore, the movement of birds from the spacious rearing system (floor) to a space-restricted environment in a laying system (FC) may have been stressful, whereas moving hens from a rearing cage environment to a layer cage environment may have been less stressful. This supports the suggestion that rearing birds in a system that closely resembles their future housing environment helps facilitate the transition to production housing (van Emous, 2003; Colson *et al.*, 2008).

Rearing conditions significantly affected the antibody responses to SRBC. Our results are in agreement with Shini (2003) who showed that hens kept on floor pens had higher antibody responses when compared with FC. Furthermore, we indicate that the effects on humoral immunity are long-lasting regardless of later housing conditions. An interaction between rearing and housing and KLH titre was found in FR hens that had the highest titre when they were subsequently housed in FC. This may indicate lack of stress and improved immunity as a result of early adaptation in floor-reared hens.

In summary, the results on H/L did not support the assumption that hens in FR may experience less stress. In contrast, the H/L ratio was highest in FC. This inconsistency suggests that other factors may have been involved in the humoral and cellular immune response to SRBC and H/L ratio in FR hens, respectively. Litter floor and enrichment of FC (i.e. nest, perches and litter) present a more complex environment than CC regarding hygiene and microbial contamination, which can influence heterophils and stress levels (Shini *et al.*, 2008). Indeed, heterophilia and lymphopenia are natural defence mechanisms against bacterial infection in avians (Maxwell and Robertson, 1998). FR and subsequent housing in FC with access to litter (in a litter box) represent the highest opportunity for earlier and/or long-lasting exposure to environmental antigens, that is, pathogens. This could be the explanation for altered H/L ratio and SRBC titre found in the FR/FC hens. Similarly, the alteration of H/L ratio in FC could be the result of a higher pathogenic contamination present in this system rather than stress response to cage housing, or a combination of both factors. It is therefore likely that effects of early rearing environment on immune response may not unambiguously reflect stress and hen welfare, but rather early and/or long-lasting exposure to environmental pathogens. It has to be emphasized that depriving hens of litter leads to stress and an increased H/L ratio too (El-Lethey *et al.*, 2003), and that further experiments are needed to draw firmer conclusions. Recently, Shini *et al.* (2008) showed that corticosterone treatment and bacterial exposure both increase H/L ratios, but alter the ultrastructure of heterophils and lymphocytes differently. The possibility to use other cellular and molecular parameters of stress and immune response, that is, heterophil ultrastructure evaluation (Shini *et al.*, 2008), measurement of acute phase proteins (Rath *et al.*, 2009) and cytokine and chemokine gene expression levels in heterophils and lymphocytes (Shini and Kaiser, 2009; Shini *et al.*, 2010) could be explored to differentiate effects between non-infectious stress (induced by housing and social factors) and bacterial stress (induced by environmental pathogens). Further studies are needed to avoid potential misinterpretation involving the H/L ratio as an index of hen welfare (e.g. Nicol *et al.*, 2009).

H/L ratios measured under all experimental conditions were lower than previously reported (e.g. El-Lethey *et al.*, 2000; Wall *et al.*, 2004; Shini *et al.*, 2008). As suggested by Gross and Siegel (1983), H/L ratios of about 0.20, 0.50 and 0.80 are characteristic of low, optimal and high degrees of stress. Our results indicated that hens in this study did not experience severe stress. Hens might probably have experienced acute stress when they were transferred to the layer house (Craig *et al.*, 1988). However, this was not measured in this study and did not have any long-term effect at later stages of the laying period.

Conclusion

This study shows that early rearing environment affects stress and immunological response of hens in the subsequent laying period. The H/L ratio was increased in FR birds and in laying hens housed in FC during the laying period, indicating some degree of stress, but the immune response to SRBC was higher only in FR hens. These results suggest that the effects on immune response may reflect pathogenic load due to environmental complexity rather than stress due to housing system or rearing system *per se*. As a result, a degree of caution should be exercised when making interpretations based on these indicators of hen welfare. In order to further explore the impact of rearing and housing conditions on hen welfare, there is a need to investigate the role of pathogens *v.* stress on immune measurements that are commonly used as stress indicators before being able to draw firm conclusions.

