Measurement of corticosterone metabolites in chicken droppings

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Abstract 1. A non-invasive technique for stress assessment is needed. Therefore, an enzyme immunoassay (EIA) for measurement of glucocorticoid metabolites in chicken droppings was established and validated.

2. Radiolabelled corticosterone was administered intravenously to detect the time course of excreted metabolites. The metabolites were then characterised by chemical and immunological methods to find a suitable antibody.

3. Reversed-phase high-performance liquid chromatography (RP-HPLC) separations of the peak concentration samples revealed that corticosterone was extensively metabolised, mainly to more polar substances.

4. HPLC fractions were tested in several EIAs for glucocorticoid metabolites, where the highest quantities were detected by a newly established cortisone assay, measuring metabolites with a 3,11-dione structure.

5. The biological relevance of this cortisone EIA was confirmed by stimulation of adrenocortical activity by adrenocorticotropic hormone (ACTH).

6. With this newly developed EIA it should be possible to measure adrenocortical activity non-invasively in chickens and other galliformes, thus providing a tool for a variety of research fields, such as poultry production, ethology and behavioural ecology.

INTRODUCTION

In recent years there has been a growing interest in and concern about animal welfare, not only in scientific but also in public discussion. The questions, how to define animal welfare and how to measure it, are still under debate. The absence of stress is one of the most important indicators of animal welfare. Stress is well known to have a significant impact on a variety of physiological and behavioural variables. Its disruptive effect on immune functions, reproduction and behaviour of mammals has been shown (Riley, 1981). Assessing an animal's stress physiology is therefore essential for the understanding and improvement of animal welfare, health and reproduction. As the assessment of animal well-being is a complex matter, misinterpretations are in part prevented by measuring a wide range of physiological, endocrine and behavioural variables simultaneously (Ladewig and Matthews, 1992; Clark *et al.*, 1997a–d).

Stressful situations usually evoke an increased glucocorticoid production and output from the adrenal cortex, which is mediated by the release of adrenocorticotropic hormone (ACTH) from the pituitary gland (Axelrod and Reisine, 1984; Munck *et al.*, 1984). Plasma glucocorticoid concentrations are therefore widely used to diagnose stress responses in various species (Beuving, 1983; Munck *et al.*, 1984; Broom and Johnson, 1993; von Holst, 1998). Although the concentration of plasma corticosterone is a reliable indicator of stress in chickens (Beuving and Vonder, 1986), difficulties in collecting blood samples and negative effects of the sampling procedure itself pose serious limitations to this approach. In birds, handling and bleeding are stressful events, which markedly influence the glucocorticoid concentrations (Harvey *et al.*, 1980). Therefore, one main problem of plasma sampling is the determination of basal values, especially in wild animals (Stead *et al.*, 2000). Many authors recommend catching and sampling within a few minutes, but it is

only assumed that these values reflect true basal concentrations of plasma corticosterone (Silverin, 1998; Littin and Cockrem, 2001). In sparrows, plasma corticosterone rises within a few minutes after capture (Wingfield *et al.*, 1982). In chickens, Beuving and Vonder (1978) described an increase 45 s after restraint. Droppings offer the advantage that they can be easily collected without stressing the animal, the sampling procedure is feedback-free and not limited in amount or duration. Therefore, measuring excreted corticosterone metabolites is a much better tool for long-term studies than plasma samples. A non-invasive technique to measure faecal steroid metabolites has been established successfully in an increasing number of mammals and is already used to investigate questions in the fields of 'stress and animal welfare' and behavioural ecology (Teskey-Gerstl *et al.*, 2000; Schatz and Palme, 2001; Möstl and Palme, 2002). Previously established enzyme immunoassays (EIAs) have also proven useful in some birds (geese: Kotrschal *et al.*, 1998; Adélie penguins: Nakagawa *et al.*, 2003 and storm-petrels: Quillfeldt and Möstl, 2003). In chicken, so far, none of our EIAs could be used to monitor adrenocortical activity. Although a corticosterone assay suitable for chicken droppings is described by Dehnhard *et al.* (2003), basic information about metabolism and excretion of corticosterone in chicken is lacking.

The aim of this study was to establish and validate a non-invasive method for investigating adrenocortical activity by measuring corticosterone metabolites in chicken droppings. Therefore, the excretion pattern of injected radiolabelled corticosterone was determined and metabolites were characterised in order to establish an EIA for their quantification. Furthermore, the biological relevance of this method was assessed by stimulation and suppression of adrenocortical activity by ACTH and dexamethasone, respectively.

