



SSPE-CT-2004-502315

LAYWEL

Welfare implications of changes in production systems for laying hens

Specific Targeted Research Project (STReP)

Thematic Priority: Integrating and strengthening the ERA, Area 8.1.B.1.4, task 7

Deliverable 5.4

Final report for WP5

**Physiology and stress indicators,
Annexes**

Due date of deliverable: 23

Actual submission date: 24

Start date of project: 1/1/2004

Duration: 24 months

Organisation name of lead contractor for this deliverable: INRA

Revision [Final]

Project co-funded by the European Commission within the Sixth Framework Programme (2002-2006)		
Dissemination Level		
PU	Public	
PP	Restricted to other programme participants (including the Commission Services)	x
RE	Restricted to a group specified by the consortium (including the Commission Services)	
CO	Confidential, only for members of the consortium (including the Commission Services)	

**LayWel Work Package 5 - Physiology and stress indicators
Annexes Final Report (20/01/06)**

Manuscript 1 (INRA-SRA Partner 5)

Animal Research (2004) 53: 235-243.

**Humeral quality and adrenal responsiveness in laying hens reared
in standard and furnished cages**

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Abstract – In order to find out whether furnished cages contribute to improving the welfare of laying hens, humerus quality and adrenal responsiveness were evaluated in laying hens reared in standard (S) and furnished cages (F). Four cage models were used: S5, a standard cage model with 5 hens per cage; S6, a standard cage model with 6 hens per cage; F7, a furnished cage model with 7 hens per cage (with a nest, dust-bathing box, two perches, and claw-shortening) and F15, a furnished cage model with 15 hens per cage (with a nest, dust-bathing box, two perches, and claw-shortening). At 72 weeks of age, maximal adrenal responsiveness was evaluated by measuring the changes in blood corticosterone level induced by the i.m. injection of 10µg per hen of 1-24 ACTH (n = 15 hens per cage model). Hens (n = 15 to 23 hens per cage model) were slaughtered and the left and right humeri were used for measurement of weight, biomechanical characteristics in a flexion test, dry matter and ash percentage. Basal corticosterone levels did not differ significantly while the injection of ACTH produced a significant rise in corticosterone levels ($P < 0.001$) of similar amplitude for all cage models. Humeri weights, biomechanical characteristics (elastic strain, bioyield point, stiffness and breaking strength), dry weight and percentage of dry matter were not significantly different between cage models. The humeri ash percentage was significantly ($P = 0.03$) lower in birds from the S6 cage model (57.4%,) than in birds from other cage models (S5: 59.0%; F7: 58.9%; F15: 59.7%). Adrenal responsiveness and major humeral characteristics were not significantly improved in furnished compared to standard cages in our experimental conditions.

laying hen / furnished cages / exercise/ bone / corticosterone / welfare

Résumé : Qualité de l'os et capacité de réponse de la glande surrénale chez des poules pondeuses élevées en cages standard et en cages aménagées. Afin d'analyser si les dispositifs d'enrichissement apportés dans des cages aménagées contribuent à l'amélioration du bien-être chez la poule pondeuse, nous avons mesuré la réactivité de la glande surrénale et la qualité des humérus de poules pondeuses élevées en cages standard et en cages aménagées. Quatre modèles de cages ont été comparés : une cage standard à 5 poules S5, une cage standard à 6 poules S6, une cage aménagée à 7 poules F7 (avec un nid, un bac à poussière, 2 perchoirs, un système raccourcisseur de griffes) et une cage aménagée à 15 poules F15 (avec un nid, un bac à poussière, 2 perchoirs, un système raccourcisseur de griffes). La capacité de réponse maximale a été testée en comparant les corticostéronémies mesurées avant et après l'injection i.m. de 10µg par poule d'ACTH 1-24 (n = 15 poules par modèle de cage). Quinze à 23 poules par modèle de cage ont été abattues à l'âge de 72 semaines. Le poids, les caractéristiques biomécaniques et la composition des humérus droit et gauche ont été mesurés. Les taux

de bases de la corticostéronémie ne différaient pas significativement tandis que l'injection d'ACTH induisait une augmentation significative de la corticostéronémie ($P < 0,001$) dont l'amplitude était comparable pour chaque modèle de cage.

Le poids des humérus, leurs caractéristiques biomécaniques (déformation élastique, résistance élastique, rigidité, résistance à la rupture), leur poids sec et leur pourcentage de matière sèche n'étaient pas significativement différents entre les modèles de cage. Le pourcentage de cendres de l'humérus était significativement ($P = 0,03$) plus faible pour les humérus des oiseaux du modèle de cage S6 (57,4 %) comparés aux humérus des poules des autres modèles de cage (S5: 59,0 %; F7: 58,9 %; F15: 59,7 %). La réactivité de la glande surrénale ainsi que les caractéristiques principales des humérus n'ont pas été significativement améliorées dans les cages aménagées par rapport aux cages standard dans nos conditions expérimentales.

poule pondeuse / cage aménagée / exercice / qualité de l'os / corticostérone / bien-être.

1. INTRODUCTION

The rearing of laying hens in standard cages has been the focus of discussion for several years, especially since the adoption of the 1999 European Directive [10]. Two main criticisms are addressed to this type of cage: the living space is too small and too uniform. These rearing conditions are reported to have a direct impact on the welfare of laying hens [8, 39]. Such space restriction also limits the possibility of bird movement and consequently appears to be at the origin of weak skeletons [17, 36, 41]. In standard cages, hens are housed in an extremely bare environment, without a nest, litter and perches. Hens therefore cannot fully perform laying, dust-bathing and perching behaviours. This impoverishment of the behavioural repertoire may be at the origin of stress and stereotyped behaviour [5, 16, 34, 38, 46, 47, 50]. It might be possible to improve hen welfare with rearing systems that include a larger living space and an enriched environment. Two new systems have been proposed: aviaries and furnished cages [29]. Aviaries have disadvantages since the mortality rate is increased by cannibalism, and certain sanitary problems are enhanced for the animal and affect eggshell quality [2, 33]. The furnished cage might be an acceptable compromise between the standard cage and the aviary because it combines several advantages of both systems and minimises the disadvantages.

The aim of adding furniture in the cages is to increase the possibility that hens express their behavioural repertoire. Providing new items allows hens to perform laying behaviour and dust-bathing but it is also believed to increase their possibility of having physical activities. The effect of exercise on bone has been widely documented in human osteoporosis [51] and rats [13] where physical activity increases bone apposition while, on the contrary, a reduction in mechanical stresses by spaceflight decreases bone density [11]. Improvement of bone apposition via exercise has also been reported in chickens [42, 52] and in laying hens [32]. Increasing bone apposition is of particular interest in laying hens since many of them are affected by cage layer osteoporosis which consists of bone loss and is also considered to be the primary cause of bone fractures during processing. In a survey of a commercial flock, McCoy et al [31] considered that 35% of deaths were attributable to osteoporosis and death occurred earlier in osteoporotic hens (45.5 weeks of age) than in non-osteoporotic hens (51.6 weeks of age). Giving access to perches has been shown to increase tarsometatarsus bone volume in laying hens [23, 53]. Enrichment with perches, nests and dust baths also increased the

maximum strength of the humerus at slaughter in 80-week-old laying hens [3]. However, giving access to perches in cages and systems such as aviaries has sometimes failed to improve tibial breaking strength [23, 48].

Standard cages limit physical activity and, as a boring environment, are also considered to be a source of frustration and consequently a chronically stressful environment [5, 12, 16, 24, 37]. Activation of the adreno-corticotropic axis in response to acute stress has been demonstrated in birds [18, 35, 45] indicated by a rise in plasma corticosterone levels in the peripheral circulation in birds [9, 21, 26, 35]. On the contrary, chronic stress or repeated acute stress such as repeated handling can lead to a progressive decrease in corticosterone response or fear in various species [12, 14, 25]. One approach to investigating chronic stress consists of using ACTH stimulation [49] to measure adreno-corticotropic responsiveness [18, 28, 30, 45].

The data reported here complement zootechnical data [20] obtained in different cage systems in the context of the laying hen directive [10]. In the present study, we focused on two physiological and welfare indicators, bone quality and adrenal responsiveness. The ACTH stimulation test [19, 49] was used to investigate chronic stress in hens reared in standard and furnished cages. Since the humerus is the bone showing the greatest response to husbandry systems [17, 27, 36], humeral characteristics were measured for morphology, biomechanics and composition in order to investigate bone quality.

2. MATERIALS AND METHODS

2.1. Animals and rearing conditions

Standard cages (S) and furnished cages (F) were used according to directive 1999/74/CE. Two models of each type were used. The maximum of hens was housed in each cage, respecting the different limiting factors according to EU-law such as food trough length per hen, area per hen and so on. The cages differed mainly by the cage design and group sizes (see [20] for details). Four cage models were used: S5 (n = 96), a standard cage model with 5 hens per cage (Length 59.5 cm × Depth 55.5 cm × Height 41.5 cm, no extra-furniture); S6 (n = 108), a standard cage model with 6 hens per cage (L 60 cm × D 63.5 cm × H 51 cm, no extra-furniture); F7 (n = 72), a furnished cage model with 7 hens per cage (L 91 cm × D 63.5 cm × H 51 cm, with a nest, dust-bathing box, two perches and claw-shortening) and F15 (n = 24), a furnished cage model with 15 hens per cage (L 233 cm × D 73 cm × H 54 cm, with a nest, a dust-bathing box, two perches, and claw-shortening). The furnished cages provided two plastic perches across the length of the cage.

At 18 weeks of age, beak trimmed ISA-Brown hens were housed in standard cages and furnished cages. The lighting schedule was 15 hours light / 9 hours dark and the room temperature was maintained at 20-22 °C. The hens were fed a standard diet (EM = 2800 kcal, CP = 16.3%, Ca 3.6%, available P = 0.3%). Food and water were available *ad libitum*.

2.2. Adrenal responsiveness

Sixty laying hens from 60 different cages (15 hens for each specific cage model, one randomly chosen hen per cage) were selected at 72 weeks of age. The hens received a single i.m. injection of 10µg per hen (approximately 5 µg·kg⁻¹ BW) 1-24 ACTH (Immediate Synacthen, Norvatis, 2 & 4 Rue Lionel Terray, B. P. 308, F-92506 Rueil Malmaison Cedex) diluted in saline solution (400 µL, 0.9% NaCl w/v). This dose has been shown to induce maximal HPA reactivity 15 min post injection in both laying hens (unpublished data) and in other bird species (ducks, [40]; turkeys and quails, unpublished data). Blood samples (3mL) were collected from the wing vein into heparinised tubes prior to the injection and 15 min post-injection and the hens were placed in a crate during the period between the two samplings.

The plasma was separated by centrifugation, and stored at -20 °C before being assayed. Plasma corticosterone levels were measured in duplicate using a specific radioimmunoassay [15]. All samples from a specific trial were assayed within the same specific assay. Calculations of the radioimmunoassay were performed using the RIASmart Programme (Packard Instrument Co., Camberra, 1989).

2.3. Humeral quality

One randomly chosen hen per cage from 23 furnished cages of each model and 15 standard cages of each model were identified. The marked birds were slaughtered at 72 weeks of age. The right and left humeri bones were removed from the carcasses and were frozen at -20 °C until processing. Humeri were weighed when thawed to obtain a hydrated weight.

A three-point flexure test was then carried out on the bones (Instron Number 1102, High Wycombe, UK). The rate of travel of the mobile anvil was 5mm per min and the width of the bearer was 45 mm. Stiffness was calculated as the slope of the loading curve before the bioyield point [22], i.e. the inflection point of the loading curve.

The humeri were then defatted in ether for 24 h, dried (110 °C for 12 h) and weighed. The bones were ashed (550 °C for 14 h) and ash weight was calculated relative to dry weight in order to obtain the ash percentage.

2.4. Statistical analysis

Mean values between left and right humeri were used. Humeral data were compared using one way ANOVA followed by the PLSD Fisher test. Body weight was not introduced as a covariate in the ANOVA since the humeral weights and the mechanical characteristics of the humeri were not correlated with body weight. The introduction of body weight as a covariate did not modify the ANOVA results. Corticosterone concentrations were compared by repeated ANOVA measures.

3. RESULTS

The ACTH injection effect (= time effect) was highly significant ($P < 0.001$), whereas the cage model effect ($P = 0.49$) and the interaction ($P = 0.25$) were not. Mean basal levels ranged from 1.5 to 3.0 ng·mL⁻¹ of plasma and the mean responses measured 15min ACTH post-injection ranged from 21.5 to 24.0 ng·mL⁻¹ of plasma (Tab. I).

The responses of the humeri to the flexion test were not significantly different between the four models during the elastic part of the loading curve (elastic strain, bioyield point, stiffness, Tab. II). The breaking strength was not affected by the cage model effect (Tab. II).

There was no significant cage model effect on the hydrated weights, dry weights of the humeri, nor on the percentage of dry matter (Tab. III). The ash percentage was significantly lower in the S6 birds compared to the other cage models (Tab. III).

4. DISCUSSION

All the results but one were comparable for standard and furnished cages in our experimental conditions. Thus the parameters related to humeral morphology and quality were not significantly different between the cage models, except in hens reared in one model of the standard cage (S6) in which there was a reduced ash percentage compared to the other cage models. Corticosteroids have well known osteoporotic effects, however, the relationship between stress, blood corticosterone and bone quality remains unclear in birds [7, 44]. This reduction in ash content in S6 cannot be related to an increase in corticosterone level since basal levels did not differ between the cage models. The lower ash percentage for S6 could be due to the higher mortality rate with this cage model, possibly due to the excessively high ambient temperature during the summer months (up to 30 °C in the building) [20]. The heat dissipation was limited in standard cages, especially with 6 hens per cage, and may have had metabolic consequences on the hens and reduction of mineral feed intake (not measured in our experiment). Reduced ash percentage and mortality rate might have been related to clinical or sub-clinical osteoporosis but this cannot be confirmed since no other observations such as broken bones corroborated this hypothesis. Because only one type of standard cage resulted in a reduction in ash content, the lack of enrichment cannot by itself explain this reduction. The higher number of birds in addition to the lack of enrichment may also have contributed to a possible reduction in wing movement: a low number of birds in a cage is more effective in increasing wing movement than the types of cage used [6]. Changes in bone quality are closely related to the patterns of behaviour that are modified since they induce various mechanical strains [43]. Perchery systems have been shown to increase wing flapping and thus to considerably improve breaking strength in the humerus, while terrace systems with ramps from one tier to another increase stepping and breaking strength in the tibiotarsus [27]. In our experimental conditions, the changes in behaviour induced by the furnished cages were possibly too small to enhance bone composition or biomechanical characteristics.

The reason why the decreased ash percentage did not modify the biomechanical characteristics can be explained by the wide range of parameters involved in the flexion test. The flexion curve is dependent on bone composition as well as on bone size (outer and inner dimensions). In the present experiment, the difference in ash percentage appears to have been too slight to induce changes in stiffness or breaking strength, since the bone dimensions may vary in a different way between cage models and counteract the effect of the composition. In the present experiment, we were expecting differences because the perching rate was high (almost 100% at night, Guesdon unpublished data) in both furnished cage models and the humerus has been reported to be stronger in cages with perches [1]. The fact that biomechanical properties of the humeri were not different between cage models could be due to an insufficient power of the statistical

analyses. Because intra-group variability was higher than expected, the tests were also less powerful than expected. However, with our data, an average 1.22% difference can be detected with a sample number of 15 and an average 0.98% difference can be detected with a sample number of 23. These percentages can be observed in the various parameters we studied. We can then assume that the non-existence of the differences between cage models appears to be related to the fact that furnished and standard cages may be considered as very similar systems when compared to aviaries in which more space is available for movement and flying. In some cases, although low stocking densities were used (3045 cm² per bird including the nest box compared to 1524 cm² per bird), the furnished cages used did not allow the hens to perform wing flapping [4]. In battery-caged birds, the strength and the radiographic density of the humerus were lowered by 40 to 50% compared to data obtained in various aviary systems [17]. When Wilson *et al* [53] compared cages with and without perches, they noticed a difference in the trabecular bone but they also noticed that osteopenia was widespread in both types of cages, suggesting that other factors must be studied to improve bone quality in laying hens since the enrichment of cages was not effective enough to achieve this.

Furnished cages were also not effective in modifying adrenal responsiveness whereas a bare environment has been reported to induce chronic stress [12, 24]. Moreover adrenal reactivity has been shown to differ in ducks raised in different rearing conditions (collective vs. individual cages), making it possible to conclude upon a chronically stressful environment [19]. In the present experimental conditions, it was only feasible to measure basal levels and to investigate maximal adrenal reactivity since the birds had to be removed from their cage in order to be injected and bled. The results from our laboratory and those from the literature indicate that a single measurement of plasma corticosterone taken 15 minutes post-injection of a dose of 10µg per hen or higher can be used to test full adrenal gland reactivity. Under our present experimental conditions, we did not observe any difference in corticosterone changes that could have indicated differing states of adrenal reactivity related to a different stress status.

