Welfare Indicators in Laying Hens

Malin Alm
Faculty of Veterinary Medicine and Animal Science
Department of Animal Nutrition and Management
Uppsala

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Abstract

There is a growing concern regarding the welfare of laying hens worldwide and there are both ethical and economic reasons for improving their welfare. Although several different welfare indicators are used today, their ability to accurately reflect welfare status is sometimes questioned. This thesis sought to increase knowledge regarding a number of these welfare indicators by investigating whether and to what extent they were affected when layers were exposed to different stressors. Three different commercial laying hen genotypes were used and birds were challenged by being excluded either from their nests in furnished cages or from the litter area in a single-tier floor system.

Excluding birds from their nests resulted in an increased stress response that was detectable in corticosterone metabolites in droppings, corticosterone concentration in egg yolk, heterophil to lymphocyte ratio and egg shell irregularities. Excluding birds from the litter area during the first two weeks in the laying facility resulted in differences in feather cover, approaches towards a novel object, tonic immobility duration and egg shell irregularities measured later in the laying period. Interestingly, according to the welfare indicators used, birds previously excluded from the litter area, and consequently deprived of litter and available area, had better welfare than non-excluded birds. In addition, levels of corticosterone metabolites in droppings were influenced by factors such as diet, genotype, bird age, cage tier, droppings mass, time of day and the kind of assay used.

The results showed that several, but not all, indicators were able to detect different stress responses, suggesting that they are more or less appropriate to use depending on the situation prevailing. Many factors influenced the results obtained and there were few and inconsistent correlations, displaying a complex relationship between indicators. Overall, this indicates that welfare assessment should preferably be based on results from several indicators and that careful interpretation of the results is required before stating firm conclusions regarding laying hen welfare.

Keywords: Laying hens, welfare indicators, stress, corticosterone, feather cover, tonic immobility, novel object, H/L ratio, egg shell irregularities, behaviour, production.

Author's address: Malin Alm, SLU, Department of Animal Nutrition and Management, P.O. Box 7024, 750 07 Uppsala, Sweden
E-mail: Malin.Alm@slu.se

A hen is only an egg's way of making another egg.
Samuel Butler (1835-1902)

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Alm, M., Holm, L., Tauson, R. and Wall, H. (2014). Corticosterone metabolites in laying hen droppings- Effects of fiber enrichment, genotype, and daily variations. *Poultry Science* 93, 2615-2621.

II Alm, M., Tauson, R., Holm, L., Wichman, A., Kalliokoski, O. and Wall, H. A comparison of potential welfare indicators measured in laying hens in relation to nest exclusion (manuscript).

III Alm, M., Wall, H., Holm, L., Wichman, A., Palme, R. and Tauson, R. (2015). Welfare and performance in layers following temporary exclusion from the litter area on introduction to the layer facility. *Poultry Science* 94, 565-573.

Abbreviations

DM	Dry matter
DW	Dekalb White
EIA	Enzyme immunoassay
FAWC	Farm Animal Welfare Council
FCM	Faecal corticosterone metabolites
H/L	Heterophil to lymphocyte ratio
HPA	Hypothalamic-pituitary-adrenal
LB	Lohmann Brown
LSL	Lohmann Selected Leghorn
NO	Novel object
TI	Tonic immobility

1 Introduction

In the 17th century, the French philosopher Descartes described animals as complicated self-automated machines without the ability to feel pain and hence unable to suffer (Steiner, 2005). Since then, the moral status of animals has changed considerably. Ruth Harrison's book "*Animal Machines*" in 1964 started the debate and concern for farm animal welfare in the intensive farming practices that had developed during the 1960s (van de Weerd & Sandilands, 2008). The report by the Brambell Committee in 1965, where views regarding the feelings of animals, health and natural behaviour were described, then had a major influence on animal welfare research and also on animal welfare legislation in Europe (Keeling *et al.*, 2011). With the aim of improving the welfare of domesticated animals, applied research within different disciplines followed, where *e.g.* ethologists studied animal behaviour and described behavioural needs, while the concept of stress was studied within physiological research (Veissier & Miele, 2014). Although the meaning of welfare and stress can vary greatly depending on the person asked, the fact that they are connected and influenced by both physiological and behavioural responses is now commonly accepted (Moberg, 2000).

Today, public awareness regarding the quality, safety and ethical issues of food production is greater than ever. In general, animal-related food products are now expected to be produced and processed with increased consideration for animal welfare (Blokhuis *et al.*, 2003). Within the European Union, 77% of consumers believe that further improvements in animal welfare are necessary (European Commission, 2007). Animal welfare is in turn closely linked to the quality of the product, according to the perception of most consumers (Ingenbleek & Immink, 2011). Since poor welfare often reduces productivity, product quality and profitability, there are economic, ethical and safety reasons for monitoring and improving the welfare of farmed animals (Blokhuis *et al.*, 2013).

Improvements regarding the welfare of laying hens can be achieved by development and enhancement of environmental factors such as production systems, management practices, procedures for transportation and slaughter, diet formulation and allocation of enrichment. Laying hen welfare may also be greatly affected by breeding goals and legislation. In order to evaluate how these factors affect laying hens, assessments of stress and welfare are needed. If stress and welfare can be monitored, this makes it possible to avoid specific stressful situations and reduce inevitable causes of poor welfare (Broom & Johnson, 1993). How stress and welfare assessments should be performed is not an easy question, however, and opinions in this area vary considerably (Butterworth, 2009). Several different indicators are now being used, or have been suggested for use, in experimental studies and on commercial farms, to evaluate welfare (Welfare Quality, 2009; Nicol *et al.*, 2011; Cook, 2012). The results obtained can form the basis for changes in management practices, breeding goals, legislation or perhaps specific welfare labels on food products. However, whether the indicators adequately reflect the welfare in different situations is not fully understood and hence this issue needs further investigation.

2 Background

2.1 Welfare and stress

Welfare is a multidimensional concept (Botreau *et al.*, 2007) and there are many different opinions regarding how it should be defined. For example, Broom (1986) stated that "the welfare of an individual is its state as regards its attempts to cope with its environment". In more recent years, the concept of allostasis, *i.e*. the ability of an animal to cope with physical and mental stress, was introduced (Korte *et al.*, 2007). The Farm Animal Welfare Council (FAWC) describes the five freedoms, which define ideal states of welfare, as: 1) Freedom from hunger and thirst; 2) freedom from discomfort; 3) freedom from pain, injury or disease; 4) freedom from fear and distress; and 5) freedom to express normal behaviour. The scientific term 'animal welfare' refers to the actual and current state of the animal, involving both mental and physical status (Keeling *et al.*, 2011; Backus *et al.*, 2014). Hence, the welfare concept has altered over the years and will most likely continue to do so in the future (Backus *et al.*, 2014).

According to (Backus *et al.*, 2014), stress is a biological response to internal or external events, real or perceived, that disrupt homeostasis. The stimulus or threat that elicits a stress response is referred to as a 'stressor'. As is the case for welfare, stress is a complex concept, with several opinions regarding its definition (Jensen & Toates, 1997). However, a connection between welfare and stress is difficult to ignore (Moberg, 2000; Mormède *et al.*, 2007). Therefore, a common approach when evaluating welfare is to assess the stress response of the animals. When animals are faced with a physical or emotional stressor, different biological systems are activated, causing stress responses in order to cope with the potential threat to homeostasis. These responses can cause behavioural and immunological changes, responses in the autonomous nervous system and responses in the neuroendocrine system (hypothalamic-

pituitary-adrenal (HPA) axis) (Moberg, 2000). For example, the stress hormones adrenalin and noradrenalin are regulated by the autonomic nervous system, whereas cortisol and corticosterone (glucocorticoids) are regulated by the neuroendocrine system. The stress response is complex and can vary greatly between different individuals depending on prior experience, hormonal status and type of stressor (Cook *et al.*, 2000). For most people, stress is associated with something negative. However, it should be borne in mind that it helps the individual cope with their surroundings and is also displayed *e.g.* during excitement. Yet, if the stress response is very strong or continues for a long period, it can have very negative effects on the individual. This is due to redistribution of resources, resulting in impairment of the biological functions involved *e.g.* in immune responses, reproduction and growth (Moberg, 2000). If a stressful stimulus is repeated frequently or over an extended period, the adrenal cortex (producing glucocorticoids) may adapt, which results in cessation of the response. Hence, an increased glucocortical response indicates that an outer stimulus exists, but a decreased response may not be evidence of disappearance of that stimulus (Broom & Johnson, 1993). In general, short-term or acute stress has been designated as stress lasting minutes to hours, whereas long-term or chronic stress lasts for several hours a day over weeks (Dhabhar & McEwen, 1997).

In order to assess and improve the welfare of laying hens, a wide range of different indicators are suggested to be monitored and recorded (Welfare Quality, 2009; Nicol *et al.*, 2011; Cook, 2012). Some of these indicators were chosen for further investigation in this thesis and are introduced below.

2.2 Corticosterone

In birds, corticosterone is the main end product of the HPA axis (deRoos, 1961). In response to a stressor, plasma levels of corticosterone are elevated within minutes, with the aim of mobilising energy stores through protein catabolism (Harvey *et al.*, 1984). Measuring these circulating levels of corticosterone has long been considered a standard way of assessing stress (Mormède *et al.*, 2007). However, the method is invasive and requires blood sampling of a hen within minutes after capture to avoid disruptive effects from the procedure itself (Radke *et al.*, 1985). Other non-invasive ways to measure the corticosterone response have therefore been developed.

After being distributed in the body, corticosterone is metabolised in the liver and excreted via the kidneys into urine or via the bile into the gut (Palme *et al.*, 1996; Möstl & Palme, 2002). Hence, faecal corticosterone metabolites (FCM) can be measured in bird droppings and reflect the levels found in the

plasma around 1-4 hours earlier (Rettenbacher *et al.*, 2004). This method offers the advantage of sample collection without disturbance of the birds and has been shown to be capable of detecting increased stress levels (Rettenbacher *et al.*, 2004; Rettenbacher & Palme, 2009).