MATERIALS AND METHODS

Animals

In all experiments, laying hybrids, ISA Brown, obtained from a commercial breeder (R. Schropper PLC, Gloggnitz, Austria) were used. Twenty animals, 10 of each sex, were housed individually in cages, but had olfactory, visual and vocal contact with each other. They were accustomed to their cages one week prior to the experiments. Males and females were not housed together in the same room. Food and water were supplied *ad libitum* throughout. The daily light period was from 06:00 to 20:00 h. All investigations were conducted between 20

and 34 weeks of age. The average body weight was 2·6 and 2 kg in males and females, respectively.

Experiment 1. Radiometabolism study

Each bird was given 1·7 MBq (= 46 µCi) of radiolabelled corticosterone, dissolved in 1 ml of 0·9% NaCl solution containing 10% (v/v) ethanol into the *vena cutanea ulnaris*. The ^{3}H-corticosterone (NET-399; [1,2,6,7-^{3}H(N)]-corticosterone; 2830·5 GBq/mmole) was obtained from New England Nuclear (Dreieich, Germany). The purity was checked by thin layer chromatography (Palme *et al.*, 1996). Before injection, droppings of each bird were collected to determine background levels of radioactivity. During the first 24 h after injection, samples were collected immediately after voidance, put into plastic freezer bags and stored at −24°C. For the next 3 d, samples were drawn at intervals of 3 h. After 4 d, collections were done every 12 h for another 4 d.

To measure the radioactivity, 0·5 g of the homogenised sample was extracted with 5 ml of 60% (v/v) methanol by shaking for 30 min. After centrifugation, aliquots (0·5 ml in duplicates) of the supernatant were mixed with 6 ml of scintillation fluid (Quicksafe A®, No. 100800, Zinsser Analytic, Maidenhead, UK) and measured in a liquid scintillation counter (Packard Tri-Carb 2100TR, Meriden, CT, USA). Radioactivity was expressed as MBq per kg sample.

In one female animal, we tried to separate the faecal from the urinary components of the droppings before analysis. Therefore, the white uric acid of the excrement was simply peeled off with a pair of forceps and referred to as 'urine'. Because this method is not practicable for a large number of samples and the two components could not be separated completely, this was performed only in one animal.

To characterise the excreted metabolites, reversed-phase high-performance liquid chromatography (RP-HPLC) separations were performed. The samples containing the highest amounts of radioactive metabolites (usually the first peak, but in two animals, samples from the second peak were also measured) were subjected to a clean-up procedure with Sep-Pak C_{18} columns (Touma *et al.*, 2003). After diluting the extracted samples 1:10 with water, samples were passed through a Sep-Pak C_{18} cartridge (1 g, Waters, Milford, MA, USA). The cartridge was washed with 10 ml of aqua bidest. and eluted with 5 ml of 80% methanol. The eluate was dried under a stream of N_2 and resuspended in 100 µl of 20% methanol. RP-HPLC was performed as described by Schatz and Palme (2001). In addition, immunoreactivity of the fractions (diluted 1:5 with assay buffer) was checked

in a corticosterone assay (Palme and Möstl, 1997), a newly established cortisone assay (see later) and a tetrahydrocorticosterone assay (Quillfeldt and Möstl, 2003). Extracts of droppings were also incubated with β-glucuronidase/arylsulfatase as described by Teskey-Gerstl *et al.* (2000), to determine the amount of hydrolysable steroids.

Experiment 2. Biological relevance of the developed method for evaluating adrenocortical activity—administration of ACTH and dexamethasone

To determine the diurnal rhythm of the basal values, samples were collected after spontaneous defecation over a period of 24 h one week prior to the experiments. To stimulate adrenocortical activity, the animals received 2 ml (0·25 mg) of ACTH (Synacthen®, Ciba-Geigy, Basel, Switzerland). To suppress adrenocortical activity, each bird was injected with 3 ml (2 mg per kg) of dexamethasone (Dexa TAD, Lohmann Animal Health, Cuxhaven, Germany) intravenously. All samples were collected immediately after voidance for 24 h, then every 3 h for one day and every 6 h for 2 d. All samples were frozen and extracted as described in experiment 1. Following extraction, aliquots (after dilution with assay buffer 1:10) of the supernatant were measured with the corticosterone EIA, the cortisone EIA and the tetrahydrocorticosterone EIA.