We conclude that furnished cages were not effective in improving humeral quality, possibly because frequent wing movements cannot be performed in these rearing conditions. The present results concerning the investigation of HPA reactivity also gave no indication that these cages were perceived as less stressful than standard cages by the hens.

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Table I. Corticosterone concentration (ng·mL⁻¹ plasma) prior (T0) and after (T15 min) i.m. injection of ACTH (10µg per laying hen at 72 weeks of age) in different cage models.

	Cage models					<i>P</i> -value ¹		
	S5	S6	F7	F15	SEM	Cage	Time	Cage × Time
Number of hens	15	15	15	15				
T0	2.3	3.0	2.2	1.5	0.24	0.49	< 0.001	0.25
T15 (min)	21.6	22.5	23.9	23.5	2.01			

¹One ANOVA with repeated measures.

Table II. Mean values for humeral biomechanical parameters in the 72 week-old laying hen in different cage models.

	Cage models				SEM	<i>P</i> -value ¹
	S5	S6	F7	F15		
Number of hens	15	15	23	23		
Elastic Strain (mm)	0.96	0.91	0.88	0.85	0.04	0.78
Bioyield Point (N)	89.1	93.2	87.6	92.8	2.9	0.87
Stiffness (N per mm)	98.8	115.2	113.4	118.4	3.8	0.34
Breaking Strength (N)	137.8	152.7	150.8	155.0	3.7	0.44

¹One way ANOVA

Table III. Mean values for humeral weights and composition in the 72 week-old laying hen in different cage models.

	Cage models				SEM	<i>P</i> -value ¹
	S5	S6	F7	F15		
Number of hens	15	15	23	23		
Hydrated Weight (g)	48.8	54.5	50.0	49.7	0.86	0.15
Dry Weight (g)	29.6	32.4	30.5	30.1	0.52	0.32
Dry Matter (%)	60.7	59.8	61.3	60.7	0.39	0.67
Ash Percentage (%)	59 ^b	57.4 ^a	58.9 ^b	59.7 ^b	0.26	0.03

¹One way ANOVA, mean values labelled with the same letter do not differ significantly (PLSD Fisher test, $P < 0.05$)

Manuscript 2 (INRA-SRA Partner 5)

In : 22nd World's Poultry Congress; Istanbul (TUR); 2004/06/08-13, Proceedings:321(abstract), CDROM:[/1225.pdf] 4p. WPSA, turkish branch, Izmir (TUR).

Production and stress parameters in laying hens, beak-trimmed or not, raised in standard or furnished cages

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Abstract: ISA Brown laying hens (n = 2028) were reared on litter floor and placed in four types of cages, two standard and two furnished ones, during the productive period. Groups of 5 (S5) or 6 (S6) hens were initially housed per standard cages, whereas 15 birds were housed in each model of the furnished cages (F15M and F15P). The number of birds per cage was calculated in order to fulfil all the criteria of the directive 1999/74 EC of the European Commission (1999). Half of the birds were beak-trimmed at 9 days of age.

The number of eggs laid, the place where they were laid in furnished cages (nest, dust-bath or rest of the cage) and egg quality (dirty, broken or normal) were recorded, as well as the mortality. Feather scores were recorded at 72 weeks of age. Basal corticosterone levels, adrenal sensitivity and maximal reactivity, heterophyl/lymphocyte ratio and immune competence were assessed at the end of the productive period (70 weeks of age and over).

Mortality rates were normal for beak-trimmed hens but were unacceptably high (between 36 and 52%) in non beak-trimmed hens, in both standard and furnished cages. Mortality was mainly due to cannibalism whose occurrence was initiated as soon as the birds were placed in cages. Non beak-trimmed hens also showed poorest feather conditions, than beak-trimmed hens, whereas cage effect was not related to the type per se (furnished vs. standard). Egg production was relatively similar in the four experimental conditions when expressed as hen-day egg production. In furnished cages, eggs were mostly laid in the nest (over 80%), however the proportion of eggs laid out of the nest was still too high as these eggs were more frequently broken or dirty and also showed higher bacterial load (Mallet *et al.*, 2003).

Corticosterone measurements, heterophyl/lymphocyte ratio and antibody production in response to immune challenge show that stress levels might be higher in enriched than in standard cages and in non beak-trimmed than in beak-trimmed hens in our experimental conditions, i.e. with this specific genotype. Indeed, our results show that cannibalism can be a problem when the hens are not beak-trimmed, even if they are housed in furnished cages and/or in small group size. Moreover, nest design should be improved in order to reduce the proportion of second grade eggs.

Key words: Laying hen, rearing conditions, egg laying, stress, welfare.

Introduction

In Europe, usage of standard battery cages will be phased out in 2012 (Directive 1999/74/CE). Among the different possible alternative systems, the furnished cages are appealing because they retain some of the practical advantages of the standard cage while they simultaneously offer a richer environment allowing the bird to express a wider range of behaviours. The present experiment was undertaken to compare results obtained for both production parameters and physiological stress indicators, for hens which were beak trimmed or not at 9 days of age and placed in four different models of standard (2) and furnished (2) cages. The hypothesis was that if furnished cages were going to be an appropriate alternative, they should provide better welfare conditions for the birds. A

direct comparison of behaviour expressed in the two systems, i.e. standard versus furnished, and a quantification of equipment use would probably not provide much pertinent information. It has already been observed that the equipment are used by some hens and not by others (Appleby and Mc Rae, 1983). Performances such as egg production and mortality should be comparable or better to what is obtained in standard cages as they are also relevant welfare indicators. We therefore used both production parameters and physiological stress indicators for welfare assessment. Among the physiological indicators, adrenal reactivity and the activity of the immune system seems to be the best indicators of chronic stress. Thus, in case of chronic stress, a hyper-sensitivity followed by a depletion of the adrenal maximal reactivity can successively be observed depending upon duration and intensity (Dantzer and Mormède, 1979; Koelkebeck *et al.*, 1986). Furthermore, the immune system of chronically stressed animals is characterised by higher heterophyl/lymphocyte ratio (Wolford and Ringer, 1962) and by a depressed antibody production (Gross and Siegel, 1973; 1980). These different physiological stress indicators were therefore used in order to evaluate welfare consequences of housing hens in standard or furnished types of cages and beak-trimming practice.

Material and methods

ISA brown female chicks, from which half of them were beak-trimmed at 9 days of age, were reared on floor until 18 weeks of age, then transferred in cages for the productive period (n=2028). Four models of cages were used; two models of standard cages in which we housed 5 or 6 hens, respectively (S5: n= 96 (480 hens) and S6: n=108 (648 hens)), and two models of furnished cages in which we housed 15 hens (F15P: n=36 (540 hens) and F15M: n=24 (360 hens)). These standard and furnished cages were located in two similar rooms of a unique building. Densities in the cages were adjusted in order to fulfil all criteria of 1999/74/EC Directive (1999) and are described in details in Guesdon (2004). Fluorescent lighting was used and on 15 hours daily with intensity ranging between 1.5 and 3 lux in the front side of the cages.

Mortality and egg laying were recorded daily during the 52 week productive period. On the weekdays, eggs were recorded according to where they were laid in the furnished cages (nest, dust-bath and rest of the cage) and to their quality (dirty, broken and normal). Eggs were also candled to record the proportion of cracked eggs four times during the 52-week production period. Mortality was summed over the whole 52-week period whereas egg production was grouped in 13 four-week periods. Feather conditions were appreciated for the hens included in the physiological stress indicator assessment at the age of 72 weeks using a technique derived from Tauson (1984) as described in Guesdon (2004). Five body areas (neck, breast, wing, back, and tail) were scored from 0 (low ranking) to 6 (high) taking into account both the quality and the quantity of the feathers.

In order to assess basal corticosterone levels, as well as adrenal sensitivity and maximum reactivity, corticosterone concentrations were measured, using a specific radioimmunoassay (Etches, 1976), in plasma extracted from samples (n = 48 x 2) collected immediately before (basal level) and 15 minutes after intra-muscular injection of .625 or 10 µg/kg body weight of 1-24 ACTH (Immediate Synacthen, Novartis). Heterophyl/Lymphocyte ratio (H/L) were estimated from the same initial blood samples, after establishing leukocyte percentages from a smeared drop of blood stained with May-Gründwald and Giemsa stains (Lucas and Jamroz, 1961). Humoral immunity were assessed by immunising the same hens (n = 128, 16 (2x8) per cage model and beak status) with human IgG (100 µg/hen), sheep red blood cells (SRBC; 1 ml of a 2% solution/hen) and Key Hole Limpet Hemocyanin (KLH; 250µg/hen), at 70 weeks of age. Blood samples for the assessment of immune responsiveness were collected immediately before immunisation to determine presence of antibodies/cross reacting antibodies, then 3 and 7 days after and thereafter once weekly for 4 weeks. Detailed dynamic of serum antibody levels will be available for the 3 antigens but only the measures related to human IgG antibodies are available at present (Figure 1 & table 1: 0, 8 and 28 days after immunisation). Antibody titres against human IgG were determined using an enzyme-linked immunosorbent assay (ELISA) (Michel *et al.*, Unpublished).

Physiological (corticosterone and antibody levels) and production measures (13 periods) were analysed with an ANOVA for repeated measures, whereas H/L ratio and plumage condition were with a multifactorial ANOVA. Whenever P values reached significance ($P < .05$), post-hoc Fisher PLSD were used for pair comparisons.

Results and discussion

All the results are summarised in table 1 and antibody responses are detailed in figure 1. Mortality was highly influenced by beak trimming. In beak-trimmed hen cages, mortality rates ranged between 3 to 7% while there were over 35% in cages housing non beak-trimmed hens. This mortality was mostly due to cannibalism whereas no death could be attributed to cannibalism in beak-trimmed hens. Somehow, lower mortality rates were observed in furnished than in standard cages for non beak-trimmed hens. This is surprising as it is commonly accepted that larger group size leads to higher risk of cannibalism (Hughes, 1982), however a beneficial effect of enrichment and/or of the much lower densities cannot be excluded. It should be stated that although our results were very poor when hens were not beak-trimmed, good results have been reported elsewhere using another genotype (Moe *et al.*, 2004). We cannot exclude therefore that different genotypes may differ in their adaptability per se or capacity to cope with environmental factors. We cannot exclude either an effect of the fluorescent nature of the light although the light intensity was really low, being below 3 lux.

Due to these very high mortality rates, hen housed egg production was very low in non beak-trimmed cages. Hen day egg production was affected by cage model, but this was mostly due to the lower laying rate observed for the F15P cages. We cannot exclude that the number of collected eggs did not correspond exactly to the egg output and we did not observed any effect of beak-trimming on hen day egg production.

The percentages of eggs laid in the nest were rather low in the two models of furnished cages (80 and 85% in F15P and F15M, respectively). This leads to both economical and sanitary problems as eggs laid outside the nest are more frequently broken or dirty. These eggs also had a higher bacterial load on the shell (Mallet *et al.*, 2003). Low egg laying rates in the nest was at least partly due to the attractiveness of the Astroturf mat used as the dust-bath. Therefore, nest design should be improved and/or dust-bath mat better differentiated from the floor nest in order to reduce the proportion of second grade eggs.

Plumage condition was significantly influenced by beak condition and by cage model. Feathering was clearly higher in beak-trimmed, than in non beak-trimmed hens. The cage effect was less clear and seemed to reflect model effects rather than a type (standard vs. furnished) effect. Beak trimming by such can give rise to acute and chronic pain (Gentle, 1986; Duncan *et al.*, 1989). On the other hand, the absence of beak trimming can lead as presently observed to feather pecking and cannibalism, which may also lead to chronic stress state. This aspect was assessed using various physiological indicators of chronic stress. Basal levels of corticosterone were low but significantly affected by cage with the hens kept in F15P cages showing a significantly higher basal level. On the other hand, beak-trimming effect were close from significance with non beak-trimmed birds which had higher basal levels of corticosterone. This last observation further emphasised our hypothesis, regarding the possible detrimental consequences of feather pecking (feather cover and cannibalism) in the non beak-trimmed hens.

Table 1: Cages main characteristics and results for the different production and physiological measurements. (BT = beak trimmed hens, Intact = Intact beak). P values = probability of the null hypothesis.

Cage models	Standard S5		Standard S6		Furnished F15M		Furnished F15P		P values	
Hens housed (Day 0)	5		6		15		15			
Cage size (cm ²)	3302		3780		17520		11340			
Density (cm ² /hen)	660		630		1168		756			
Beak	BT	Intact	BT	Intact	BT	Intact	BT	Intact	Cage	Beak
Mortality	3	51	5	52	3	43	7	36	<.001	<.001
Eggs/Hen Housed	82.4	57.0	82.8	53.5	83.1	56.9	80.5	59.1	NS	<.001
Eggs/Hen Day	83.5	83.8	84.8	83.1	84.4	82.6	82.2	76.1	<.002	NS
% Eggs in Nest	-	-	-	-	80	-	85	-	NS	-
% Eggs in Dust-Bath	-	-	-	-	13	-	9	-	NS	-
% Eggs elsewhere	-	-	-	-	7	-	6	-	NS	-
% Broken Eggs	4.1	-	1.9	-	5.6	-	3.8	-	<.001	-
% Cracked Eggs	8.1	-	6.2	-	9.8	-	15.8	-	<.001	-
% Dirty Eggs	4.9	-	4.9	-	3.0	-	7.1	-	<.001	-
Feather cover	11.1	3.5	23.0	6.0	17.5	10.3	12.6	5.1	<.001	<.001
Corticosterone (ng/ml)	1.6	1.7	2.2	3.2	1.5	1.6	1.6	2.2	<.01	=.08
After .625µg/kg ACTH	9.5	10.5	6.4	10.2	13.0	13.7	6.8	8.5	<.05	NS
After 10µg/kg ACTH	12.0	16.9	18.8	17.7	16.3	20.1	18.1	16.4	NS	NS
H/L	.39	.28	0.79	.26	.92	.89	1.22	.89	<.05	=.19
AB Day 0	64	85	77	24	61	47	43	31	NS	<.001
AB Day 8	119	1108	113	933	1132	914	1217	1009		
AB Day 28	5	5	7	7	7	7	7	7		
AB Day 28	688	332	527	292	491	328	484	486		

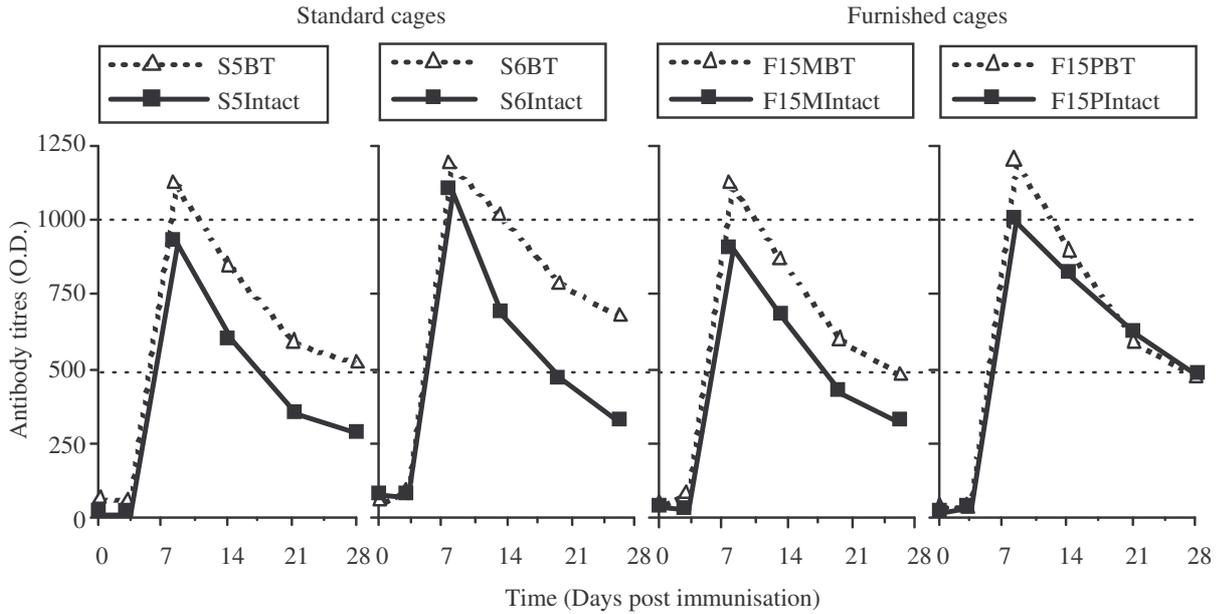


Figure 1: Antibody titres in laying hens, beak-trimmed or not and raised in standard (S5 & S6) or furnished cages (F15M & F15P), before (0) and after immunisation with Human IgG at 70 weeks of age.