Another proposed non-invasive way to measure corticosterone response is in the eggs, where the majority of the egg corticosterone (80%) is found in the yolk (Royo *et al.*, 2008). Corticosterone has been shown to accumulate in the yolk during egg formation (Rettenbacher *et al.*, 2009), but the exact method of deposition is not yet fully understood (Groothuis & Schwabl, 2008). The interest in investigating egg corticosterone is increasing and measuring corticosterone in the yolk has been proposed as a possible indicator of stress (Royo *et al.*, 2008; Singh *et al.*, 2009). Measurement of corticosterone in the albumen is also sometimes performed (Downing & Bryden, 2008; Cook *et al.*, 2009), but was not investigated in this thesis.

2.3 Heterophil to lymphocyte ratio

The leucocytes in the blood are involved in the immunological functions of the body. Heterophils and leucocytes make up the majority of leucocytes in birds, with heterophils playing a phagocytic role in the acute inflammatory response (Harmon, 1998) and leukocytes being involved in the adaptive part of the immune response (Alberts *et al.*, 2002). In birds, the number of heterophils has been shown to increase and the number of lymphocytes to decrease in response to a number of different stressors (Maxwell, 1993; Davis *et al.*, 2008). Therefore, the relative proportion of heterophils to lymphocytes (H/L ratio) is often used as a measure of the stress response. Despite the more general usage of H/L ratio to determine long-term stress, there are also studies showing quite a rapid increase in H/L ratio in relation to different short-term stressors or glucocorticoid treatment (Gross, 1990; Mitchell *et al.*, 1992; Shini *et al.*, 2008).

2.4 Feather cover

Feather pecking in commercial laying hen flocks is an unwanted behaviour that results in impaired welfare (Rodenburg *et al.*, 2013). It also leads to economic losses by increased feed intake in hens with poor feather cover, which have to compensate for their body heat loss due to insufficient insulation (Tauson & Svensson, 1980). The behaviour is multi-factorial but has been associated with stress, among other things (El-Lethey *et al.*, 2000). The occurrence of feather pecking can be estimated indirectly by scoring bird feather cover, since feather pecking results in feather damage. Scoring of feather cover is therefore widely

used as an indicator of welfare (*e.g.* Welfare Quality, 2009; Freire & Cowling, 2013) and in a survey was ranked the most important welfare indicator by many experts (Rodenburg *et al.*, 2008).

2.5 Behaviour – observations and tests

Observing the behaviour of animals is one way of assessing welfare that can offer a large range of information regarding the animals' needs, preferences and internal states (Olsson *et al.*, 2011). In the home environment of birds, specific behaviours can be quantified by ethological approaches, but can also be investigated by different behaviour tests. For example, estimating the fearfulness of birds is a commonly used welfare approach because fear can be defined as an animal's avoidance of danger (Jones, 1996) and therefore considered a state of suffering. Fearfulness can be assessed in a number of different ways in laying hens, two of which are the tonic immobility (TI) test and the novel object (NO) test. Tonic immobility is an unlearned anti-predation response (Jones, 1996) displayed as a state of immobility after manual restraint. In the NO test, birds' avoidance of a conspicuous novel stimulus (*e.g.* a multi-coloured stick or pencil) is measured. A longer duration of TI and higher avoidance of NO are believed to indicate higher fearfulness (Jones, 1986).

2.6 Egg shell irregularities

During the production period in a laying hen flock, a proportion of the eggs normally display a variety of shell irregularities (Wolc *et al.*, 2012). There can be several reasons why different irregularities occur, as some have been shown to be caused by delayed oviposition due to stress (Hughes *et al.*, 1986; Mazzuco & Bertechini, 2014). Therefore, assessment of different kinds of irregularities can be used as an indicator of environmental stress (Reynard & Savory, 1999), and hence may serve as an indicator of welfare status (Sherwin *et al.*, 2010).

2.7 Production parameters

Production traits such as egg production, feed and water consumption are carefully recorded in commercial laying hen flocks. Whether these parameters can serve as an indicator of welfare is debatable. There are studies showing clear associations between welfare and production parameters, *e.g.* increased fearfulness related to decreased egg production (Barnett *et al.*, 1994) and lower egg weight (De Haas *et al.*, 2013), inferior feather cover related to increased feed consumption (Tauson & Svensson, 1980) and higher basal plasma levels of corticosterone related to lower egg weight (De Haas *et al.*, 2013). However, the relationship between welfare and production is not clear, because birds may still have high production levels but poor welfare. Therefore, changes in production parameters are suggested to be used as a possible first sign of impaired welfare, but it is claimed that they are inappropriate to use as important general indicators of welfare (Blokhuis *et al.*, 2007; Keeling *et al.*, 2011).

2.8 Use of several indicators in the same study

Due to the complexity of stress responses, resulting in both behavioural and physiological responses when adapting to environmental challenges, it is generally recommended that more than one variable be investigated in order to understand the context (Cook *et al.*, 2000). Thus, nowadays there is increased interest in using multiple welfare indicators within the same study (Nicol *et al.*, 2011). However, in some studies the indicators can be in agreement, but in others they may disagree. For instance in one previous study, birds that were kept under different housing conditions showed the highest prevalence of feather pecking, longest TI duration and highest H/L ratio in the same groups (kept without access to straw and given pelleted feed) (El-Lethey *et al.*, 2000). In another study, birds with inferior feather cover instead showed shorter duration of TI and lower H/L ratio compared with birds having superior feather cover (Campo *et al.*, 2001). Hence, including a wide range of indicators may lead to difficulties in interpreting the outcome, since different indicators can show contradictory results (Nicol *et al.*, 2006; Moe *et al.*, 2010). The reasons for these discrepancies can be many, but in some cases they may be due to misleading results that have been elevated or changed due to other factors not directly connected to stress responses and welfare.

3 Aims of the thesis

The overall aim of this thesis was to obtain better knowledge on a number of indicators more or less commonly used for assessment of welfare in laying hens, by studying the influence of different factors and challenges. Specific objectives were to:

- Gain more knowledge regarding FCM levels in droppings as a measure of stress by investigating whether factors such as diet, time of day, genotype, cage tier, dropping mass or bird age affect these levels.

- Study whether, and to what extent, different welfare indicators are affected when hens are:

  a) Exposed to a relatively short-term stress challenge, such as exclusion from nests.

  b) Excluded from their litter area during the initial part of the laying period.

- Investigate the relationship between different welfare indicators.

- Evaluate how exclusion from the litter area, a management procedure sometimes applied on commercial farms, affects overall welfare and production in laying hens.

4 Materials and Methods

This thesis is based on three experiments (described in Papers I-III, respectively) conducted at the Swedish Livestock Research Centre at Lövsta, Uppsala, Sweden, between 2011 and 2013. The experimental facilities have been certified for research purposes by the Swedish Board of Agriculture and the studies and all procedures included were approved by the Uppsala Local Ethics Committee before commencement.

Three commonly used commercial laying hen genotypes were included in the experiments; Lohmann Selected Leghorn (LSL), Lohmann Brown (LB) and Dekalb White (DW). The Lohmann genotypes were obtained from the breeder Gimranäs AB (Sweden) and the DW genotype from the breeder Swedfarm AB (Sweden). The birds were housed in either furnished 8-hen cages described by Wall & Tauson (2013) (Figure 1) or in a single-tier floor laying system with 100 birds in each pen (Figure 2). No birds were beak-trimmed. An overall description of the studies and the indicators investigated are listed below and a summary is provided in Table 1. More detailed descriptions are found in the specific papers (I-III) describing each of the three studies.

4.1 Paper I

The aim of the study described in Paper I was to investigate whether and how FCM levels and mass of droppings were affected by dietary treatment, genotype and diurnal rhythm. Two genotypes, LSL and LB, were housed in furnished cages and the study was performed during three consecutive days at the age of 40 weeks. Droppings were sampled four times per day and analysed with a corticosterone enzyme immunoassay (EIA). One experimental unit consisted of 10 cages, which resulted in three replicates per combination of diet and genotype.

4.2 Paper II

The aim of the study presented in Paper II was to measure several welfare indicators before, during and after a stressful challenge, in order to investigate to what extent they were affected and the degree of correlation between different indicators. Laying hens (LSL genotype) housed in furnished cages were used in this study. At 66 weeks of age, they were either excluded from their nests for a period of five consecutive days or housed as prior to the study, *i.e.* with unlimited access to nests. Exclusion from the nests was hypothesised as being very stressful, because layers are highly motivated to lay their eggs in a nest (Weeks & Nicol, 2006; Kruschwitz *et al.*, 2008). The indicators tested were FCM levels in droppings, corticosterone concentration in plasma and egg yolk, feather cover, TI duration, H/L ratio and egg shell irregularities. Some behaviour recordings were also performed. Cage was considered the experimental unit, giving eight replicates per treatment.

4.3 Paper III

The aim of the study reported in Paper III was to investigate how the welfare and performance of layers were affected by the hens being excluded from the litter area when introduced to the laying facility. This is a routine sometimes adopted (Lambton *et al.*, 2010) in order to help the layers quickly find food and water and to minimise the number of floor-laid eggs later on. The procedure is permitted in most EU countries but not in Sweden, since it results in deprivation from both litter and a substantial amount of space, which is thought to have a negative impact on bird welfare. However, whether and how this procedure actually affects welfare and production have never been investigated previously. Pullets (DW genotype) housed in single-tier floor pens were used in this study. On introduction to the laying facility, birds were either excluded from the litter area for two weeks (16-18 weeks of age) or given unlimited access to the litter area. Production performance and welfare indicators were measured between 16 and 72 weeks of age. The indicators tested were FCM levels in droppings, feather cover, TI duration, NO test and egg shell irregularities. Some behaviour recordings were also performed. Pen was considered the experimental unit, giving three replicates per treatment.