Determination of metabolites

EIAs were performed as described by Palme and Möstl (1997) on anti-rabbit-IgG-coated microtitre plates using the double antibody technique and biotinylated steroids as labels. For the newly developed cortisone assay, the antibody was raised in a rabbit immunised against 4-pregnene-17α,21-diol-3,11,20-trione-21-HS, which had been bound to bovine serum albumin. Working dilution of the antibody was 1:20 000. 4-Pregnene-17α,21-diol-3,11,20-trione, linked at position C20 to carboxymethyloxime (CMO), was used as biotinylated label (Möstl *et al.*, 2002). Working dilution of the label was 1:5 000 000.

Cortisone (4-pregnene-17α,21-diol-3,11,20-trione) was used as standard (range of the curve from 2 to 500 pg/well); the 50% intercept was at about 60 pg. The cross reactions were as follows: 4-pregnene-17α,21-diol-3,11,20-trione, 100%; 4-androstene-3,11,17-trione, 30%; 5α-androstane-3,11,17-trione, 20%; 4-pregnene-11β,17α,20α,21-tetrol-3-one,9%;4-pregnene-3,20-dione, 2·3%; 5β-androstane-3,11,17-trione, 2·2%; 4-pregnene-11β,21-diol-3,20-dione, 1·8%; 4-androstane-3,17-dione,0·9%; 5α-androstane-3,17-dione,0·5%;

5α-androstane-3α-ol-11,17-dione; 5α-androstane-3β-ol-11,17-dione;5β-androstane-3α-ol-11,17-dione had cross-reactions below 0·1%. The intra- and interassay coefficients of variation were 17 and 20%, respectively.

Details of the assay procedure are described by Touma *et al.* (2003). Standards or samples (50 µl) were incubated in duplicate with label (100 µl) and antibody (100 µl) overnight at 4°C. After incubation, the plates were washed with 0·02% Tween 20 solution (Merck, Darmstadt, Germany 822184) and blotted dry, before 250 µl streptavidin horseradish peroxidase conjugate (4·2 mU, Art. No. 1089153, Boehringer, Mannheim, Germany) were added to each well. Plates were left in the dark on stirring tables for 45 min at 4°C. After another washing step, 250 µl of tetramethylbenzidine (69·4 nmoles/well; Art. No. 1089153, Boehringer) were added to each well and the plates were again incubated at 4°C. After 45 min the enzymatic reaction was stopped with 50 µl of 2 moles/l sulphuric acid. Adsorbance was measured at a wavelength of 450 nm (reference filter: 620 nm) on an automatic plate reader (Labsystems Multiscan, MCC/340; Szabo-Scandic, Vienna, Austria).

Statistical analysis

For data which are not normally distributed, the range and the median are given. Because all droppings were collected after spontaneous defecation, time and frequency differed between individuals. For comparison between the ACTH experiment and diurnal rhythm, all values were allocated to timeframes and compared with repeated ANOVA and *post hoc* tests. Results of the dexamethasone experiment were compared to basal values using the *t*-test. All statistical analysis was performed with Sigma-Stat® (SPSS Inc., Munich, Germany).

