THESIS OF A DOCTORAL (PhD) ESSAY

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MOSONMAGYARÓVÁR
INSTITUTE OF ANIMAL SCIENCE

Head of the Doctorial School:
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A NEW METHOD FOR CORTICOSTERONE DETECTION IN EGGS AND MEASUREMENT OF CORTICOSTERONE IN EGG YOLK AND BLOODPLASMA

Written by:

GYIMÓTHY – WILLMANN ILSE MARIA

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1. Introduction

As modern animal keeping systems continue to gain importance, objective means for determining their adequacy in relation to animal well-being are becoming increasingly necessary. Furthermore, the prohibition of the use of conventional cages in the laying hen industry in the European Union as of 2012 makes the need for development of alternative keeping systems – keeping in mind ethological as well as health and economic aspects – and the control of their ability to meet the demands of the animals housed indisputable. This is why the search for parameters which enable the objective and reliable measurement of well-being and stress exposure of animals is being enforced. In this respect, methods focusing on the benefit of non invasive stress measurement are preferred, since disturbances arising through the stress inflicted by sampling itself are avoided.

In laying hens, corticosterone and its metabolites are suitable tools for measuring stressload, as their detection in the bloodplasma, feces as well as in the egg is possible. Using eggs for the non invasive assessment of stress offers the advantage, that, as opposed to blood samples, they do not represent just a

single sample, but reflect the circulating amounts of corticosterone during the period of egg formation, which lasts approximately 24 hours. Furthermore, eggs offer a stable sample material – unlike in the feces, corticosterone is not further metabolized in the eggs and the storage of samples is possible.

2. Own studies

2.1 Objectives of the experiments

The preliminary objective of this thesis was to develop a practicable method for the detection of corticosterone in the egg. Furthermore, this thesis aimed at comparing the well-being and stress exposure of laying hens kept in free range- and cage keeping systems on the basis of the hens´ health status and the results of corticosterone measurements in both the bloodplasma and the egg. In this respect, a correlation between blood and egg corticosterone levels was searched for.

The hypothesis was that it is possible to develop a method that is sensitive enough to enable the detection of corticosterone in the egg yolk. In addition, owing to the pulsatile nature of corticosterone excretion and its quick rise in the blood in stressful situations, the author did not expect to find a correlation between blood and egg corticosterone levels. A difference between the stress exposure in free range- and cage keeping systems was hypothesized, given accordance with the requirements posed by the European Union. Considering the adequacy of both systems and their ability to meet the physiological demands of laying hens in comparison, cage

keeping systems should impose a greater stress load than free range keeping systems, since they offer less freedom for hens to perform characteristic activities representing important elements of their social-, nesting-, feed intake-, comfort-, locomotory- and resting behavior.

2.2 Material and Method

2.2.1. Experimental animals

Hens belonging to the breed "Yellow Hungarian Chicken" (Mosonmagyaróvár, Hungary) were used as experimental animals. The laying hens used in the framework of this thesis were kept at the breeding station in Mosonmagyaróvár, an integral part of the University of West Hungary, Faculty for Agricultural and Food Sciences, Institute for Animal Breeding. They were raised in a collective and uniform herd enclosing a total of 6000 animals up to 10 weeks of age. The space allowance during this period was 12chickens/m². At 11 weeks of age, after the first selection process, they were transferred to a so-called post-breeding centre, were they remained until the 26th week. The keeping system of this post-breeding centre was similar to a free range keeping system – a herd totalling 1408 animals (1280 hens and 128 cockrels) was divided into 32

groups, each consisting of 40 hens and 4 cockrels. The space allowance during this period was increased to reach 1,2 chickens/m². A second selection at 26 weeks of age decreased the herd size further – 544 animals (480 hens and 64 cockrels) were again grouped into 32 elite stocks – each consisting of 15 hens and 2 cockrels – at a space allowance of 0,48 chickens/m².

At 45 weeks of age 40 hens coming from this herd were transferred into two different keeping systems. 20 hens were stalled into cages, the other 20 were stalled into a free range keeping system. None of the hens had shortened beaks or clipped wings. All hens received the same routine vaccination program and were fed with identical feed during the trial.

2.2.2. The Cage Keeping System

The battery cages (Type: HTK KHÜNE, Mosonmagyaróvár) used in the framework of this trial were arranged in 5 rows, each row consisting of 4 cages positioned next to one another at one level. One hen was kept per cage, each hen receiving a space allowance of 2250 cm². The microclimate in this keeping system was regulated by means of natural ventilation. The lighting, too, was natural (total window area: 5,4 m²) and the hens received 14 hours light per day.

2.2.3. The Free Range Keeping System

This keeping system encompassed a floor keeping system with a free-run, developed and built at the University of West Hungary, Faculty for Agricultural and Food Sciences, Mosonmagyaróvár. Hens received 20 dropnests and 4 perches per group. The free range keeping system consisted of a total of 544 chickens (480 hens and 64 cockrels), grouped in 32 elite stocks of 17 chickens (15 hens and 2 cockrels) each and kept at a stocking density of 0,48 chickens/m² useable floor area. In addition, the hens received daily access to a free-run (0,72 chickens/m²) bedded with sand. Ventilation was natural and regulated by an outlet ventilation system. The lighting program was set at 12 hours artificial light per day. In addition, hens received natural sunlight.

