# Non-invasive measurement of adrenocortical activity in male and female rats

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## Summary

Rats are widely used in biomedical research as animal models for human diseases. However, due to their small body size, blood sampling is complicated and invasive and thereby can seriously interfere with endocrine functions and possibly compromise the animals' welfare. Therefore, a non-invasive technique to monitor stress hormones in these animals is highly desired. Our study aimed to gain general information about corticosterone metabolism and excretion and to validate a 5$\alpha$-pregnane-3$\beta$,11$\beta$,21-triol-20-one enzyme immunoassay (EIA) to reliably measure faecal corticosterone metabolites (CMs) in laboratory rats. In total, 18 rats were administered 2.3 MBq of $^{3}$H-corticosterone intravenously and *per os*, respectively (intravenous: 6 males and 6 females; *per os*: 3 males and 3 females). Subsequently, all voided excreta were frequently collected for five days. About $75 \pm 9$% of the recovered CMs were found in the faeces. Peak concentrations of radiolabelled steroids appeared in the urine after $1.7 \pm 0.6$ h in males and after $6.0 \pm 3.5$ h in females. In faeces, maxima were observed after $14.7 \pm 2.4$ h in both sexes. In principle, the time course and delay for both routes of administration (intravenous or *per os*) were the same, except for a delay of peak concentrations in urine ($4.5 \pm 2.1$ h) in *per os* administered males. Using high-performance liquid chromatography (HPLC), faecal $^{3}$H-CMs were characterized and differences were found between the sexes. In both sexes, corticosterone was extensively metabolized, but while males showed only minor variations in their CM patterns, those of females differed largely between individuals. To validate the mentioned EIA, we investigated the diurnal variation (DV) of glucocorticoids as well as effects of the injection procedure itself and conducted an adrenocorticotropic hormone challenge test and a dexamethasone suppression test, using six male and six female rats each. Our results demonstrated that pharmacological stimulation, suppression and DV of adrenocortical activity were accurately reflected by means of CM measurement in faeces. By successful physiological validation, we proved for the first time the suitability of an immunoassay to non-invasively monitor adrenocortical activity in rats of both sexes. This method opens up new perspectives for biomedical and pharmacological investigations as well as for animal welfare related issues.

**Keywords** Rodents; glucocorticoids; sexual dimorphism; faeces; non-invasive; enzyme immunoassay; stress

Laboratory rats (*Rattus norvegicus*) are commonly used in biomedical research, e.g. as animal models for human disorders. In addition, almost all pharmaceutical companies rely on them for toxicity testing. Although scientific knowledge about the rat has grown constantly during the last

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decades, it is still common practice to use exclusively male rats in research, ignoring possible sex differences. Additionally, due to the animals' relatively small body size, physiological data, which are still mainly measured from blood samples, are difficult to obtain. Blood collection in laboratory rats is usually performed by tail vein nicking, tail clipping, bleeding after decapitation or implantation of indwelling catheters (Vahl *et al*. 2005). However, these methods raise serious problems, especially when adrenocortical activity should be monitored. It is difficult to obtain frequent samples from individuals (for example, to monitor endocrine changes during the course of a disease, a social encounter or during the diurnal rhythm), and the circulating hormone levels are influenced rapidly in response to the stressful handling, physical restraint and the blood sampling procedure itself. So, besides animal welfare issues, experimental results can be seriously confounded by these manipulations (Gärtner *et al*. 1980, Riley 1981, Haemisch *et al*. 1999, Vahl *et al*. 2005). Furthermore, blood samples represent concentrations at only one point of time, i.e. a very narrow time frame.

Analysing glucocorticoid (GC) metabolites in faecal samples can be an alternative that offers several advantages (for review, see Möstl & Palme 2002, Palme 2005, Touma & Palme 2005). This technique might be used as a completely non-invasive method to monitor adrenocortical activity in the laboratory rat. Collecting faeces is easy and can be done without disturbing the animal. Therefore, repeated sampling from the same individual is possible and does not interfere with the animal's endocrine status. Further, faecal samples do not represent a single point of time, as circulating levels of blood hormones are integrated over a certain period of time (Palme *et al*. 1999, Touma & Palme 2005). In recent years, different enzyme immunoassays (EIAs) for measuring faecal GC have been successfully established and validated in a growing number of laboratory, domestic, zoo and feral animals (for a review, see Touma & Palme 2005).

In rats, Bamberg *et al*. (2001) were the first to publish on a measurement of corticosterone metabolites (CMs) in the faeces. However, although they clearly demonstrated the presence of a diurnal variation (DV) and even the reflection of a dexamethasone suppression in the concentrations of the faecal metabolites measured (they used a corticosterone and an 11$\beta$-hydroxyaetiocholanolone EIA), they could not find any increase in the metabolites following an adrenocorticotropic hormone (ACTH) stimulation test. The latter clearly disqualified the assays suitable for evaluating adrenocortical activity. Since then a total of five papers on that topic have been published (Pihl & Hau 2003, Eriksson *et al*. 2004, Knox *et al*. 2004 Royo *et al*. 2004, Cavigelli *et al*. 2005). They confirmed the presence of a clear diurnal rhythm in excreted CMs in both sexes. However, they all lacked a sound validation of the methods used (see Palme 2005, Touma & Palme 2005), as none of those papers could demonstrate that changes in adrenocortical activity are well reflected by concentrations of faecal CMs measured by the applied corticosterone immunoassays. However, that would be especially important, as Bamberg *et al*. (2001) found a corticosterone EIA to be unsuited.

As there is only limited information about the metabolism and excretion of corticosterone in rats (Bamberg *et al*. 2001, Cavigelli *et al*. 2005), we first conducted radiometabolism studies to characterize the excreted CMs in the faeces, to reveal the impact of the route of administration and to investigate the possible effects of the animal's sex. According to recently published guidelines (Palme 2005), we also stimulated and suppressed adrenocortical activity pharmacologically, investigated the effect of the injection procedure (saline injection [SI]) itself and checked the DV of corticosterone by leaving the animals untreated. Taken together, all our experiments served the final goal of proving the suitability of the 5$\alpha$-pregnane-3$\beta$,11$\beta$,21-triol-20-one EIA to reliably monitor adrenocortical activity from the faecal samples of laboratory rats of both sexes.

## Materials and methods

*Animals and general housing conditions*

To investigate the metabolism and excretion of corticosterone, several experiments were performed using a total of 30 adult rats. (Fischer, Crl:CD(SD), Charles River, Germany). At the age of 12 weeks and four days before starting the experiments, the animals arrived at the laboratory, where they were separated and housed individually in standard Makrolon cage type IV (Tecniplast, Buguggiate, Varese, Italy) with sawdust as bedding material. The animal housing room was maintained under standard laboratory conditions (light–dark cycle: 12:12 h, lights on at 06:00 h; temperature: $21 \pm 1^{\circ}$C; relative humidity: $50 \pm 10\%$). Commercial rat diet and bottled tap water were available *ad libitum*. Permission for performing the animal experiments was obtained from the provincial government of Lower Austria (LF1-TVG-5/075-2004).