High chronic stress level is usually associated with adrenal hyper-sensitivity, followed by adrenal depletion, illustrated by a decrease in maximal reactivity, depending on intensity and chronicity of the stressor (Dantzer and Mormède, 1979; Koelkebeck *et al.*, 1986) and decreased immune-competence (Gross and Siegel, 1980). With the low dose of 1-24 ACTH used to assess adrenal hypersensitivity, the cage effect was significant but it was then the furnished F15M cage that showed the highest level of corticosterone. There is also a tendency for a beak effect ($P=0.08$) with highest levels of corticosterone being measured once again in non beak-trimmed hens. Housing conditions were not perceived as a major source of chronic stress since, although we observed significant increases in corticosterone following injection of the highest dose of 1-24 ACTH, we did not observe any significant effect of the cage and beak factors.

A significant effect of cage on H/L ratio was found with birds from furnished cages showing significantly higher values. Alternately, while cage effect was not significant, beak-trimmed birds showed significantly higher antibody titres and somehow different time course responses after immunisation. These two effects could suggest higher stress levels in furnished cages and in non beak-trimmed hens.

Conclusions

We did not observe any systematic effect of cage type (standard vs. furnished) on the various stress indicators studied however, when significant differences are observed, they are indicating higher stress levels in furnished than in standard cages, even when used at very low densities exceeding the official requirements. It is likely that this observation does not result from a difference between cage type *per se* but of a single model effect and/or of an interaction between different factors such as cage type, group size and genotypes. Our results also indicated that, when tendencies or significant beak effects are observed, beak-trimmed hens are showing lower stress levels. This result is further emphasised by behavioural data, showing that these beak-trimmed hens also expressed lower fear reactions than intact hens (Guesdon, 2004). Taken together, these results would suggest that beak trimming results in possibly better welfare for this specific genotype.

Evidences from this specific study and the literature suggest that genotypes respond differently to environmental factors. Genotype's adaptability may therefore be the most important

single factor to ensure welfare in hens to be housed in cage alternatives with large group sizes. Genetic adaptation to rearing conditions needs to be considered as one of the major breeding goals in order to place the "adequate" hybrid in the right environment and to avoid disaster in terms of welfare.

Acknowledgements: This project and V. Guesdon were financially supported by grants from CNPO, DGAL and OFIVAL. We thank M. Couty, B. Gaultier, D. Boulay and N. Wacrenier for their expert technical assistance.

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Manuscript 3 (INRA-SRA; Partner 5)

(unpublished data)

**The effects of cage model, density, group size and genotype
by Guémené et al.,**

The aim of the current study was to examine the differential effects of cage models, stocking density, group size, genotype and their interaction on corticosterone basal levels as well as adrenal sensitivity and maximal reactivity. One model of standard cage (SC) and 3 models of furnished cages (FC) were located in two different rooms of a same barn. Group size in SC ranged of 4 to 6 on a surface of 3302cm². In FC, group size were of 11, 15 or 20 in one model (FCPi A, B, C, D & E:11340cm²), 30 in a second model (FCPi C: 22680cm²) and, 23 and 31 in a third model (FCM: 17375cm²). FC differed in their equipment: perches, nest, scratching area. On the 32 week of lay, adrenal sensitivity and maximal adrenal responsiveness was evaluated 10 and 60 min post-injection by measuring the changes in blood corticosterone from basal level induced by the i.m. injection of 0.5 and 10µg/kg BW of 1-24 ACTH (n = 16 to 24 hens per cage model), respectively. Responses to the injections did not differ between genotypes at 10min post injection but levels remained higher after 60min for one genotype indicating a higher adrenal reactivity (Fig. 1). Likewise, a higher sensitivity was measured in group of 30 compared to 15 birds per cage, although they had a similar surface per hen (Fig. 2). Stocking density in interaction with cage model (FCPi C & D differing only in the position of the perchs) affected corticosterone basal level (Fig. 3). In conclusion, larger stocking density or group size, as well as their respective interactions with the cage model, can affect HPA axis functionality, a result which suggest a higher sensitivity to stressful events under these housing conditions. On the other hand, it remains to be explored if the differences observed between genotypes are due to differential responsiveness capacity per se and if it can result in different sensitivity to stressful conditions.

Figure 1: Corticosterone levels (ng/ml plasma) measured 10 and 60 min post-injection of 1-24 ACTH at a dose of 10µg/kg B.W. in laying hens from two different genotypes and housed in conventional or furnished cages. (Mean ± S.D.)

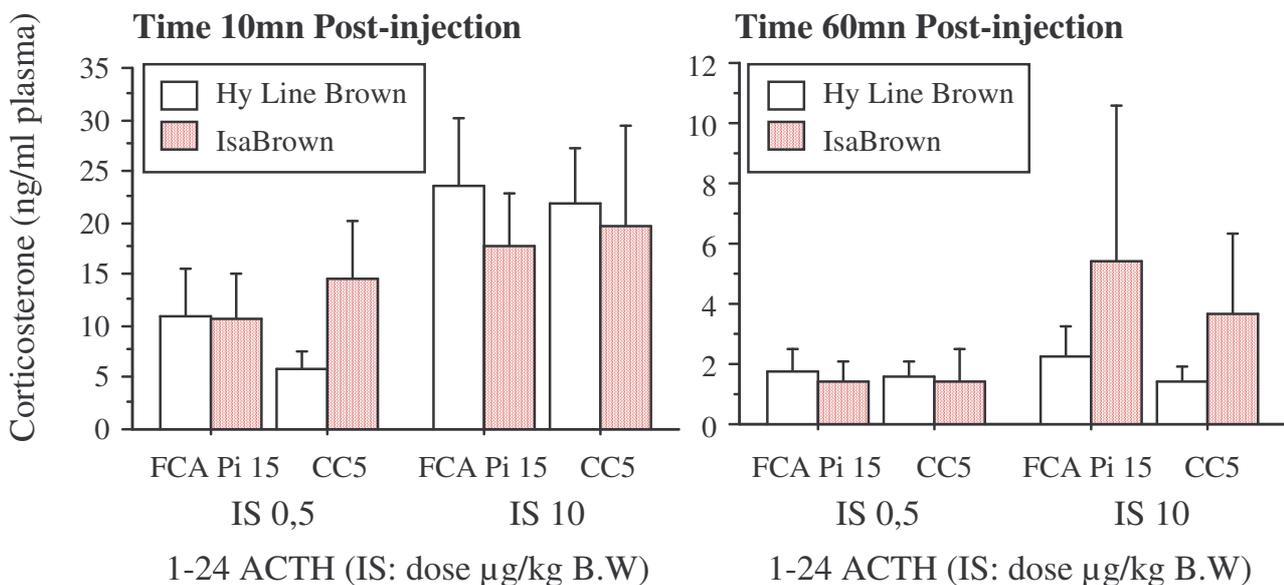


Figure 2: Corticosterone levels (ng/ml plasma) measured 10 min post-injection of 1-24 ACTH at dose of 0.5 or 10 µg/kg B.W. in laying hens housed in furnished cages at two different group sizes. (Mean ± S.D.)

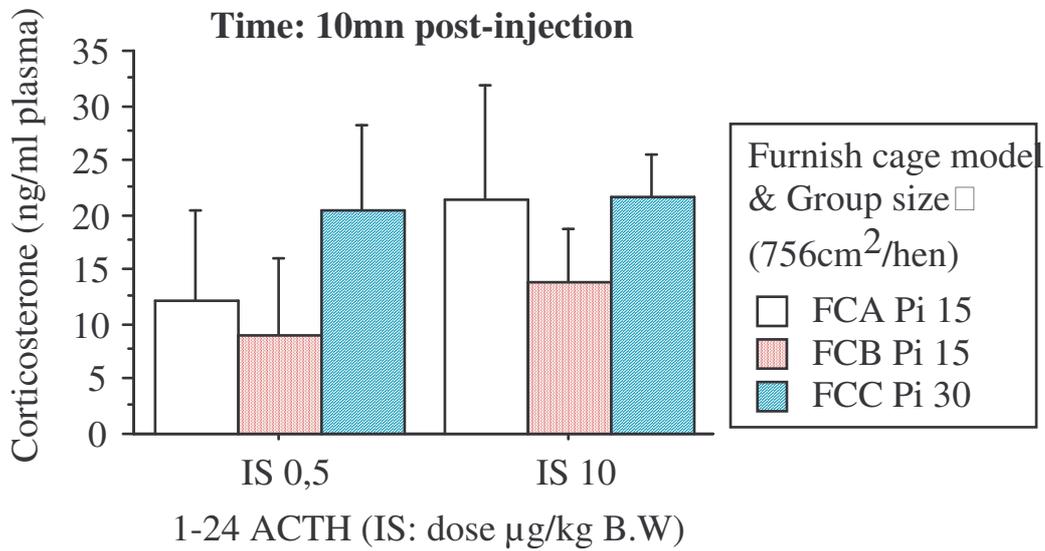
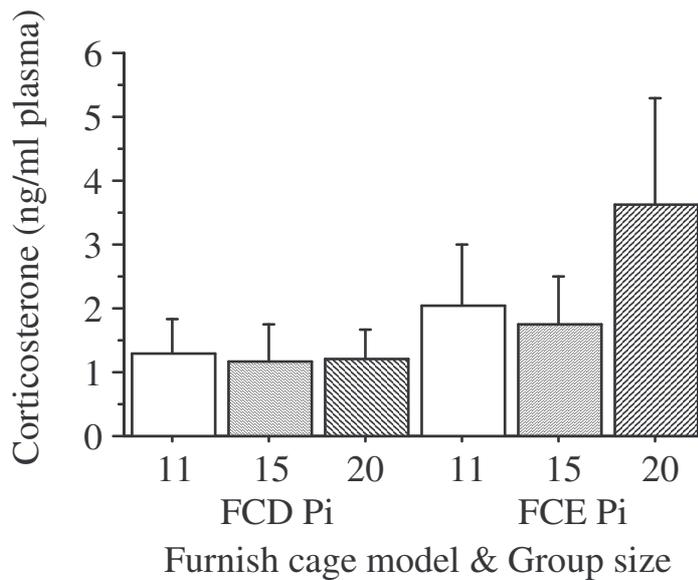


Figure 3: Corticosterone basal levels (ng/ml plasma) measured in laying hens housed in furnished cages at 3 different densities. (Mean ± S.D.)



Manuscript 4 (UNIZAR; Partner 9)

(unpublished data)

The effects of cage model, genotype and rearing treatment upon faecal corticosterone

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The aim of the current study was to examine the differential effects of housing system, rearing system and breeds by measuring corticosterone metabolites levels in faeces, a physiological indicator of chronic stress. Four experiments were realized. In the first one, brown hens from two different breeds were placed in three different housing systems (2 models of furnished cages and 1 conventional one; $n \geq 240 \times 3 \times 2$). In the second one, hens from a unique breeds ($n = 320$) were originally reared on floor or in cages and placed in a single model of furnished cage. In the third one, hens from two different breeds (White (Leghorn) vs. Brown (Rhode Island); $n = 160 \times 2$) were reared in cage and placed a single model of furnished cage. In experiment 4, two breeds and two housing conditions (Furnished and conventional cages) were look at in a factorial approach. Corticosterone levels were evaluated after extraction and measurement of its metabolites present in faeces, using an antibody directed against 5β -pregnane, 3α , 11β -diol 20one. In experiment 1, higher levels were measured in conventional than in furnished cages, and within the two models of furnished cages, the lowest data were measured in the “Spanish” model (Table 1). It could have been concluded from these results that the conventional cages are a more stressful environment and that differing stress states can results from the use of furnished cages. However, although the same situation prevailed by the end of the laying period for one genotype out of two in experiment 4, the reverse situation was observed for both genotypes earlier in the laying period (Table 4). Therefore, it is not to exclude either that the present data resulted from differences in the bird metabolic states due, for example, to differing levels of physical activity and/or feed intake, two parameters that were not looked at in the course of these studies. In experiment 2, floor-rearing hens presented significantly higher corticosterone levels than cage rearing hens when placed in cages during the laying period. These results suggest that hens reared in cages will better adapt to cage systems latter than those reared in floor systems. Data from experiments 3 and 4 showed significant breed effects with white breeds having higher corticosterone levels than brown ones (Table 3) and differences between brown breeds with interaction between the age and housing system factors (Table 4). The question being whether hens from a specific breed have higher basal levels, related from example to different overall metabolites rates, or if they are more stressful/stressed in these specific experimental conditions? From the present data, it is thus not possible to conclude from the differential results obtain with different breeds, but the existence of such differences should be taken into account when evaluating and comparing adaptation of hens to different housing systems across laboratories. On the other hand, the harmonisation of the pre-laying rearing environment with the one used later appears to be a major focus to ensure a better adaptability of the hens during their productive period.

Table 1: Faecal corticosterone levels (ng/g) in laying hens housed in 3 different housing systems.

Housing system	faecal corticosterone (ng/g)
Conventional cage	36.3 ± 2.4 c
Furnished Spanish cage	12.7 ± 3.0 a
Furnished German cage	18.7 ± 2.4 b

Table 2: Faecal corticosterone levels (ng/g) in laying hens reared in floor system and cage.

Rearing system	faecal corticosterone (ng/g)
Floor	32.4 ± 3.6 a
Cage	18.7 ± 3.6 b

Table 3: Faecal corticosterone levels (ng/g) in two breeds of laying hens housed in furnished cages.

Breed	faecal corticosterone (ng/g)
Hy Line White	45.6 ± 4.9 a
Isa Brown	18.7 ± 3.6 b

Table 4: Corticosterone plasma levels (ng/ml) levels in two breeds of laying hens (Isa Brown & Hy Line Brown) housed in furnished cages (Big Dutchman) or in conventional cages (Zucami, Spanish model)

Age	Breed	Cage type			
		Furnished		Conventional	
		Hy-Line	Isa Brown	Hy-Line	Isa Brown
1/3 of the laying period		19.9 ± 1.1 aA	13.0 ± 2.0 bA	6.8 ± 1.1 cA	8.6 ± 1.1 cA
End of the Laying period		11.8 ± 1.6 aB	4.6 ± 0.6 bB	12.8 ± 1.6 aB	17.5 ± 1.7 cB

Different small letters means the significant differences in each line. $p \leq 0.01$
 Different capital letters means the significant differences in each row. $p \leq 0.01$

Manuscript 5 (UNIBRIS & ADAS; Partners 3 & 7)

Accepted for publication in British Poultry Science

Effects of Stocking Density, Flock Size and Management on The Welfare of Laying Hens in Single-Tier Aviaries.

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Short Title: Welfare of Hens in Aviaries

Abstract

1. Management practices, stocking rate and flock size may affect laying hen welfare but there have been few replicated studies in commercial non-cage systems. This study used a broad range of physical and physiological indicators to assess the welfare of hens in 36 commercial flocks.
2. Six laying period treatments were examined with each treatment replicated six times. Three stocking rates were compared (7, 9 and 12 birds m⁻²). At the highest stocking rate birds were kept in small (n= 2450) or large (n=4200) flocks. Additionally, at 12 birds m⁻², in both small and large flocks, birds were subjected to either standard (SM) or modified (MM) management.
3. Bone strength, fracture incidence, heterophil:lympocyte (H:L) ratio, live weight, organ weights, serum creatine, serum osmolality, muscle pH and faecal corticosterone were measured on samples of birds at the end of the rearing period and at the end of lay. During the laying period, mortality, production and integument condition were recorded at regular intervals.
4. Birds housed at 7 and 9 birds m⁻² had higher mortality, lower % liver weight, and worse plumage condition than birds housed at 12 birds m⁻² by the end of lay. There were no clear effects of flock size on the welfare indicators recorded. Modified management resulted in improved plumage condition.
5. At the end of the rearing period, fracture incidence was negligible and H:L ratio was within a normal range. By the end of lay fracture incidence was 60% and H:L ratio was high across all treatments, suggesting an overall deterioration in the welfare of these birds.

Introduction

The use of non-cage housing systems for laying hens is likely to increase as the 2012 EU ban on conventional cages approaches. Council Directive 1999/74/EC (Communities, 1999) proposes a maximum stocking rate of nine birds per m² for hens in non-cage systems after 2012. However, few studies have examined the effects of stocking rate on bird welfare in commercial systems and there is no clear scientific evidence for this recommendation.