Figure 1. Pictures of the furnished cage system used in Papers I and II. The left picture shows the set-up of the cage system in three tiers. The right picture shows one furnished cage including eight hens, a feed trough in front, a perch to sit on, water nipples in the back, an enclosed nest with a blue plastic curtain and a litter bath on top filled with sawdust. Photo: M. Alm.

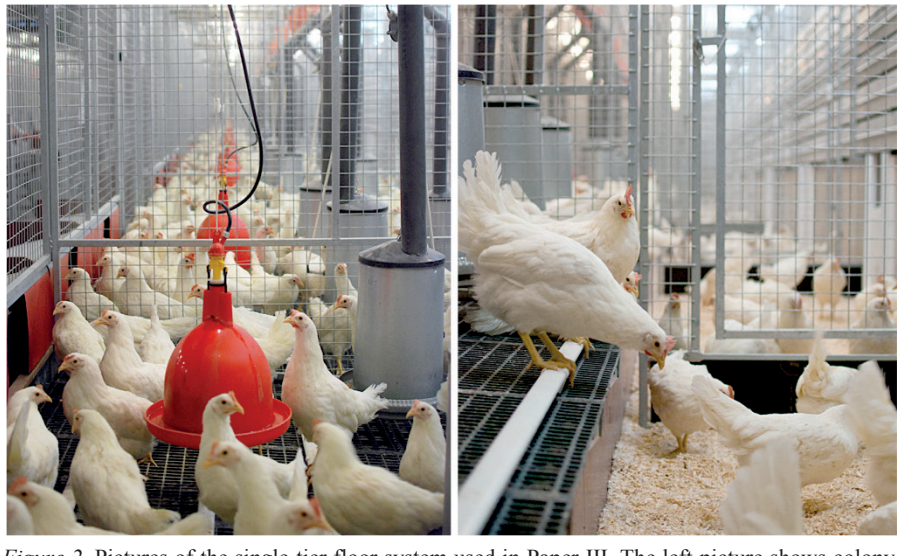


Figure 2. Pictures of the single-tier floor system used in Paper III. The left picture shows colony nests enclosed by red plastic curtains in the front and food and water supplied on the slatted elevated floor area. The right picture shows integrated perches on the slatted floor and the litter area, which consisted of wood shavings. Photo: M. Alm.

Table 1. *Summary of the experimental structure and indicators included in Papers I-III.*

	Paper I	**Paper II**	**Paper III**
Housing:	Furnished cages	Furnished cages	Single-tier floor pens
Genotype:	LSL and LB	LSL	DW
Replicates:	3 per genotype and treatment	8 per treatment	3 per treatment
Number of birds in total:	960	128	600
Age of the birds:	40 weeks	61-70 weeks	16-72 weeks
Experimental period:	3 days	10 weeks	57 weeks
Treatment:	Control: Basal diet	Open: Unlimited access to nests	Open: Unlimited access to litter area
	Fibre: Basal diet + 3% fibre	Closed: Exclusion from nest for 5 days	Closed: Exclusion from litter area for 2 weeks
Indicators:			
FCM in droppings	X	X	X
Corticosterone in egg yolk		X	
Corticosterone in plasma		X	
H/L ratio		X	
TI duration		X	X
NO test			X
Egg shell irregularities		X	X
Feather cover		X	X
Behaviour observations		X	X
Production parameters			X

LSL = Lohmann Selected Leghorn; LB = Lohmann Brown; DW = Dekalb White; FCM = Faecal corticosterone metabolites; H/L = heterophil to lymphocyte ratio; TI = tonic immobility; NO = novel object.

4.4 Measures included in the studies

4.4.1 Faecal corticosterone metabolites in droppings

Bird droppings were collected, placed in sealed plastic bags and frozen (-20 °C) before an extraction procedure was performed. In all three studies, the droppings were analysed by a corticosterone EIA and in Paper III a cortisone EIA was performed in parallel. Before analysis with the corticosterone EIA (Arbor Assays, Ann Arbor, MI), the samples were thawed, homogenised, dried, ground and extracted according to the manufacturer's instructions using 0.2 g dried sample and 95% ethanol. Before analysis with the cortisone EIA (Rettenbacher *et al.*, 2004), the samples were instead thawed, homogenised and 0.5 g wet sample was extracted with 60% methanol (Palme *et al.*, 2013).

4.4.2 Corticosterone in egg yolks

Eggs were collected and egg yolks separated from the albumen (Paper II). All yolks from one cage were pooled and a 4 g sample was frozen (-20 °C). Prior to analyses, the samples were thawed and extracted with ethanol according to the protocol by Kozlowski *et al.* (2009). The extracts were then analysed with a corticosterone EIA (DRG Instruments GmbH, Marburg, Germany).

4.4.3 Corticosterone in plasma

Blood samples were drawn from the bird's wing vein using a 0.8 mm needle and a syringe (Paper II). On average, it took 1.5 min from catching until the blood was collected. Blood samples were poured into tubes (coated with potassium- ethylene diamine tetraacetic acid) and stored (at 8 °C) for a couple of hours before centrifugation. The plasma was collected and frozen (-20 °C) until analysis with the same corticosterone EIA as was used for droppings samples (Arbor Assays, Ann Arbor, MI).

4.4.4 Heterophil to lymphocyte ratio

Blood for analysis of H/L ratio was collected simultaneously with the samples for plasma corticosterone analysis (Paper II). Blood smears on glass slides were stained and 200 leukocytes counted, of which the number of heterophils was divided by the number of lymphocytes.

4.4.5 Feather cover

For assessment of feather cover and body condition, the integument scoring protocol devised by Tauson *et al.* (2005) was used in Papers II and III, but bird weight, foot condition and keel bone deviations were omitted from the protocol in Paper II. Birds were scored using a four-point scale with respect to feather cover on the neck, breast, cloaca, back, wings and tail, where 1 equals totally denuded and 4 a fully feathered body part. A total feather cover score ranging from 6 to 24 was calculated from the six body parts. Pecking wounds on comb and rear body part, keel bone deviations and foot condition were also assessed using a graded scale ranging from 1 (worst condition) to 4 (best condition).

4.4.6 Tonic immobility duration

The TI test for assessment of fearfulness was performed in Papers II and III. The procedure was performed as described by Jones & Faure (1981) in an adjacent room. The bird was placed on its back in a U-shaped cradle and TI was induced by carefully restraining the bird for 15 s. If the bird stayed motionless for 10 s following the restraint, induction of TI was considered successful and the latency to self-righting (duration of TI) was recorded. No more than three attempts per bird were made to induce TI and a bird was allowed to stay in TI for a maximum of 15 (Paper III) or 20 min (Paper II). All tests were performed by the same operator.

4.4.7 Novel object test

The NO test was performed as described in the Welfare Quality® (2009) assessment protocol for poultry, with some adjustments due to the design of the experimental facilities (Paper III). A 20 cm long multi-coloured stick was placed in the middle of the litter area in each pen. The number of hens within one hen length of the NO was then recorded every 10 s for a total period of 2 min. All tests were performed by the same operator.

4.4.8 Behaviour observations

Some behaviour observations were made by analysing video recordings from Papers II and III. In Paper II, scan sampling was performed where the number of birds eating, sitting inside the nests or giving their attention to the nests was recorded. In Paper III, the activity of the birds during the time of exclusion from the litter area was investigated by counting the number of runs (a hen running for a minimum distance of two hen lengths). Utilisation of the litter area was also investigated by counting birds in this area using snapshots from the video recordings.

4.4.9 Egg shell irregularities

The number of eggs displaying different shell irregularities was recorded in Papers II and III. One egg at a time was visually examined and categorised as having any of the following irregularities: wrinkled top, pimpled (with small bumps), spotted (areas with thinner shell), striped (longitudinal grooves), thin-shelled, or without any irregularity.

4.4.10 Production parameters

In Paper III, egg production, laying percentage, number of floor-laid eggs, mortality, feed intake and feed conversion ratio (FCR) were calculated from 20 to 72 weeks of age. The proportion of cracked and dirty eggs was assessed by candling on seven occasions during the laying cycle.

4.5 Correlations between indicators

In Paper II, correlations between the indicators FCM levels in droppings, corticosterone concentration in egg yolk and plasma, egg shell irregularities (sum), H/L ratio, TI duration and feather cover were analysed based on group means with different parts of the dataset included (*e.g.* basal levels alone or together with measures during the nest exclusion). In addition, individual values per hen and day were tested for H/L ratio versus corticosterone in plasma and feather cover versus TI duration.

Correlations between the indicators used in the third study (not included in Paper III) were calculated. Correlations between indicators for which data from more than one occasion were available were compared based on group means. These indicators were FCM in droppings (from the cortisone EIA), NO approaches and feather cover. In addition, individual values per hen and day were tested for feather cover (not presented in Paper III) versus TI duration.

4.6 Statistical analysis

Data analysis in all studies was performed using SAS statistical software (version 9.2, SAS Institute Inc., Cary, NC). Tukey-Kramer adjustment for multiple comparisons was included in pair-wise comparisons and the level of significance was set at $P<0.05$. The Mixed procedure was used for production parameters, FCM levels in droppings, corticosterone in egg yolk, corticosterone in plasma, feather cover, NO approaches, H/L ratio and behaviour recordings. The TI latency in Paper II was analysed with the Mixed procedure, but TI latency values in Paper III were not normally distributed and were therefore instead compared using the Kruskal-Wallis test. Egg shell

irregularities were analysed with the Glimmix procedure using a logistic regression distribution. The fixed effects of treatment and time of sampling were included in the statistical models of all studies, as well as interactions between fixed effects. In addition, the fixed effect of tier level was included in Papers I and II and that of genotype in Paper I. Pearson's product-moment correlation was used to investigate the correlations between indicators in Papers II and III.

