Excretion of corticosteroid metabolites in urine and faeces of rats

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Summary

Stress enhances the production of corticosteroids by the adrenal cortex, resulting in the increased excretion of their metabolites in urine and faeces. An intraperitoneal injection of radioactive corticosterone was applied to adult, male Sprague-Dawley rats to monitor the route and delay of excreted metabolites in urine and faeces. Peak concentrations appeared in urine after 3.2 ± 1.9 h and in faeces after 16.7 ± 4.3 h. Altogether about 20% of the recovered metabolites were found in urine and about 80% in faeces. Using high-performance liquid chromatography (HPLC), several peaks of radioactive metabolites were found. Some metabolites were detected by enzyme immunoassay (EIA) using two different antibodies (corticosterone, 11β-OH-aetiocholanolone). There was a marked diurnal variation with low levels of faecal corticosterone metabolites in the evening and higher values in the morning. This diurnal variation was influenced neither by the intraperitoneal injection of isotonic saline nor by ACTH. However, the administration of dexamethasone eliminated the morning peak for 2 days.

Keywords Rat; urine; faeces; corticosterone; ACTH; dexamethasone

Adaptation to stressful events is associated with an increased production and secretion of glucocorticoids from the adrenal cortex into the blood. Various specific and non-specific stimuli are able to induce increased secretion of glucocorticoids (Clark *et al.* 1997a,b). When the hypothalamic-pituitary-adrenal axis is suddenly activated, glucocorticoids significantly increase and, in rats, maximum values are usually seen about 20 min later, although the time sequence depends on the stimulus intensity. However, the glucocorticoid concentration in the blood is not an appropriate indicator of long-term aversive stimulation, because animals may become less anxious or concerned or may get used to the stimulus. Also, the feedback regulatory system tends to reduce hormone values (Manser 1992). High plasma concentrations of glucocorticoids inhibit the release of ACTH from the pituitary gland, which in turn causes a decreased hormone secretion by the adrenal cortex. Therefore, high concentrations of glucocorticoids do not usually persist for a long time (no more than 90 min) in the circulation. Nevertheless, an increase in glucocorticoid concentrations in peripheral blood can represent a sensitive indicator of the intensity of discomfort or distress experienced by the animals (Van de Kar *et al.* 1991).

In contrast to the concentration in blood, which is influenced by the stressful sampling itself (Cook *et al.* 1973) and which reflects a momentary situation, the collection of urine or faeces allows the monitoring of previous

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stressful conditions, without needing to handle the animals. However, the determination of metabolites in urine samples is hampered by the difficulty of obtaining the samples. Faecal samples can easily be collected from the ground, and the contents have been mixed in the gut, thus providing an integrated measure of a few hours. An enzyme immunoassay (EIA) has been successfully established for the determination of corticosteroid metabolites in faecal samples of farm animals (Palme & Möstl 1997, Palme *et al.* 1999, 2000). This method has been used for other species as well (hares: Teskey-Gerstl *et al.* 2000, cats and dogs: Schatz & Palme 2001).

The present study was aimed at the determination of corticosterone metabolites in faecal and urine samples of male rats for possible non-invasive monitoring of stressful situations. Therefore, in the first experiment the percentage and the time course of the excretion of radioactive corticosterone in urine and faeces was determined. The immunoreactivity of the faecal metabolites was checked after HPLC separation by the use of two different EIAs. In order to study the applicability of this non-invasive method, the effect of an intraperitoneal injection of saline, ACTH or dexamethasone on faecal concentrations of corticosterone metabolites was investigated in another experiment.

Materials and methods

Animals

Groups of 8-week-old male Sprague-Dawley rats (Crl:CD® (SD) IGSBR) with a body weight of 150–180 g were obtained from Charles River, Germany. After arrival, the animals were maintained in groups of four in standard Makrolon cages, type IV (Tecniplast, Buguggiate, Varese, Italy) ($56 \times 33 \times 20$ cm) with wood shavings in a separate room under conventional conditions (12:12 h day/night conditions, 21 ± 1°C room temperature, $55 \pm 5\%$ relative humidity, 6–8 changes of air per hour) for 7 days. After this period the animals were transferred either into individual cages for the study of their metabolisms (type ACC-5062, UNO, NL; $12 \times 20 \times 11$ cm) or into steel wire net cages (type III, $38 \times 22 \times 15$ cm). Pelleted food (sniff® R/M-H, sniff, Spezialdiäte, Soest, Germany) and bottled tap water was supplied *ad libitum*. For feeding in cages used for metabolism studies, the pellets were soaked in water and offered as mush.