*Radiometabolism study*

*Administration of radiolabelled corticosterone and sample collection* On day 0 of the experiment at 09:00 h, each rat received 2.3 MBq ($= 62.1\ \mu$Ci) of $^{3}$H-corticosterone (1,2,6,7-$^{3}$H-corticosterone, specific activity: 2.61 TBq/mmol, NEN, Boston, MA, USA) diluted in 0.75 mL of sterile isotonic saline solution, containing 10% (v/v) ethanol. In order to investigate the influence of the route of administration, 12 rats (6 of each sex) were injected intravenously and six rats (3 of each sex) received the corticosterone solution *per os* via probe. The whole procedures of catching, fixation, intravenous injection or administration *per os*, respectively, and transferring the rat into its individual metabolism cage (steel wire, cylindrical, 20 cm height, 20 cm diameter) did not exceed 4 min per animal in all experiments.

In these metabolic cages, all excreta could drop through the bars of the wire floor and were separated afterwards by two pipes, one covered by a mesh so that only the urine could run through it. So, all excreta could be easily collected without disturbing the animals. All sample collections performed during the dark phase were done under dimmed light conditions to avoid disturbing the animals' natural activity rhythm. During the first 4 h, the rats were kept without water and pelleted food to strictly avoid contamination of the urine and faeces. After this period, bottled water was supplied *ad libitum*. After 12 h, food was given *ad libitum* as well. During the first 24 h, every voided urine and faecal sample was collected separately, stored at $-30^{\circ}$C and the time of sampling was documented. Subsequently, sampling was done 30, 36, 48, 72 and 96 h after administration.

*Extraction and determination of $^{3}$H-CMs*

A total of 0.1 mL of each urine sample was filled into a scintillation vial (Art. No. 6008117, Packard Instruments, Meriden, CT, USA); 6 mL of scintillation fluid (Quicksafe A, No. 100800, Zinnser Analytic, Maidenhead, UK) was added and the radioactivity was measured in a liquid scintillation counter (Tri-Carb 2100TR, Packard Instruments, Meriden, CT, USA) for 5 min while running a quench compensation programme.

Each faecal sample was well homogenized (with mortar and pestle), and an aliquot of 0.1 g was extracted with 2 mL of 80% methanol for 30 min on a vortex (Touma *et al*. 2003). After centrifugation, a 0.1 mL aliquot of the supernatant (in duplicate) was mixed with 6 mL scintillation fluid and was measured in the liquid scintillation counter as described above. The amount of radioactivity was then calculated by adjusting the total weight of the sample. The total recovery of radioactivity was calculated as the ratio of the combined sums of the recovered radiolabelled metabolites in urine and faeces divided by the total amount of administered radioactivity.

*High-performance liquid chromatography*

To characterize the excreted $^{3}$H-CMs in the faeces of rats, extracts of samples, containing peak radioactivity, were subjected to a reverse-phase high-performance liquid

chromatography (HPLC) and the collected fractions were then analysed with different EIAs. The analyses were performed with samples of 10 animals (intravenous: 3 males, 3 females; *per os*: 2 males, 2 females) to reveal – besides individual variation – impacts of sex and route of administration on the metabolism of corticosterone in rats. Details of the method used are described by Touma *et al*. (2003). However, we used double the amount of each faecal sample (0.1 g instead of 0.05 g) for the extraction. The immunoreactivity of the HPLC fractions was determined with the 5α-pregnane-3β,11β,21-triol-20-one EIA, described by Touma *et al*. (2003), and the corticosterone EIA, described by Palme and Möstl (1997) and already used in male rats' faeces by Bamberg *et al*. (2001).

### *Physiological validation of the EIA*

*Animal housing conditions and sample collection procedure* To enable individual sampling and quantitative collection of all voided faeces, without the need to handle the animal, the rats were housed singly in stainless steel wire cages, which were placed in standard Makrolon cages type IV. The rats were habituated to this housing system four days before starting the experiment. In total, 12 rats (6 males and 6 females) were used for all biological validation experiments. As rats are nocturnal animals and their steroid secretion pattern is known to show a circadian variation, all sample collections during the dark phase were performed under dimmed light conditions to avoid disturbing the animals' natural activity rhythm.

Faecal samples were collected quantitatively and stored at −30°C until analysis. Sampling times were: 0, 2, 4, 6, 8, 10, 12, 14, 16, 20, 24, 30, 48, 72 and 96 h after administration. All excreta dropped through the bars of the wire cage and could be easily collected from the floor of the lower Makrolon cage. The floors of the cages were completely covered with filter paper to immediately absorb the urine. As already proved in studies on laboratory mice (Touma *et al*. 2003, 2004), this technique achieved a minimum contamination of the faeces with urine. During each sampling, the filter paper was renewed and the amount of faeces voided during the sampling interval was documented.

### *Evaluation of the DV*

In order to investigate rats' circadian corticosterone rhythm, a control experiment was set up to distinguish between effects of the pharmacological stimulation/suppression of adrenocortical activity and the physiological circadian rhythm. The rats were left undisturbed in their cages and did not receive any injection or handling. Faecal samples were collected as described above.

### *Saline injection*

Since the handling procedure of capture, fixation and injection may be a stressful event itself, a second control experiment was performed to investigate its effects on the pattern of excreted faecal GC metabolites. Starting the experiments at 09:00 h, each rat was injected with sterile isotonic saline solution (0.5 mL). Faeces were collected and stored as described above.

### *ACTH challenge and dexamethasone (dex) suppression test*

To stimulate adrenocortical activity, we performed an ACTH challenge experiment, using a synthetic ACTH (100 μg/kg body weight; Synacthen, Novartis AG, Basel, Switzerland) dissolved in 1 mL of sterile isotonic saline solution. To suppress the animals' adrenocortical activity, a dex suppression test was performed, using dex (1 mg/kg body weight; Dexa TAD, aniMedica GmbH, Senden-Bösensell, Germany). Starting the experiments at 09:00 h, each rat was injected with ACTH or dex, respectively. Faeces were collected and stored as described above.

### *Extraction and determination of faecal CMs*

Faecal CMs were extracted as described above. A 10 μL aliquot of the methanolic supernatant was diluted (1:100) with assay

buffer (Tris/HCl 20 mmol, pH 7.5) and frozen at −20°C until analysis. To determine the amount of faecal CMs, we used the 5α-pregnane-3β,11β,21-triol-20-one EIA described in detail by Touma *et al*. (2003, 2004) – this EIA is available for research collaborations. Based on its cross-reactions, this group-specific EIA measures steroids with a 5α-3β,11β-diol structure (Touma *et al*. 2003).