5 Results

5.1 Faecal corticosterone metabolites in droppings

5.1.1 Effect of changes in dropping mass

In Papers I and II, total collection of droppings produced during a specified period was carried out, allowing expression of FCM in terms of both concentration (ng/g) and excretion rate (total amount/h). The mass of droppings produced differed between genotypes, diets (in one genotype), times of day and tier levels (Paper I), resulting in different outcomes depending on how FCM were expressed. The excretion rate, taking differences in droppings mass into account, differed between genotypes and there were interactions between genotype and diet, whereas no such differences were observed for concentration of FCM. In Paper II, however, mass of droppings was not affected by nest exclusion or sampling day and hence, results are only presented as concentrations.

5.1.2 Effect of diurnal rhythm, diet, genotype and cage tier

Both FCM excretion rate and concentration varied during the day, but did not display a distinct and recurring diurnal rhythm (Paper I). Mean values for three days showed their highest levels 0 and 4 h after onset of light and their lowest levels 8 h after onset of light, when measured 0, 4, 8 and 12 hours after lights on. A diet containing 3% more fibre resulted in increased FCM concentration in both genotypes, but excretion rate was increased only in LB hens (Figure 3). No effect of cage tier was seen in Paper I, but in Paper II the middle cage tier displayed higher FCM levels than the top tier, with the bottom tier having intermediate FCM levels.

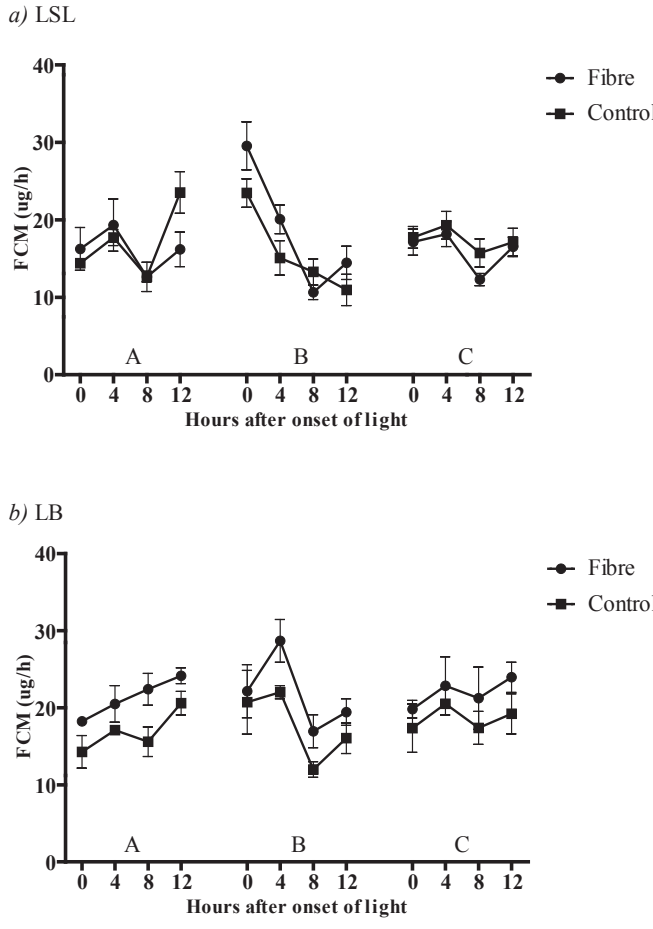


Figure 3. Excretion rate (mean ± SEM) of faecal corticosterone metabolites (FCM) measured in droppings from a) Lohmann Selected Leghorn (LSL) and b) Lohmann Brown (LB) hens from each feed treatment during three consecutive days (A, B, and C). Control = basal diet; Fibre = basal diet supplemented with 3% ground straw pellets replacing a fraction of the wheat.

5.1.3 Effect of exclusion from nests and exclusion from the litter area

Exclusion from the nests resulted in elevated FCM levels in droppings, which served as biological validation of the immunoassay used (Paper II). However, birds that had unlimited access to the nests displayed the same elevated pattern as birds excluded from the nests (Figure 4). For significant differences between days, see Figure 1 in Paper II.

Exclusion from the litter area after introduction to the laying facility (Paper III) did not affect FCM levels (in either of the two EIAs performed), either at the time of exclusion or later in the laying period, in comparison with birds with unlimited access to the litter area throughout (Figures 5a and 5b).

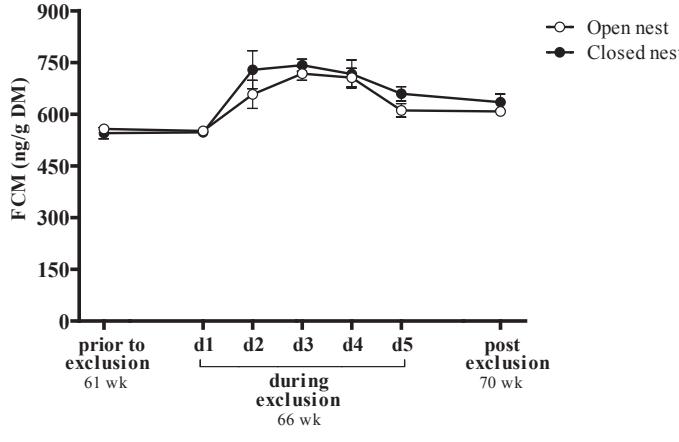
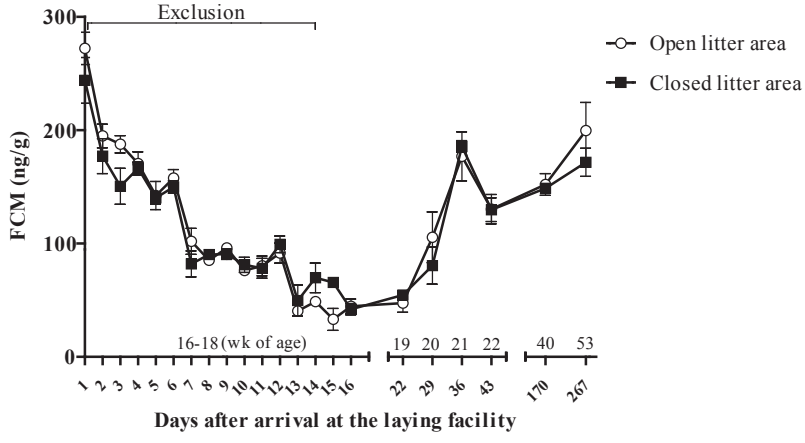


Figure 4. Concentration (mean ± SEM) of faecal corticosterone metabolites (FCM) measured in droppings from hens in each treatment prior to, during and post exclusion from nests. Open nest = hens with unlimited access to the nest; closed nest = hens excluded from the nest during five consecutive days.

a) Cortisone EIA



b) Corticosterone EIA

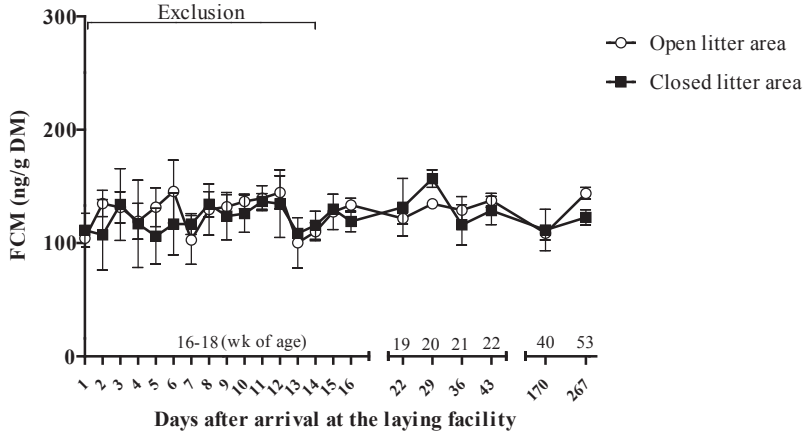


Figure 5. Concentrations (mean ± SEM) of faecal corticosterone metabolites (FCM) measured by *a)* a cortisone enzyme immunoassay (EIA) and *b)* a corticosterone EIA in droppings from hens with unlimited access to the litter area (open litter area) and hens excluded from the litter area during the first two weeks after transfer to the laying facility (closed litter area). FCM levels were measured on 22 occasions between days 1 and 267 after arrival at the laying facility.

5.1.4 Effect of different assays

In Paper III, where droppings samples were collected throughout almost a whole laying period, the outcome from the two different EIAs displayed different patterns. The cortisone EIA showed the highest levels directly after introduction to the laying facility, the lowest levels around 18-20 weeks of age and then increased levels later in the laying period (Figure 5a). The corticosterone EIA, on the other hand, showed a more even level, with very few significant changes between days (Figure 5b; data not included in Paper III).

5.2 Egg shell irregularities

During exclusion from the nests, the percentage of eggs with a wrinkled top and the sum of all egg shell irregularities were elevated (Paper II). However, similar elevations were apparent in eggs from birds not excluded from their nests (Figure 6). For significant differences between days, see Figure 3 in Paper II.

Exclusion from the litter area during the first two weeks in the laying facility resulted in a lower percentage of eggs with a wrinkled top and total shell irregularities measured later in the laying period (40 weeks of age) in comparison with birds given unlimited access to the litter area from the start (Paper III).