Experimental set-up

Injection of ^{3}H-corticosterone

Six rats were injected with 16 μCi of ^{3}H-corticosterone (NET 399, 70 Ci = 2590 GBq/mmol, NEN, Boston, MA, USA) each on day 1 at 09:00 h and transferred to cages for metabolism studies in order to collect urine and faecal samples. The total amount of voided urine was collected via a plastic tube at the bottom of the metabolic cage in measuring cylinders, which were changed after 1, 2, 3, 4, 6 and 8 h. Faecal samples were collected after 4 and 8 h. On day 2 the samples were collected at 09:00 and 17:00 and on day 3 at 09:00. Faecal samples were also collected on days 4, 5 and 6 at 09:00.

According to the results obtained from this experiment, the study was repeated in six animals but modified in that the ^{3}H-corticosterone injection was performed at 20:00 in order to detect maximal excretion in faeces during day time on day 2. Urine and faecal samples were collected every 2 h from 06:00 to 22:00 on day 2, and at 08:00 on days 3, 4 and 5. Faecal samples were also collected once on days 6, 7 and 8.

Administration of isotonic saline, ACTH or dexamethasone

Twenty-four male rats were transferred into steel wire net cages (type III) and housed individually. On day 1 (7 days after transfer to wire net cages) groups of six animals were sham treated (Group a), injected intraperitoneally with isotonic saline (Group b), with 100 μg/kg body weight ACTH (Synacthen™, Ciba-Geigy, Basel) (Group c) or with dexamethasone at 1 mg/kg body weight (Group d). Injection of 1 ml per kg body weight was performed at 18:00. For sham treatment, which should mimic injection-related stress by handling, the animals were picked up,

held in a position for sham injection and put back into the cage.

Faecal samples were collected twice daily at 08:00 and 18:00 on days -5, -4, -3, and on days 3, 4 and 5. On day 2 (one day after injection) faecal samples were collected every 2 h from 08:00 to 20:00. All samples were stored in plastic vials at -24°C until analysis.

Determination of radioactive metabolites

After thawing the urine and faecal samples, an aliquot of 0.1 ml of urine was measured directly by the addition of 2 ml of scintillation fluid (Quicksafe A^{TM}, No. 1008000, Zinsser Analytic, Maidenhead, UK) and the determination of the radioactivity (dpm) was performed with a Packard Scintillation counter (Packard Tri-Carb 2100TR, Meriden, USA). From the faeces, an aliquot of 0.2 g was extracted with 0.4 ml of distilled water and 1.6 ml of methanol, as described by Palme *et al.* (1996). After centrifugation, 0.5 ml of the supernatant was mixed with 10 ml of scintillation fluid and the radioactivity (dpm) determined as in the urine.

Characterization of radioactive metabolites

The amount of ether soluble/insoluble steroids was determined as described by Palme *et al.* (1996). After extraction from the faeces with methanol, the supernatant was concentrated (to approximately 1.5 ml) and extracted with 3 times 5 ml diethyl-ether. Radioactivity was measured in the combined ether extracts and in the remaining aqueous phases.

HPLC of corticosterone metabolites

The extraction, separation on reverse-phase high-performance liquid chromatography (RP-HPLC) and characterization of the radioactive metabolites were achieved according to the method that has been described in detail by Teskey-Gerstl *et al.* (2000). From selected faecal samples with peak radioactivity ($n = 6$), 0.2 g was suspended in 10 ml of methanol (80%) and the supernatant diluted with 30 ml of sodium acetate buffer (0.2 M, pH 4.8) and passed through a Sep-Pak C_{18} cartridge (1 g, Waters, Milford, MA, USA). The cartridge was washed with 10 ml of double-distilled water and dried with a stream of nitrogen. Elution was performed using 10 ml of dichloromethane, ethylacetate/methanol (5/1) and methanol successively. The dichloromethane fraction (containing unconjugated metabolites) and the ethylacetate/methanol fraction (containing mainly conjugated or polar unconjugated steroids) were separately injected onto a Novapak C_{18} column (3.9 × 150 mm) with a Mini-Guard-column (C_{18}). The RP-HPLC was performed as described by Teskey-Gerstl *et al.* (2000), using different methanol/water gradients for unconjugated and conjugated metabolites. In addition, the immunoreactivity of the metabolites was tested in a corticosterone- and an 11β-OH-aetiocholanolone-EIA (Palme & Möstl 1997).