*Statistical analysis*

Since parts of the data obtained were not normally distributed, the data were analysed using non-parametric statistics (Siegel & Castellan 1988). All statistical tests were applied two-tailed using the software package SPSS (11.5.1). Analysis of variance (ANOVA) on ranks was used to evaluate differences between more than two dependent samples. Two independent samples were compared using the Mann–Whitney *U*-test (MWU; two-tailed), if not normally distributed, and using the Students *t*-test if normally distributed, while differences between more than two independent samples were calculated with the Kruskal–Wallis *H*-test (KWH). Differences were considered significant if their probability of occurring by chance was less than 5%.

## Results

*Radiometabolism study*

*Time course and route of corticosterone excretion* The mean total recovery (urine and faeces) of intravenous administered radioactivity was 87.0±14.6% for males and females. A total of 25.2±9.2% was excreted in the urine and 74.8±9.2% in the faeces. No differences were detected between sexes, concerning either the recovery (MWU test, $n_{\text{males}} = n_{\text{females}} = 6$; $P = 0.699$) or the route (*t*-test, $n_{\text{males}} = n_{\text{females}} = 6$; $P = 0.536$) of excretion. While the time course of radioactivity excreted in faeces was similar for both sexes, the time course of radioactivity excreted in urine showed significant differences concerning the peak excretion (MWU test, $n_{\text{males}} = n_{\text{females}} = 6$; $P = 0.041$). In males, peak radioactivity was recovered in the first urinary samples after the administration (1.7±0.6 h). About 4–8 h after injection, a second, smaller peak was observed, which was, however, maximum in females (6.0±3.5 h after administration). In females too, a smaller peak could be observed up to 4 h after administration, at the same time as the main peak occurred in the males (Figure 1). Subsequently, in both sexes, the decrease of radioactivity was slightly protracted and showed additional minor peaks during this phase. In the faeces, maximum radioactivity was reached later. In both sexes, peak concentrations of $^3$H-CMs were observed after 14.8±2.4 h and declined afterwards (Figure 1). Thus, the vast majority of radioactive metabolites was excreted via the urine and faeces within the first 24 h after administration and background levels were reached within the next few days.

Concerning the total amount of recovered $^3$H-CMs and the proportions excreted via the urine and faeces, no statistically significant differences were found between intravenous and *per os* administered $^3$H-corticosterone (total recovery: 84.8±3.7%; excretion in the faeces 78.2±4.7%). However, peak concentrations in the urine of male rats were significantly delayed (peak concentration *per os* after 4.5±2.1 h), while no differences were found for other parameters of the excretion patterns (Figure 2).

*Characterization of CMs by HPLC immunograms*

The HPLC separations revealed more than 20 peaks of radioactivity, indicating a large number of different metabolites being formed by males and females (Figures 3 and 4). All males showed three dominant peaks of CMs eluting around the position of corticosterone (fractions 68–80), indicating rather apolar metabolites. The relative proportion of these three peaks varied strongly according to the route of administration. One of these major peaks elutes at fraction 74, which is identical with the eluting point of 5α-pregnane-3β,11β,21-triol-20-one. Additionally, some less prominent peaks were found between

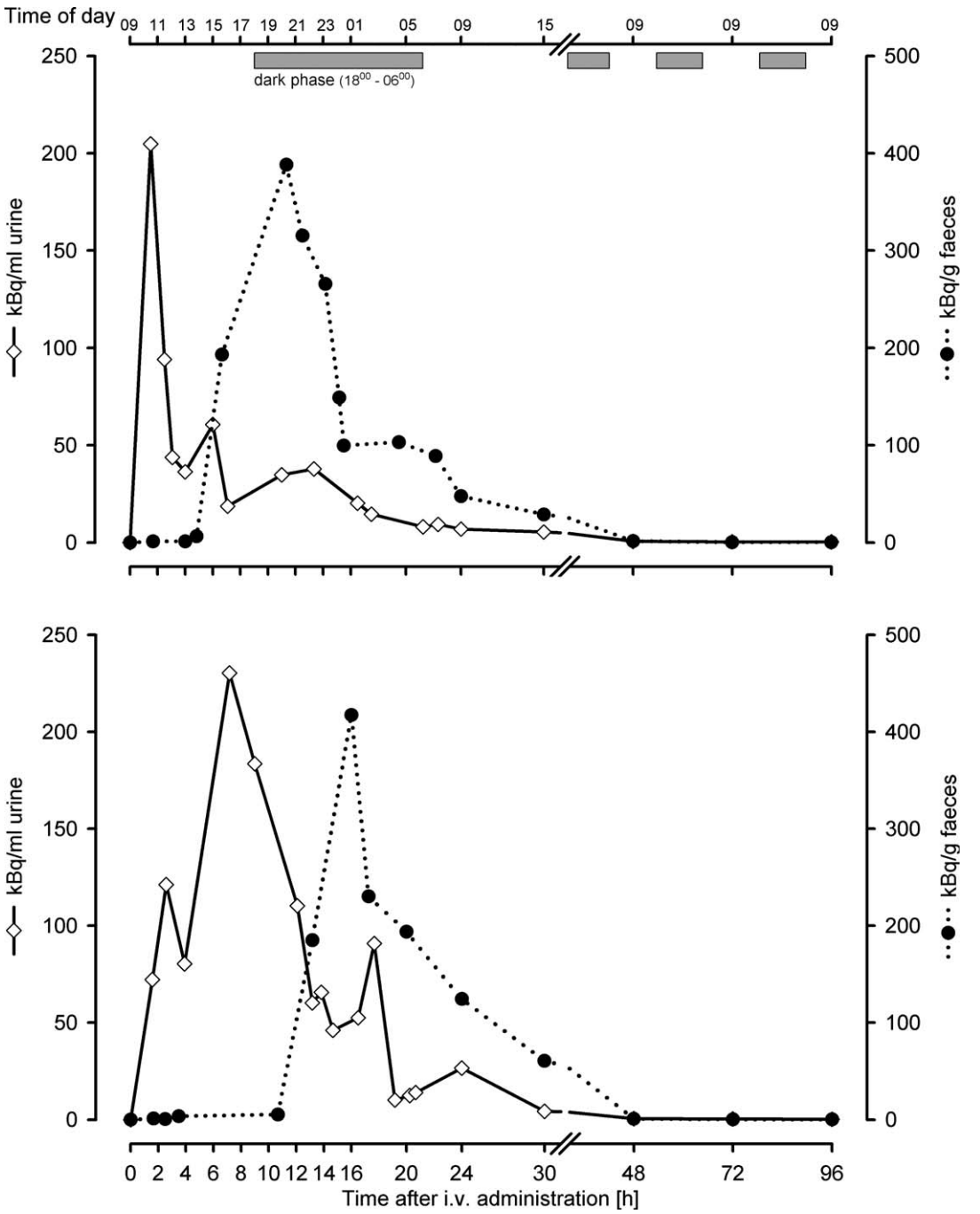


**Figure 1 Time course of excreted $^{3}$H-corticosterone metabolites (kBq/mL or g) in urine and faeces of a representative male (upper panel) and female (lower panel) rat after intravenous injection at 09:00 h**

fractions 35 and 55, i.e. showing a higher polarity than the three major peaks. Only a few very small peaks were detected in fractions 1–25 (Figure 3).