RESULTS

Experiment 1

The total recovery rate of radioactivity ranged from 52 to 67% (median: 60%) and from 61 to 97% (median: 78%) in males and females, respectively. Excretion of radioactivity started almost immediately after injection (Figure 1). Two main peaks could be observed, followed by several small peaks that seemed to occur periodically every 24 h during the protracted decline. The first peak was reached at about 1·5 h (min. 1 h, max. 5·2 h) and 1 h (0·4 to 1·5 h) in males and females, respectively. The second peak appeared after 4·7 h (2·2 to 8·3 h) and 3·4 h (2·3 to 4·6 h) in males and females, respectively. In the single hen, where urine and faeces were analysed separately,
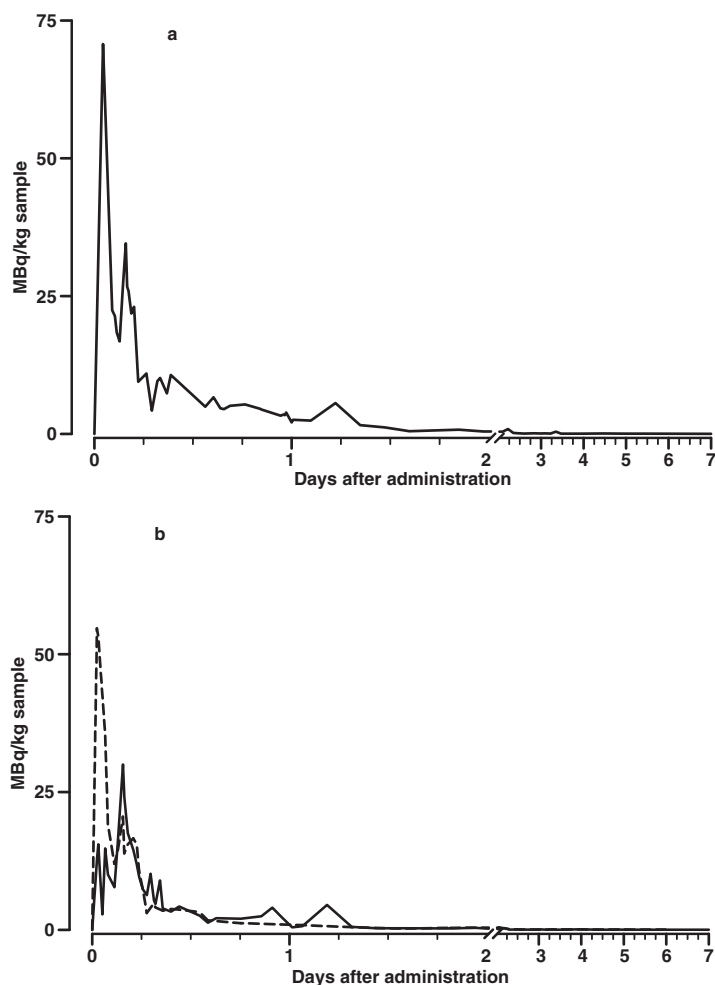
Figure 1. *Time course of excretion of [³H]corticosterone in droppings of one male (a) and in separated droppings of one female (b). The dashed line represents results from the urine, the solid line shows results from the faecal components of the droppings. Data are given in MBq/kg sample.*

the urinary components showed a peak after 0·6 h and a smaller one after 3·8 h. The faecal components reached peak concentrations after 0·8 and 3·8 h (main peak), and also showed smaller peaks that occurred periodically (Figure 1b).

In both sexes, only small amounts of the radioactive metabolites were ether extractable (1 to 36%, median 9%) and even less could be extracted after treatment with β-glucuronidase/arylsulfatase (2 to 34%, median 5%).

RP-HPLC separations revealed the presence of more than 15 radioactive peaks, indicating a large number of different metabolites. Although some individual differences (quantitatively) were found, the results were consistent within males and females, respectively. In males, one dominant peak was found between the elution positions of oestrone sulphate and cortisol (fractions 30 and 40) and there were also some smaller peaks with higher polarity (fractions 10 to 20). In females, the main peaks were from fraction 40 to 50, but there were also radioactive peaks with higher polarity (fractions 30 to 40). In

both sexes, no radioactive peaks were found at the elution position of cortisol and corticosterone (Figure 2). There were neither differences in the chemical characterisation between the samples from the first and the second peak, nor in the separated samples from the female animal. In contrast to the other two assays, which measured only insignificant amounts (data not shown), the cortisone assay was able to detect some of the radioactive metabolites in both sexes (Figure 2).