EIA of corticosterone metabolites

For the quantitative determination of the metabolites of corticosterone, 0.5 g of homogenized faeces was suspended in 5 ml methanol (80%) and centrifuged, and the supernatant diluted 1:10 with assay buffer. An aliquot (10 μl) was used in the EIA, which was performed as described by Palme and Möstl (1997). Two antibodies were used, which displayed major cross-reactions with corticosterone and with 11β-OH-aetiocholanolone, respectively. The range of the standard curve was 2–500 pg/well for both steroids, leading to limits of detection of 28.8 ± 2.9 nmol/kg faeces for corticosterone and 7.2 ± 0.7 nmol/kg for 11β-OH-aetiocholanolone respectively. The characterization of the antibody against 11β-OH-aetiocholanolone will be described by Spendier *et al.* (2001).

Statistical analysis

Statistical analysis (paired t-test and repeated measures ANOVA, using group and time as variables) was performed using the Statistica^{TM} software package (StatSoft, Tulsa, OK, USA).

Table 1 Recovery (%) and time lag (h) of the peak excretion of radioactive metabolites in urine and faecal samples of male rats after intraperitoneal administration of ^{3}H-corticosterone

	Percentage		Hours	
Rat No.	Urine	Faeces	Urine	Faeces
1	20.5	79.5	6.0	8–24
2	18.9	81.2	1.0	8–24
3	24.6	75.4	4.2	8–24
4	19.9	80.1	2.0	8–24
5	21.3	78.7	4.1	8–24
6	12.9	87.1	2.0	8–24
Mean	19.7	80.3	3.2	
SD	3.9	3.9	1.9	
7	10.2	89.8	< 10	14.0
8	10.2	89.8	< 10	24.0
9	19.5	80.5	< 10	18.0
10	13.2	86.8	< 10	18.0
11	10.4	89.6	< 10	14.0
12	12.3	87.7	< 10	12.0
Mean	12.6	87.4		16.7
SD	3.6	3.6		4.3

SD = standard deviation

Results

Percentage and delay of excreted corticosterone metabolites in urine and faeces (Table 1, Fig 1)

After injection of ^{3}H-corticosterone, 12.9–24.3 (19.7 ± 3.9) % of the radioactivity was excreted in the urine and 75.4–87.1 (80.3 ± 3.9) % in the faeces. Peak concentrations in the urine were found after an average of 3.2 ± 1.9 h and in the faeces between 8 and 24 h (Table 1).

As Fig 1a shows, the monitoring of the time course of the excretion in urine was possible in the first experiment, but the maximal excretion in faeces occurred between the last sample of the first day and the first sample of the next day (8–24 h after administration). Therefore, an administration in the evening was chosen for the next trial (Fig 1b). The route of excretion was similar to that of the first experiment, with 10.2–19.5 (12.6 ± 3.6) % in urine and 80.5–89.8 (87.4 ± 3.6) % in faeces. The maximal excretion in urine was within the first 10 h and in faeces it was found between 12 and 24 (16.7 ± 4.3)h.

Characterization of radioactive corticosterone metabolites (Fig 2)

In the faecal samples the percentage of ether extractable metabolites ranged between 18.1–66.5 (median 42.5) % of the total faecal radioactivity. The separation of the faecal metabolites on HPLC revealed several peaks and a high individual variability of the relative amounts of the metabolites present. In the dichloromethane fractions, eight peaks could be found with a polarity between cor-

