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Hormones as indicators of stress

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Abstract

Animal welfare is of increasing importance and absence of chronic stress is one of its prerequisites. During stress, various endocrine responses are involved to improve the fitness of the individual. The front-line hormones to overcome stressful situations are the glucocorticoids and catecholamines. These hormones are determined as a parameter of adrenal activity and thus of disturbance. The concentration of glucocorticoids (or their metabolites) can be measured in various body fluids or excreta. Above all, fecal samples offer the advantage that they can be easily collected and this procedure is feedback free. Recently, enzyme immunoassays (EIA) have been developed and successfully tested, to enable the measurement of groups of cortisol metabolites in animal feces. The determination of these metabolites in fecal samples is a practical method to monitor glucocorticoid production.

1. Animal welfare and stress

In vertebrates, normally habitats are not static and animals have adapted to predictable situations by physiological, morphological and behavioural modifications. The unpredictable components of life cause an “emergency life history stage” [1], which results in changes in the endocrine and metabolic status of an organism. There is increasing interest in animal welfare topics not only in science but also in public discussion. The questions how to define animal welfare and how to measure it are still under debate. A potential indicator of animal welfare is the absence of stress, but there is no standard definition of stress and no single biochemical assay system to measure stress [2]. Thus, there is a need for additional biochemical or endocrine parameters for detection of disturbances.

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The most often used nomenclature defines environmental stimuli that lead to an imbalance of homeostasis as "stressor" and the corresponding defense reaction of an animal as "stress response."

2. Stress as a physiological mechanism

The discovery of metabolic, immunological and neuroendocrine mechanisms make it possible to describe the stress reaction in physiological terms [3]. A multitude of hormones (e.g., ACTH, glucocorticoids, catecholamines, prolactin, etc.) is involved in the stress response (for review [4]). The adrenal glands have a key-role in hormonal reactions to stress as they are involved both in the hypothalamic–pituitary–adrenocortical axis and the symphatho-adrenomedullary system [5]. Adverse situations trigger responses of the adrenals, which result in an increase in glucocorticoid and/or catecholamine secretion. These increases are the front-line endocrine mechanisms to defend the organism against the stressful conditions.

As a physiological mechanism, stress *per se* is not inherently bad [5]. For example, glucocorticoids are released in response to situations that are not normally regarded as stressful, including courtship, copulation and hunting [6]. In addition hormones, which increase during stress periods, are also part of the hormonal cascade causing parturition in some species [7,8]. During short-term stress, glucocorticoids improve fitness by energy mobilisation [9] and may change behaviour [10]. However, severe chronic stress (prolonged periods of high cortisol concentrations) may decrease individual fitness by immunosuppression and atrophy of tissues [11]. In addition, the reproductive success of the animal is decreased [12,13]. There are also indications that stereotypies might be related to stress [14].

As data concerning the metabolism and the excretion of catecholamines are almost lacking so far in farm animals and relatively few studies have reported concentrations in sample materials other than the urine (e.g., [15]) we have concentrated on glucocorticoids in feces. However, more investigations concerning these basic questions should be fostered to enable an evaluation of both "stress axes" in the future.

3. Sample materials for determining glucocorticoids (or their metabolites)

The concentration of cortisol in blood is widely used as an indicator of stress, although caution is advised, since an increase does not occur with every type of stressor [6,16]. Like many other hormones, glucocorticoids have a circadian rhythm in many species. Frequent blood sampling demonstrated circadian rhythms in growing pigs [17], bulls [18] and horses [19] but such rhythms may be abolished by prolonged stress [20]. Thus, rhythmicity and episodic secretion demand frequent sampling.

One has to consider that sample collection, which often involves confinement or handling of animals may by itself be stressful and may confound the results [21,22]. Therefore, feedback-free sampling methods are preferential. To overcome these problems, some researchers have made use of special remote blood sampling devices [22]. Alternatively, several authors have investigated non-invasive sampling procedures such as a corticoid (metabolite)

determination in the urine [15], saliva [23] or milk [24]. However, there are some major drawbacks related to each: saliva or urine collection also needs some manipulation of the animal and can be used only to a limited extent in free moving animals and milk is limited to lactating animals.