Stocking rate has been examined as a factor in a number of epidemiological surveys of laying hen behaviour (e.g. Gunnarsson et al., 1999; Oden et al., 2002) but most studies have been conducted in

relatively small-scale experimental facilities (e.g. Nicol et al., 1999; Estevez et al., 2002). Drawing overall conclusions is difficult due to the wide range of stocking rates examined (1.3 - 30 birds / m²) and the two ways by which these have been manipulated. This is either by altering group size (e.g. Nicol et al., 1999), or by changing spatial allowance (e.g. Carmichael et al., 1999), two variables that may themselves have differential effects on bird welfare (Cooper and Albentosa, 2003). Indeed, the results of experiments conducted on small flocks of birds (tens or hundreds) cannot necessarily be generalised to the commercial situation where birds are housed in flocks of many thousands, due to scaling effects. Aggression, for example, is relatively high in small flocks but lower in large flocks, as birds adopt strategies to avoid negative social interactions (Carmichael et al., 1999; Hughes et al., 1997; Keeling et al., 2003; Nicol et al., 1999). There may be many other effects of this kind, suggesting that research on a commercial scale is required if the results are to be directly relevant and applicable to the commercial situation.

One of the biggest difficulties when working on a commercial scale is in achieving full replication of flock size or stocking density treatments. Sometimes replication is not attempted and detailed observational studies of just one commercial flock are published (e.g. Oden et al., 2004). Other studies, even when conducted on experimental farms, sometimes report no (n = 1), or minimal (n= 2), treatment replication (Carmichael, et al., 1999; Channing et al. 2001). Full treatment replication on a commercial scale can probably only be achieved by an extended study over many flock cycles.

It should also be noted that, although average stocking density can be prescribed, bird distribution on the ground can be very uneven. A study of birds in small percheries at an average stocking rate of 18 birds/m² found that the localised distribution of birds varied from 9 to 41 birds per m² (Channing et al. 2001). Uneven bird distribution gives increased freedom of movement for some birds and decreased freedom for others (Appleby 2004) but difficulties in accessing resources are not borne equally. Evidence suggests that some individuals may fare particularly badly (Freire et al., 2003).

There is a general consensus that animal welfare should be assessed using a variety of different indicators (Mendl, 2001) and this approach has been taken by the European scientific working group reviewing the impact of housing on laying hen welfare (Blokhus et al., 2005). Candidate indicators include overall mortality, physical health, physiological status and behaviour. However, studies of stocking rate have so far concentrated almost exclusively on behaviour, particularly feather pecking and cannibalism. (Nicol et al., 1999; Huber-Eicher and Audigé, 1999; Carmichael et al., 1999; Gunnarsson et al., 1999; Oden et al., 2002). There is a need for studies that take an integrated approach to the assessment of hen welfare, by measuring a variety of different parameters. The aim of the current study was therefore to examine the differential effects of stocking rate, flock size and management on the welfare of laying hens, using a range of indices under commercial conditions. This paper reports the results of the measurements of non-behavioural parameters. Mortality, production, and integument condition were assessed during lay. Additional measures of stress and bone condition were taken at the onset of lay and at the end of lay. Observations of bird behaviour and distribution during the laying period were also taken, but these are reported separately (Zimmerman et al., 2005; Zimmerman et al., submitted). The benefits of working on a commercial farm were tempered by practical constraints, which limited the independence of some replicates, and produced house x treatment confounding. Despite these difficulties, the results enabled us to assess laying hen welfare under current commercial conditions with a broader range of measures and a greater degree of replication than is normally achieved.

Materials and Methods

The study was conducted on a commercial farm that had six, structurally identical, single-tier aviaries available. Six different laying period treatments manipulating stocking density, flock size and management type, were examined with each treatment being replicated six times. Three stocking rates were compared (7, 9 and 12 birds m⁻²). At the highest stocking rate birds were kept in small (n= 2450) or large (n=4200) flocks. Additionally, at 12 birds m⁻², in both small and large

flocks, birds were subjected to either standard (SM) or modified (MM) management. A summary of each treatment is given in Table 1. Overall, the study design used 113,400 birds.

Table 1 here

Shaver pullets were obtained from commercial suppliers. The birds had been beak-trimmed at approximately 10 days of age, and fully vaccinated before transport to laying houses at 16 weeks of age. Eight rearing flocks from five different farms contributed birds to the study. Four of the rearing farms provided perches for the pullets but one farm, which contributed two flocks to each of Treatments 1, 2 and 3, did not.

Standard Management and Housing

Management and husbandry followed the company's standard procedures except where the experimental protocol required a departure from normal practice. Food and water were available *ad libitum* through a chain feeder and bell drinker system respectively. The amount of feeder and drinker space per bird was kept constant in all treatments. Communal Vencomatic nestboxes were provided and eggs were collected automatically on a motorised belt. The nests were provided at a constant ratio across treatments, in one tier in Treatments 1 and 2 and in two tiers in the other treatments, with an alighting rail to allow proper access. Hens had on average 10 cm feeder space, and one bell drinker per 88 birds was available. Environment control was achieved through an automated ridge extraction system operating on a minimum interval timer with thermostatic override. The target ambient temperature was 20° C. Lighting was provided by fluorescent tubes, supplemented by tungsten bulbs. The light period was between 0500 and 1900 hours, with the lights in the nest boxes on between 0400 and 1800 hrs. The target light intensity was 10 lux.

Modified Management

In Treatments 4 and 6, the birds had nipple lines (1 nipple cup per 10 hens) instead of bell drinkers and there were no lights in the nest boxes. These modifications had been shown to decrease the risk of feather and vent pecking (Green et al., 2000; Pöttsch et al., 2001).

House Design

Each house was divided into two separate pens (A and B) by a wire mesh barrier (see Figure 1), such that each pen housed a separate flock undergoing the same treatment. Occasionally birds managed to fly or squeeze through the barrier, but this was uncommon. The length of the pens differed between treatments. So, for example, the house with Treatment 1 contained a total of 4,900 birds (2,450 in each pen) at the start of the treatment. Pens (measuring a maximum of 30 x 14 m for the large flocks) consisted of a litter area (width: ± 2.20 m) over one third of the house area, and a raised (± 1 m high) slatted platform (width ± 3.70 m) which allowed droppings to accumulate underneath, over two thirds of the house area. Two rows of nest boxes subdivided a pen into two sides. Both sides were identical in layout but mirrored. The slatted platform contained feeders and drinkers along the whole length of the pen.

Study Constraints

The experiment took four years to complete. Due to logistical difficulties treatments were not run simultaneously, but were staggered across seasons. It was also not possible randomly to assign treatments to houses, because of financial and practical constraints, e.g. the expensive additional tiers of nest boxes required for the higher stocking density treatments could only be installed in some houses. So, treatment was confounded with house, except for Treatment 5 in which the first four replicates were run at the same time in two separate houses (this was due to a delay in populating the house for the first round, caused by a fire in the rearing flock accommodation), and Treatment 3 in which the last two replicates were run in two separate houses. Close monitoring of environmental conditions at the start and throughout the experiment was undertaken to ensure that any apparent house effects were considered.

Assessment of Rearing Flock Condition

Prior to placement 50 birds from each rearing flock were individually caught from random positions within the house. These were manually restrained and rendered immediately unconscious using a percussive device designed for humane casualty slaughter (ref). Following stunning, birds were bled using a full ventral neck cut, which severed both carotid arteries and jugular veins. During bleeding, blood samples were collected from 25 birds per flock for haematological and biochemical analysis. After bleeding a small piece of breast muscle was also collected and immediately frozen in liquid nitrogen. All birds were transported to the University and held at +5°C. During dissection liver, heart, spleen and the Bursa of Fabricius, were carefully removed and weighed. The presence or absence of old breaks was recorded in the keel (sternum) and furcula (fused clavicles), and the severity of damage to the keel was categorised using a 5-point photographic scale (Wilkins et al., 2004). The limb (humerus and tibia) and keel bones were removed, cleaned of soft tissues, and the peak breaking strength determined from a three-point compression test using a Stevens CR analyzer. The distance between the two fixed supports was 52 mm for the tibia and 36 mm for the humerus. The bones were positioned so that the third compression point made contact at the midpoint. Compression was achieved by applying force at this third point at a constant head speed of 50mm/minute. Breaking strength of the keel was determined using the same apparatus modified to support the keel while applying a round head (8 mm) probe to two positions on the bone, directly below the manubrial spine (Point A) and on the lateral surface (point B).

Blood samples were collected into EDTA tubes for the analysis of heterophil:lymphocyte ratio. Manual blood smears were prepared and stained with modified Wright stain. The smears were then examined under the microscope by two experienced veterinary haematologists, 100 heterophil/lymphocyte cells were counted and the cell ratio calculated. Blood was collected into a heparinised tube, centrifuged immediately and the plasma frozen in liquid nitrogen for subsequent analysis. The plasma was analysed for Creatine phosphokinase (CPK) with a Randox CK-NAC kit (Randox Laboratories), and for osmolality (Camlab Micro-Osmometer). Faecal samples were collected, freeze-dried and sieved. Faecal corticosterone was extracted using 90% methanol/10% distilled water. The supernatant was vortexed for 2 hours. Diluted samples were assayed using an ICN ImmunoChem double antibody 125 I RIA kit and the precipitate counted in a Gamma counter. pH was measured in pectoralis major muscle that had been left for 24 hrs at +2°C to reach ultimate pH. Duplicate 1 gm samples of muscle were then homogenised in 10 ml of distilled water and measurements made using a Radiometer (Copenhagen) PHM80 meter and an Ingold combined electrode (lot 406-M6-DXK-57/25) calibrated with standard buffers.

Assessment of Laying Flock Condition

Mortality

The producer recorded bird deaths daily on standard record sheets for each flock. Mortality was calculated for each flock on a period basis of 4 weeks.

Production parameters

The producer recorded the number of eggs laid, whether they were laid in the nests, on the slatted floor or on the litter, and whether they were dirty, cracked or soft each day. The rate of lay was calculated for each pen on a period basis of four weeks. Feed was allocated automatically into the house, via chain feeder hoppers. Feed usage was recorded weekly. Egg weights were calculated periodically by weighing samples of 90 eggs from each flock.

Physical Condition of Birds

Thirty birds per pen were randomly selected, weighed individually and assessed for feather and foot condition. This paper reports data from assessments made at 32-35, 48-51, 56-59 and 68-70 weeks of age. Feather loss was assessed using the following five-point scoring system for the different areas of the body (head, neck, back, wings, tail, abdomen and breast):

- 1 Complete feather cover
- 2 Worn feathers detectable

- 3 Badly worn feathers detectable or small bare patches ≤ 25 mm diameter.
- 4 Badly worn feathers detectable or large bare patches up to 75% of the area
- 5 Badly worn feathers over most of the area, or mostly devoid of feathers.

In addition, at the same time periods, each bird was allocated a score for the number of pecks to the vent or the number of peck marks visible on the comb. Each bird was then assessed for foot condition. The left foot of each bird was observed and the following areas assessed for damage: pad, digit and claw matrix on a five-point scoring system:

- 1 Good condition – no lesions
- 2 Lesions which are clearly visible but of minor importance and/or frequency
- 3 Lesions which are considered severe but not widespread
- 4 Poor foot condition, several lesions over much of the area, no evidence of bleeding.
- 5 Very poor foot condition with inflamed and/or bleeding lesions visible over much of the area.

Environmental Conditions

Litter moisture content and litter friability were assessed from seven different sampling points per flock. A 5cm square core of litter was weighed before and after drying at 100°C to assess % moisture. Litter friability was assessed on a five-point subjective scale from 1 (fully friable) to 5 (extensive capping over >80% of assessment area). This paper reports data from assessments made at 32-35, 48-51, 56-59 and 68-70 weeks of age. Light intensity was measured in lux in each house at onset of lay and approximately every eight weeks thereafter. Repeated readings were taken at nest-box entrances (using both tiers of nest-boxes in treatments 4 and 6) and at the highest value obtained when the photosensitive probe was incident to the respective light source. Atmospheric ammonia determinations were made using Draeger tubes held approximately 1m from the ground, in a range of locations throughout the house. Ammonia and relative humidity measurements were taken approximately every 8 weeks. Temperature was recorded by suspending maximum/minimum thermometers on each side of the houses at bird level.

End of Lay Assessment of Bird Condition

At the end of the laying period 50 birds selected at random from each flock were caught and euthanased as described for the rearing flocks, with the exception that the Bursa of Fabricus could not be dissected as it was generally too small and difficult to find. Blood and muscle samples were obtained from 25 birds per flock as described previously. A faecal sample was additionally taken for the assessment of corticosterone metabolite concentration.

Statistical Analysis

Descriptive data are presented for the condition of the birds at the end of the rearing period. Lack of replication did not permit a formal analysis of differences between the five rearing farms. However, because we knew which rearing flocks contributed to the various laying flocks, we were able to take some account of possible differences in initial bird condition, before drawing conclusions about treatment effects. For parameters such as bone strength and organ weight we conducted analyses of variance at the end of lay to examine treatment effects, with the inclusion and exclusion of average rearing flock values as co-variates. The validity of including rearing flock values as co-variates depends on a judgement as to the extent to which initial differences in laying flock condition may still be influential at end of lay. There is no obvious answer, and our experiment was not designed to address this issue, therefore we conducted both types of analysis.

During the laying period, we selected key periods for analysis (32-35, 48-51, 56-59 and 68-70 weeks of age). Behavioural observations were also taken at these times, allowing comparisons with behavioural data (Zimmerman et al., 2005). Analysis of variance was performed on transformed data, where appropriate. Repeated measures ANOVA was generally used when independent samples were taken on the same flock at different times (e.g. bodyweight, plumage scores) to examine treatment and time

effects. Differences between treatments were examined using Tukey post-hoc comparisons. For the cumulative data on mortality, repeated measures analysis was not appropriate.

Environmental measures of temperature, light, and air quality could be assessed only on a house, not flock, basis and descriptive results only are given for these parameters. Litter condition was measured on a flock basis, as relatively independent readings were obtained for the two different flocks housed within each house. These data were subjected to repeated measures analysis of variance.

RESULTS

Assessment of Rearing Flock Condition

At the end of the rearing period, only 1 bird out of the 400 sampled had sustained a fracture. Overall mean values for the parameters assessed at the end of the rearing period are given in Table 2.

Assessment of Flock Condition During Lay

Mortality

Cumulative mortality increased with time in all flocks and all treatments (Figure 2). There were no significant differences between treatments at the key periods of 32-35 and 48-51 weeks of age. However, by 56-59 weeks of age, there was a significant effect of treatment ($F= 3.00$; d.f. 5, 30; $p = 0.026$). Post-hoc analysis of treatment differences showed that mortality in T2 was significantly higher than mortality in T3, T4, T5 and T6 but not significantly different than mortality in T1. At the end of lay this difference was even more apparent ($F= 3.44$; d.f. 5,30; $p=0.014$), with T2 having significantly higher mortality than T3, T4, T5 and T6, but not than T1. Variation in mortality was greater for T1 and T2 than for the other treatments, and mortality was particularly high in Year 1. However, even in Years 2 and 3 mortality tended to be higher in T1 and T2 than in the other treatments.

Production

As expected, egg production (total eggs/bird numbers*days) declined steadily with time after the onset of lay in all flocks and treatments (Figure 3). Overall production was low, varying between 69.9% and 72% across the different treatments. There were no significant treatment differences ($F= 1.52$ d.f. 5, 30; NS), although there was a highly significant decline in production over the key periods ($F= 34.2$, d.f 3, 90; $p<0.001$). Average egg weight varied from 61.5g (sd 3.7) to 62.4g (sd 4.8) across the different treatments, with no significant treatment differences. The percentage of eggs laid in the nests was above 90% in all treatments.

Bodyweight

Repeated measures analysis of variance on bird bodyweight, using weights taken at the key periods revealed no significant differences between treatments ($F= 1.34$; d.f. 5,26; NS). However, bird bodyweight increased with age ($F= 45.86$; df 3,78; $p<0.001$) (Figure 4).

Plumage and integument condition

Repeated measures analysis of variance on average plumage score, using scores taken at the key periods (Fig 5) revealed significant differences between treatments ($F= 4.66$; d.f. 5,26; $p<0.01$) and plumage condition deteriorated over time ($F= 57.85$; d.f. 3,78; $p<0.001$). Post-hoc analysis revealed that the plumage condition of birds in T1, T2 and T3 was worse than that for other treatments at all time periods. Averaging plumage score across different body areas can mask differential effects due to abrasion or pecking damage so the plumage scores for the separate body areas were therefore examined at 60 weeks of age when differences were most apparent (Table 3). Significant treatment differences existed for plumage scores in the neck, breast, wing and tail regions. Treatments 1 to 3 generally had worse plumage scores than the other treatments for all these regions, supporting the conclusion from the analysis of overall plumage scores.

There were no significant treatment differences for vent pecking, pecks to the comb or foot condition. Vent pecking was very rare. The overall average number of pecks to the comb at 60 weeks was 1.82. Foot condition score deteriorated with time ($F= 4.81$; d.f. 3,78; $p<0.01$), but never exceeded an average value of 1.5. Hyperkeratosis was rarely noted. No flock had a mean score greater than 1.3. There were no significant treatment differences and no apparent increase with time.