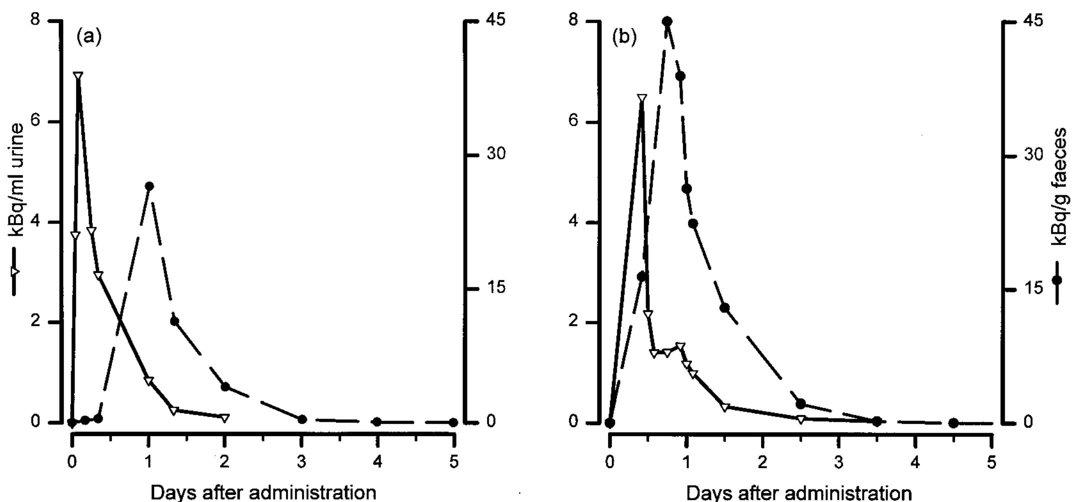

Fig 1 Excretion of radioactive metabolites in urine (—▽—) and faeces (—●—) of male rats after intraperitoneal administration of ^{3}H-corticosterone