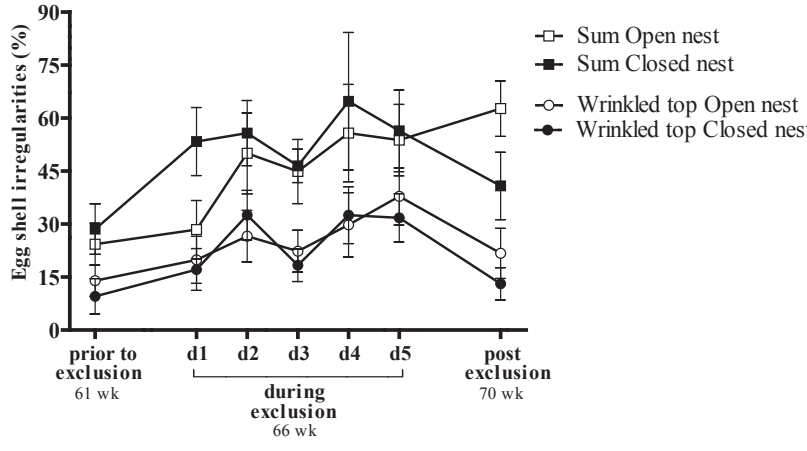


Figure 6. Percentage (mean ± SEM) of eggs with a wrinkled top and sum of all shell irregularities (wrinkled top, pimpled, spotted, striped and thin-shelled) found on eggs from hens in each treatment; Open nest = hens with unlimited access to the nest; closed nest = hens excluded from the nest during five consecutive days.

5.3 Feather cover

Exclusion from the nests did not affect feather cover (Paper II), but exclusion from the litter area in the first two weeks in the laying facility resulted in superior feather cover at both 40 and 54 weeks of age compared with birds with unlimited access to the litter area (Paper III). For both treatments, feather cover was inferior at 54 weeks of age compared with 40 weeks.

5.4 Tonic immobility duration

Exclusion from nests did not affect duration of TI (Paper II). However, exclusion from the litter area in the first two weeks in the laying facility affected duration of TI in birds later on. Birds that had previously been excluded from the litter area had shorter duration of TI at 49 weeks of age, indicating lower fearfulness, compared with birds that had unlimited access to the litter area from the start (Paper III).

5.5 Novel object test

Exclusion from the litter area in the first two weeks in the laying facility led to higher willingness to approach a NO at 38 to 57 weeks of age (Paper III), in comparison with birds that had unlimited access to the litter area from the start (Figure 7). There was no difference between the treatments in the beginning or end of the study and the approaches towards NO generally decreased with age, indicating increased fearfulness.

5.6 Heterophil to lymphocyte ratio

During exclusion from the nests, H/L ratio was elevated compared with the value measured 4 weeks prior to exclusion (Paper II). However, birds that were not excluded from their nests displayed the same pattern.

Some birds suffered from foot injuries due to the non-optimal leg rings and they all displayed extremely high H/L ratios. In addition, some high H/L ratios that could not be correlated with any visible health disorder were also present. Individual values for both categories were omitted from the dataset prior to analysis.

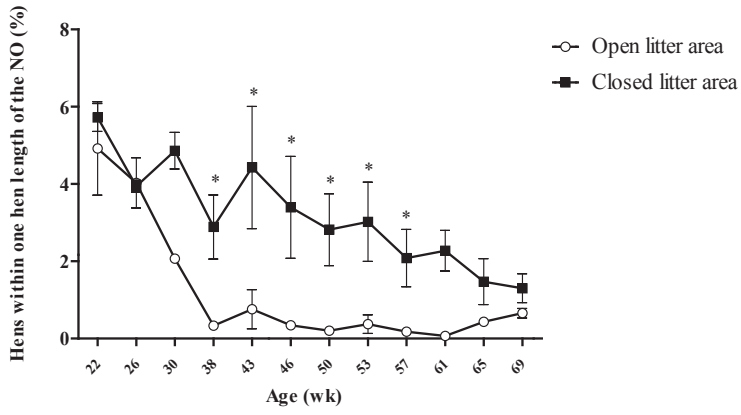


Figure 7. Percentage of hens (mean ± SEM) within one hen length of the novel object (NO) recorded at different ages (22 to 69 weeks) for hens with unlimited access to the litter area (open litter area) and hens excluded from the litter area during the first two weeks after transfer to the laying facility (closed litter area). The two treatments differ significantly ($P<0.05$) at ages marked with an asterisk.

5.7 Corticosterone in egg yolks

Exclusion from the nests resulted in elevated corticosterone levels in egg yolk (Paper II). However, egg yolks from birds that were not excluded also showed increased levels during this period (Figure 8). For significant differences between days, see Figure 2 in Paper II.

5.8 Corticosterone in plasma

In contrast to some other indicators, corticosterone levels in plasma were not elevated during the period of exclusion from the nests. Instead, increased plasma levels were seen four weeks after exclusion, both in birds previously excluded and in those that had not been excluded from their nests (Paper II).

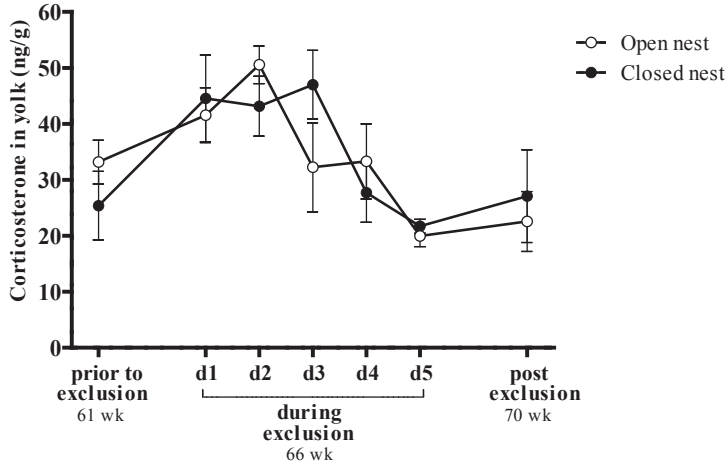


Figure 8. Concentration (mean ± SEM) of corticosterone in egg yolk measured in eggs from hens in each treatment before, during and after exclusion from nests. Open nest = hens with unlimited access to the nests; closed nest = hens excluded from the nests during five consecutive days.

5.9 Behaviour observations

When birds were excluded from the nests, they directed increased attention towards the nests compared with before exclusion and occasionally also displayed behaviours such as pecking and clawing at the nest door, head bobbing and attempts to access the nests from outside (Paper II).

During exclusion from the litter area, birds that were excluded performed fewer runs than birds with access to the litter area (Paper III). No difference in utilisation of the litter area was seen between the previously excluded and not excluded birds when the litter area became available for all groups of layers.

5.10 Production parameters

Exclusion from the litter area in the first two weeks in the laying facility resulted in lower feed intake and superior FCR than for birds with unlimited access to the litter area from start (Paper III). No differences were observed in other production parameters.

5.11 Correlations between indicators

In Paper II (nest exclusion), high basal levels of FCM were related to inferior feather cover (r = -0.37; P = 0.042; n = 30) and to increased percentage of the sum of all egg shell irregularities (r = 0.40; P = 0.027; n = 30). On including values collected during the challenge (nest exclusion), a correlation between FCM levels and egg shell irregularities also emerged. Three other significant correlations were found, but they were inconsistent and changed depending on which part of the dataset was compared. When comparing individual hen values, a positive relationship was observed between basal levels, *i.e*. prior to and post nest exclusion, of TI duration and feather cover score (r = 0.22; P = 0.017; n = 121), but no relationship was found between H/L ratio and plasma corticosterone concentration.

Correlations calculated based on data from the study described in Paper III (exclusion from the litter area), also revealed some relationships depending on which part of the dataset was compared (Table 2; not included in Paper III). High FCM levels in droppings were related to low willingness to approach the NO when comparing data for three different ages, but not when comparing only the latter two ages. Here, a low willingness to approach the NO was strongly related to inferior feather cover. There was a significant negative relationship between individual levels of TI duration and feather cover score (r = -0.40; P = 0.001; n = 60), but the relationship differed within the different treatments (Open: r = -0.33; P = 0.079; n = 30; Closed: r = 0.14; P = 0.472; n = 30).

Table 2. *Pearson's correlation coefficient (r, upper values) and P value (lower values) between indicators*[1]*. Significant correlations are highlighted in bold type.*

	Age when samples were collected	FCM in droppings	NO approaches	Feather cover
Three occasions (n = 18)				
FCM in droppings	(22, 40 and 53 wk)	1.00		
NO approaches	(22, 38 and 53 wk)	**-0.49** **0.038**	1.00	
Two occasions (n = 12)				
FCM in droppings	(40 and 53 wk)	1.00		
NO approaches	(38 and 53 wk)	-0.26 0.414	1.00	
Feather cover	(40 and 54 wk)	-0.43 0.159	**0.81** **0.002**	1.00

[1]FCM = Faecal corticosterone metabolites; NO = novel object

6 Discussion

6.1 Faecal corticosterone metabolites in droppings

6.1.1 Effect of diet, genotype and cage tier

The fact that addition of 3% fibre to the feed resulted in increased FCM levels (both concentration and excretion rate) in Paper I shows that rather small changes in the diet can affect FCM levels in laying hens. The reason for this increase is unknown, but it is probably not due to increased stress levels (Goymann, 2012). Microbial composition can affect the steroid hormones in droppings to a high extent (Klasing, 2005). Therefore, differences in FCM levels as a result of diet can instead be the result of formation of different types of corticoid metabolites, *e.g.* due to diet-derived variations in microbial composition (Goymann, 2012).

The differences between genotypes in terms of characteristics of droppings and FCM levels in Paper I may be explained by differences in consumption of feed and litter substrate between the two genotypes reported by Kalmendal *et al.* (2013). Besides genetic differences most likely explaining parts of the effect, differing microbial composition may also play a role in this case, as previously discussed.

The results regarding the effect of cage tier are difficult to explain, especially because the highest levels were seen in the middle cage tier and because an effect was only present in Paper II and not in Paper I.

6.1.2 Effect of diurnal rhythm

A diurnal rhythm in plasma corticosterone levels has been shown in laying hens with generally high levels at the beginning of the light period and low levels at the end of the light period (Beuving & Vonder, 1977; Etches, 1979; Johnson & van Tienhoven, 1981). Consequently, one could expect a diurnal rhythm also in FCM levels. In Paper I, the levels of FCM were found to fluctuate during the day, but no distinct and recurring diurnal pattern was obvious. The reason may be that FCM is excreted in the droppings over a specific period, leading to a smoother pattern compared with levels in the blood (Rettenbacher & Palme, 2009). Previous studies have also not found a diurnal rhythm (Rettenbacher *et al.*, 2004; Rettenbacher & Palme, 2009).