Above all, fecal samples offer the advantage that they can be easily collected without stressing the animals. Methods for measuring fecal steroid metabolites of placental or gonadal origin are well established to evaluate reproductive function [25]. As the measurement of physiological stress also has importance in wildlife management, conservation biology and behavioural ecology, the measurement of fecal glucocorticoid metabolites is gaining increased importance.

4. Metabolism and excretion of glucocorticoids (Fig. 1)

For the development of non-invasive techniques to monitor adrenocortical activity, basic knowledge of the metabolism and excretion of glucocorticoids is necessary. As early as 1972, Lindner [26] investigated the excretion of infused ^{14}C-cortisol in the sheep. He stated that two-thirds of the radioactivity was subsequently found in the bile. The main metabolites were glucuronides of tetrahydrocortisol, tetrahydrocortisone and cortolones. Also $C_{19}O_3$ steroids (androstanes) were formed. Additionally, enterohepatic circulation of these metabolites occurred and cortisol metabolites were voided *via* the feces.

To investigate the percentage of cortisol excretion *via* urine and feces of farm animals, Palme *et al.* [27] infused radioactive cortisol intravenously into sheep, ponies and pigs. Urine and feces were sampled and the radioactivity was measured. High interspecies differences were found regarding the amount of radioactivity excreted *via* urine or feces and the time course of the excretion (for other species see also [28]). The highest radioactivity in urine was seen

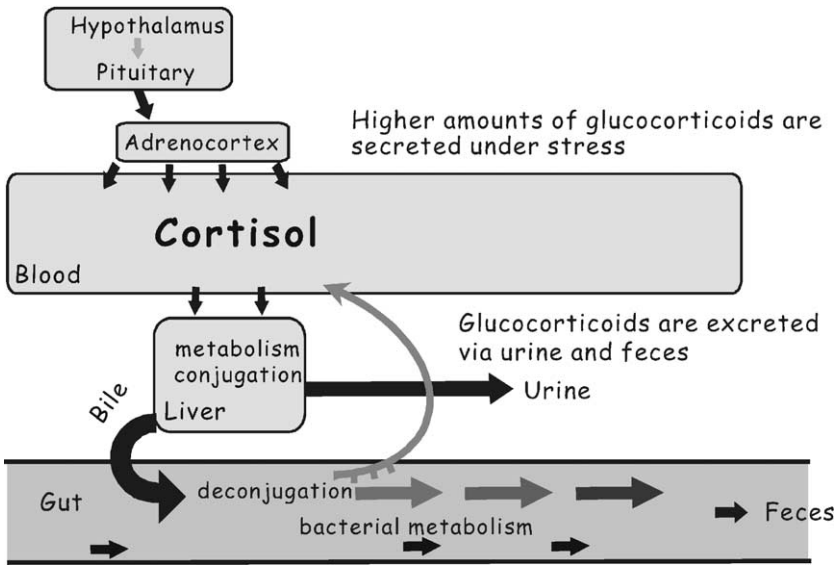

Fig. 1. Scheme of the secretion, metabolism and excretion of glucocorticoids.

close to the time of infusion, whereas in feces maximum concentrations were measured about 12 h (sheep), 24 h (ponies) or 48 h (pigs) later (reflecting the intestinal passage time in these species). The predominating excretory route was *via* the urine, but to different degrees (sheep, 72%; pony, 59%; and pig, 93%).

