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Biological validation of a non-invasive method for stress assessment in chickens

Biologische Validierung einer nichtinvasiven Methode zur Stressmessung beim Huhn

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Summary

Non-invasive methods to monitor adrenocortical activity need thorough validation. Besides analytical issues, the ability of the chosen test system to detect small changes in hormone concentrations triggered by stress perception must be evaluated. In this study, we biologically validated a previously established enzyme immunoassay (EIA) for corticosterone metabolites (CM) in chicken droppings. Adult laying hens were subjected to one hour of transport and in another experiment to 10 min of restraint. Droppings were collected subsequently after each stressor, over 36 h in total. Additionally, we analysed droppings that were collected after the birds first arrived at the experimental site and encountered unfamiliar housing conditions. Transporting the hens caused significantly increased mean CM concentrations for 3 h (328 nmol/kg droppings; $p = 0.02$) compared to baseline values (101 nmol/kg), whereas after 10 min of restraint, elevated levels of CM were not detected (166 nmol/kg; $p = 0.87$). When chickens were confronted with the new environment, CM concentrations stayed significantly elevated over the whole 36 h sampling period (> 313 nmol/kg; $p < 0.05$). In conclusion, this method is suitable to evaluate disturbances such as transport non-invasively in chickens.

Keywords: corticosterone metabolites, chicken, glucocorticoids, stress

Zusammenfassung

Nichtinvasive Methoden zur Erhebung der Nebennierenrindenaktivität benötigen umfassende Validierung. Neben analytischen Gesichtspunkten muss auch evaluiert werden, wie gut das Testsystem kleinere, durch Stress ausgelöste Veränderungen der Hormonkonzentration erfasst. In der vorliegenden Studie führten wir eine biologische Validierung eines zuvor etablierten Enzymimmunoassays (EIA) für Kortikosteronmetaboliten (CM) im Geflügelkot durch. Adulte Legehennen wurden eine Stunde lang transportiert und in einem weiteren Experiment zehn Minuten fixiert. Nach jedem Stressor wurden individuelle Kotproben über 36 h kontinuierlich gesammelt. Zusätzlich wurden unmittelbar nach dem Eintreffen der Tiere am Versuchsgelände, bei der Einstallung in ein nicht vertrautes Haltungssystem, Kotproben gesammelt. Im Vergleich zu den Basalwerten waren die CM Konzentrationen nach Transport 3 h lang erhöht, während nach 10 min Fixation keine erhöhten CM Konzentrationen gefunden wurden. In dem neuen Haltungssystem blieben die CM Konzentrationen während der gesamten 36 h erhöht. Zusammenfassend ist diese nichtinvasive Methode zur Erhebung von Belastungen wie beispielsweise Transport beim Huhn gut geeignet.

Schlüsselwörter: Kortikosteronmetaboliten, Huhn, Glukokortikoide, Stress

Introduction

Physical and psychological stressors have the potential to activate the hypothalamic-pituitary-adrenocortical-axis and trigger stress hormone secretion (Axelrod and Reisine, 1984). The concentration of glucocorticoids (GC) in plasma is therefore widely used as an indicator for the amount of stress which an animal experiences (Möstl and Palme, 2002). However, corticosterone concentrations in the plasma can be influenced by the blood sampling procedure itself, which might be stressful (Harvey et al., 1980). Therefore, non-invasive methods are receiving more and more attention. Assessment of hormonal levels via faeces minimises handling of the animals and results are therefore unaffected by the investigator or the sampling method. This technique also enables frequent sampling over a longer time period. However, before embarking on any research involving non-invasive techniques, several validation processes have to be carried out (Touma and Palme, 2005).

In almost every species investigated so far, the native GC (cortisol or corticosterone) is not present in the excreta because of metabolism (Palme et al., 2005). GC are metabolized in the liver usually by being converted into water-soluble compounds (Taylor, 1971). These metabolites then are excreted via faeces and urine in mammals (Brownie, 1992; Palme et. al., 2005) or via droppings in birds (Rettenbacher et al., 2006). Due to this metabolism, enzyme immunoassays (EIA) that were developed to determine the original hormone in plasma are often not applicable for faeces, as their antibodies have a high specificy for the native GC and therefore might not detect GC metabolites (Möstl et al., 2005). Therefore, EIA with group-specific antibodies have proven more successful in several species. These antibodies react with a certain group of steroids and are more likely to bind the GC metabolites (Möstl and Palme, 2002). However, due to their broader "reaction-spectrum", these antibodies may also cross-react with other steroids or steroid metabolites (e. g. from sex steroids), which are present in the excreta. To ensure that relevant GC metabolites are detected by the utilised antibody, a validation of the used EIA is therefore required (Möstl et al., 2005).