The results were consistent among males except for minor quantitative individual variations and the administration differences mentioned. On the contrary, patterns among

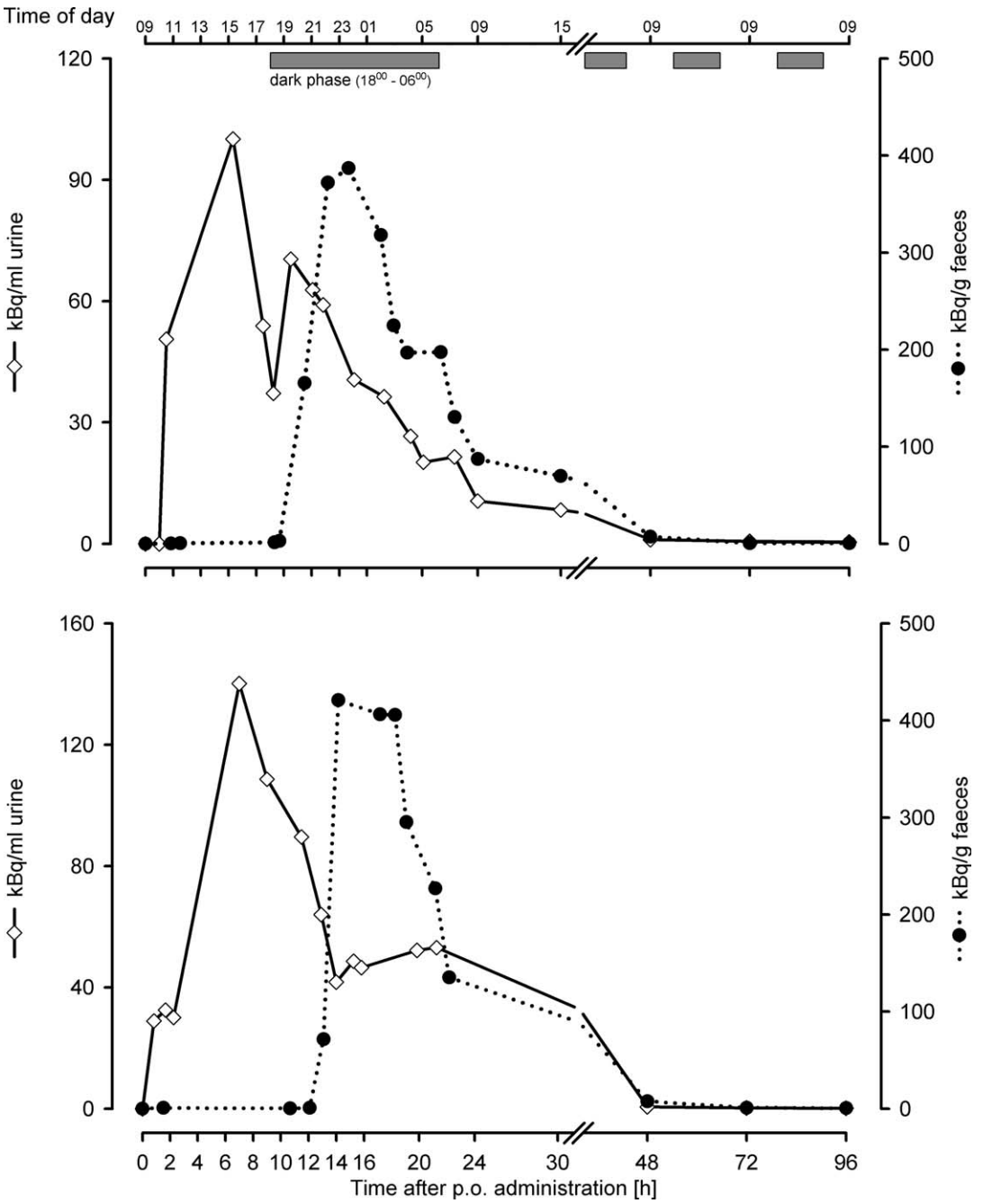


**Figure 2 Time course of excreted $^{3}$H-corticosterone metabolites (kBq/mL or g) in urine and faeces of a representative male (upper panel) and female (lower panel) rat after *per os* administration at 09:00 h**

females were inconsistent. Differences were observed between the two groups of administration (intravenous and *per os*) as well as among individuals of the same group. Only two peaks (fractions 52 and 61) showed certain continuity, although their relative proportions varied. No further similarities were detected, concerning either the amounts of polar and apolar metabolites, or the position of the eluted peaks (Figure 4).