2.2.4. Assessment of the Health Status

A clinical examination of the same hens from which blood and egg samples were taken was performed at days 0, 2, 7 and 16 of the trial. Clinical parameters included the condition of plumage, nails and footpads as well as the evaluation of injuries of the appendages, cloaca, feet or other body parts. To assess the health status, 5 hens per keeping system were examined and defects were evaluated using a scoring system developed by

Tauson (1986) and Tauson et al. (1984) in the course of sampling.

2.2.5. Corticosterone in bloodplasma

On day 0, hens were transferred to their respective keeping systems. Since this transfer posed a considerable amount of stress to the hens, distorting plasma corticosterone levels, no blood samples were taken on this day. Blood samples were taken from 5 hens per keeping system on days 2, 7, and 16 of the trial. Since plasma corticosterone levels increase within 1 to 2 minutes subsequent to the initiation of stress, which in this case included the catching, fixation and blood sampling, care was taken to keep the time interval between the catching of the hens and the taking of the blood sample at a minimum (preferably less than 1 minute). 2 ml of blood were collected from the wing vein and collected in a sampling tube coated with Na-EDTA. Blood samples were then centrifuged at 2500g for 15 minutes to separate the plasma from the corpuscular elements. The plasma was then transferred to an Eppendorf vial and stored at -20°C. The extraction of corticosterone and the enzyme immunoassay (EIA) used to measure plasmacorticosterone levels was performed at the University of Veterinary Medicine in Vienna, Austria, at the Institute for Biochemistry using the method developed by Palme and Möstl (1997) and Palme et al. (1999).

2.2.6. Egg Collection and Assessment of Corticosterone Levels in the Egg Yolk

Freshly laid eggs were collected from the same 5 hens per keeping system which underwent blood sampling and / or clinical examinations on days 0, 2, 7 and 16 of the trial. Eggs were immediately cooled and transferred to the State Centre for Food Control and -Analysis in Budapest.

The method used to extract and measure corticosterone in the egg yolk is a newly established technique that can be used to evaluate stress exposure of laying hens based on the assessment of corticosterone levels in the egg yolk using HPLC-MS/MS (High Performance Liquid Chromatography – Tandem Massspectrometry) following a special extraction procedure.

Following homogenisation and drying, the egg yolk samples were exposed to a solvent clean-up and extraction process using an Accelerated Solvent Extractor (ASE 200). The extracted samples were then evaporated, spiked with a dexamethasone standard and redisolved in an eluent.

The HPLC was interfaced with MS/MS using an atmospheric pressure chemical ionisation (APCI) ion source working in its positive mode. HPLC was performed on a SUPELCO Hypersil BDS C_{18} analytical column as a stationary phase. The

composition of the mobile phase (acetonitrile and 0,005 M ammonium acetate) varied according to a time – dependent gradient program. The effluent of the HPLC column was delivered to the APCI interface and ion source of the MS/MS system using nitrogen as the probe / cone and solvation gas and helium as the auxiliary collision gas. Methodological runs were performed to obtain data on APCI – related ionisation modes for the detection and confirmation of corticosterone and its internal standard (ISTD) dexamethasone. Collision energies for the afore mentioned analytes were determined to obtain molecular ions and related ion fragments. Selected ion monitoring was performed in scan mode from 50 to 450 m/z. A standard amount of dexamethasone was added to the samples to facilitate the quantification of corticosterone. A validation procedure was performed to determine the response function of the corticosterone calibration curve, extraction efficiency, limit of detection (0,025 ppb), limit of quantification (0,04 ppb), repeatability, accuracy and selectivity (Sas et al. 2006).

This method is the first known functional method based on HPLC-MS/MS that enables the reliable detection of corticosterone in the egg yolk.

3. New scientific results

On the basis of the new method for corticosterone detection in eggs developed in the course of this thesis and the measurements performed, following new scientific results could be established:

1. The method based on HPLC-MS/MS (High Performance Liquid Chromatography – Tandem Massspectrometry) which was developed in the course of this thesis is functional and sensitive enough to enable the detection and measurement of corticosterone amounts as small as 0,025 ppb (limit of detection) and 0,040 ppb (limit of quantification), identifying corticosterone not only by its mass and mass / charge ratio, but also according to the fragmentation pattern of its ions, a property which is characteristic and unique for each substance. This method is the first known functional method based on LC-MS/MS that enables the reliable detection of corticosterone in the egg yolk.

2. Measurements showed that corticosterone is transferred to the egg yolk, providing a sample material which can

be used for the non invasive assessment of stress load. This creates the possibility to measure corticosterone and in the consequence stress exposure by non invasive means of sampling, eggs presenting a more stable sample material than feces.