Aside from analytical issues such as ensuring that the antibody cross-reacts with the excreted metabolites, Touma and Palme (2005) also strongly recommend both a physiological validation (comprising stimulation of the adrenal cortex via ACTH and suppression via dexamethasone) as well as a biological validation. The latter implies sampling after known stressful events, in order to document the applicability of the EIA to detect small alterations in adrenocortical activity.

In this study, a previously established and physiologically validated EIA for chicken droppings (Rettenbacher et al., 2004) was biologically validated by subjecting adult laying hens to different stressors. Animals were transported for one hour and in another experiment restrained for ten minutes. Droppings were collected, the excreted GC metabolites were quantified and compared to baseline concentrations. In addition, we also collected samples after the birds were translocated to the experimental site. Our aim was to assess the potential of the assay system for evaluating stressful conditions in domestic chickens.

Material and Methods

Ten healthy female laying hybrids, ISA Brown, obtained from a commercial breeder (Schropper PLC, Gloggnitz, Austria) were kept in groups on deep litter until 26 weeks of age and were then moved to a different housing system. The animals were caught, each five animals put into crates and transported by car for one hour. At the new location, hens were housed in single cages, where they had visual and acoustic contact with each other. Food and water were supplied *ad libidum*. The 14 h light period was from 06:00 a. m. to 08:00 p. m., temperatur was maintained at 21 °C. To enable individual collection of droppings, plastic panels were placed under each cage. During the first 36 hours in the new environment, all droppings were collected immediately after defaecation, put into plastic freezer bags and frozen at –24 °C. During the dark phase, sampling was done under dimmed light. In total, 23 ± 4 samples per individual (mean ± SD) were collected.

The birds were given ten days to acclimatise to the new environment. During this period, the room was entered twice a day for approximately 15 min by either an animal caretaker or S. R., birds were visually inspected, plastic panels were cleaned from excreta, eggs were collected and the feed trough was refilled, all done without opening the cages. After this 10 d period, individual droppings (17 ± 1) were collected for a total of 36 h to determine baseline concentrations of glucocorticoid metabolites. Samples were stored as described above. The following experiments were performed in weekly intervals: For the transport experiment, chickens were taken out of their cages, put into crates, transported for one hour by car and then put back into their single cages. Individual droppings (21 ± 1) were collected as described above. In the restraint experiment, animals were taken out of their cages and placed on a table where they were manually restrained for ten minutes. Birds were put in right lateral recumbency, their legs retracted caudally, their wings not retracted but gently pressed to the body. After ten minutes, birds were put back into their cages, and samples (22 ± 1) were collected. In both the transport and the restraint experiment, one sample per bird was also collected right before each experiment. Permission for the animal experiment was obtained from the Austrian Federal Ministry for Education, Science and Culture (GZ 68.205/59-Pr/4/2002).

In the lab, each dropping was homogenised and 0.5 g were suspended in 5 ml of 60% (v/v) methanol by shaking for 30 min on a multi-vortex. After centrifugation, aliquots of the supernatant were diluted 1:10 with assay buffer and concentrations of faecal corticosterone metabolites (CM) were determined with a non-commercial EIA, developed in our lab (Rettenbacher et al., 2004). Cortisone, linked at position C-21 to bovine serum albumin using hemisuccinate as a spacer, was utilized as the immunogen. Due to cross-reactions, the antibody detects metabolites with a 3,11-dioxo structure. As corticosterone is the major glucocorticoid in adult birds, the fact that cortisone itself (a cortisol metabolite), is present in chicken droppings can be excluded. Details of the assay procedure and cross-reactions are described by Rettenbacher et al. (2004). Cortisone was used as a standard and values were expressed as cortisone equivalents in nmoles per kg droppings.