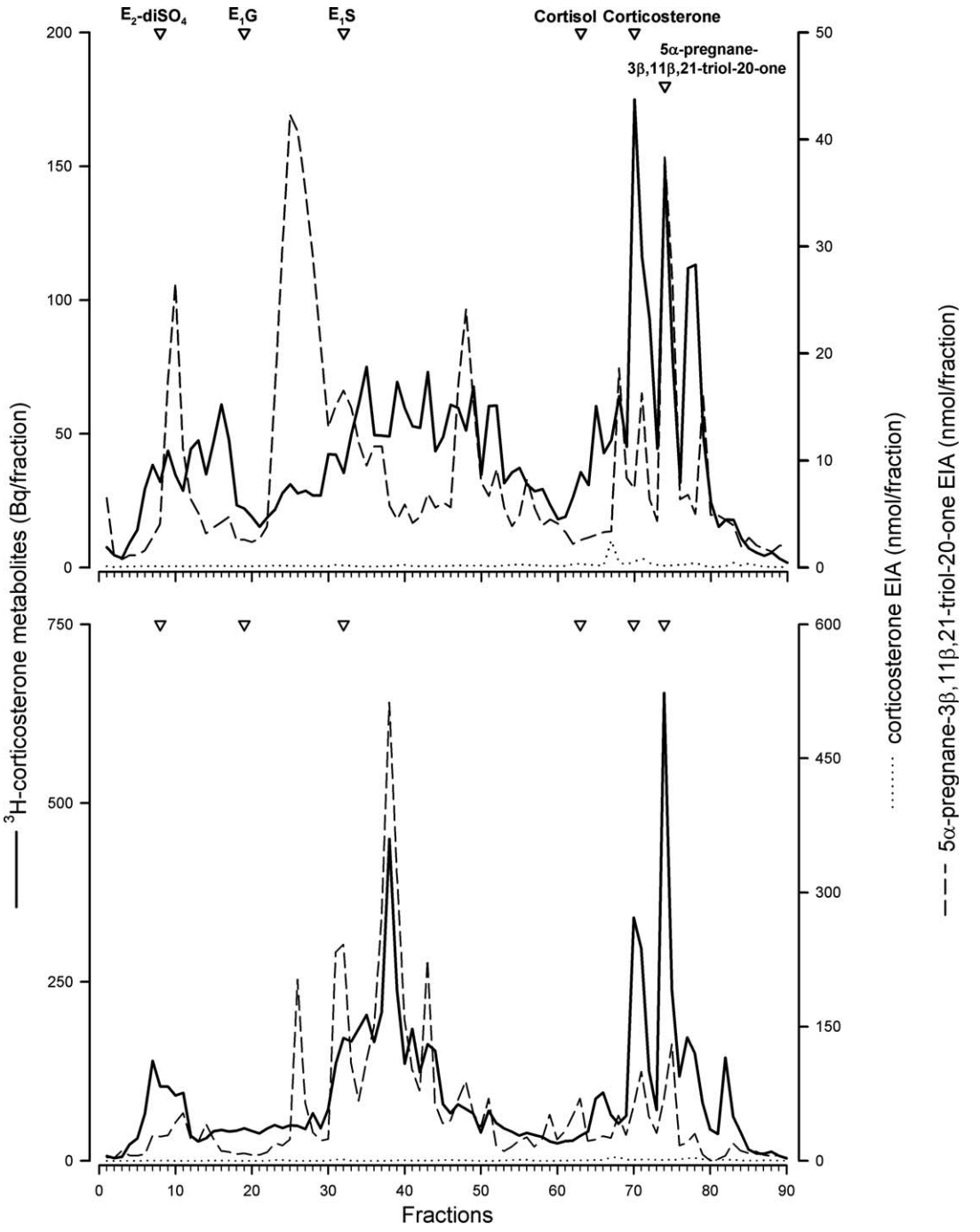


**Figure 3 Reverse-phase high-performance liquid chromatographic (RP-HPLC) separation of faecal $^{3}$H-corticosterone metabolites (peak sample) in the faeces of a representative male after intravenous (upper) and *per os* (lower panel) administration.** Radioactivity of each fraction was determined by liquid scintillation counting, and immunoreactivity was measured in a corticosterone enzyme immunoassay (EIA) and a 5$\alpha$-pregnane-3$\beta$,11$\beta$,21-triol-20-one EIA. Open triangles mark the approximate elution positions of respective standards ($E_2\beta$-di$SO_4$ = 17$\beta$-oestradiol-disulphate, $E_1G$ = oestrone-glucuronide, $E_1S$ = oestrone-sulphate)

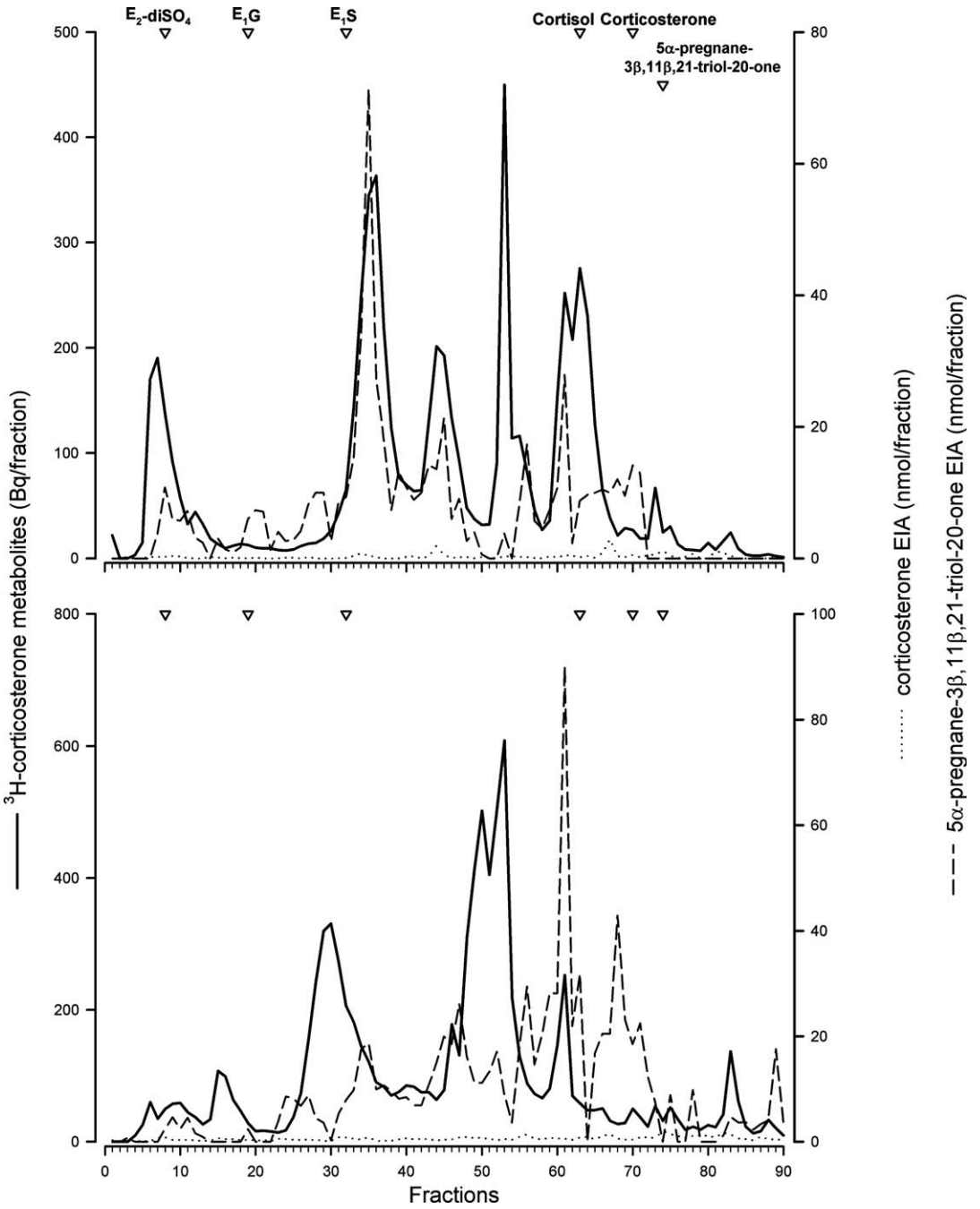


**Figure 4 Reverse-phase high-performance liquid chromatographic (RP-HPLC) separation of faecal $^{3}$H-corticosterone metabolites (peak sample) in the faeces of a female rat after intravenous (upper) and *per os* (lower panel) administration. For further details, see legend of Figure 3**