Experiment 2

The total amount of metabolites and the increase after ACTH administration measured were higher in the cortisone assay than in the corticosterone and the tetrahydrocorticosterone EIA. In that EIA, the administration of ACTH resulted in an increase of 844 ± 501% and 662 ± 317% compared to basal values after 1·7 h (±0·4) and 1·1 h (±0·7) in males and females, respectively. The peak values ranged from 753 to 3529 nmoles/kg and from 508 to 1641 nmoles/kg in males and females, respectively. There was a
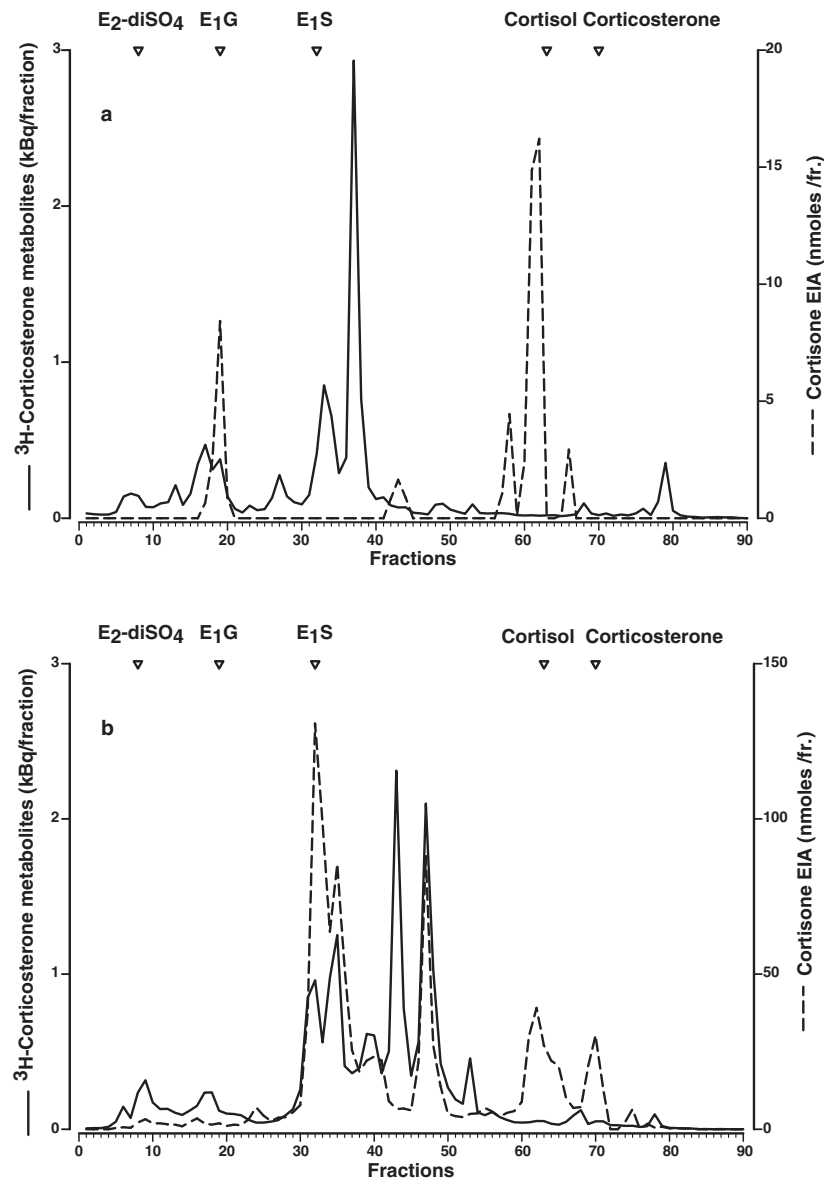
Figure 2. *Reversed-phase high-performance liquid chromatographic separation of [³H]corticosterone metabolites in droppings of one male (a) and one female (b) chicken. Radioactivity of each fraction was determined by liquid scintillation counting. Immunoreactivity was measured in the cortisone immunoassay. Open triangles mark the approximate elution positions of respective standards (E_2-diSO$_4$ = oestradiol-17β-disulphate, E_1G = oestroneglucuronide, E_1S = oestronesulphate, cortisol, corticosterone).*

significant difference between males and females with regard to the height of the ACTH peaks ($P = 0.004$); time did not differ significantly ($P = 0.131$; Figure 3).

Basal values did not reveal a distinct diurnal rhythm in excretion of the metabolites measured by the cortisone assay. The results served mainly as reference for the ACTH and dexamethasone experiment. The mean values were 221 ± 59 (mean $\pm$ SD) and 124 ± 38 nmoles/kg in males and females, respectively. There was a significant difference between male and female basal concentrations ($P < 0.001$).

No suppressive effect of dexamethasone was observed. The mean values after injection of dexamethasone were 249 ± 126 and 79 ± 47 nmoles/kg in males and females, respectively,

and did not differ significantly from 24 h basal values ($P = 0.75$ and 0.41 in males and females, respectively).