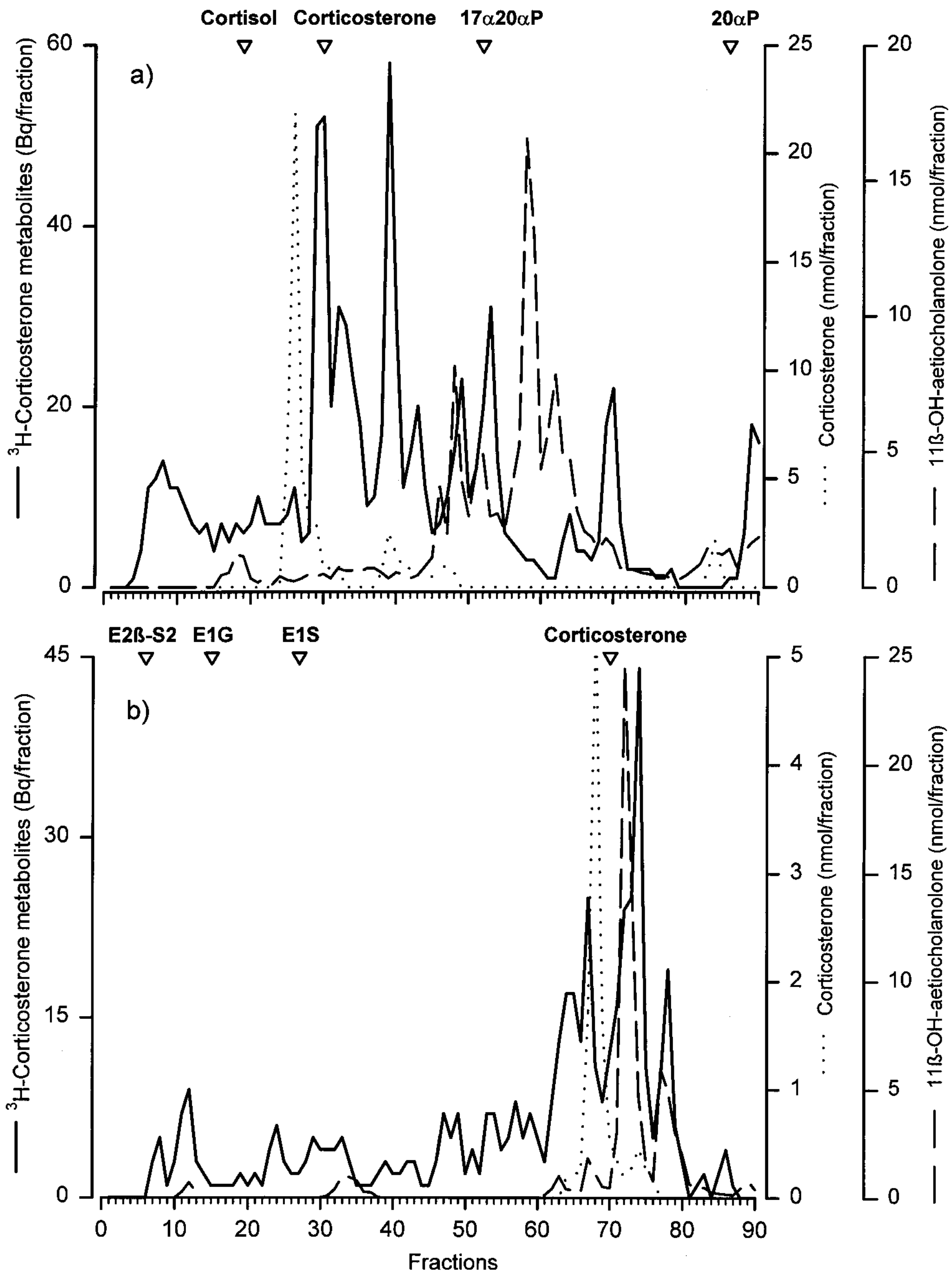

Fig 2 Reverse-phase high-performance liquid chromatography (RP-HPLC)—immunogram of unconjugated (a) and conjugated (b) metabolites of ^{3}H-corticosterone in a faecal sample of a male rat. The immunoreactivity of the metabolites was determined with two different enzyme immunoassays using antibodies, which had cross-reactions with corticosterone (·····) or 11β-OH-aetiocholanolone (– –), respectively. Fractions marked with ▽ represent the approximate elution time of respective standards (17α,20αP = 17α,20α-dihydroxyprogesterone, E1 = oestrone, E2β = oestradiol-17β, E1G = oestrone-glucuronide)

ticosterone and 20α-OH-progesterone. One of these peaks had a retention time in the vicinity of corticosterone, the other metabolites were less polar. However, corticosterone itself was present only in very small amounts. In the ethyl acetate/methanol fractions three major peaks were present in the vicinity of corticosterone. Conjugated metabolites were detectable only in very small amounts.

Effect of an intraperitoneal injection of isotonic saline, ACTH and dexamethasone (Fig 3)

In the control group, which did not receive any injection, there was a marked diurnal variation in the amount of corticosteroid metabolites measured in the faecal samples (Fig 3a). This diurnal variation could be observed in all the animals in all 4 groups. Although there were individual variations, in all groups the samples collected in the evening had lower values, whilst those collected in the morning had significantly higher values ($P < 0.0001$ in the paired t-test). The concentration of corticosterone was 989 ± 310 nmol/kg faeces in the morning and 443 ± 111 nmol/kg in the evening sample, and that of 11β-OH-aetiocholanolone was 3817 ± 1232 nmol/kg and 2327 ± 768 nmol/kg, respectively.

The administration of isotonic saline (Fig 3b) or of ACTH (Fig 3c) did not alter the