In samples of both routes of administration with the 5$\alpha$-pregnane-3$\beta$,11$\beta$,21-triol-20-one EIA, immunoreactive metabolites were detected in several HPLC fractions with radioactivity, indicating that the respective $^{3}$H-CMs are recognized by the EIA. In males, e.g. immunoreactivity was found with three peaks in fractions 68–80

and up to five peaks in fractions 30–50 (Figure 3). Again, at fraction 74, a prominent immunoreactive peak could be measured. The assay showed further variations between the routes of administration in males. Especially in the samples of intravenous administrations, fractions 8–11 showed very high immunoreactivity, while in the samples of *per os* administrations, immunoreactivity was almost absent. The reactivity with the already mentioned three apolar peaks (fractions 68–80) showed large differences concerning the relative proportions. Although the patterns of radioactive metabolites of female HPLC separations varied, the EIA was still able to detect at least four peaks of radioactive metabolites (Figure 4). On the other hand, a corticosterone EIA showed almost no reactivity with the CMs found in the faeces of males and females, except one minor peak in fractions 68 and 69 (Figures 3 and 4).

*Physiological validation*

*Evaluation of DV* The experiment to evaluate diurnal changes of corticosterone concentrations revealed a significant variation of excreted faecal CMs in male and female rats over time (ANOVA on ranks, $n_{\text{males}} = n_{\text{females}} = 6$, $\chi^2_r = 60.1$ and 58.1, df = 14, both $P < 0.001$). A clear pattern of highly increasing and decreasing concentrations was observed reaching maximal values (measuring CM concentrations of 95.2 and 17.4 $\mu$mol/kg faeces in males and females, respectively) in the second third of the dark phase (16 h after start of the experiment, i.e. in the sampling interval between 23:00 and 01:00 h) and minimal values (8.6 $\mu$mol/kg in males, 1.3 $\mu$mol/kg in females) at the beginning of the light phase (2 h after start of the experiment, i.e. in the sampling interval between 09:00 and 11:00 h). Relatively high concentrations of CMs were also found in samples representing larger sampling intervals (samples collected over periods of 18 and 24 h; sample points at $T = 30$, 48, 72 and 96 h). This pattern of diurnally varying CM concentrations was in principle identical for both sexes, although measured faecal CMs were about six times higher in males than in females (Figure 5).

*Effects of the injection procedure*

Very similar to the DV of CM concentrations, significant changes were also observed in faecal samples of males and females used in this control experiment evaluating effects of the injection procedure itself (ANOVA on ranks, $n_{\text{males}} = n_{\text{females}} = 6$, $\chi^2_r = 64.3$ and 47, df = 13, both $P < 0.001$). In males, the time course of excretion paralleled that of the DV (Figure 5). Concentrations were slightly lower in general, but significant (MWU test, $n_1 = n_2 = 6$; $U = 5$; $P < 0.05$) only at sampling points $T = 14$ and 30 h. In females, at sample points $T = 4$, 6, 8 and 10 h, significantly higher values ($U = 0$–5; $0.001 < P < 0.05$) could be measured.

*ACTH challenge test*

In the ACTH challenge experiment, a significant variation of CMs was found for both sexes (ANOVA on ranks, $n_{\text{males}} = n_{\text{females}} = 6$, $\chi^2_r = 66.1$ and 56.7, df = 14, both $P < 0.001$). The injection of ACTH caused a rise of CM concentrations in the faeces 4–12 h later and stayed elevated over a period of 4–10 h (KWH test, $n_1 = n_2 = n_3 = 6$, df = 2; males: sample points at $T = 12$ and 14 h, $H = 6.9$–11; $0.001 < P < 0.05$; females: sample points at $T = 4$, 8, 12 and 14 h, $H = 6$–10.3; $0.001 < P < 0.05$). During this time period, significant differences between the two control experiments and the CM excretion pattern of the ACTH challenge experiment could be observed. Although males generally showed much higher absolute concentrations of excreted CMs, the relative increase and decrease did not differ significantly between the sexes (Figure 5).

*Dex suppression test*

Also, in the dex suppression experiment, a significant variation of CMs was found for both sexes (ANOVA on ranks, $n_{\text{males}} = n_{\text{females}} = 6$, $\chi^2_r = 71.7$ and 44.1, df = 14, both $P < 0.001$). For the first 12 h in