DISCUSSION

The aim of this study was to establish and validate an assay for the measurement of corticosterone metabolites in chicken droppings. The radio-metabolism study, which is the first reported in chickens, was conducted because knowledge about the species-specific excretion and metabolism of glucocorticoids is relevant to developing such a technique. The course of excretion is essential for setting up the best sampling regimen. In this study, the samples were drawn
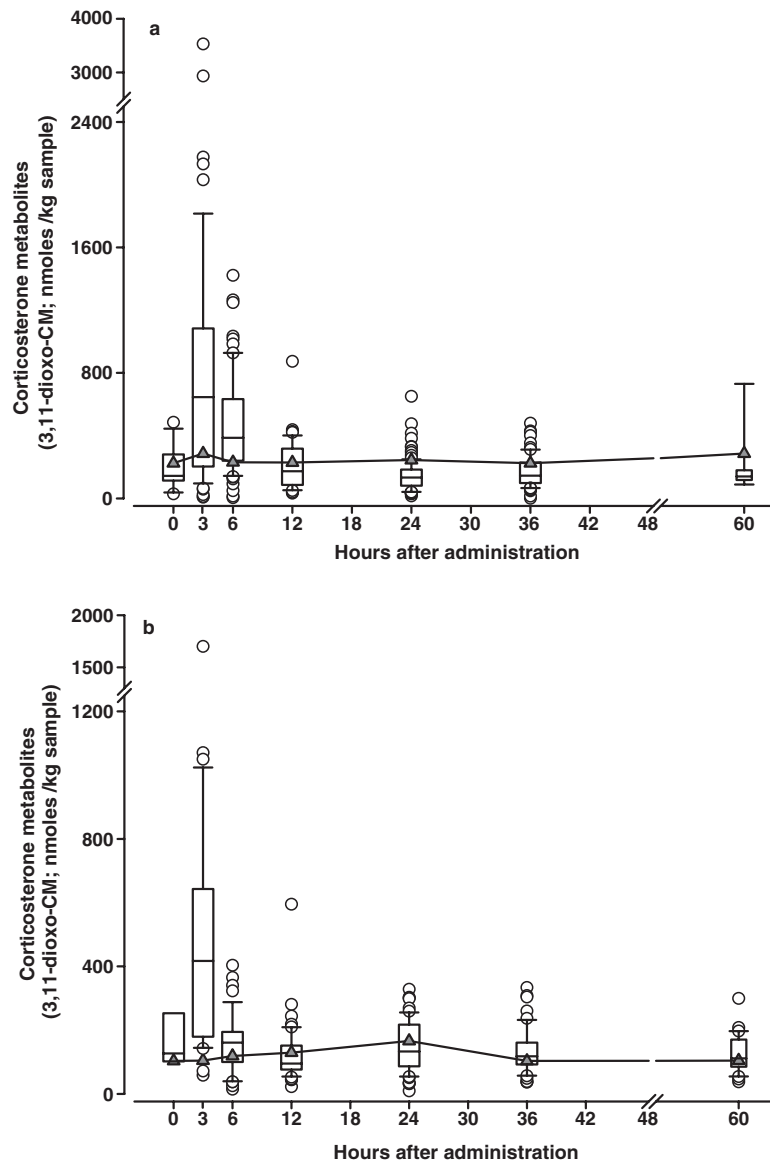
Figure 3. *Concentration of corticosterone metabolites after administration of adrenocorticotropic hormone (ACTH) (at time zero) in droppings of males (a) and females (b) in comparison to basal values. Data resulting from ACTH administration are given as boxplot diagrams showing medians (lines in the boxes), 25 and 75% quartiles (boxes), 10 and 90% ranges (whiskers) and outliers (dots). Basal values are given as line plots showing medians of 10 animals.*

continuously for the first 24 h; after analysis the results were allocated to timeframes, to compare the individuals. For future investigations, it is important to know the exact time lag between the corticosterone blood peak and the metabolites' peak in the droppings. This may help to associate a suspected stressor with its corresponding faecal correlate.

In addition, the number, the polarity and the immunological characteristics of the excreted metabolites were determined to establish an EIA for their quantification. Plasma samples were not taken, as several authors have already reported a correlation between plasma steroid concentrations and faecal metabolites in mammals (Möstl *et al.*, 1999; Palme *et al.*, 1999; Stead *et al.*, 2000), as well as in chicken (Dehnhard *et al.*, 2003).