The lowest mean FCM excretion rate (based on mean values of all three days) was seen 8 h after onset of light in Paper I. Higher levels were found 0, 4 and 12 h after onset of light. Partly based on these findings, droppings samples were collected within the range 5.5-10 h after onset of light in Papers II and III. However, due to the inconsistency shown in FCM levels, the optimum time for collection of droppings was difficult to determine.

6.1.3 Concentration versus excretion rate

There are differing opinions regarding how to correctly express FCM. In most studies FCM levels are expressed in terms of concentration, which has been shown to give reliable results (Lepschy *et al.*, 2010). However, the concentration of FCM will incorrectly estimate FCM levels if the mass of droppings produced per time interval or the interval between defecation events is not relatively constant (Goymann & Trappschuh, 2011). Hence, some authors suggest expression of FCM as excretion rate (total amount FCM per hour), because it accounts for variations in dropping mass (Goymann *et al.*, 2006; Carlsson *et al.*, 2009). However, in many studies total collection of droppings produced in a given time interval is not possible. In this thesis work, total collection of droppings was not possible in Paper III, but was performed in both Papers I and II. The mass of droppings was not influenced by day of sampling or by exclusion from the nests in Paper II, whereas an effect of genotype, fibre-enriched feed (in one genotype), cage tier and time of day was seen in Paper I. Hence, in many cases concentration probably gives an accurate estimate of FCM. However, under certain conditions where the production of droppings may vary, *e.g.* between sampling occasions or treatments, the

excretion rate of FCM may be preferable because it accounts for changes in droppings production.

6.1.4 Comparison of FCM levels

In general, it is very difficult to compare FCM levels measured in different studies because of the many factors that may differ, both regarding the type of assay used and the range of different preparation steps (*e.g.* extraction and dilution). The difference regarding changes in FCM over time between the two EIAs in Paper III may be due to several factors, such as the different extraction methods used (Palme *et al.*, 2013) and the fact that the two assays detect different groups of corticosterone metabolites (Möstl *et al.*, 2005). The levels found in the cortisone EIA, especially the first high values directly after transfer from the rearing to the layer facility, seem credible, as transportation and relocation have been shown to increase FCM levels (Rettenbacher & Palme, 2009). Therefore, the cortisone EIA probably has higher biological sensitivity and can therefore detect smaller differences in adrenocortical activity (Touma & Palme, 2005), in comparison to the corticosterone EIA. This is also supported by the fact that the cortisone EIA is group-specific, *i.e.* especially developed for detecting a range of different corticosterone metabolites (Rettenbacher *et al.*, 2004). The corticosterone EIA, on the other hand, relies on cross-reactions with corticosterone metabolites, because corticosterone in its native form seems to be absent in droppings (Palme *et al.*, 2005). Methanol used as extraction medium prior to the cortisone EIA may also be a more efficient extractant compared with the ethanol used prior to the corticosterone EIA.

Despite the same extraction procedure and assay being used in Papers I and II, FCM levels differed when comparing birds of the same genotype (LSL). The birds were 40 and 61-70 weeks respectively, but the difference seemed too large to be the result of that factor alone. This difference remains unexplained and further highlights the difficulty in comparing FCM levels between studies.

6.1.5 FCM as an indicator of stress

Corticosterone metabolites in droppings have been shown to be a good indicator of stress in many studies (*e.g.* Carlsson *et al.*, 2009; Rettenbacher & Palme, 2009). Some of the results in this thesis support this finding, *e.g.* the elevation of FCM levels during exclusion from the nests in Paper II. In addition, the high levels of FCM displayed during the first days after transfer

of birds to the laying facility, a known stressor (Rettenbacher & Palme, 2009), in Paper III indicates an ability of FCM to reflect a relatively short-term stress response in laying hens.

In the latter part of the laying period in Paper III, the birds in the two treatment groups (excluded or not from the litter area) displayed large differences regarding feather cover, fearfulness and egg shell irregularities, suggesting that the stress level and welfare differed between treatment groups. However, no difference in FCM levels was detected in that case. This suggests that some care is needed when using FCM as a single measure of long-term stress and welfare. The results presented in this thesis also showed that several factors can influence FCM levels, *e.g*. feed, genotype, time of day, cage tier, bird age, mass of droppings produced and the kind of assay used. If these factors are not accounted for, this may affect the reliability of the FCM parameter for comparing stress levels between different groups of birds.

6.2 Egg shell irregularities

Previous studies have reported an increase in egg shell irregularities as a response to stress (Hughes *et al.*, 1986; Reynard & Savory, 1999), which was confirmed by the findings in this thesis. Egg shell irregularities were shown to increase during nest exclusion and were also positively correlated with FCM levels measured on the same day. A higher percentage of egg shell irregularities was also found later in the laying period in groups of birds (previously not excluded from the litter area) where other indicators (TI duration, NO test and feather cover) suggested decreased welfare. Of the irregularities studied in this thesis, 'wrinkled top' displayed significantly increased levels as a single measure in both studies (Papers II and III). These results support possible use of egg shell irregularities as a welfare indicator, as suggested previously (Sherwin *et al.*, 2010). In particular, percentage of eggs with wrinkled tops, a parameter not investigated previously, may serve as an easily measured and cheap indicator if further studies can show similar connections to stress and welfare.

6.3 Feather cover

As many experts have pointed out (Rodenburg *et al.*, 2008), feather cover seems to be an important and useful indicator of welfare and this was supported by some of the results in this thesis. In the latter part of the laying period, birds previously excluded from the litter area in Paper III displayed superior feather cover, which was accompanied by differences in other indicators (TI duration, NO test and egg shell irregularities), also suggesting increased welfare compared with non-excluded groups of birds.

The duration of the relatively short-term challenge of excluding birds from their nests in Paper II, on the other hand, was probably not enough to have an effect on feather cover. However, superior feather cover was correlated with lower FCM levels, suggesting an ability to indicate a stress response in a similar way.

6.4 Tonic immobility duration and novel object test

Fearfulness is often used as a welfare indicator and there is a general assumption that increased fearfulness is associated with increased stress and often feather pecking. However, a number of studies have shown that the relationship seems much more complex, with individual differences between birds and contradictory results depending on the type of fear test used and the age at which it was performed (Cockrem, 2007; Nicol *et al.*, 2011; Bögelein *et al.*, 2014). In Paper III, birds that had been excluded from the litter area displayed shorter duration of TI, higher willingness to approach a NO, superior feather cover and less egg shell irregularities later in the laying period. Superior feather cover was also correlated with higher willingness to approach a NO (at group level) and shorter duration of TI (at individual level), indicating that superior welfare was connected to lower fearfulness in this study. In Paper II, superior feather cover at individual level was instead correlated with longer duration of TI, and the stress response associated with nest exclusion did not affect TI duration. These results suggest an opposing connection with feather cover compared with the results in Paper III and that the stress from nest exclusion did not result in increased fearfulness. Hence, the results obtained in one of the studies in this thesis regarding fearfulness support the general assumption of a connection with stress and welfare. However, in another study the connection with stress was absent and an unclear relationship with feather pecking was displayed at individual bird level.

6.5 Corticosterone in egg yolk

Opinion regarding the use of corticosterone levels in yolk as a measure of stress is somewhat divided. Some authors propose it as a possible indicator of stress (Royo *et al.*, 2008; Singh *et al.*, 2009), while others argue that it is not an appropriate method (Rettenbacher *et al.*, 2013). The latter group argue that only a very small amount of corticosterone is present in the yolk and that the antibody in most assays cross-reacts with gestagens such as progesterone present in the yolk in much higher concentration (Rettenbacher *et al.*, 2005, 2009, 2013). Consequently, many assays may display incorrect levels of corticosterone in the yolk, according to those arguments. The hens in Paper II in this thesis showed elevated levels of corticosterone in yolk during exclusion from the nests, which supports use of this parameter as a possible indicator of stress. However, the levels measured were high and the assay used showed quite high cross-reactivity with progesterone (7.4%). Hence, the possibility cannot be excluded that this fact confounded the results. In addition, corticosterone seems to be deposited in the yolk over a period of 11 days (Rettenbacher *et al.*, 2005) and may be influenced by several factors during this period. In all, using corticosterone in yolk as a biomarker of stress in laying hens is controversial and further research is needed in order to investigate its usefulness.

6.6 Heterophil to lymphocyte ratio

Investigating the H/L ratio in the blood as a measure of stress and welfare is now a commonly used method in birds, because it has been proven to increase in response to a large number of stressors (Maxwell, 1993; Davis *et al.*, 2008). The increased H/L ratios observed during exclusion from the nests in this thesis support this finding. However, when detecting an effect on H/L ratio, there are some difficulties in distinguishing a stress response from a response due to infection or inflammation (Moe *et al.*, 2010; Cotter, 2014). This issue was clearly illustrated in Paper II, where birds with foot injuries due to the non-optimal leg rings displayed highly increased H/L ratios. In addition, there were some very high H/L ratios that could not be linked to any visible injury or other health issues and were consequently difficult to interpret. One way to distinguish the reasons for increased H/L ratios in future studies could be to investigate the cell structure of the leucocytes on a very detailed level (ultrastructure). Leucocyte ultrastructure has been shown to differ depending

on the challenge used (representing stress or bacterial infection) to elicit an increased H/L ratio (Shini *et al.*, 2008).