3. Comparing the health status of the hens in the caged- and free range system no significant differences could be detected. Though the state of the plumage was relatively worse in hens kept in the free range system, indicating that the incidence of feather pecking was higher, this finding was statistically not significant. Feather pecking could not be observed in hens stalled in the cage keeping system, but this is not surprising, considering that the hens were kept individually in cages, having no possibility for social contact. Footpad- and nail health were not significantly better in hens kept in the free range keeping system, though the duration of the trial was too short to draw conclusions what concerns the long term effects of cage keeping without bedding on the state of these two health parameters.

4. Corticosterone values in the bloodplasma showed no significant differences when comparing hens kept in the caged- and free range system. The arithmetic means of

plasmacorticosterone for hens stalled in the cage keeping system ranged from 0,850 ± 0,336 ng/ml to 2,100 ± 0,780 ng/ml with a total arithmetic mean of 1,590 ± 0,734 ng/ml and a wide range of individual values from 0,53 ng/ml to 3,81 ng/ml. In the hens stalled in the free range keeping system, the arithmetic means of plasmacorticosterone values ranged from 0,804 ± 0,037 ng/ml to 1,644 ± 0,502 ng/ml, with a total arithmetic mean of 1,110 ± 0,474 ng/ml and a narrower range of individual values from 0,33 ng/ml to 2,90 ng/ml. Comparing the single arithmetic means calculated for each day of sampling for plasmacorticosterone in both keeping systems, all daily means were higher in hens kept in the cage keeping system, excepting day 2. Likewise, the total arithmetic mean for plasmacorticosterone was higher in the cage keeping system as compared to the free range keeping system. Comparing the two keeping systems, statistical analysis proved that the differences in the plasmacorticosterone values were not significant.

5. Corticosterone values in the egg yolk showed no significant differences when comparing hens kept in the caged- and free range system. The arithmetic means of corticosterone in the egg yolk of hens stalled in the cage

keeping system ranged from 0,133 ± 0,093 ppb to 0,209 ± 0,143 ppb, with a total arithmetic mean of 0,179 ± 0,133 ppb and individual values ranging from 0,040 ppb to 0,668 ppb. In the hens stalled in the free range keeping system, arithmetic means of the corticosterone values in the egg yolk ranged from 0,071 ± 0,041 ppb to 0,571 ± 0,395 ppb, with a total arithmetic mean of 0,314 ± 0,211 ppb and a wider range of individual values from 0,040 ppb to 1,361 ppb. Hens stalled in the free range keeping system showed higher daily means than did the hens kept in the cage system, except on day 2. In addition, the total arithmetic mean of corticosterone was higher in the egg yolk of hens kept in the free range keeping system as compared to the cage keeping system. Comparing the two keeping systems, statistical analysis proved that differences in the corticosterone values in the egg yolk were not significant.

6. There was no correlation between corticosterone levels measured in the bloodplasma and the egg yolk in both keeping systems. Plasma corticosterone fluctuated greatly due to its episodic and pulsatile release, levels rising very fast as a response to a stressor (for example blood sampling). Eggs, in this case egg yolk, on the other hand allowed the non invasive long term

measurement of corticosterone and reflected the levels of corticosterone in the plasma during the 24 hours of egg formation. In this respect, eggs offered collective samples that evened out fluctuations and are distortions arising through invasive sampling procedures.

7. The results of corticosterone measurements in plasma and egg yolk do not indicate a difference in the stress experienced by hens kept in cages and free range keeping systems in comparison. Though a difference between the stress exposure of hens kept in free range and cage keeping systems was hypothesized and an increased stress load in the cage keeping system was expected, this could not be verified in the framework of this thesis. Given the circumstances of this study, however, a significant increase of stress exposure in hens kept in cages was not likely, since both the cage keeping system as well as the free range keeping system were modified and did not correspond with the requirements of the European Union, allowing only a comparison between an “improved cage keeping system” and a “reduced free range keeping system”.

4. List of publications made in the theme of the dissertation

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GYIMÓTHY, I.M. (2004): Stresstényezök és stressválaszok a baromfitartásban, Magyar Állatorvosok Lapja, 126, 65-128, 2004/2, 101-106.

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GYIMÓTHY, I.M. (2004): Entwicklung neuer Methoden zur nicht invasiven Messung der Stressbelastung bei Hühnern, 17th IGN-Symposium – 11th FREILAND-Meeting, Vienna.

GYIMÓTHY, I.M., GÁAL, E. (2004): Non-invasive methods for the assessment and comparison of stress exposure in poultry kept in intensive and free range keeping systems, 30th scientific days 2004, Mosonmagyaróvár.

SAS, B., DOMANYI, G., GYIMOTHY, I., GAAL KOVACSNE, K., SÜTH, M. (2006): Influence of the type of management system on corticosterone transfer into eggs in laying hens. Acta Veterinaria Hungaria 54 (3), 343-352.

Austrian Parliament, 2003 till present

Consultative expert in animal welfare with focus on the development and establishment of a new law for animal protection in accordance with the European Union in Austria.

Advisory member of the Austrian parliamentary subcommittee especially in the field of protection of animals kept for agricultural purposes with respect to animal well being in different keeping systems.