Environmental Monitoring

Environmental ammonia concentrations for individual flocks ranged from 3.0 ppm to 32.5 ppm in early lay, and from 5.0 ppm to 41 ppm towards the end of lay. The range of relative humidity for individual flocks was from 54% to 91% in early lay, and 60% to 87% towards end of lay. There were no obvious associations with treatment, or consistent effects of time. The values for light intensity near the light source were close to the target value of 10 lux, ranging from 8.63 to 9.54 across the different treatments. There was very little variation in weekly readings. Light readings near the nest boxes ranged from 5 to 7 lux in T1, T2, T3, T5, and 1 to 3 lux in T4 and T6. This confirms that the removal of lights from nest-boxes in Treatments 4 and 6 was effective in reducing light intensity just outside the nest boxes. Stocking density did not affect recorded house temperature. Direct comparisons can most easily be made using Treatments 1, 2 and 3, which were run at the same time of year, in adjacent houses. In Year 1, all three treatments experienced a maximum temperature of 34° C, in Year 2 the maximum was either 28 or 29° C and in Year 3 the maximum was either 35 or 36° C. Minimum recorded temperature also hardly differed between these three treatments. In Year 1 the minimum was either 2 or 3° C, in Year 2 it was between 6 and 8° C, and in Year 3 it was between 10 and 12° C

Litter friability varied greatly with time, usually declining but sometimes improving as the flock aged (Fig 6). There was a significant interaction between treatment and flock age ($F= 2,69$; d.f. 15; 54; $p<0.001$) with litter condition in Treatment 5 tending to be better towards the end of lay. Average litter moisture content varied from 25% to 49% across the experiment, with no obvious treatment effect. The most direct comparison of the effect of stocking density on litter moisture content can be made by comparing T1, T2 and T3 as these birds were housed at the same time of year in adjacent houses. The overall average figures were T1 31.7%, T2 32.0% and T3 36.2% indicating only slight increase in moisture content with increased stocking density.

End of Lay Bird Condition

The most striking finding of the post-mortem analyses at the end of lay was the high prevalence of old fractures in birds from all treatment (Table 4). Bone breakage prevalence was not significantly influenced by treatment but was much higher level than the negligible level observed at the end of the rearing period.

Faecal corticosterone, CPK, bone strengths and heterophil:lymphocyte ratios were not significantly influenced by treatment. Table 2 shows that bone strengths were on average higher at the end of lay than at the end of the rearing period. When average rearing flock values were entered as co-variates, average keel bone strength for birds in T5 became significantly higher than for birds in any other treatment ($F = 4.42$, $p < 0.01$).

Heterophil:lymphocyte ratios were much higher at the end of lay than at the end of the rearing period and CPK values tended to be lower (Table 2).

There was a highly significant treatment difference in liver weight, either as an absolute value ($F = 8.06$; $p < 0.0001$) or when expressed as a percentage of body weight ($F = 6.301$; $p = 0.0005$). Liver weight as a % of bodyweight, was significantly lower for birds from T1 than T5, for birds from T2 than birds from T3, T4, T5 and T6. Birds from T5 also had higher liver weights than birds from T3 and T6. However, when average rearing flock values for % liver weight were added as a co-variate, there were no significant treatment differences. There was also a significant treatment effect on spleen weight either as an absolute value ($F = 4.72$; $p = 0.003$) or when expressed as a percentage of body weight ($F = 2.48$; $p = 0.05$). Spleen weight was significantly higher in T4 and T6 than in T1, T2 and T3 and this was not affected by adding rearing flock values as co-variates.

There was a strong treatment effect on osmolality ($F= 5.49$; $p = 0.001$), which was significantly lower in Treatment 5 than any other treatment. Finally, muscle pH was also affected by treatment ($F=3.04$; $p = 0.03$) and was lower in Treatments 4 and 5 than Treatments 1 and 3.

Discussion

It is generally accepted that there is no single measure of welfare (Dawkins, 2003) and that information should be sought using a variety of indicators of health, physiology and behaviour. Information from these diverse sources has to be combined to draw overall conclusions about the benefits and drawbacks of different housing systems (e.g. Blokhuis et al., 2005). This is difficult because indicators may not co-vary and some may be more valid than others. As yet there is little agreement between experts on the specific weightings that should be assigned to each indicator. Also, although there have been many laboratory studies of laying hen welfare, there are few replicated commercial studies that use a sufficiently broad range of welfare indicators to allow us to address these issues in practice. The primary aim of this experiment was to examine the effects of stocking density on bird welfare using a range of indicators. There was unavoidable confounding of house and treatment, but environmental monitoring did not reveal any biologically significant differences in temperature, air quality or light between the houses.

Mortality is an important indicator of welfare, and the overall average mortality rate in this study was within the wide range reported for other commercial non-cage systems (e.g. Tauson and Holm, 1999; Ekstrand et al., 1997; both cited in Blokhuis et al., 2005). Mortality was significantly higher in the low stocking-rate treatments in the current study (T1 and T2, with an average of 18.7% across 12 flocks). These rates were higher than reported for beak-trimmed flocks housed at 7 birds m^{-2} in single-tier floor systems in Denmark where mortality varied from 8.7 to 12.1 % (Jensen et al., 2003 cited in Blokhuis et al., 2005). In our study, the timing of the increased mortality was between 40 and 43 weeks for Treatment 1, and between 48 and 51 weeks for Treatment 2. Cause of death was not recorded systematically by the participating company, and so it is difficult to know how low stocking rate influenced mortality. An important indicator that co-varied with mortality, was plumage damage. Plumage condition was worse in Treatment 1 than any other treatment during early lay. Plumage damage in Treatment 2 increased to a similar level as Treatment 1 by weeks 48 to 51 and, in both of these low-density flocks, plumage continued to deteriorate as the end of lay approached. Zimmerman et al. (2005) reported higher levels of feather pecking and aggression in Treatment 1 than other treatments. However, the association between low stocking rate and reduced plumage condition is not totally clear, as overall plumage damage was also poor in one of the four high-density flocks (T3).

Green et al (2000) noted a strong association between farmer-reported feather pecking and the occurrence of egg peritonitis and infectious bronchitis, although these authors could not determine whether feather pecking preceded or followed disease. In our study, the onset of increased mortality, above the experimental baseline, was co-incident with an increase in overall plumage score to an average of approximately 3.5. There may be common risk factors for disease and feather pecking, both of which may then contribute to increased mortality. It is worth noting, however, that the egg production of birds in the low-density flocks was not significantly lower than that of other flocks, even with increased mortality. Thus, the birds that did not die in the low-density flocks performed well. The % liver weight of the birds in T1 and T2 at the end of lay was significantly lower than of birds from other treatments, possibly due to the additional metabolic load involved in maintaining a relatively higher egg production rate than birds in other treatments at this time. The lower % liver weight might also suggest that these birds were not chronically stressed as, in broilers, liver weight tends to increase due to lipid and moisture accumulation in response to ACTH (Puvadolpirod and Thaxton, 2000). However, caution is required in extrapolating these experimental results to laying hens, where detailed interactions between physiological stress, egg production and organ weights have not been examined. In addition, when rearing flock values were included as co-variates in the analysis, the significant treatment effect on liver weight was no longer apparent.

The association between low stocking rate, increased mortality and reduced plumage condition is counter to the results of previous experimental studies where flock sizes were much lower. For example, in a study of hens housed in flock sizes of between 72 and 368 Nicol et al., (1999) feather pecking *increased* with increasing stocking density. Results from small-scale experiments cannot necessarily be extrapolated to the commercial situation. Paradoxical effects may also result if birds housed at low average stocking rates cluster disproportionately around key resources, resulting in an actual increase in localised stocking rate.

In this study, we found very few effects of flock size. Sometimes one treatment showed a statistically higher or lower value of a particular indicator but no overall picture emerged. For example, osmolality was low in hens in the high-density small flocks of Treatment 5, but this was not the case for hens housed in high-density small flocks with modified management (T6), or in low-density small flocks (T1). A more consistent picture emerged in relation to modified management. Changes in drinker type and nest-box lighting were implemented with the express intention of reducing feather pecking and improving plumage condition. Zimmerman et al. (2005) showed that feather pecking was indeed reduced in the MM flocks, and this was confirmed by the improved plumage scores of birds in Treatments 4 and 6, reported here.

Our finding that some 60% of birds across all treatments had sustained a fracture of the keel or furculum by the end of lay was a cause for concern. It confirms a recent report that the incidence of old breaks in end of lay hens is high (Wilkins et al., 2004) but also shows that the fractures were sustained during lay. Only 1 bird out of 400 sampled at the end of the rearing period had a fracture. By the end of lay, absolute bone strength was higher than at the end of the rearing period, although the ratio of bone strength to average body weight was slightly lower (e.g. keel A 19.8 at end of rear, 18.2 at end of lay; keel B 8.9 at end of rear, 8.3 at end of lay). Research is needed to ascertain when fractures occur, and to identify risk factors and preventive strategies. Another notable change between the end of the rearing period and the end of lay was the large increase in H:L ratio. The values for H:L ratio at the end of the rearing period were similar to those reported previously for cage-reared pullets at a similar age (Patterson & Siegel, 1998) but the average end of lay H:L ratio of 1.67 in the current study (Table 2) was greatly above values reported previously in adult birds (e.g. Mahboub et al, 2004; Campo and Davila, 2001). It is unlikely that the increase observed in our study was simply a function of normal ageing. Davis et al (2000) monitored caged laying hens from the onset of lay to 63 weeks and found H:L ratios increased from approximately 0.1 to approximately 0.3. At 64 weeks of age the hens were forcibly moulted by food deprivation for 14 days, when H:L ratio increased to approximately 0.7. An increase in H:L ratio is thought to be a reliable and valid indicator of stress in poultry (e.g. Gross and Siegel, 1983; Maxwell and Robinson, 1998). Faecal corticosterone levels were also twice those found at the end of the rearing period. It is therefore possible that all of the hens in our study were experiencing high levels of stress, associated perhaps with plumage loss (Campo et al., 2001) or fracture incidence. It is also possible that they were mounting a response to a background disease challenge. If so, any treatment differences due to stocking rate or flock size would be masked.

Stocking rate is frequently selected as a parameter when legislators attempt to set minimum welfare standards, probably because it is relatively easy to regulate. However, other housing and management factors may have a more profound influence on bird welfare (Dawkins et al, 2004). The indicators used in this study did not show that the welfare of laying hens was compromised by housing at 12 birds per m⁻², in comparison with birds housed at 9 or 7 birds per m⁻² in single-tier aviaries. Modified management had a beneficial effect in improving plumage condition and the welfare of the birds at the end of the rearing period appeared good. Overall, however, high levels of mortality, H:L ratio, faecal corticosterone, incidence of bone fractures and poor plumage scores, suggested that the welfare of the birds in all treatments was relatively poor by the end of lay.

Acknowledgements

We would like to thank the participating company, Deans Foods, for their full co-operation throughout this long study. The work was funded by Defra (AW0223).

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Table 1 Summary of the six treatments used

Treatment	Stocking rate (birds m ⁻²)	Flock size (no. of birds)	Management type
1	7	small (2,450)	Standard (SM)
2	9	small (3,150)	Standard
3	12	large (4,200)	Standard
4	12	large (4,200)	Modified (MM)
5	12	small (2,450)	Standard
6	12	small (2,450)	Modified

Table 2.

Body condition of birds at the end of the rearing period and at the end of the laying period.

The rearing flock values are the average of 8 rearing flocks. The laying flock values are the average of 34 laying flocks (2 missing values from Treatment 6). Fifty birds were dissected from every flock to give values for bone strength and organ weights. Twenty five birds were blood or tissue sampled from every flock to give values for biochemical parameters.

Measure	Average value at the end of rearing	Standard deviation	Average value at the end of lay	Standard deviation
H/L Ratio	0.55	0.31	1.67	0.75
Live weight (kg)	1.27	0.68	1.85	0.06
Liver as % of body weight	2.12	0.13	2.53	0.23
Heart as % of bodyweight	0.42	0.04	0.43	0.05
Spleen as % of bodyweight	0.19	0.01	0.11	0.01
Bursa of Fabricius % bodyweight	0.18	0.025	n/a	n/a
Humerus bone strength (kg)	16.8	1.3	19.2	1.1
Tibia bone strength (kg)	17.0	2.0	25.2	2.1
Keel point A bone strength (kg)	25.1	5.1	33.7	3.4
Keel point B bone strength (kg)	11.0	2.4	15.3	2.4
Log ₁₀ CPK (u/L)	2.64	0.15	2.44	0.12
Osmolality (mosmol)	314	4.2	316	6.2
Muscle pH	5.63	0.13	5.74	0.06
Faecal Corticosterone (ng/g dry matter)	28.2	5.9	55.2	21.5

Table 3.

Average feather scores for different body areas at 60 weeks of age. * Indicates a body region where treatment differences were significant at $p < 0.05$

Plumage score for body region	T1	T2	T3	T4	T5	T6
Head	3.39	3.41	3.29	2.91	2.91	2.76
Neck *	3.39	3.46	3.35	3.02	3.23	2.91
Breast *	4.05	3.89	4.23	3.82	3.55	3.62
Back	3.75	3.25	3.85	3.66	3.80	3.48
Wing *	2.77	2.82	3.12	2.40	2.74	2.52
Abdomen	4.26	3.81	4.09	4.03	3.81	3.81
Tail *	3.57	3.32	3.58	3.01	3.04	3.17

Table 4.

Prevalence of old bone breaks in birds in birds dissected at the end of lay. Severity was assessed using a standard scale (Wilkins et al., 2004).

	T1	T2	T3	T4	T5	T6
Keel breaks (% of birds)	52	56	59	57	55	65
Furculum breaks (% of birds)	8	11	8	7	5	2
Total % birds with breaks	56	62	60	59	56	68
Severity (standard scale)	0.83	0.93	0.86	0.87	0.88	1.02

Figure Captions

Figure 1. Schematic drawing of a house. The slatted platform contained feeders and drinkers. Nest boxes ran along the whole length of the house.

Figure 2. Average cumulative mortality per treatment (6 replicates per treatment).

Figure 3. Average percent production per treatment (6 replicates per treatment).

Figure 4. Interaction between bodyweight and time (4 key periods) for each treatment.

Figure 5 Average feather score (derived from scoring of 6 different bird area's: head, neck, back/wings, tail/vent, abdomen and breast) plotted for each of Treatments 1-6, at 4 key periods (weeks).