6.7 Corticosterone in plasma

The most frequently used indicator of stress is probably corticosterone level in plasma. However, plasma corticosterone level may increase rapidly (Radke *et al.*, 1985), and its use can be limited because catching a bird and being able to draw blood within minutes may be difficult in non-cage production systems. In Paper II, the procedure (from catching to finishing blood collection) was done within 2 minutes and hence the results should not have been affected by the procedure. However, the results did not support the ability of plasma corticosterone to detect the relatively short-term stress associated with exclusion from the nests. Moreover, it is probable that increased levels would have been detected if the samples had been taken closer to the time of lay, when the stress from nest exclusion was likely to be higher. The increased plasma levels found at the end of the laying period in Paper III indicated increased basal stress level at this time. However, due to its ability to increase rapidly and also to be up-regulated or down-regulated by different environmental conditions, it has been claimed that "extreme caution should be the rule before stating firm conclusions in term of stress and thus welfare assessment, after measurement of circulating corticosterone levels" (Mormède *et al.*, 2007). General use of plasma corticosterone levels as a welfare indicator has also been questioned by D'Eath *et al.* (2009) and Moneva *et al.* (2009). Hence, despite measurement of plasma corticosterone levels being widely used as an indicator of stress and welfare, it is probably not the most optimal way when assessing long-term stress responses or general welfare status.

6.8 Behaviour observations

Besides behaviour tests to assess fearfulness, behaviour observations in the birds' home environment were carried out in this thesis. These were performed in addition to the other indicators, with the aim of gaining a better understanding of the results obtained. In Paper II, the increased interest directed towards the nests by the hens, including some active attempts to forcibly enter the nests, suggested that the exclusion was perceived as an undesirable experience, which supported the associated stress response

detected in other indicators. Similarly, the decreased number of runs among birds during exclusion from the litter area in Paper III provided an indication of less aggression among these birds, possibly explaining the positive effects seen in other indicators following the exclusion procedure. However, the use of runs as a criterion for aggression was slightly limited in this case, because fewer runs could have been a result of the birds having less available space. Another suggested explanation for the positive effects seen with the exclusion procedure was that it resulted in increased occupation (access to the litter area) at the critical time of onset of lay. However, the fact that there was no difference between excluded and non-excluded birds in terms of number of hens utilising the litter area when the exclusion period ended did not support this suggestion. Overall, behavioural observations can be a useful tool when trying to investigate the welfare of laying hens, but can also be very time-consuming.

6.9 Production parameters

Production parameters were recorded in Paper III, but not with the aim of using them as welfare indicators, although some relationships with welfare indicators were found. No differences regarding egg production parameters or mortality were seen, but birds that were not excluded from the litter area displayed increased feed consumption and inferior FCR compared with excluded birds. These birds also displayed inferior feather cover and increased fearfulness. This most likely influenced the results by increasing the energy maintenance of the birds, both by increased body heat losses due to poorer insulation (Tauson & Svensson, 1980) and possibly also by an increased activity level due to the higher fearfulness. These results reveal a clear connection between bird welfare and production economics, with both the birds and the farmer benefiting from good animal welfare.

6.10 Correlations between indicators

This thesis showed that despite indicators being in agreement, correlations between indicators can be absent. Correlations were found between some indicators (as discussed earlier), but in general the occurrence of correlations was low and they also differed depending on the part of the dataset that was compared. One explanation may be differences in the time lag from when a

stressor is introduced until the stress reaction is reflected in the respective indicator. Consequently, this complicates the selection of samples to compare. Similarly to this thesis, Engel *et al.* (2011) found no correlations between corticosterone in plasma and corticosterone in yolk or FCM levels, while Nicol *et al.* (2011) found no correlation between corticosterone levels in plasma and any of the other indicators included in their study.

Previous studies have shown that individual laying hens differ consistently in the way they cope with stress, both with regard to behaviour and physiology. In general, passive, non-aggressive and shy individuals respond to stress with higher increases in corticosterone levels and lower noradrenergic responses compared with individuals that are proactive, aggressive and bold (Carere *et al.*, 2010). The occurrence of the two personality types in different treatment groups may affect the outcome of measured indicators and may also explain why correlations between indicators varied depending on whether group or individual values were compared.

6.11 Overall effects of exclusion from the nest (Paper II)

Because laying hens have a strong desire to access to a suitable nest site (Cooper & Albentosa, 2003), denying access to nests for hens that are accustomed nest layers was hypothesised as being a stressful procedure. Therefore this procedure was used as a challenge in order to compare potential effects in different welfare indicators.

During the period of exclusion from the nests, FCM in droppings, egg shell irregularities, H/L ratio and corticosterone in yolk displayed elevated levels compared with values measured prior to exclusion. However, control birds that still had access to their nests displayed the same elevated levels during this period. These results suggest that stress was induced by the exclusion procedure and that this stress spread to birds in adjacent cages with access to the nests. Behaviour recordings revealed a desire to enter the nest when access was denied. If audio recordings had been performed too, it is possible that increased occurrence of gakel-calls and alarm cackles would have been detected, as in a previous study on nest deprivation (Zimmerman *et al.*, 2000). This may have been one way in which stress responses were transferred between excluded and non-excluded birds, since the experimental set-up allowed visual, auditory and also some tactile contact between treatment groups.

Some of the effects seen in FCM levels, corticosterone levels in yolk and egg shell irregularities during the exclusion period may have been partly affected by the procedure of blood sampling and manual restraint during the TI tests. However, increased levels were in most cases seen already on day 1 or day 2 of the exclusion (before disturbance of the other sample collection had occurred) and hence were most likely caused by the exclusion procedure alone. Despite elevated levels in several indicators, no effect on plasma corticosterone levels or TI duration was seen during the exclusion (discussed elsewhere).

6.12 Overall effects of exclusion from the litter area (Paper III)

When layers were introduced to the laying facility, the two-week period of exclusion from the litter area resulted in deprivation from both litter and a substantial amount of space. Because litter is an important resource for layers that may reduce the risk of feather pecking (Blokhuis & Van der Haar, 1989; Gunnarsson *et al.*, 2000; Van de Weerd & Elson, 2006), there is general concern regarding the welfare impacts of such a procedure, which is therefore not permitted in Sweden. However, in this thesis there was no difference between excluded and non-excluded birds during the exclusion period regarding FCM levels in droppings, suggesting that the exclusion itself did not increase stress levels in the birds. Later in the laying period there were still no differences in FCM levels, but birds that had been excluded displayed superior feather cover, were less fearful according to both TI latency and NO test and produced eggs with fewer shell irregularities. These findings suggest that in contrast to existing beliefs, the exclusion procedure for the first two weeks in the laying facility had a positive impact on bird welfare.

One of the reasons for performing the exclusion procedure in practice is to help the birds find food and water, which may have played a role in the results. On the other hand, the limited pen size in the experimental facility resulted in a rather close distance to both food and water, irrespective of treatment, and may therefore have had less effect. The reduction in available space during this period possibly played a role in the positive impact. This would be in line with findings by Nicol *et al.* (2006) of superior feather cover in birds kept at high stocking density (12 hens/m^2) compared with low stocking density (7 and 9 hens/m^2). However, the opposite has also been observed (Nicol *et al.*, 1999; Bestman *et al.*, 2009). Decreased social distance between birds may decrease aggression (Hughes & Wood-Gush, 1977) and the lower number of runs

observed in this study among the excluded birds during the exclusion may be an indication of that. Moreover, the higher stocking density for the excluded birds (12.2 hens/m^2) may have resulted in a smoother transition from the rearing system (15 hens/m^2) than for the non-excluded birds, which suffered an abrupt decrease in their stocking density (to 7.8 hens/m^2). Hence, excluded birds might have adapted more easily after transfer, in accordance with Colson *et al.* (2008), who pointed out the importance of similar environments during rearing and laying. Van de Weerd & Elson (2006) also suggested that a seamless transition between rearing and laying conditions might reduce feather pecking in flocks.

The non-excluded birds had increased feed consumption and inferior FCR compared with the excluded birds (discussed elsewhere), but otherwise no difference in production performance or mortality was seen. An additional reason why the exclusion procedure is applied is to reduce the number of floor-laid eggs. One might have expected a difference in this parameter, but no such difference was found. However, this may be linked to the fact that the conditions in a small-scale experiment differ from those in large-scale production. Furthermore, if the exclusion had lasted longer, a higher percentage of hens would have started laying during the exclusion period, which would probably have resulted in a different outcome.

The increased FCM levels in droppings, increased fearfulness according to NO observations and inferior feather cover seen towards the end of the laying period suggest that welfare generally declined with age. These results are similar to findings in a study by Nicol *et al.* (2006), where higher FCM levels and decreased feather cover were seen at the end of the laying period.

Having access to litter is important for laying hens (Gunnarsson *et al.*, 2000; Widowski & Duncan, 2000) and early access may decrease feather pecking (Blokhuis & Van der Haar, 1989). Consequently, denying the hens access to litter may be a risk factor for development of feather pecking (de Haas *et al.*, 2014). In Paper III, the two-week period without access to litter may have been unfavourable for the hens (although not indicated in FCM levels), but was perhaps overshadowed by positive aspects of the exclusion possibly connected to density, as discussed earlier. The ideal would perhaps be increased stocking density in the first period after transfer to the laying facility, but still with access to litter, and then gradually decreasing density later on. This would follow the suggestion by Zimmerman *et al.* (2006) that one way to

reduce aggression and feather pecking is to keep the hens at a higher stocking density initially and then gradually decrease the density with age.

7 Conclusions

The overall conclusion from the work presented in this thesis is that numerous factors can influence the outcome of different welfare indicators used in laying hens. Thus this calls for both careful planning of sample collection and careful interpretation of the results before stating firm conclusions regarding laying hen welfare. Further conclusions were that:

- FCM levels in droppings are affected by diet composition, genotype, bird age, cage tier, dropping mass, time of day and the kind of assay used. Consequently, these factors need to be taken into account in order to accurately estimate differences in stress responses.

- Depending on the situation tested, some indicators may be more or less suitable for detecting differences in stress and welfare, suggesting that welfare assessment should be based on results from several indicators.

- The outcome of the studies in this thesis suggests that:

  - FCM in droppings, yolk corticosterone concentration, H/L ratio and egg shell irregularities have a similar ability to reflect differences arising from a relatively short-term stress challenge.