As all droppings were collected after spontaneous defecation, time and frequency differed between individuals. Therefore, for each experiment, data were allocated in time intervals (0–3 h; 3–6 h; 6–12 h; 12–24 h, 24–36 h) and for each animal the mean concentration of all samples that were excreted within the respective time frame was calculated. The mean CM concentration of the droppings (n = 10) that were collected right before the experiments are presented at time point 0, except for the translocation experiment, where the chickens were kept in groups on deep litter and individual sampling was therefore not possible. Differences within each experiment were assessed via RM ANOVA. To assess differences between the experiments, the means of all animals were compared at identical time points via one way RM ANOVA and post-hoc tests (Bonferroni t-tests). Test rejection probability levels were set at 5%. All statistical analyses were conducted with SigmaStat (Systat Software Inc., San Jose, CA).

Results

No changes in consistence or colouration of the droppings were observed during any of the experiments. Mean baseline concentrations of excreted CM ranged from 101 nmol/kg droppings (0–3 h interval) to 148 nmol/kg (12–24 h), but differences between time intervals were not significant. Translocation resulted in significantly higher mean concentrations of CM compared to baseline values ($p < 0.05$ in all intervals). The highest values were found during the first six hours. The mean concentrations were 428 nmol/kg ($p < 0.001$) during the first three hours after translocation and 495 nmol/kg ($p = 0.006$) during the 3–6 h interval. Compared to baseline values, mean CM concentrations stayed elevated throughout the whole sampling period and ranged from 313 nmol/kg ($p = 0.025$) during the 6–12 h interval to 333 nmol/kg (24–36 h interval; $p = 0.008$). Transporting the chickens significantly elevated CM concentrations during the first three hours to 328 nmol/kg ($p = 0.015$), but not 3–6 h after transport (296 nmol/kg; $p = 0.44$) and returned to baseline values during the 6–12 h interval (125 nmol/kg; $p = 1$). There was a significant difference ($p = 0.04$) between the 0–3 h interval and the 6–12 h interval within the transport experiment. Restraining the animals for 10 minutes did not result in elevated concentrations of CM (0–3 h interval: 166 nmol/kg; $p = 0.87$) compared to baseline levels. Time intervals within the relocation and within the restraint experiment did not differ significantly ($p > 0.05$ for both experiments). Mean CM concentrations of the droppings at time point 0 did not differ significantly from baseline values ($p = 0.36$). The measured concentrations of CM (means and standard errors) of all experiments are presented in Figure 1.

Discussion

Several assays have been applied to evaluate disturbances via non-invasive methods in the chicken (Dehnhard et al., 2003; Fraisse and Cockrem, 2006). For each immunoassay, a careful validation is necessary, in order to determine the applicability and usefulness of the method in question. The aim of this study was to evaluate, if we can detect increased adrenocortical activity caused by disturbances with our EIA. We therefore chose manipulations that are known to elevate blood corticosterone concentrations and quantified the excreted CM. This procedure is referred to as biological validation and is the last step of the validation process recommended by Touma and Palme (2005), which comprises analytical, physiological and biological validation of an EIA.

Our assay system proved to be sensitive enough to detect biologically meaningful alterations in adrenocortical activity of adult laying hens after one hour of transport, whereas after a manual restraint for ten minutes, CM