Acknowledgements

This study was financially supported by grants from the Norwegian Research Council (NFR 144384/110). Financial support from the LayWel Research project (European FP6- No. 502315-STReP) to perform the corticosterone assay is also greatly acknowledged. The authors thank the staff of the Norwegian Poultry Association for the technical support, in particular B. Siliart and D. Soyer (Veterinary School of Nantes, France) for performing the differential leucocyte count, M. Couty (INRA, France) for expert technical assistance in assaying corticosterone, and M. Borg and G. M. Johansen (Norwegian School of Veterinary Science, Norway) for technical assistance with assays for the antibody response evaluation.

References

Appleby MC, Walker AW, Nicol CJ, Lindberg AC, Freire R, Hughes BO and Elson HA 2002. Development of furnished cages for laying hens. British Poultry Science 43, 489–500.

Barnett JL, Tauson R, Downing JA, Janardhana V, Lowenthal JW, Butler KL and Cronin GM 2009. The effects of a perch, dust bath, and nest box, either alone or in combination as used in furnished cages, on the welfare of laying hens. Poultry Science 88, 456–470.

Blokhuis HJ, van Niekerk TF, Bessei W, Elson A, Guemene D, Kjaer JB, Levrino GAM, Nicol CJ, Tauson R, Weeks CA and van de Weerd HA 2007. The LayWel project: welfare implications of changes in production systems for laying hens. World's Poultry Science Journal 63, 101–114.

Colson S, Arnould C and Michel V 2008. Influence of rearing conditions of pullets on space use and performance of hens placed in aviaries at the beginning of the laying period. Applied Animal Behaviour Science 111, 286–300.

Council Directive 1999 of 19 July 1999 laying down minimum standards for the protection of laying hens. Off J EU L 203, 53-57. Retrieved March 26, 2010, from http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:1999:203:0053:0057:EN:PDF

Craig JV and Muir WM 1996. Group selection for adaptation to multiple-hen cages: beak-related mortality, feathering, and body weight responses. Poultry Science 75, 294–302.

Craig JV, Okpokho NA and Milliken GA 1988. Floor- and cage-rearing effects on pullets' initial adaptation to multiple-hen cages. Applied Animal Behaviour Science 20, 319–333.

Dohms JE and Metz A 1991. Stress – mechanisms of immunosuppression. Veterinary Immunology and Immunopathology 30, 89–109.

EFSA (European Food Safety Authority) 2005. The welfare aspects of various systems of keeping laying hens. The EFSA Journal 197, 1–23.

El-Lethey H, Huber-Eicher B and Jungi TW 2003. Exploration of stress-induced immunosuppression in chickens reveals both stress-resistant and stress-susceptible antigen responses. Veterinary Immunology and Immunopathology 95, 91–101.

El-Lethey H, Aerni V, Jungi TW and Wechsler B 2000. Stress and feather pecking in laying hens in relation to housing conditions. British Poultry Science 41, 22–28.

Etches RJ 1976. A radioimmunoassay for corticosterone and its application to the measurement of stress in poultry. Steroids 28, 763–773.

Faure JM 1991. Rearing conditions and needs for space and litter in laying hens. Applied Animal Behaviour Science 31, 111–117.

Gore ER 2006. Immune function tests for hazard identification: a paradigm shift in drug development. Basic & Clinical Pharmacology & Toxicology 98, 331–335.

Gross WB and Siegel PB 1980. Effects of early environmental stresses on chicken body weight, antibody response to RBC antigens, feed efficiency, and response to fasting. Avian Diseases 24, 569–579.

Gross WB and Siegel HS 1983. Evaluation of the heterophil/lymphocyte ratio as a measure of stress in chickens. Avian Diseases 27, 972–979.

Guémené D, Guy G, Noirault J, Destombes N and Faure J-M 2006. Rearing conditions during the force-feeding period in male mule ducks and their impact upon stress and welfare. Animal Research 55, 443–458.

Hester PY, Muir WM, Craig JV and Albright JL 1996. Group selection for adaptation to multiple-hen cages: hematology and adrenal function. Poultry Science 75, 1295–1307.

Hetland H, Moe RO, Tauson R, Lervik S and Svihus B 2004. Effect of including whole oats into pellets on performance and plumage condition in laying hens housed in conventional and furnished cages. Acta Agriculturae Scandinavica A 54, 206–212.

Kjaer JB and Guémené D 2009. Adrenal reactivity in lines of domestic fowl selected on feather pecking behavior. Physiology & Behavior 96, 370–373.

Korver K, Zeijlemaker WP, Schellekens PTA and Vossen JM 1984. Measurement of primary in vivo IgM- and IgG-antibody response to KLH in humans: implications of pre-immune IgM binding in antigen-specific ELISA. Journal of Immunological Methods 74, 241–251.