  - Feather cover, approaches towards a NO, TI duration and egg shell irregularities have a similar ability to reflect differences regarding stress and welfare evolving during a longer period of time.

- Despite agreement between indicators regarding how they changed in response to different situations, statistically significant correlations were generally few and inconsistent, showing that the relationship between different indicators is complex.

- Excluding layers from the litter area on introduction to the laying facility for two weeks did not compromise their welfare, according to the indicators tested. On the contrary, several indicators suggested that welfare was improved, which also resulted in lower feed intake and better feed conversion ratio.

- Decreased welfare can result in economic losses due to an increased energy maintenance requirement in the hens, leading to higher feed intake. This demonstrates the multiple reasons for maintaining good animal welfare.

8 Personal reflections on practical applications

The results obtained in this thesis, together with previous findings in the area, resulted in the following personal reflections regarding the current practical use of the different indicators included in this thesis:

- When investigating the effects of short-term stressors such as transportation and regrouping, physiological indicators such as FCM levels in droppings, H/L ratio and corticosterone in plasma are probably convenient and reliable, if samples are collected properly.

- When investigating long-term stress responses and differences in welfare between farms or housing systems, evaluating feather cover, approaches towards a NO and observations of specific behaviours are likely to be more relevant indicators. In these situations, physiological indicators can be difficult to interpret, both due to the ability of the HPA axis to adapt and because factors such as feed, litter substrate, bird age, activity level, temperature and bacterial pressure can influence the results and most likely differ between farms and housing systems.

- An indicator that might be used for both short-term and long-term stress responses is egg shell irregularities. In particular, irregularity displayed as small wrinkles on the top of the egg showed potential to be used as a welfare indicator. If this appearance can also be correlated with stress in future studies, it could serve as a non-invasive, easy to perform and cheap welfare indicator.

- Duration of TI and corticosterone in yolk may be useful as welfare indicators in specific situations. However, some confounding and uncertain results have been found for these indicators, warranting further investigations before drawing firm conclusions regarding their ability to reflect the welfare status in laying hens.

9 Future perspectives

To more accurately assess and interpret results obtained from different welfare indicators, and perhaps also improve welfare, the following suggestions for further research are proposed:

- Further examine the possibility of using egg shells with wrinkled tops as a welfare indicator, by studying how the percentage of eggs displaying this irregularity changes in response to other kinds of short-term and long-term stress challenges.

- Investigate whether keeping hens in the beginning of the laying period at a similar stocking density as during rearing, with access to litter, has beneficial effects on welfare due to the smoother transition between the rearing and laying systems.

- Investigate the impact of individual differences, for example the occurrence of the passive and proactive personality traits, when using different welfare indicators such as tonic immobility duration.

- Examine in detail how corticosterone is integrated into the egg yolk and whether these levels reflect a general stress response and are thus meaningful to assess when investigating stress and welfare.

10 Svensk sammanfattning

Att ha ägg på sitt frukostbord ser många som en självklarhet och i Sverige äter vi runt 220 ägg per person och år. Hur hönorna som värper äggen mår är det allt fler som intresserar sig för. En god djurvälfärd främjar inte bara hönorna utan kan ha stor påverkan på såväl produktkvalité som ekonomi. Hur ska man då veta hur hönorna mår? Det är ingen lätt fråga att besvara, men det finns idag ett flertal olika välfärdsparametrar som används för att försöka ta reda på detta. Ett vanligt sätt att skatta välfärd är att mäta olika stressresponser hos hönorna, eftersom stress och välfärd är nära kopplade till varandra. Hönorna har stresshormonet kortikosteron, som motsvarar människans kortisol, vilket kan mätas till exempel i blodet, gödseln och äggen. I gödseln är det egentligen inte kortikosteron i sig som mäts utan de ämnen som hormonet brutits ner till i levern. Eftersom immunsystemet påverkas av stress kan man även mäta förhållandet mellan olika typer av vita blodkroppar i blodet.

Det är också vanligt att hönornas rädsla undersöks till exempel genom att mäta så kallad tonisk immobilitet. Tonisk immobilitet är ett slags ”spela död”-beteende som fåglar reflexmässigt utför när de fångas av ett rovdjur. När tonisk immobilitet mäts läggs hönan försiktigt på rygg och hålls stilla en kort stund. Ju längre hönan sedan ligger orörlig desto räddare är hon. En annan variant att mäta rädsla är att lägga in ett främmande föremål till hönorna i deras hemmiljö och registrera hur lång tid det tar innan de vågar närma sig objektet. Registrering av beteenden som hönorna naturligt gör i sin hemmiljö kan också göras genom beteendeobservationer. Att titta på hönornas befjädring är ett indirekt sätt att upptäcka om hönorna ägnar sig åt att plocka fjädrarna av varandra vilket stressade hönor kan göra.

Utseendet på äggskalen kan variera och ibland kan man se olika typer av avvikelser så som en knölig yta eller annorlunda form. Vissa avvikelser har

visats förekomma mer när hönorna utsatts för stress och kan därmed användas för att indikera hur hönorna mår. Hur många ägg hönorna producerar och hur mycket foder som går åt och så vidare, registreras noggrant av lantbrukarna. Förändringar i dessa parametrar kan vara ett första tecken på att djurens välmående ändras men de bör inte användas som enskilda mått på att djuren mår bra eftersom djur med dålig välfärd fortfarande kan ha en hög produktion.

Trots att det finns flera sätt att undersöka hönors välmående råder viss osäkerhet kring hur väl olika parametrar verkligen reflekterar stress och välfärd. Syftet med denna doktorsavhandling var därför att öka kunskapen kring några av dessa välfärdsparametrar. Detta har gjorts genom att undersöka hur olika faktorer kan påverka resultatet från dem och hur parametrarna förändras när hönorna blir utsatta för olika typer av påfrestande situationer.

Tre olika experiment genomfördes på Sveriges lantbruksuniversitets forskningscentrum i Lövsta, utanför Uppsala, med tre vanligt förekommande värphönshybrider. I de två första experimenten inhystes hönorna i inredda burar dvs. med tillgång till värprede, ströbad och sittpinne och i det sista experimentet i ett envåningssystem för frigående hönor med tillgång till värpreden, sittpinnar och en golvyta med strö.

I det första experimentet undersöktes hur nivåerna av stresshormonet kortikosteron i gödseln påverkas av olika faktorer. Det visade sig att nivåerna varierade beroende på vilken av hybriderna som testades, vilket foder hönorna åt, tid på dygnet, mängden gödsel som producerades samt på vilken av de tre burvåningarna som hönorna befann sig. Helt klart är att det, förutom stress, även finns många andra faktorer som påverkar nivåerna av kortikosteron i gödseln.

I det andra experimentet jämfördes hur ett antal parametrar påverkades när hönorna i en inredd bur blev utsatta för en stress. Stressen bestod i att hönorna nekades tillträde till sina reden. En höna värper ett ägg nästan varje dag och vill då gärna lägga det på ett lite undangömt ställe såsom i ett rede. När hönorna var utestängda från sina reden försökte de ändå ta sig in vilket tyder på en stark vilja att använda redet. Under utestängningen uppmättes också förhöjda nivåer av kortikosteron i både gödseln och i äggen. Även förhållandet mellan olika vita blodkroppar ändrades vilket indikerade en ökad stress. Samtidigt noterades en högre andel ägg med avvikelser på äggskalen. Lite förvånande var dock att även de hönor som inte utestängdes från sina reden visade samma förändringar. Därmed verkar stressen till följd av redesutestängningen påverkat även hönorna i de omkringliggande burarna som inte var utestängda från sina reden.

Inga hönor visade dock någon effekt på kortikosteron i blodet, tonisk immobilitet eller befjädring.

I det tredje experimentet anlände hönorna till värpstallet vid 16 veckors ålder. Under de två första veckorna stängdes de då ute från ströbädden för att se om och hur detta påverkade olika välfärdsparametrar och hönornas produktion. I kommersiell äggproduktion tillämpas ibland denna procedur för att hönorna ska lära sig hitta foder och vatten i den nya miljön och även lära sig lägga ägg i redena istället för i ströbädden. Proceduren är dock inte tillåten i Sverige i och med att hönorna blir utan strö och måste vistas på en mindre yta, men den praktiseras i andra länder. Det visade sig att hönorna som blev temporärt utestängda från sin ströbädd hade bättre befjädring, var mindre rädda (både enligt test med ett främmande föremål och tonisk immobilitet) och lade färre ägg med avvikelser på skalen när detta undersöktes senare under produktionsperioden. Detta tyder alltså på att utestängningen från ströbädden hade en positiv inverkan på djurens välfärd, kanske genom att de lättare anpassade sig till den nya främmande miljön när ytan begränsades och närheten till de andra hönorna var mer påtaglig. Däremot sågs ingen skillnad mellan behandlingarna i nivåerna av kortikosteron i gödseln varken under utestängningen eller senare. Ingen skillnad kunde ses i äggproduktionen men de hönor som inte varit utestängda, och visade tecken på sämre välfärd, åt mer foder än de som varit utestängda. Detta berodde sannolikt på att de behövde kompensera för en högre värmeförlust till följd av en sämre isolerande befjädring. Eftersom foder står för cirka 60 % av produktionskostnaden i äggproduktionen, finns här en tydlig koppling mellan välfärd och ekonomi som visar att det finns både etiska och ekonomiska fördelar med en god djurvälfärd.

Sammanfattningsvis visar resultaten i denna doktorsavhandling att det finns många faktorer som kan påverka resultaten när man mäter olika välfärdsparametrar. Parametrarna verkar också fungera olika bra för att detektera skillnader i stress och välfärd, beroende på i vilken situation de används. Detta talar för att flera indikatorer bör användas för att på ett så tillförlitligt sätt som möjligt kunna bedöma nivåer av stress och välfärd.

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