The metabolism of cortisol in the various species and the further conversions of these substances have not been fully investigated. Metabolism includes (de)conjugation, oxidation at C-11, reduction at C-3 and/or C-20 and/or C-21 and formation of ring A saturated steroids [29]. In addition, a side-chain cleavage was found for cortisol [26,30], which resulted in the formation of androstanes. There are also considerable species differences in the metabolites formed [28]. Möstl *et al.* [31] showed that in ruminants at least 21 cortisol metabolites can be detected in fecal samples using high performance liquid chromatography (HPLC)/mass spectroscopy but the route of metabolism and the participation of bacterial enzymes are unknown. Möstl *et al.* [32] compared chromatograms of fecal cortisol metabolites after infusion of ^{14}C-cortisol into ponies and pigs. In ponies, one dominating metabolite was present, whereas in pigs a total of five more prominent radioactive peaks were measured after HPLC. Using HPLC and enzyme immunoassays (EIA), Palme and Möstl [30] showed that almost no authentic cortisol was excreted in sheep, even after intravenous infusion of 1 g of cortisol. A similar situation was described in ponies and pigs [32]. In some other species, polar (conjugated) cortisol metabolites also occur (e.g., cats, primates and hares [33–35]).

5. Extraction

As nearly all cortisol metabolites present in the feces of farm animals have a polarity similar to unconjugated steroids, an extraction procedure for this group of steroids has to be used. Palme and Möstl [30] showed that the suspension of wet feces (0.5 g) in 5 ml of methanol (80%) yielded highest recoveries. As similar results were obtained in ponies [32], extracting fecal samples with a high concentration of methanol is recommended. Wasser *et al.* [36] described an extraction system based on 0.2 g fully lyophilised, powdered feces, boiled in 90 or 100% ethanol that was also suitable for a variety of species. The two extraction methods (wet feces/methanol or dried feces/ethanol) seem to be quite similar [36]. Till now, the necessity of correction for the water content of fecal samples is under discussion, but one has to keep in mind that, under physiological conditions, the water content of feces in farm animals kept under constant feeding conditions only varies within a small range.

6. Immunoassays

For measurement of fecal cortisol metabolites, Wasser *et al.* [36] investigated three different commercially available radioimmunoassays for cortisol and one assay for corticosterone in a variety of wildlife mammals. A corticosterone antibody (ICN Biomedicals, Costa Mesa, CA) gave best results. This antibody may not only be used for radioimmunoassay but also for EIA as shown by Goymann *et al.* [37].[1] The cross-reactions of this antibody are still not

[1] The biotinylated corticosterone label.

fully evaluated. As authentic cortisol/corticosterone are not present in fecal samples of many species, the test system measures substances structurally related to this hormone.

A second analytical possibility is to use immunoassays specially developed for glucocorticoid metabolites. Palme and Möstl [30] and Möstl *et al.* [31] described group specific EIAs for cortisol metabolites using 11-oxoetiocholanolone as immunogen and a biotinylated steroid as label. The difference between these cortisol metabolites and gonadal androgen metabolites is the functional group at position C-11 of the molecule (a characteristic of glucocorticoid metabolites).

The assay system described by Palme and Möstl [30] used an antibody against 11-oxoetiocholanolone coupled at position C-3. This EIA (measuring 11,17-dioxoandrostanes-11,17-DOA) proved suited for evaluating adrenocortical activity in sheep, cows and horses [32,38,39] and also for some zoo and wildlife animals (e.g., roe deer, hares and elephants [35,40,41]). Möstl *et al.* [31] raised an antibody against 11-oxoetiocholanolone coupled at C-17. This EIA records higher concentrations of cortisol metabolites in cows compared to that described by Palme and Möstl [30], most probably because the new antibody has also cross-reactions with $C_{21}O_4$ cortisol metabolites. Till now, no assay system has been developed for measuring fecal cortisol metabolites on a routine basis in pigs. The reason may be the long passage time of the metabolites in the gut and, therefore, an expanded time for bacterial conversion.