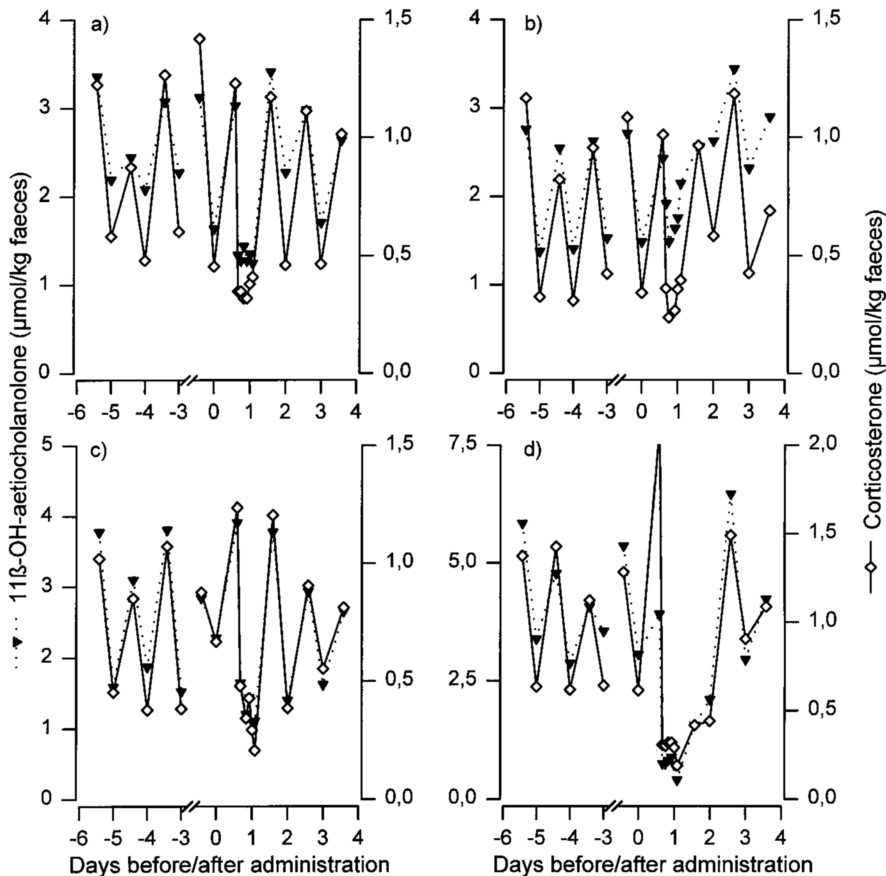

Fig 3 Concentration (μmol/kg) of immunoreactive metabolites in faecal samples of individual male rats before and after (a) sham treatment (handling), or an intraperitoneal administration of (b) isotonic saline, (c) ACTH (0.1 mg/kg, (d) dexamethasone (1 mg/kg). The concentration was determined by two different enzyme immunoassays, using antibodies against corticosterone (—◇—) or 11β-OH-aetiocholanolone (···▾···), respectively

pattern of diurnal variation. The small increase (seen in some animals) on the day after the administration of ACTH was not statistically significant. However, the injection of dexamethasone (Fig 3d) depressed the morning values of the following 2 days, with the exception that, in the first morning sample, which was the first sample collected after the injection, the concentration of metabolites measured by the corticosterone EIA was higher than in any other sample.

Although the group had no effect, a significant time effect (pre-treatment, days 2, 3 and 4 to 5) was detected for the two steroids in both the morning and evening samples (repeated measures ANOVA, $P < 0.02$ for all). Most importantly, however, there was an interaction between time and treatment group ($P < 0.02$ for all), suggesting the time effect to be different for different groups. *Post hoc* comparisons (Fisher's LSD test) revealed that this was due to Group d being different ($P < 0.05$) from all the others.

Discussion

Some 30 years ago the excretion of radioactive labelled corticosterone was monitored in rats. After intraperitoneal administration, the radioactive metabolites were determined in urine and faecal samples by gas liquid chromatography (GLC) and mass spectrometry. The metabolites in faecal samples of male rats were more polar than those of females. Male rats excreted mainly unconjugated metabolites (Eriksson & Gustafsson 1970a), and it was found that in conventionally-kept rats the steroid sulphates were already hydrolysed by microbial enzymes in the intestinal tract (Eriksson & Gustafsson 1970b).

Our results confirm the findings by Eriksson and Gustafsson (1970a), that most of the corticosterone metabolites are excreted via the faeces. They are present in the unconjugated form, just as they could be found mainly in the ether extractable fractions. However, no attempt was made in the present investigation to elucidate the type of conjugation, as the conjugated metabolites were present only in minor amounts. As it is shown in Fig 2 the two antibodies used in the EIAs cross-reacted with different metabolites. However, from Fig 3 it can be seen that both antibodies allowed the measurement of diurnal variations, as their pattern was quite similar, although quantitatively different.

Several methods of blood sampling in chronically-stressed rats have been described in the literature (Sarlis 1991). However, sampling of blood always causes an increase in corticosterone levels, if it is not performed within 2 min of the animal being handled. Mende (1999) found peak values of corticosterone in blood samples taken 20 min after several types of stressful manipulations.

Haemisch *et al.* (1999) reported that in response to four repeated blood samples drawn within 2 h from the tail vein of conscious rats, plasma concentrations of corticosterone increased significantly in response to the first sampling. Subsequently they decreased to baseline values in the fourth sample. The single blood sampling elicited an adrenocortical response comparable to other laboratory procedures (handling, restraint, new environment). The subsequent decline to basal levels was probably due to the feedback inhibition of the hypothalamic-pituitary-adrenocortical axis. As the half-life time of corticosterone in plasma is about 60 min, this may explain the time course of the decrease. The sampling procedure itself was, therefore, not severely stressful.

In our experiment, a short increase of corticosterone in blood, which may have been caused by the administration of ACTH (as was seen in another experiment, using the same dose and the same breed and age of rats), was not reflected by an increase in the corticosteroid metabolites in faeces. Therefore, with the antibodies currently used it would not be possible to monitor any stressor which acts for only a short period of time. It may be that the determination of other metabolites (using different antibodies in the EIAs) would gain better results. On the other hand, the frequency of sampling of the faeces would have to be increased, and should be performed, at best, at the time of each defecation. The long intervals between faecal

samples in the present study could have masked the presence of peaks of short duration.