Figure 6 Litter friability score

Figure 1

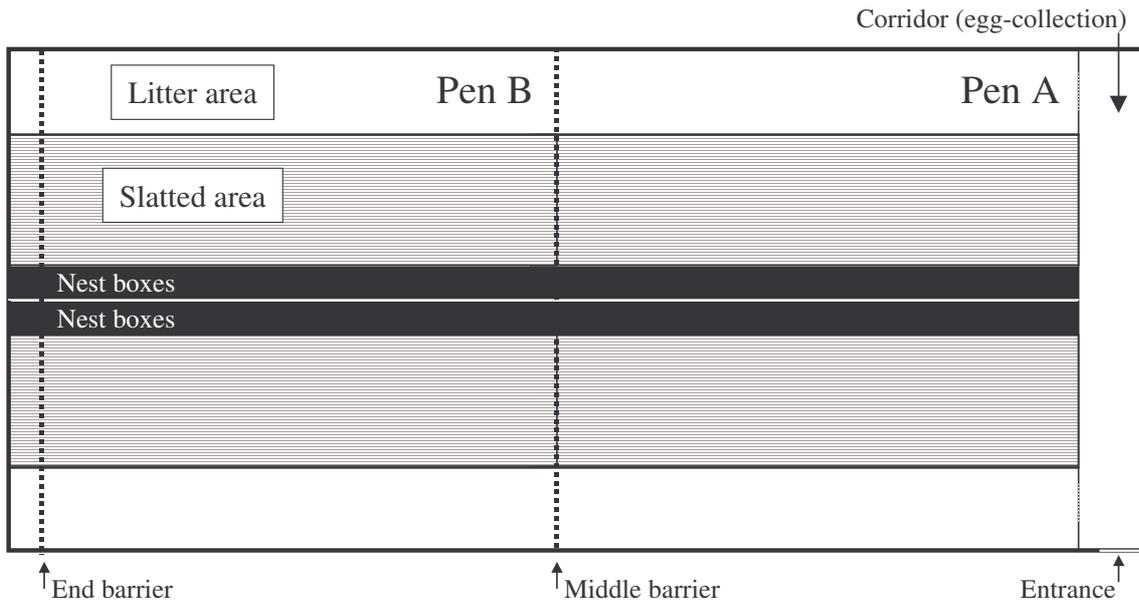


Figure 2

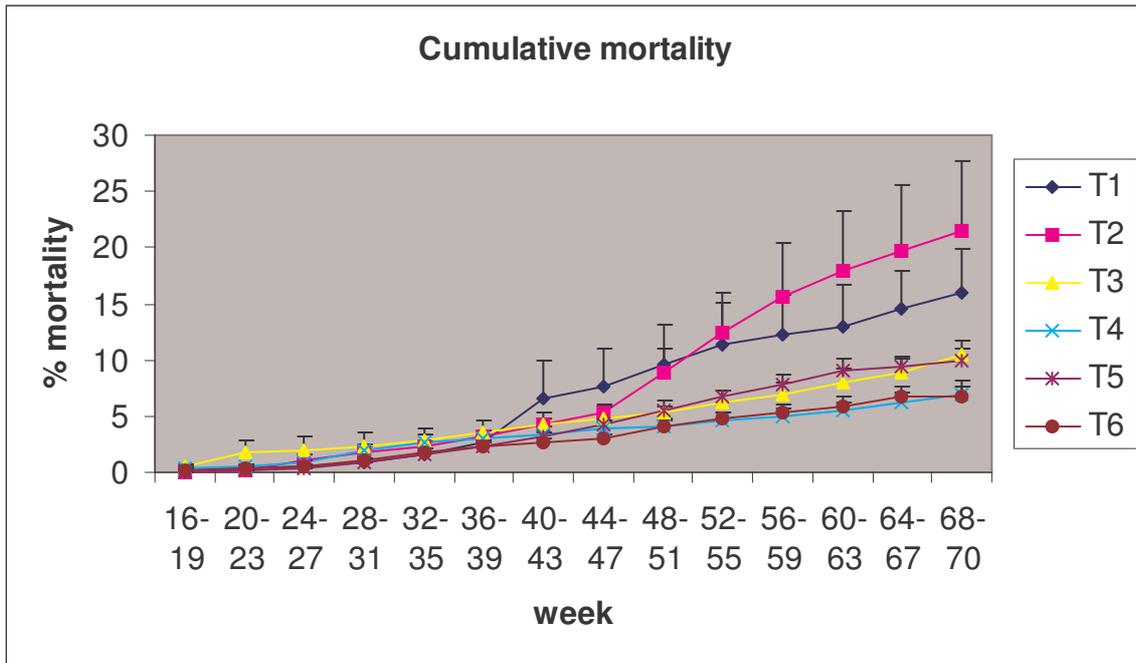


Figure 3

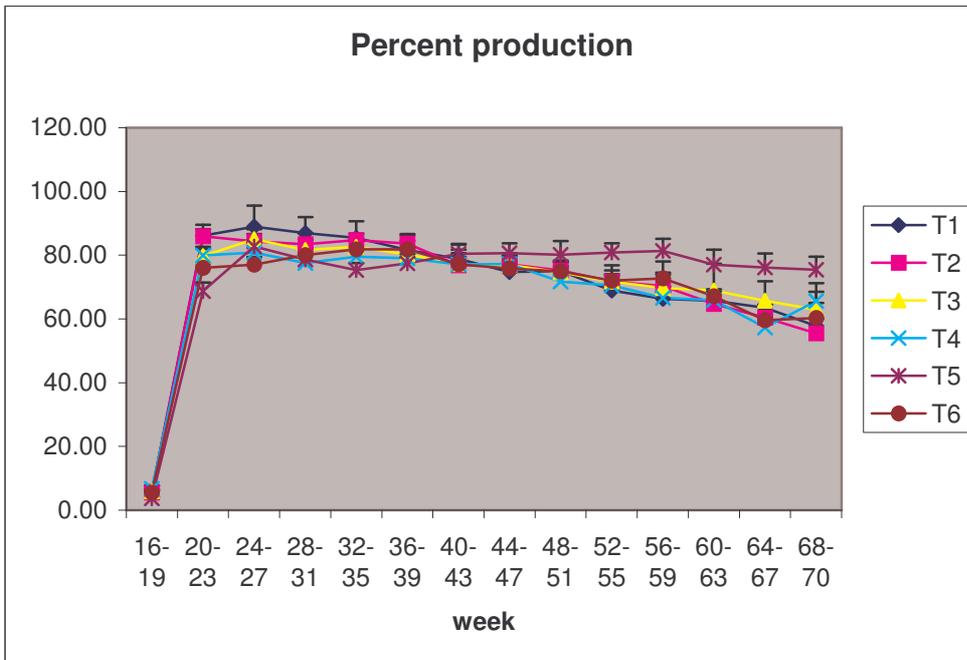


Figure 4

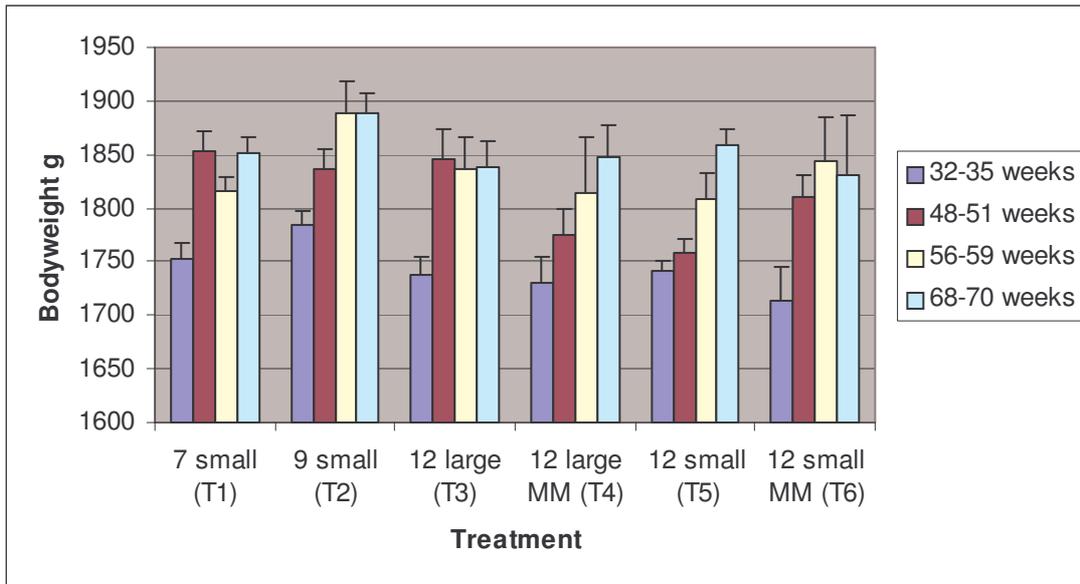


Figure 5

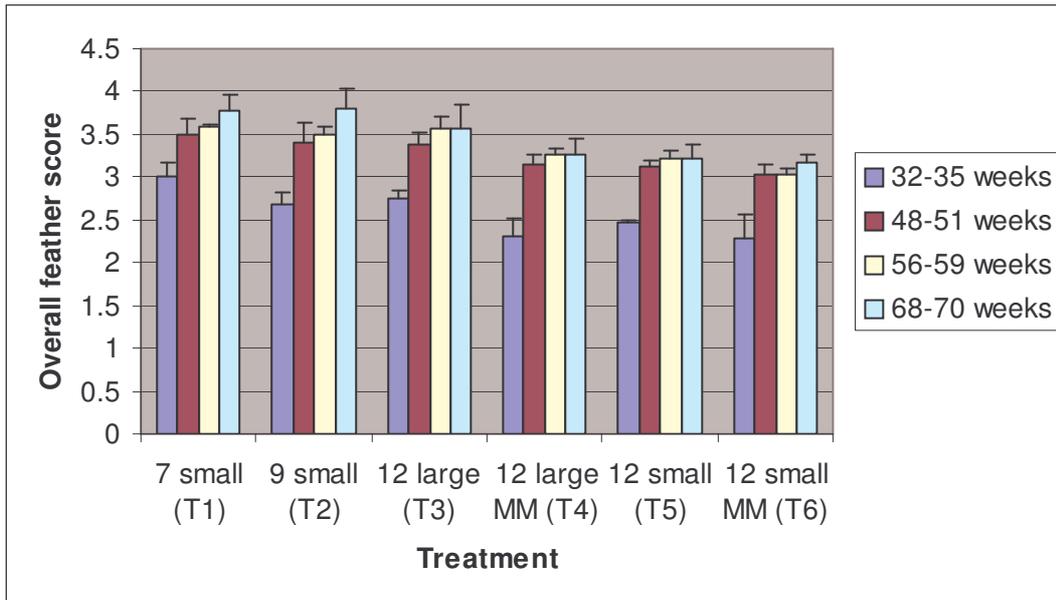
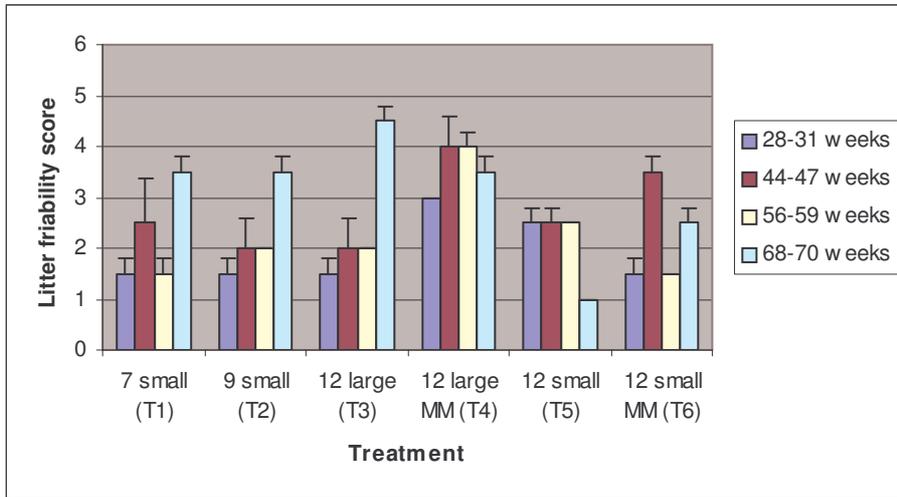


Figure 6



Manuscript 6 (AFSSA & INRA-SRA; Partner 5)

From : 6^{èmes} Journées de la Recherche Avicole, St Malo, France, March 30-31 Ed ITAVI, Paris.

Welfare of laying hens kept in cages or in aviaries: physiological indicators of stress and fearfulness

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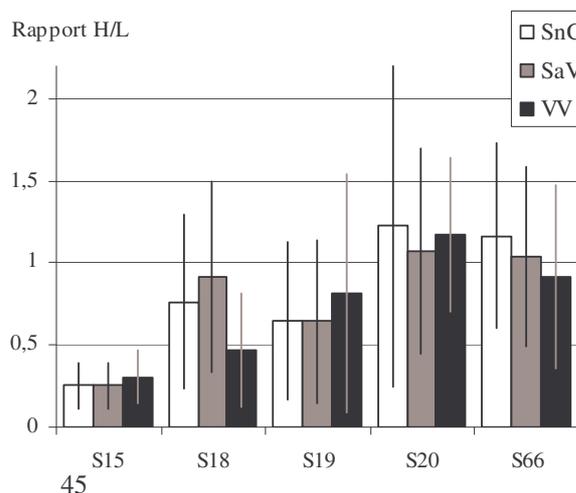
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With the setting of the directive 1999/74/EC, housing systems for laying hens will have to change. Conventional cages will be banned and only furnished cages or alternative systems (floor, aviaries...) will be allowed. The aim of this study was to compare welfare of hens kept in conventional cages after being reared in floor pens, with hens in laying aviaries that were previously reared either in rearing-aviaries (recommended) or in furnished-floor pens (intermediate solution). This paper reports results concerning physiological indicators of stress and fearfulness. Zootechnical parameters, body condition on farm and at slaughter and mortality during transport are presented in two complementary papers (COLSON *et al.*, 2005; MICHEL *et al.*, 2005).

10180 ISA Brown hens were reared from one day old, beak-trimmed at nine days old, transferred from rearing to laying systems at 17 weeks old (W17) and slaughtered at 69 weeks old (W69). They were placed in three housing alternatives: 5060 hens Floor-reared then kept in conventional Cages (FC), 2560 hens Furnished-floor reared (with perches and platforms) then kept in one laying-Aviary (FA) and 2560 hens Aviary-reared then kept in one laying-Aviary (AA). Stress response to transfer in laying system was assessed by Heterophiles to Lymphocytes ratio (H:L) measured before and after transfer (W15, W18, W19, W20; n=20 to 30). Stress response to housing conditions was assessed by the H:L at the end of the laying period (W66; n=30) and by the increase of corticosterone level 15 min after injection of a high dose (10µg/kg) of ACTH_{1-24}} (W15, W20, W66; n=24 to 30). Plasmatic corticosterone levels were assayed by Radio Immuno Assay (ETCHES, 1976). Fearfulness was assessed by a novel environment (NE) test (W52-59, n=12 groups of 4 hens). The NE (100 × 100 cm, width × depth) contained water, food and a piece of Astroturf®. We registered during 10 min indicators of mobility, exploration and frustration by focal sampling and indicators of activity by scan sampling.

H:L around transfer differed between treatments only in W18 (FC: 0.8±0.5, FA: 0.9±0.6, AA: 0.5±0.3; p<0.001). We verified that it was not an effect of age, therefore the transfer seemed to stress more FC and FA hens than AA hens (GROSS & SIEGEL, 1983) (Fig. 1).

Figure 1: H/L ratio (means ± SD) around the time of transfert and by the end of the productive periode .



Basal plasmatic levels of corticosterone were always low. These levels were higher in FC and AA hens in W15 (FC: 3.2 ± 2.2 , FA: 1.5 ± 1.0 , AA: 3.5 ± 2.6 ng/mL; $p < 0.001$), but higher only in FC hens in W66 (FC: 4.5 ± 3.8 , FA: 2.9 ± 2.0 , AA: 2.6 ± 2.4 ng/mL; $p = 0.041$). As elevated basal levels indicate an acute stress response (BEUVING & VONDER, 1978) or hypersensitivity, manipulations seemed more stressful for FC hens (Fig. 2). After the injection of ACTH, increases of corticosterone level were always high. These increases had a tendency to be lower in FC in W15 (FC: 24.3 ± 8.0 , FA: 29.9 ± 8.8 , AA: 28.9 ± 10.1 ng/mL; $p = 0.094$) and lower in FC and AA hens in W20 (FC: 20.1 ± 11.0 , FA: 25.5 ± 10.4 , AA: 19.3 ± 10.7 ng/mL; $p = 0.053$). As a lower increase indicates a chronic stress response (KOELKEBECK *et al.*, 1986), it seemed that FC hens were more stressed by their housing conditions than FA hens. We found no explanation to the low increase obtained in AA hens in W20. H:L measured in W66 had a tendency to be higher in FC hens (FC: 1.2 ± 0.6 , FA: 1.0 ± 0.6 , AA: 0.9 ± 0.6 ; $p = 0.071$), which indicated that FC hens were a little bit more stressed by their housing conditions. By putting these two results together, FC hens seemed more stressed by their housing conditions than FA and AA hens.

In NE test, for all the variables measured, there was a difference between treatments ($p \leq 0.001$): FC hens were less mobile, less active and explored less the NE than FA and AA hens; and they showed less frustration behaviours (Fig. 3). These results indicated a higher fearfulness of hens kept in cages compared to hens kept in aviaries (FAURE, 1975; JONES, 1996), probably because cages are a less enriched housing environment than aviaries (REED *et al.*, 1993).