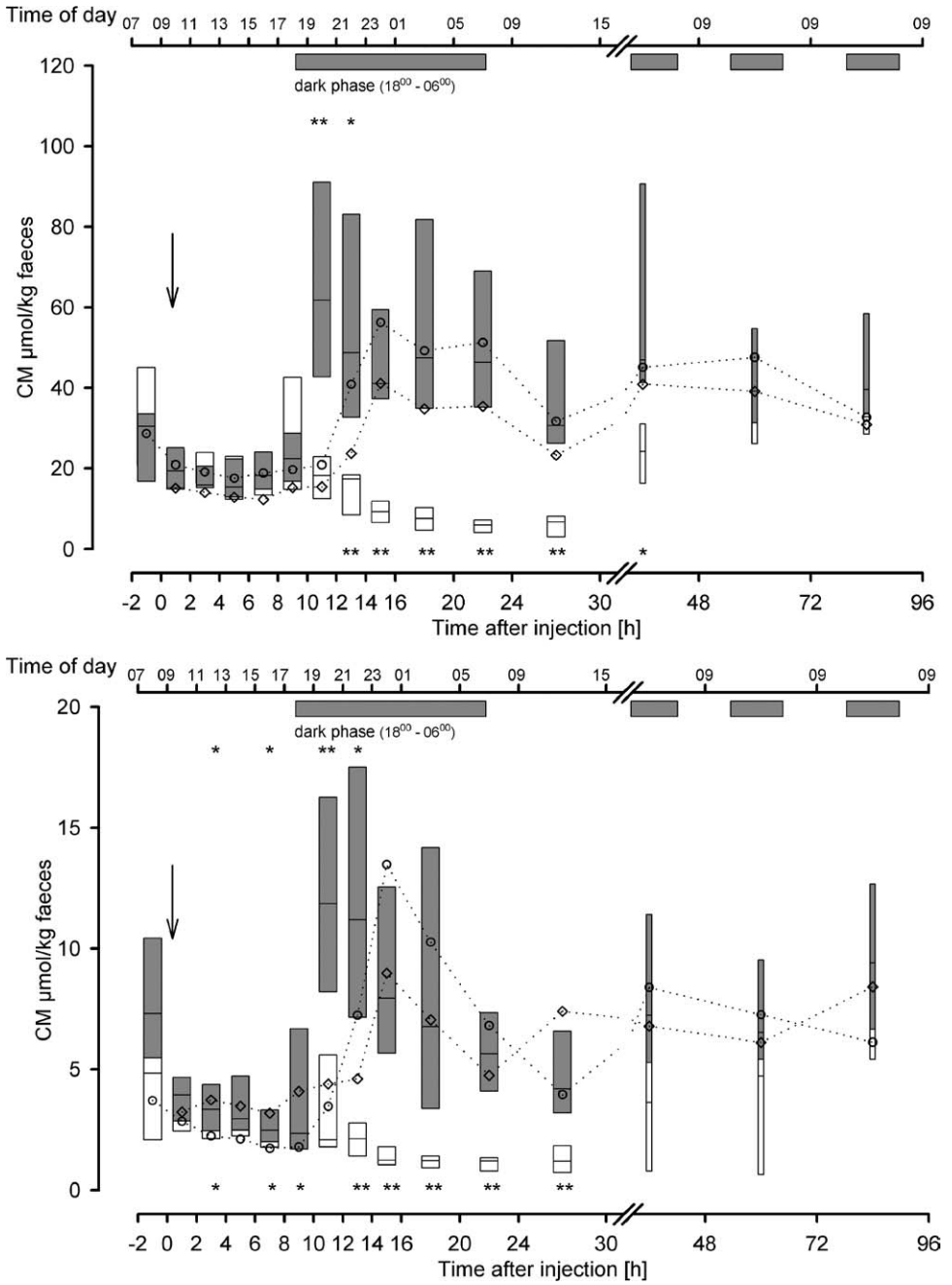


**Figure 5 Corticosterone metabolites (CMs), measured with the 5α-pregnane-3β,11β,21-triol-20-one enzyme immunoassay (EIA), in faecal samples of male (upper panel) and female (lower panel) rats treated with adrenocorticotrophic hormone (ACTH) (grey) and dexamethasone (white), respectively.** Data are given as box plots (medians: lines in the boxes; 25 and 75% quartiles: boxes). Time of day and dark phase (horizontal bars) are indicated at the top of each panel. The arrows mark the time of injection. Significant differences ($^{*}P<0.05$; $^{**}P<0.01$) between the ACTH challenge and the two control experiments 'diurnal variation' (medians given as circles) and 'saline injection' (medians, given as diamonds, are connected with a dotted line for ease of traceability) are marked with asterisks, placed on top of the boxes. Significant differences between the dexamethasone suppression and the two control experiments are marked with asterisks, placed below the boxes

males and for the first 10 h in females, the time course of excreted CMs was similar to the time course observed in the DV and SI experiments. However, from this time on, significant differences were observed over an extended period for both sexes. In males, significantly lower CM concentrations were found between $T = 12$ and 72 h (KWH test, $n_1 = n_2 = n_3 = 6$, df = 2; sample points at $T = 14$, 16, 20, 24, 30 and 48 h, $H = 8.6$–13.3; all $P < 0.05$). In females, significantly lower CM concentrations were found at sample points $T = 10$, 16, 20, 24 and 30 h (KWH test, $n_1 = n_2 = n_3 = 6$, df = 2, $H = 7.1$–12.1, all $P < 0.05$). Thus, suppression of adrenocortical activity lasted for about 34 h in males and 20 h in females. Subsequently, CM secretion returned to levels measured in the control groups (Figure 5).

## Discussion

As is known from other studies investigating faecal GC metabolites, the route and delay of excretion, as well as the metabolites formed, differ largely not only between species but also between the sexes (Palme *et al*. 1996, Touma *et al*. 2003, Rettenbacher *et al*. 2004; for a review, see Palme *et al*. 2005). In addition, to our knowledge, possible effects caused by the route of administration have not been reported so far in any species. Therefore, this study aimed to gain information about the metabolism and excretion of intravenous and *per os* administered $^3$H-corticosterone in urine and faeces of male and female rats and to prove the suitability of an established EIA (Touma *et al*. 2003, 2004) to monitor stress hormone metabolites in faecal samples of laboratory rats. In addition, this study should elucidate possible sex differences regarding faecal metabolites of corticosterone and thus add valuable information to already published data of sexual dimorphism regarding GC secretion and metabolism in rats (Glenister & Yates 1961, Gala & Westphal 1965, Krieger 1973, Carey *et al*. 1995, Atkinson & Waddell 1997, Cavigelli *et al*. 2005).

In the radiometabolism experiments performed, the vast majority of administered radioactivity was recovered, as the sampling regime was rigorous and losses of voided excreta were reduced to a minimum. Thus, the simple extraction procedure for faecal samples (suspending the faeces in 80% methanol) proved to be as efficient for rats as for other species (Palme & Möstl 1997, Möstl *et al*. 1999, Schatz & Palme 2001, Touma *et al*. 2003, 2004, Touma & Palme 2005).

In contrast to other species like mice (Touma *et al*. 2003), no sex differences concerning the route of excretion of GC metabolites were found. Most of the CMs were excreted via the faeces ($75 \pm 9\%$) in both sexes. In the faeces, the time course of excreted radioactivity did not differ between the sexes either. The excretion curve of urine displayed two peaks in both sexes. In males, the first peak showed the highest values, but in females it was the second peak. The reasons for this might be a more pronounced enterohepatic circulation in females. This is underlined by the fact that this sex difference in the time course almost disappeared when the animals received the $^3$H-corticosterone *per os*, as in this case all metabolites had to pass through the liver before urinary excretion.

Concerning the metabolism of corticosterone, we focused our analyses on the faecal metabolites, as rats usually void only very small amounts of urine, which limits the practicability of its collection. HPLC separations of faecal samples revealed a large number ($> 20$) of polar and apolar radioactive CMs in male and female rats. Most of them were more polar than corticosterone (especially in female rats), which is in accordance with other studies investigating steroid metabolism in male and female rats and mice (Lowy *et al*. 1969, Eriksson & Gustafsson 1970a,b, Eriksson 1971, Bamberg *et al*. 2001, Touma *et al*. 2003). While male rats showed a relatively uniform elution pattern of radioactive metabolites with only minor variations, HPLC patterns of females showed distinct individual variations. Reasons for these differences, however, are unclear and need further investigation. Varying levels of gonadal steroids throughout the oestrous cycle of female rats might be a likely explanation, but as we did not

have any information about the cyclic state of our female rats, it was not possible to investigate this in detail.