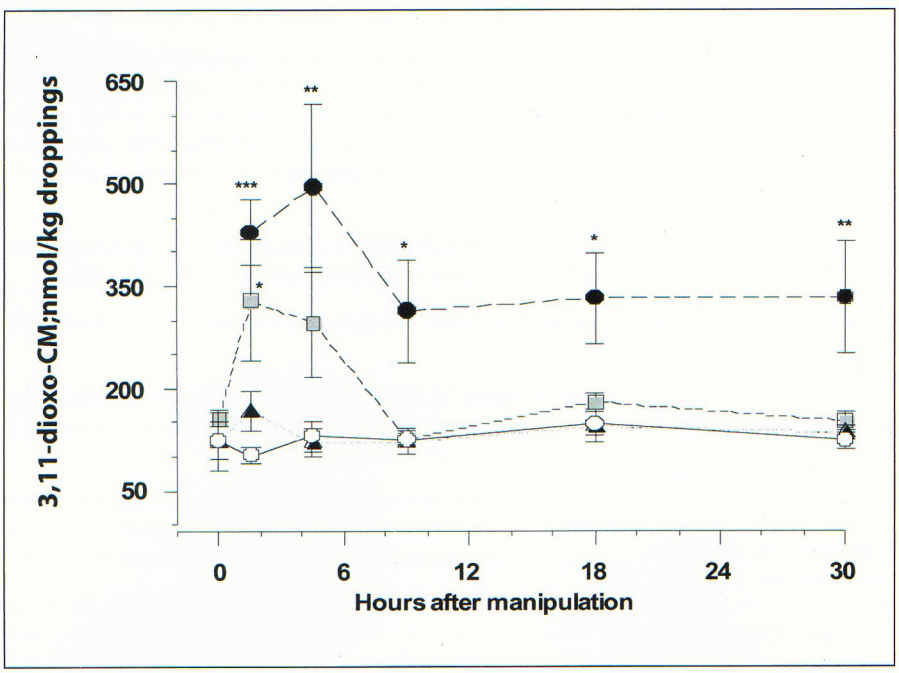

FIGURE 1: *Concentrations of corticosterone metabolites (CM) after translocation ●, transport ■, restraint ▲ and baseline values ○. Data are given as means ± sem. Asterisks indicate significant differences of experiments compared to baseline concentrations. (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$).*

concentrations were not significantly elevated. Introducing adult laying hens to an unfamiliar housing system caused a significant increase in CM, which was clearly detected by our non-invasive method: The whole procedure of catching, handling, crating and transporting the birds as well as housing them in a new environment constituted a plethora of stressful stimuli and was clearly reflected in increased adrenocortical activity during the whole sampling period. To our knowledge, this is the first study that demonstrated that it is possible to measure the stress of relocation non-invasively via droppings and it revealed that concentrations stayed elevated for more than 36 h. Based on these findings, future studies could use this assay to adress the topic in more detail and provide further knowledge about the habituation of chickens to foreign environments.

It is well established that transport constitutes a major stressor for chickens and thus enhances glucocorticoid output (Freemann, 1984), and this was also reflected in increased concentrations of CM in the droppings in our experiment. Glucocorticoid metabolites are excreted with a certain time delay, which enables to evaluate stressful procedures (e. g. transport) retrospectively via non-invasive methods. It has been demonstrated that plasma GC concentrations increase with journey duration (Chloupek et al., 2008) and this would also favour quantification of hormone levels via droppings, as CM provide a more integrated measure of adrenal activity (Dehnhard et al., 2003).

Restraint is a known stressor for poultry and its effect on plasma corticosterone concentrations is well described (Heiblum et al., 2000). In our study however, the mean CM concentrations during the first 3 h after a manual restraint were not significantly different from baseline levels. There are several indications that the way of handling the birds, (e. g. "rough or gentle" handling) and also the handling position (upright or inverted) influences the height of the corticosterone output (Kannan and Mench, 1996). As in our experiment birds were not handled in an inverted position and restrained gently, the GC output during this manipulation was probably too low or too short to be reflected in increased CM concentrations. In addition, our birds have experienced previous handling during the relocation and the transport experiment, although a habituation to handling once a week seems unlikely (Kannan and Mench, 1997). Compared to blood samples, the sensitivity of a non-invasive method to detect short or small increases in glucocorticoid output can be lower, because in the droppings, hormone levels are integrated over a certain time period and can result in a more dampened pattern. This "pooling effect" by the digestive tract (Touma et al., 2003) might also explain why a distinct diurnal rhythm was not observed in the droppings, in contrast to plasma corticosterone, where concentrations fluctuate over the day (DeJong et al., 2001). From the results of the present study we conclude that the tested method facilitates evaluation of stressors like transport, where immediate sampling is not possible, as well as investigations concerning habituation to a new environment, because a non-invasive approach enables frequent sampling over a prolonged period of time. The described assay system is the first one to detect increases in adrenocortical activity due to stressful situations non-invasively in chicken droppings. The EIA used in the present study was already successfully applied for monitoring the impact of food restriction on adult laying hens (Janczak et al, 2007) and has also proven to be a valuable tool for stress-assessment in a number of non-domestic birds (capercaillie: Thiel et al. 2008, black grouse: Arlettaz et al., 2007; blue tits and pied flycatchers: Lobato et al, 2008). Future application of the assay could address animal welfare topics in poultry as well as the extension of the method to further bird species.

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