However, it can be assumed that long-lasting effects alter the amount of excreted corticosterone metabolites in faeces, as has been shown in the present study (Fig 3d) by the depression of immunoreactive metabolites after the administration of dexamethasone. Simulating an injection, or even the intraperitoneal administration of isotonic saline, had no obvious effect on the diurnal pattern of hormone excretion in our study, which only covered 4 days before and after the administration. However, in future studies with regimes of sampling faeces over a longer period of time, it should be taken into consideration that defecation is increased on days when cages are cleaned (Saibaba *et al.* 1996). This could influence the concentration of metabolites in faeces or the ratio between conjugated and free steroids.

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References

Clark JD, Rager DR, Calpin JP (1997a) Animal well-being. III. An overview of assessment. *Laboratory Animal Science* 47, 580–5

Clark JD, Rager DR, Calpin JP (1997b) Animal well-being. IV. Specific assessment criteria. *Laboratory Animal Science* 47, 586–97

Cook DM, Kendall JW, Greer MA, Kramer RM (1973) The effect of acute or chronic ether stress on plasma ACTH concentration in the rat. *Endocrinology* 93, 1019–24

Eriksson H, Gustafsson JA (1970a) Steroids in germ-free and conventional rats. Distribution and excretion of labelled pregnenolone and corticosterone in male and female rats. *European Journal of Biochemistry* 15, 132–9

Eriksson H, Gustafsson JA (1970b) Steroids in germ-free and conventional rats. Sulpho- and glucurono-hydrolase activities of caecal contents from conventional rats. *European Journal of Biochemistry* 13, 198–202

Haemisch A, Guerra G, Furkert J (1999) Adaptation of corticosterone—but not beta-endorphin—secretion to repeated blood sampling in rats. *Laboratory Animals* 33, 185–91

Manser C (1992) *The Assessment of Stress in Laboratory Animals*. Causeway, Horsham, West Sussex, UK: Royal Society for the Prevention of Cruelty to Animals

Mende G (1999) *Effect of Simple Manipulations on the Concentration of Prolactin and Corticosterone in Laboratory Rats* (Doctoral thesis). Berlin (http://www.vetmed.fuberlin.de/online/mende)

Palme R, Fischer P, Schildorfer H, Ismail MN (1996) Excretion of infused ^{14}C-steroid hormones via faeces and urine in domestic livestock. *Animal Reproduction Science* 43, 43–63

Palme R, Möstl E (1997) Measurement of cortisol metabolites in faeces of sheep as a parameter of cortisol concentration in blood. *International Journal of Mammalian Biology* 62(suppl. II), 192–7

Palme R, Robia C, Messmann S, Hofer J, Möstl E (1999) Measurement of faecal cortisol metabolites in ruminants: a non-invasive parameter of adrenal function. *Wiener Tierärztliche Monatsschrift* 86, 237–41

Palme R, Robia C, Baumgartner W, Möstl E (2000) Transport stress in cattle as reflected by an increase in faecal cortisol metabolites. *The Veterinary Record* 146, 108–9

Sarlis NJ (1991) Chronic blood sampling techniques in stress experiments in the rat—a mini review. *Animal Technology* 42, 51–9

Saibaba P, Sales GD, Stodulski G, Hau J (1996) Behaviour of rats in their home cages: daytime variations and effects of routine husbandry procedures analysed by time sampling techniques. *Laboratory Animals* 30, 13–21

Schatz S, Palme R (2001) Measurement of faecal cortisol metabolites in cats and dogs: a non-invasive method to evaluate adrenocortical function. *Veterinary Research Communications* 25, 271–87

Spendier H, Möstl E, Kotrschal K (2001) Hatching and transport cause an increase in concentration of corticosterone metabolites in faeces of hens (in preparation)

Teskey-Gerstl A, Bamberg E, Steineck T, Palme R (2000) Excretion of corticosteroids in urine and faeces of hares (*Lepus europaeus*). *Journal of Comparative Physiology* B170, 163–8

Van de Kar LD, Richardson-Morton KD, Rittenhouse PA (1991) Stress: neuroendocrine and pharmacological mechanisms. In: *Stress Revisited 1. Neuroendocrinology of Stress* (Jasmin G, Cantin M, eds). New York: Karger, pp 133–73