The excretion pattern of radiolabelled corticosterone in chicken revealed two major peaks. The first one after 1·5 or 1 h, the second after 4·7 or 3·4 h in males and females, respectively. In mammals, which excrete urine and faeces separately, radioactivity immediately appears in the urine. After a delay, which usually corresponds roughly with the transit time from duodenum to rectum, radioactivity peaks appear in the faeces (Palme *et al.*, 1996; Schatz and Palme, 2001). From this we can draw analogous conclusions in chicken that the first peak results from renal excretion and the second peak is caused by elimination via the gut. To confirm our theory, we separated the two components of the droppings in one animal. After analysis, the first peak could be undoubtedly assigned to the urine,

the second resulted from faecal excretion. This separate analysis was only performed in one animal, as the method is very time consuming, only very small amounts of urine can be obtained and it is not possible to achieve total separation. The faeces are always contaminated to some degree with urine. Furthermore, the separation was only performed to support the assumption that excretion via urine is faster than via the intestine, and did not influence in any way the procedure of establishing an EIA for stress assessment.

Some smaller peaks that seemed to occur periodically every 24 h might result from faeces which were stored in the caeca and are voided after several hours of retention. This is supported by the fact that these peaks appeared in the faecal but not urinary components of the separated samples. Teskey-Gerstl *et al.* (2000) made similar observations in hares.

Glucocorticoids are heavily metabolised (Brownie, 1992). In contrast to findings in domestic livestock, but similar to results in geese (Kotrschal *et al.*, 1998), the majority of the metabolites were not ether-extractable, indicating the predominance of conjugated or polar unconjugated metabolites, which was confirmed by RP-HPLC separations. An enzymatic hydrolysis of conjugated metabolites did not yield higher amounts of extractable corticosterone metabolites. Similar findings have been made by Dehnhard *et al.* (2003). In our study, most of the metabolites found were more polar than corticosterone, which was virtually absent in the faeces, as confirmed by HPLC and an EIA. This is in accordance with findings in other species, where almost no native glucocorticoids (corticosterone or cortisol) were found in faeces (Palme and Möstl, 1997; Möstl *et al.*, 1999; Teskey-Gerstl *et al.*, 2000; Touma *et al.*, 2003).

In several other studies (Wasser *et al.*, 2000; Dehnhard *et al.*, 2003), test kits, designed to measure corticosterone, are used. As this study shows, corticosterone itself is not excreted by the chicken. These kits work due to antibody cross-reactions with reduced metabolites, which are, however, still not fully evaluated. Only in our study was the biochemical structure of the excreted metabolites determined to some extent, different antibodies compared and the most suitable one chosen.

Compared to the EIA used by Dehnhard *et al.* (2003) and the two other assays used in our study, the cortisone assay is more suitable, because larger amounts of metabolites are detected and also a greater increase after administration of ACTH was measured. This might be an advantage in future studies, when biological changes in adrenocortical activity should be investigated, because smaller increases can be detected.

The newly developed cortisone assay is group specific and detects metabolites with a 3,11-dioxo structure. As discussed by Palme and Möstl (1997), it is an advantage to work with group-specific antibodies, which recognise a group of metabolites rather than a specific steroid. In some species, more specific immunoassays proved inadequate, because they could not show significant cross-reactions with the excreted faecal metabolites. Following ACTH injection, faecal metabolite concentrations peaked after 1·7 h (±0·6), which resembles findings in the spotted owl, where peak concentrations were measured after 2 h, but sampling was performed hourly (Wasser *et al.*, 2000). This underlines the necessity of a strict sampling regime in birds at least during the first hours of the experiment. In chickens, Dehnhard *et al.* (2003) described an increase 4 h after stimulation with ACTH. This discrepancy can be explained by the use of two different antibodies, which detect different metabolites. One assay measures metabolites resulting from renal excretion, which appear earlier, the other one detects metabolites mainly eliminated via the gut. This is supported by the results of the radiometabolism study where two peaks appeared.

The results from the dexamethasone experiment led to the conclusion that suppression might have been incomplete. Our results confirm the findings of Dehnhard *et al.* (2003), who did not observe any suppressive effect, neither in blood nor faeces, after administration of a similar dose of dexamethasone. One explanation for these results might be that because of the mineralocorticoid effects of corticosterone the chicken need not lower the plasma corticosterone concentration to maintain homeostasis. Another reason might be that the administered dosage was too low to cause suppression.

From our study we conclude that the measurement of metabolites with a 3,11-dione structure with this newly developed cortisone assay can be used to detect adrenocortical activity in chicken. For future investigations in related species, especially galliformes, the group-specific cortisone EIA may also serve as a valuable tool. This assay can be used in basic and applied research as a non-invasive method in farm animals as well as in related wild bird species. It is relevant to behavioural ecology, stress research, zoo management and conservation biology.

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