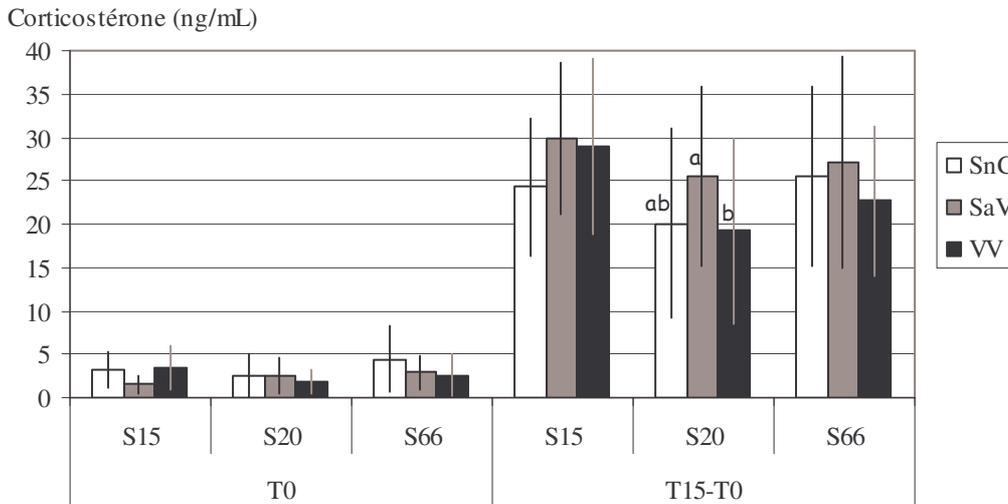
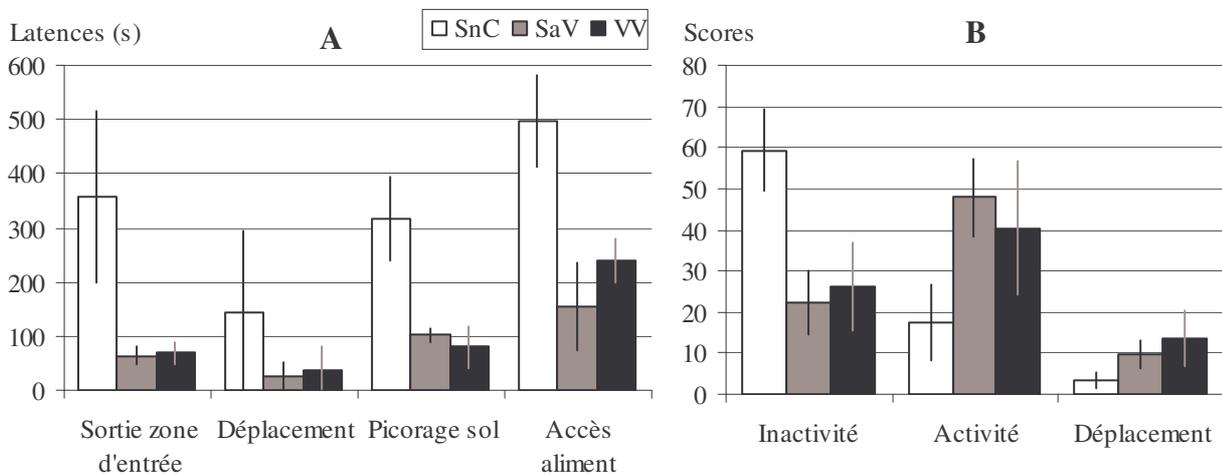


Figure 2: Corticosterone basal (T0) levels and in response to 1-24 ACTH injection at dose of $10 \mu\text{g}/\text{kg}$ B.W. ($T_{15}-T_0$) (ng/ml plasma; means \pm SD) before and after transfert to the laying house and by the end of the laying period.

a, b : different letters indicate significance for the same period and measurement ($p < 0,10$).

All these results suggest that stress is increasing between AA, FA and FC hens, and that FC hens are more fearful. Strong responses of stress or fearfulness can be the cause or the consequence of adaptation' difficulties. So, under the present conditions, adaptation' difficulties, and risks of impaired welfare that follow, seem to be increased in FC hens compared to FA hens and in FA hens compared to AA hens.

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Manuscript 7 (Norwegian School of Vet. Science, SLU & INRA – SRA; Partners 5 & 6)

In : 22nd World's Poultry Congress; Istanbul (TUR); 2004/06/08-13, Proceedings:329(abstract), CDROM:[\1619.pdf] 4p. WPSA, turkish branch, Izmir (TUR).

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Full version submitted for publication to British Poultry Science.

**EFFECTS OF PRE-LAYING REARING CONDITIONS IN LAYING HENS
HOUSED IN STANDARD OR FURNISHED CAGES
ON VARIOUS INDICATORS OF ANIMAL WELFARE**

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Abstract

The aims of the present study were to investigate possible interactions between housing systems (conventional and furnished cages) and pre-laying rearing conditions (cages or litter floor) on indicators of animal welfare related to physiology, immunology and injurious behaviour in white, not beak trimmed laying hens. Several end points of animal welfare indicators were significantly affected by the pre-laying rearing environment, some by the housing conditions, and some interactions were found. Thus, when comparing housing systems, it is most likely that indicators of welfare are influenced ambiguously by environmental complexity and, thus, a possible source of confounding interpretation and false conclusions in applied welfare research. Our results show the importance of further focus on validating indicators before using them as indicators of welfare in complex environments in applied studies. Results highly suggested that the pre-laying housing environment is of importance for hen's welfare during the productive period.

Key words

Furnished cages, pre-laying rearing conditions, animal welfare, adrenal reactivity, immune function

Introduction

In general, factors within the physical and social environment in combination with early developmental factors have an impact on animal welfare (Moberg and Mench, 2000). In furnished cages, several environmental elements (perches, dust bath and nest box) and social elements (large group size) are introduced as compared to conventional cages. Using indicators of injurious behaviours, stress physiology and immune function provide information about the hens interactions with the environment, and studies show that some of the introduced elements may ease coping ability whereas others may represent potential threats to welfare (Scientific Veterinary Committee, 1996). Thus, when comparing housing systems to get an overall picture of welfare, it is necessary to gain knowledge about end points of measures of various indicators of welfare in the particular housing systems.

Indicators of welfare may however be influenced by other elements than stress/poor welfare *per se*. As one example, stress may impede immunity (Ader *et al.*, 1991), however exposure to various environmental antigens may also interfere with measures of immunity (Maxwell and Robertson, 1998). Furthermore, early developmental factors may affect the ability to cope with an environment later in life. Early provision with structural material reduced later feather pecking (Blokhuys 1986), and early environmental complexity may affect the development of stress coping mechanisms (see Moberg and Mench, 2000) and immune responses. Thus, it is possible that indicators of welfare may be influenced ambiguously by environmental complexity and, thus, serving as a possible source of confounding interpretation and create confusion in applied welfare research.

The aims of the present study were to investigate possible interactions between housing systems (conventional cages and furnished cages) and pre-laying rearing conditions (cages or litter floor) on end points of various indicators of animal welfare in laying hens.

Materials and methods

In order to assess interactions between pre-laying rearing conditions and housing conditions during the laying period, several indicators of welfare were studied. White Lohmann Selected Leghorn (LSL) laying hens were kept on deep litter floor or in cages during the pre-laying rearing period (0-16 weeks of age) and subsequently housed in standard battery cages or in Victorsson furnished cages during the laying period (16-76 weeks of age), 100 per treatment. For deep litter floor rearing, groups of 100 hens were kept on deep litter floor (i.e. 13 hens/ m², wood shavings on floor), whereas for cage rearing 12 hens were kept in standard rearing cages from Big Dutchman (i.e. 448 cm²/bird, no enrichment). None of the rearing alternatives included perches. For housing conditions during the laying period, the standard cages contained 3 hens (730 cm²/hen) whereas the furnished cages contained 8 hens (600 cm²/hen) per cage. Hens were not beak trimmed. In the laying period, all birds were fed an oats based diet.

Mortality and production parameters (i.e. feed consumption and conversion, laying percentage and egg weight) were recorded. In order to assess HPA axis reactivity, base levels of corticosterone and the maximum secretory capacity of the adrenal glands 15 minutes following an i.m. injection of 1-24 ACTH (10µg/kg BW) were measured in 80 hens (20 per cage and rearing alternative; 2 hens randomly selected per cage) at 50 weeks of age. Plasma levels of corticosterone were measured using the RIA technique described by Etches (1976). In order to assess humoral immunity, the same hens were immunized with sheep red blood cells (SRBC; 1 ml of a 2% solution) and Key Hole Limpet Hemocyanin (KLH; 250µg/hen) at 60 weeks of age. Blood samples for the assessment of immune responsiveness were taken from these hens immediately before immunization to determine presence of antibodies/cross reacting antibodies, and thereafter once weekly for 6 weeks for humoral response. Anti-KLH IgG titres were determined using enzyme-linked immunosorbent assay (ELISA) and anti-SRBC titres were quantified using a hemagglutination assay. Physiological and immunological data were analysed using the ANOVA for repeated measure procedure (Statview). Plumage condition, pecking wounds at comb and body were recorded in hens housed in conventional cages (n=120; one group consisted of 4 cages a 3 hens, 5 groups i.e. 60 hens were used for each rearing alternative) and furnished cages (n=80; one group consisted of 8 hens within the same cage and 5 cages were use; i.e. 40 hens for each rearing alternative) at 72 weeks of age according to the method described by Tauson *et al.*, (1984). ANOVA was performed using GLM procedure (SAS), and differences between treatments were determined by using Ryan-Einot-Gabriel-Welch multiple F-test.

Results and discussion

Mortality was approximately 4% during the production period (76 weeks of age) which must be regarded as an acceptable level, and was unaffected by housing conditions and pre-laying rearing conditions. Hen-day egg production (% laying x egg weight) tended to be lower in furnished cages but numerically the difference was only 2 %. A similar numerical (NS) lower production was observed in floor reared hens compared to cage reared. Feed consumption was similar in all treatments, but due to the differences in production inferior average feed conversion ratio ($p \leq 0.05$) was observed in furnished compared to standard cages (unpublished data).

In total, housing system ($p \leq 0.04$) and pre-laying rearing conditions ($p \leq 0.002$) significantly affected plumage condition (Table 1). However, no differences were found between housing systems if hens had been reared on floor, and floor rearing represented the best alternative. Higher pecking rates may indicate a redirection of pecking due to the absence of early access to structural material when raised in cages during early development (Blokhuis, 1986). Thus, pre-laying rearing conditions with access to litter may be of importance to limit feather pecking during the productive period, even for hens to be housed in cage alternatives. Few body wounds and similar levels of comb pecking between systems were found, and no significant effects of pre-laying rearing conditions were seen.

Basal plasma corticosterone levels were low and did not differ significantly between groups. Injecting 1-24 ACTH led to significant ($p \leq 0.0001$) and comparable increases in all groups. No effects of housing alternative or pre-laying rearing conditions as such were observed, indicating that HPA axis functionality was unaffected by those factors. These results may indicate similar levels of stress in the different housing alternatives.

In general, hens responded to immunization with significant increases in anti-SRBC ($p \leq 0.0001$) and anti-KLH IgG ($p \leq 0.0001$) titres. For both immunogens, pre immunization antibody titres were present and for KLH titres were high, indicating presence of and exposure to cross reactive environmental antigens. Results are presented in Figure 1 (anti-SRBC titres) and Figure 2 (anti-KLH IgG titres).

Pre-laying rearing conditions affected anti-SRBC titre ($p \leq 0.0003$), and a time x pre-laying rearing interaction was found ($p \leq 0.0005$). Previously, it was shown that stress limits humoral antibody response in hens (Siegel, 1985), and lower antibody responses to SRBC have been interpreted as the consequence of stress related to improper housing (El-Lethey *et al.*, 2000). In the present study housing as such did not affect anti-SRBC titre, which may indicate similar levels of stress in the housing alternatives. It is not possible to conclude if differences due to pre-laying rearing conditions were a result of stress or exposure to environmental antigens. The latter is a possible explanation since hens kept in cages during the pre-laying period had lowest pre immunization and response titres compared to hens within equal housing environments during the laying period, indicating differences in sensitisation of the immune system.

Anti-KLH IgG titre was significantly influenced by pre-laying rearing environment ($p \leq 0.002$) and housing conditions ($p \leq 0.0001$), showing the highest immune response in furnished cages. Stress may deplete humoral immune response (Ader, 1991, Siegel, 1985) and, thus, the present results may indicate less stress in furnished cages. A time x rearing interaction was found ($p \leq 0.005$). However, it was not possible to conclude if differences due to pre-laying rearing conditions were a result of stress or exposure to cross reactive environmental antigens.

In conclusion, several endpoints of welfare indicators were significantly affected by the pre-laying rearing environment. To some extent, housing environment during the laying period affected some indicators, and interactions between those two factors related to rearing conditions were found. In general, these results show that when using indicators of welfare related to stress physiology and immunology it is of primary importance to gain knowledge about how these indicators are affected by single environmental elements as well as by the total physical and social

environment before being able to draw conclusions regarding animal welfare. The results show the importance of further focus on validating indicators before using them as indicators of welfare in complex environments in applied studies. Furthermore, the results emphasize the importance of knowing the life story of individual hens before being able to draw conclusions. Moreover, housing environment affected anti-KLH IgG titre but not anti-SRBC titre or adrenal responsiveness. This discrepancy shows the importance of using a broad range of indicators to gain a more complete and realistic picture on the hens ability to cope with the environment in applied animal welfare studies.

Further studies are needed to understand whether the presently observed effects on antibody response resulted from stress or were caused by the exposure to environmental antigens. Furthermore, the biological consequences of these effects on disease susceptibility are not clear.

From the present study and the chosen indicators, it was not possible to conclude that furnished cages were a better alternative to housing hens than conventional cages. However, it has to be emphasized that the chosen indicators not reflected the actual use of facilities in the furnished cages. Hens used the nest box, litter bath and perches to a large extent (unpublished data) indicating a welfare improvement due to behavioural diversity. Furthermore, as the group size differ (three hens vs. eight hens in the two housing systems), we cannot completely discard the possibility that potential improvement in welfare due to the presence of enrichments in furnished cages were counteracted by, for instance, social interactions, and further studies are needed to investigate physiological and immunological consequences of housing systems. Observed effects of pre-laying rearing conditions indicated that hens are interacting with and influenced by elements in their housing environment before transfer to the laying environment. Thus, optimising the pre-laying rearing environment is one important future focus to ensure animal welfare in hens to be housed in cage alternatives during their productive period.

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Table 1 Plumage condition, body and comb wounds in conventional and furnished cages at 72 weeks of age: effects of housing system and pre-laying rearing condition

	Conventional cages n=3 per cage		Victorsson furnished cages n=8 per cage		Effect of system (P-values)	Effect of rearing (P-values)
	Cage reared	Floor reared	Cage reared	Floor reared		
Plumage condition	10.6	11.6	9.0	11.3	0.04	0.002
Rear body wounds	3.9	3.9	3.85	3.85	0.22	0.93
Comb lesions	2.6	2.35	2.2	2.6	0.24	0.32

Plumage scoring assigned values 1, 2, 3 or 4 for each reported character, where 4 is the best. Plumage condition was recorded separately for neck, breast, wings, back and tail/area around the cloaca/vent and the five scores are added and presented as a total plumage score. Comb lesions as indicators of received aggressive pecks, and body wounds were scored according to the same scale. Score 1 indicates >5 wounds or lesions, score 4 indicates no lesions.

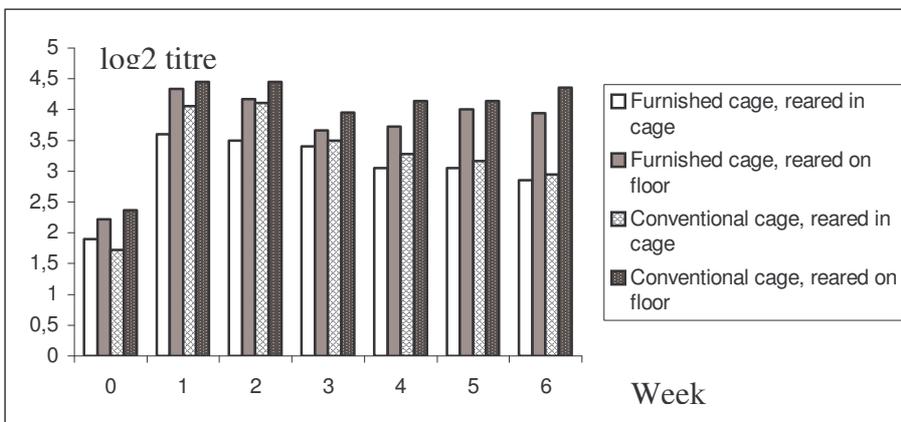


Figure 1 Anti-SRBC titre (log 2) in laying hens before (0) and after (weeks 1-6) immunization with SRBC at 60 weeks of age.

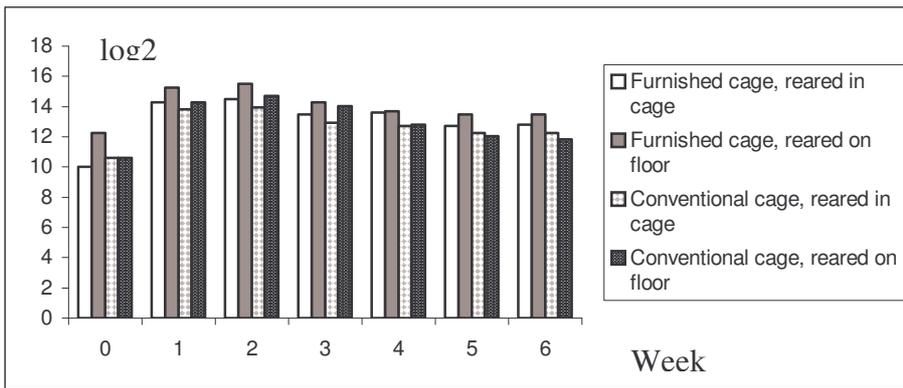


Figure 2 Anti-KLH IgG titre (log₂) in laying hens before (0) and after (weeks 1-6) immunization with KLH at 60 weeks of age.