Ling NR and Catty D 1988. Haemagglutination and haemolysis assays. In Antibodies volume I a practical approach (ed. D Catty), pp. 169–173. IRL Press, Oxford, UK.

Maxwell MH and Robertson GW 1998. The avian heterophil leucocyte: a review. World's Poultry Science Journal 54, 155–178.

Mormède P, Andanson S, Aupérin B, Beerda B, Guémené D, Malmkvist J, Manteca X, Manteuffel G, Prunet P, van Reenen CG, Richard S and Veissier I 2007. Exploration of the hypothalamic-pituitary-adrenal function as a tool to evaluate animal welfare. Physiology & Behavior 92, 317–339.

Mumma JO, Thaxton JP, Vizzier-Thaxton Y and Dodson WL 2006. Physiological stress in laying hens. Poultry Science 85, 761–769.

Nicol CJ, Caplen G, Edgar J and Browne WJ 2009. Associations between welfare indicators and environmental choice in laying hens. Animal Behaviour 78, 413–424.

Pohle K and Cheng H-W 2009. Comparative effects of furnished cages and battery cages on egg production and physiological parameters in White Leghorn hens. Poultry Science 88, 2042–2051.

Post J, Rebel JMJ and ter Huurne AAHM 2003. Physiological effects of elevated plasma corticosterone concentrations in broiler chickens. An alternative means by which to assess the physiological effects of stress. Poultry Science 82, 1313–1318.

Rath NC, Anthony NB, Kannan L, Huff WE, Huff GR, Chapman HD, Erf GF and Wakenell P 2009. Serum ovotransferrin as a biomarker of inflammatory diseases in chickens. Poultry Science 88, 2069–2074.

Rodenburg TB, Tuyttens FAM, Sonck B, De Reu K, Herman L and Zoons J 2009. Welfare, health, and hygiene of laying hens housed in furnished cages and in alternative systems. Journal of Applied Welfare Science 8, 211–226.

Scholz B, Rönchen S, Hamann H, Pendl H and Distl O 2008. Effect of housing system, group size and perch position on H/L ratio in laying hens. Archiv für Geflügelkunde 72, 174–180.

Shini S 2003. Physiological responses of laying hens to the alternative housing systems. International Journal of Poultry Science 2, 357–360.

Shini S and Kaiser P 2009. Effects of stress, mimicked by administration of corticosterone in drinking water, on the expression of chicken cytokine and chemokine genes in lymphocytes. Stress 12, 388–399.

Shini S, Shini A and Kaiser P 2010. Cytokine and chemokine gene expression profiles in heterophils from chickens treated with corticosterone. Stress: The International Journal on the Biology of Stress 13, 185–194.

Shini S, Kaiser P, Shini A and Bryden WL 2008. Differential alterations in ultrastructural morphology of chicken heterophils and lymphocytes induced by corticosterone and lipopolysaccharide. Veterinary Immunology and Immunopathology 122, 83–93.

Siegel HS 1985. Immunological responses as indicators of stress. World's Poultry Science Journal 41, 36–44.

Tactacan GB, Guenter W, Lewis NJ, Rodriguez-Lecompte JC and House JD 2009. Performance and welfare of laying hens in conventional and enriched cages. Poultry Science 88, 698–707.

Tauson R 1998. Health and production in improved cage designs. Poultry Science 77, 1820–1827.

Thorn GW, Jenkins D, Laidlaw JC, Goetz FC, Dingman JF, Arons WL, Streeten DHP and McRacken BH 1953. Pharmacologic aspects of adrenocortical steroids and ACTH in man. The New England Journal of Medicine 248, 232–245.

van de Weerd HA and Elson A 2006. Rearing factors that influence the propensity for injurious feather pecking in laying hens. World's Poultry Science Journal 62, 654–664.

van Emous R 2003. From cages to alternative systems requires different skills. World Poultry 19, 24–27.

Wall H and Tauson R 2005. Uppfödningen har betydelse för värphöns i innredda burar. Fjäderfä 8, 24–26.

Wall H, Tauson R and Elwinger K 2004. Pop hole passages and welfare in furnished cages for laying hens. British Poultry Science 45, 20–27.

Zulkifli I, Gilbert J, Liew PK and Ginsos J 2002. The effects of regular visual contact with human beings on fear, stress, antibody and growth responses in broiler chickens. Applied Animal Behaviour Science 79, 103–112.