7. Fecal sampling regime

It must be remembered that the concentration of cortisol metabolites in a fecal sample reflects the cortisol production after a species specific time period (e.g., ruminants: 10–12 h). In case of monitoring prolonged periods of elevated glucocorticoid production, the precise sampling protocol is not that critical. On the contrary, the detection of short peaks of metabolites needs frequent sample collection, otherwise they may be missed (especially in ruminants, because of the short passage time). Palme *et al.* [39] collected samples at every spontaneous defecation after a short term stress (transportation for 2 h) and showed that elevated levels of cortisol metabolites were present only in less than three to four consecutive fecal samples. Therefore, with a less strict sampling regime, peaks of cortisol metabolites might not be detected in feces.

8. Stability of the metabolites after defecation

Cortisol metabolites in the gut may be further converted by bacterial enzymes. For example, Winter *et al.* [42] described 21-dehydroxylation of glucocorticoid metabolites by anaerobic strains of bacteria isolated from human feces. Furthermore, concentrations of cortisol metabolites measured with different EIAs increase [32] or decrease [31] as was found in ruminants for the two different 11-oxoetiocholanolone EIAs. Using the ICN antibody, Morrow *et al.* [43] did not find a time dependent variation of cortisol metabolites. To avoid changes after defecation, samples should be frozen immediately or bacterial enzymes inactivated by heating or drying.

9. Measurement of fecal cortisol metabolites in various farm animals

Studies have been performed to evaluate the biological relevance of fecal cortisol metabolites. Palme *et al.* [38] injected ACTH and dexamethasone into sheep and cattle. Changes in the fecal concentration of 11,17-DOA paralleled those of cortisol in the blood with a delay time of about 10–12 h. In cattle, a good correlation ($r = 0.77$) was found between the injected dose of ACTH and the increase of fecal cortisol metabolites, but no correlation was seen between dose and the increase of plasma cortisol concentrations. Therefore, fecal concentrations of cortisol metabolites reflect the total amount of excreted and, therefore, produce better cortisol estimates than a single blood concentration, which changes quickly. High inter-animal variations, occur for both plasma cortisol and fecal cortisol metabolites' concentrations [38]. However, feces enable large scale longitudinal studies, so that an animal acts as its own control, reducing the variation.

Adverse situations increase the concentration of fecal cortisol metabolites. In cows, Palme *et al.* [39] demonstrated that road transport for 2 h was followed by an increase in 11,17-DOA concentrations in the feces. Möstl *et al.* [31] described that after transport into a new stable the cortisol excretion *via* the feces was elevated for about 1 week and declined thereafter to normal values. In mares, Merl *et al.* [44] measured the concentrations of these cortisol metabolites as a parameter of pain after castration or colic and found that abdominal pain caused a considerable increase in excretion of cortisol metabolites.

10. Standardisation of the results

The influence of feed intake may be considered as a cause of variance in fecal steroid concentrations. However, a recent study demonstrated that different amounts of grass intake did not influence fecal progesterone metabolite concentrations significantly [45]. To standardise urinary corticoid concentrations, the cortisol/creatinine ratio is usually calculated. For feces, no such standardisation procedure is necessary, but diets for farm animals are more or less constant.

All assays currently available are group specific and cross-react with different cortisol metabolites. As HPLC immunograms show, there are various cross-reacting metabolites present in fecal samples of different species. Therefore, potentially other cortisol metabolites can be used as standards in the EIAs, resulting in different absolute concentrations. In order to compare the results between various laboratories, a certain degree of standardisation of assay systems (and extraction) will be necessary.

11. Conclusion

Measuring fecal cortisol metabolites as an indicator of adrenocortical activity in animals offers the advantage of a simple sampling technique that will not interfere with the results of the study and enables even long term, longitudinal studies. Thus, such methods will be a valuable tool in a variety of research fields such as animal welfare (handling, housing and transportation) but also in ethological and environmental studies.

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The key-chemicals of these tests (coating antibody, anti-steroid antibody and biotinylated label) are offered free of charge in limited amounts by E Möstl and R Palme.

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