Manuscript 8 (INRA-SRA; Partner 5)

Plasma corticosterone levels in male chickens from two divergently selected lines of broiler for featherpecking

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Recently published data suggest that featherpecking trait is associated with specific adrenocorticotrophic axis reactivity in egg laying lines (Korte *et al.*, 1997 ; van Hierden *et al.*, 2001). However, these results originated from comparisons between poults (van Hierden *et al.*, 2001) and laying hens (Korte *et al.*, 1997) of independent lines having different genetic backgrounds. Therefore, the reported differences may not be directly associated with this behavioural trait. The present study was thus undertaken to further investigate this possible functional relationship. Male chickens originating from the 3rd generation of two divergently selected lines (FP+ & FP- : n=30x2), of a slow growth rate strain of broiler were used. Selection was performed using a slightly modified procedure of a previously reported method (Bessei, 1996). In brief, the birds are selected depending upon their tendency to peck to a fake with feathers (Picometer) at adult age and while raised in individual cages. For this study, the progenies were raised collectively in two independent rooms. Basal corticosterone levels and, responses to a physical constraint and a pharmacological challenge were compared at the age of 21 weeks. Blood samples were collected from the wing vein, before, 15 and 60 min after i.m. injection of an ACTH agonist (immediate synacthen [IS], Novartis, 40µg/birds, vol =1ml, NaCl 0,9%) and/or physical restraint. Plasma samples were assayed for corticosterone using a specific RIA (Etches, 1976).

Basal corticosterone levels were significantly ($P=0.02$) higher for chickens from the FP- group (4.5 ± 0.4 vs 3.1 ± 0.3 ng/ml). Increases were observed under both treatments and for both delays compared to basal levels (Fig. 1). Alternately, genetic origin had no significant effect at 15 min. whereas much higher levels ($P<0.001$) were measured following IS challenge (28.1 ± 2.9 vs 7.1 ± 0.9). After a delay of 60min., significant decreases ($P<0.05$) were observed but chickens from the FP- group showed higher concentrations under both treatments (9.2 ± 0.9 vs 5.9 ± 0.6). Furthermore, a complementary genetic sub-classification of the broilers (FP++, FP+, FP- & FP--) showed a good hierarchy between this classification and corticosterone levels measured at the 60min. delay under both treatments (FP -- \geq FP - \geq FP + \geq FP ++) (Fig. 2).

The present results emphasise the hypothesis that a change in the HPA axis' functionality is associated with featherpecking trait. Chickens from the higher featherpecking line showing comparable maximal reactivity but lower basal levels and different response kinetics. Further studies will be necessary in order to elucidate if these results apply to both sexes and at different physiological stages.

Figure 1: Corticosterone levels (ng/ml plasma) measured before treatment (T0) and, after 15 (T15) or 60 (T60) min of restraint or post-injection of 1-24 ACTH at a dose of 10 μ g/kg B.W. in broilers from two divergently selected genotypes for feather pecking. (Mean \pm S.D.)

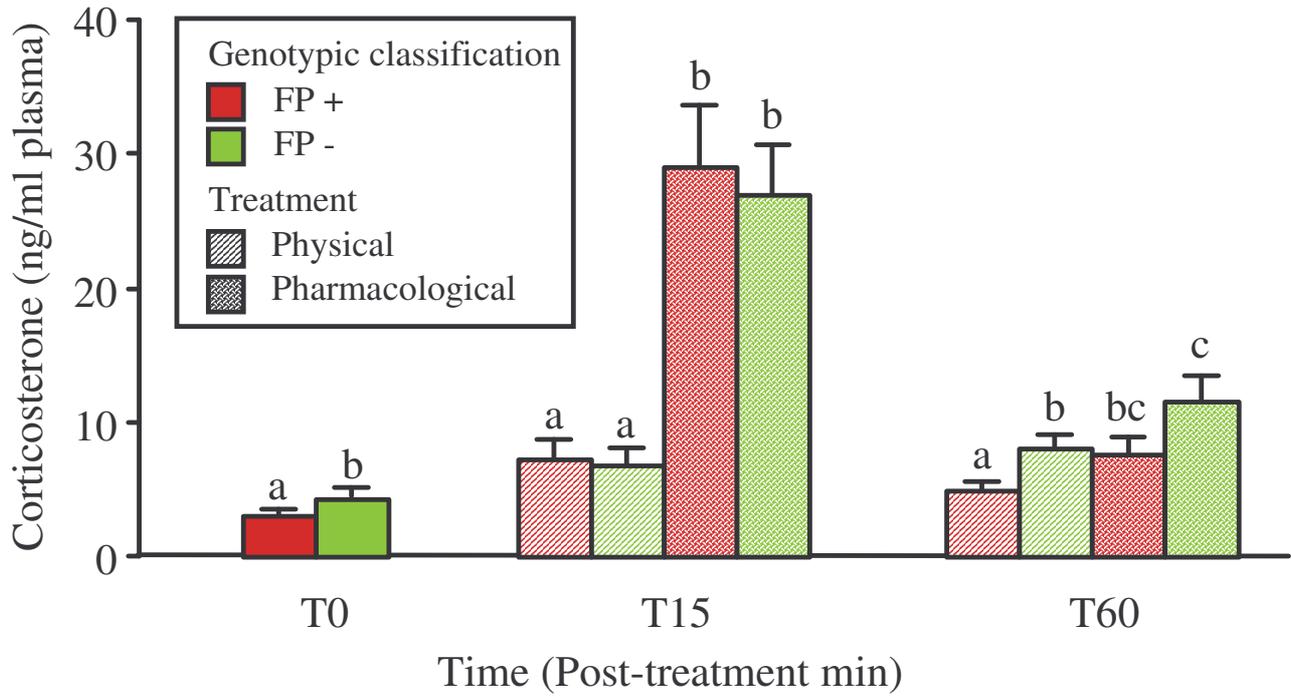
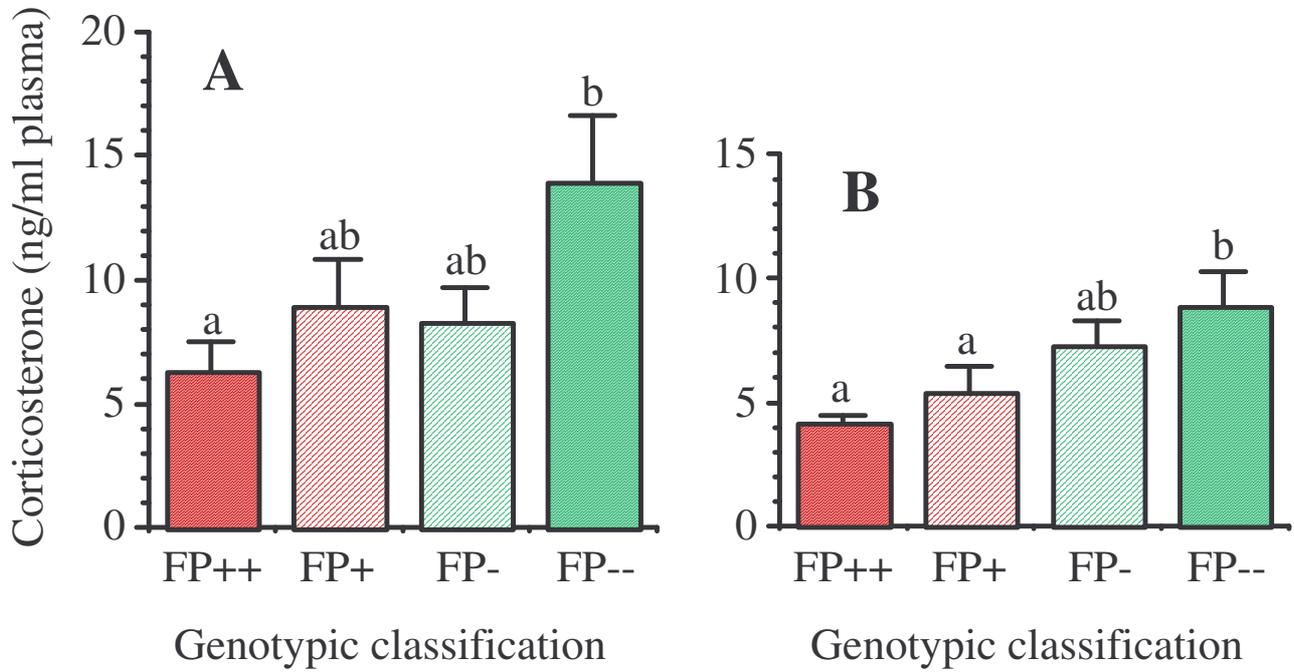


Figure 2: Corticosterone levels (ng/ml plasma) measured 60min post-injection of 1-24 ACTH at a dose of 10 μ g/kg B.W. (A) (T60) or after 60min of restraint, in broilers from divergently selected genotypes for feather pecking. (Mean \pm S.D.)



Manuscript 9 (DIAS & INRA-SRA; Partners 4 & 5)

Unpublished data

Stress reactivity in laying hens as affected by genetic selection

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Abstract

1. Introduction

Feather pecking in domestic fowl is a behavioural disorder that consists of pecking directed at and damaging the feathers of other birds. The feathers are often pulled off and may then be eaten. This behaviour can result in severe damage of the plumage, especially in laying hens (Hughes, 1982; 1985). Feather pecking is considered a "multi-factorial" problem (Hughes and Duncan, 1972; Blokhuis, 1989) because a number of causative factors have been found, environmental as well as genetic (ref til review; Kjaer and Sørensen, 1997).

The adrenocortical activity and reactivity on an acute stressor (manual restraint) has been found to differ between laying hens from lines differing in level of feather pecking. A higher level of corticosterone (cort) was found in hens from a low feather pecking line (LFP) compared to hens from a high feather pecking line (HFP), both as basal level at 3 and 56 days of age (van Hierden et al., 2002) and at 156 days (Korte et al., 1997), and after manual restraint of 5 minutes at 3 and 28 days (van Hierden et al., 2002) and after restraint in 1, 4, 6 or 8 minutes at 364 days (Korte et al., 1997). The birds in these studies originated from breeding lines with different selection history and the difference in feather pecking was a coincidental result of these commercial selection programmes, which consisted of productivity traits and mortality (Korte et al., 1997).

Recently, White Leghorn lines have been subjected to divergent selection specifically on feather pecking behaviour, and the resulting LFP and HFP lines differ significantly in level of feather pecking (Kjaer et al., 2001; Kjaer et al., in prep, Fp-gen 4 and 5). Birds from these lines are therefore very interesting subjects for the study of selection responses correlated to feather pecking, because the chance will be higher in these lines, compared to lines selected on other traits, that any correlated response are due to specific changes in genetic disposition for feather pecking. Thereby, conclusions drawn on data from these lines, with regard to the causal factors of feather pecking will

be stronger. This paper describes an investigation of basal level, reactivity to manual restraint and maximal corticosterone response in LFP and HFP birds of the sixth generation.

2. Materials and methods

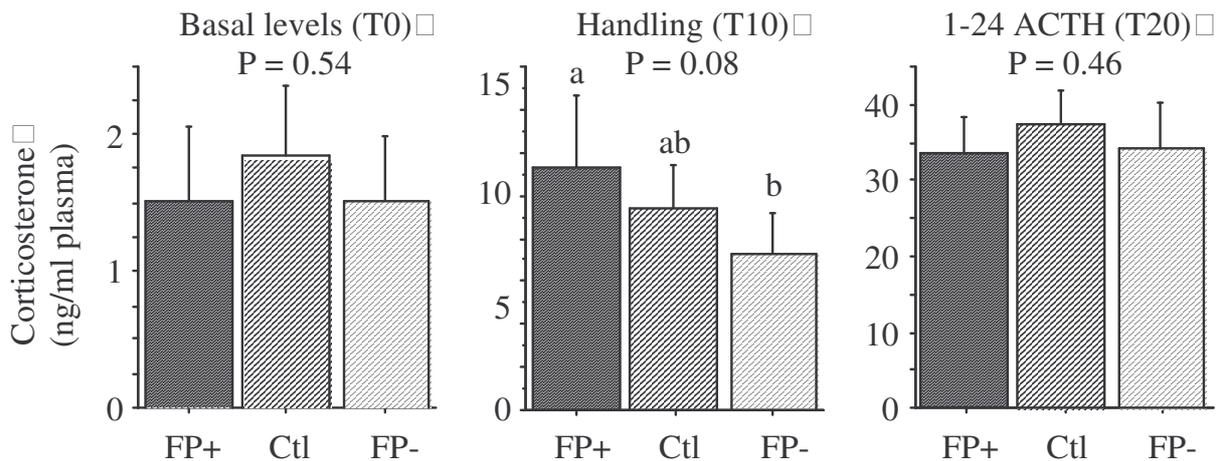
Development of genetic lines and subjects for the experiment

The data were collected from an experiment with three experimental lines of White Leghorn laying hens, among which line LP was selected for low feather pecking, line HP for high feather pecking, and line C was a random mating control line. The three lines were established in 1995 and derived from a White Leghorn layer line, which was formed in 1970. Feather pecking behaviour was measured at about 30 wk of age, by counting feather pecks (FP pecks) and then grouping pecks into bouts (events, feather pecking bouts, FP bouts). One bout was defined as a series of continuous pecking direct to the same individual. Selection was based on number of FP bouts (Kjaer et al., 2001). In each generation 30 breeder hens were selected out of approx. 220 female birds. The subjects of our study were the breeder hens of the 6th generation. They were housed in single cages at the time of the investigation at 85 weeks of age

3. Results

Hens from the three divergently selected lines had comparable basal levels of corticosterone and overall adrenal capacity, while they differed in the reactivity to handling (Fig. 1). Indeed, FP+ hens showed levels after handling during 10min than FP- hens, while controls hens showed intermediate levels.

Figure 1: Corticosterone levels (ng/ml plasma) measured before treatment, after handling during 10 min and 10 min post-injection of 1-24 ACTH at a dose of 10 μ g/kg B.W. in laying hens from two divergently selected genotypes for feather pecking and a control line. (Mean \pm S.D.)



4. Discussion

The results of this study indicated that a divergent selection program using a direct measure of feather pecking bouts had an effect on the response in corticosterone following a physical stress. This finding contradicts those of previous studies (Korte *et al.*, 1997; van Hierden *et al.*, 2001;) which reported higher responsiveness for FP- hens. However, results for poults (van Hierden *et al.*, 2001) and laying hens (Korte *et al.*, 1997) originated from independent lines having different genetic backgrounds. On the other hand, results from Guémené *et al.*, (manuscript 9) originated from comparisons between birds selected on their capacity to peck a fake. It was incidentally found that when raised collectively during the rearing period, the FP- will express significantly more feather pecks and vice versa. The results of the two studies in which hens from divergently selected strains for feather pecking were used are therefore coherent.

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Manuscript 10 (INRA-SRA, UNIVBRI & UNIZAR, Partners: 5, 7 & 9)

In: 7th European Symposium on Poultry Welfare, Lublin (Poland), 15-19 juin 2005 (Poster).
Animal Science Papers and Reports 23(1): p269-270.

Physiological indicators: contribution to the welfare assessment in various housing systems for laying hens

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Egg production, as one of the most intensive forms of animal husbandry, is considered to be a major welfare concern. Directive 1999/74/EC introduced technical modifications to the current battery cage system and will result in their ban. The housing systems for laying hens then include furnished cages, aviaries and alternative floor systems. There is still limited practical experience with production in these systems and since they are constantly developing, knowledge on their welfare implications needs continuous updating. Although not all physiological stress responses are indicative of detrimental conditions, a correlation is found between these parameters and sub-optimal conditions for the bird.

Firstly, boundaries should be drawn between acute and chronic stages of the stress response. Thus, handling an untrained hen for a brief period will be perceived as an acute stress, while prolonged confinement in over-crowded conditions might be perceived as a chronic stressor. Following an acute stress, one of the primary physiological reactions is the activation of the HPA axis, which is reflected by an increased concentration of corticosterone in the plasma. On the other hand, chronic stress can result in a progressive hypersensitivity then a decrease in adrenal capacity that can be addressed using ACTH challenges. Secondary physiological effects impede production of antibodies and effective cell-mediated immunity, while an increase in the heterophil-lymphocyte ratio (H/L) is considered as a good stress indicator. All these responses could differ in nature and intensity in interaction with the genotypes, the age, the physiological stages, etc.... These different physiological parameters have been investigated, by different partners in the EU LayWel project, in various experimental conditions which apply for the production of eggs in Europe, e.g. using different genotypes, management conditions especially furnished cages, stocking densities, group sizes, etc.

If not all, some of the physiological indicators used were somehow affected by the various experimental conditions. However, depending upon the trial and/or the indicator within a trial, controversy arises on how to interpret conflicting results. Thus, it was not possible to conclude if housing hens in furnished cages compared to conventional ones improved their welfare. Likewise, in single-tier floor systems, densities and management had no impact upon corticosterone levels and H/L ratio. Whenever it was possible to address the issue, strong interactions with the genotype as well as the rearing conditions of the pullets were observed.

In conclusion, although physiological stress parameters can provide valuable information, how an animal perceives its environment and thus how suitable this environment is to the animal, it appeared from these studies to be difficult to reach firm conclusions regarding hen's welfare in the experimented housing systems even when used together with other indicators.

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