Using HPLC immunograms, we tested the suitability of the 5α-pregnane-3β,11β,21-triol-20-one EIA for the quantification of faecal CMs in the rat. The antibody used was able to detect at least four metabolites in both sexes, although HPLC patterns of radioactive CMs present in the faeces of females showed varying patterns. One of these metabolites in males co-eluted with the 5α-pregnane-3β,11β,21-triol-20-one itself, already identified and described as one of the major CMs in the faeces (Eriksson 1971). Beside this, the EIA recognized radioactive metabolites of both the intravenous and the *per os* administered $^{3}$H-corticosterone. This indicates the presence of $^{3}$H-CMs with a 5α-3β,11β-diol structure. Taking the variations of elution patterns of radioactive metabolites and the immunoreactivity in males into account, different metabolites of GCs are likely according to the method of administration.

As expected, the corticosterone EIA showed almost no immunoreactivity with the radioactive metabolites, especially at the elution position of corticosterone, indicating that unmetabolized corticosterone is virtually absent in the faeces. This confirms previous investigations in rats (Bamberg *et al*. 2001, Cavigelli *et al*. 2005) and other animals (Palme *et al*. 1996, Palme & Möstl 1997, Möstl *et al*. 1999, Touma *et al*. 2003, Rettenbacher *et al*. 2004). Thus, our results also confirm that EIAs using group-specific antibodies rather than highly specific ones are better suited for measuring the wide array of GC metabolites found in the faeces of mammals and birds (Möstl *et al*. 2005, Palme *et al*. 2005, Touma & Palme 2005).

To evaluate the physiological relevance of the EIA for both sexes, as recommended in recent reviews (Palme 2005, Touma & Palme 2005) and so far not proven for an immunoassay in rats (Bamberg *et al*. 2001, Pihl & Hau 2003, Eriksson *et al*. 2004, Knox *et al*. 2004, Royo *et al*. 2004, Cavigelli *et al*. 2005), different validation experiments were performed, using pharmacological stimulation and suppression of the adrenal cortex as well as two control experiments. Faecal CMs measured in undisturbed rats showed a pronounced DV. In males and females, the peak-to-trough ratio of faecal CMs was about 4:1 and 9:1, respectively. This is in accordance with previous studies, describing the faecal peak-to-trough ratio as 4:1 (Bamberg *et al*. 2001) or 7:1 (Cavigelli *et al*. 2005) in male rats and between 6:1 and 18:1 in female rats (Cavigelli *et al*. 2005). Additionally, studies investigating hormone levels in plasma samples have shown a peak-to-trough ratio of about 6:1, derived from averaging the plasma levels of male and female Sprague–Dawley rats (Krieger 1973). These results support the view that blood corticosterone levels are reflected accurately by the levels of faecal CMs. However, the clear and rather strong diurnal rhythm of the faecal CM patterns might be an explanation for the problems of a previous experiment trying to detect higher CM concentrations caused by ACTH stimulation as they may have been masked by the DV (Bamberg *et al*. 2001). Thus, without investigating the natural DV and without a rigorous sampling regime, it is not possible to distinguish between the DV peak of CMs and a peak caused by an ACTH injection or a stressor.

Although time courses of excreted CMs were quite similar for males and females, absolute concentrations differed significantly between the sexes. Males generally showed about six times higher concentrations than females. A reason for this is probably that the group-specific antibody used in our EIA detects CMs formed by males to a higher extent, as faecal metabolite patterns differ largely between the sexes. At least, results of the radiometabolism study exclude the possibility that the different amounts of measured CMs in the faeces are caused by different routes of excretion. It is worth noting that the EIA we used measured about 10–20 times (Bamberg *et al*. 2001, Cavigelli *et al*. 2005), in some cases even more than 1000 times (Pihl & Hau 2003), higher concentrations of faecal CMs than the immunoassays used in previous studies. Excreted CMs might vary significantly over the females' oestrous cycle (Cavigelli *et al*. 2005). Although we have no

specific data about the oestrous cycles of the experimental females, it is unlikely that they were all in the same cyclic phase, as they were kept separately. Interestingly, although patterns of $^{3}$H-CMs differed significantly among individual females, the variations of CM excretion patterns in the validation experiments were comparable to those observed in males. This further underlines the advantage of a group-specific EIA, which detects several metabolites over a more specific one.

The SI experiment served to evaluate possible effects of the injection procedure itself, as capture, fixation and injection are likely to be stressful events for a rat and could therefore influence the animal's GC concentrations. Surprisingly, these adverse effects could not be monitored in our study at the expected point of time, as males and females showed CM excretion patterns similar to those observed in the DV group. These results are in contrast to other observations in rats (Haemisch *et al*. 1999, Vahl *et al*. 2005) and other species, as for example in mice (Touma *et al*. 2004). An explanation for this might be the relatively short manipulation time (less than 4 min), which obviously does not cause such a marked elevation of corticosterone levels. Additionally, faecal samples integrate at least a period of 2 h, in which CM levels in blood caused by short and intermediate stress might decrease again to the original level. Nevertheless, as the ACTH stimulation test demonstrated, it is still very likely that long-term or strong stressors, such as physical restraint over a longer period of time (e.g. 30 min and more, Haemisch *et al*. 1999, Vahl *et al*. 2005), are reflected in faecal CMs. Another possibility for the general lower CM levels of the SI experiment (which was performed one week after the DV experiment) might be a better habituation of the rats to the housing.

Stimulation (ACTH) and suppression (dex) of adrenocortical activity were clearly reflected in both sexes by measuring faecal CMs, thus proving the physiological validity of the EIA applied. Compared with the DV experiment, significantly increased (or decreased) CM concentrations were found about 12–14 h after the injection, which were similar to the lag time of peak radioactivity in the radiometabolism study. This is in accordance with the data of other studies in rats (Bamberg *et al*. 2001, Royo *et al*. 2004) and in mice (Touma *et al*. 2003), describing the lag time to be 10–17 h. Additionally, females already showed significant differences between the DV and the other experiments (SI, dex and ACTH challenge) at sampling points $T = 4$, 8 and 10 h. These differences cannot be caused by the administrations, as in the radiometabolism study before 8 h, virtually no radioactivity was excreted in the faeces of any animal. It is more likely that these differences are the results of natural occurring fluctuations of the DV (Krieger 1973), which would be a probable explanation for the significantly lower values in the SI experiment of males at sampling points $T = 14$ and $T = 30$ h, too. Thus, our results underline once again the need for thorough validation of an EIA (including pharmacological stimulation and suppression of adrenocortical activity) and thereby support the recommendation of recently published guidelines for rigorous validation (Palme 2005, Touma & Palme 2005).

In conclusion, our study proved that measurement of faecal CMs with the 5$\alpha$-pregnane-3$\beta$,11$\beta$,21-triol-20-one EIA is a powerful tool for monitoring adrenocortical activity in laboratory rats. This technique enables frequent sampling of individual animals, even over long periods, and avoids blood sampling. Additionally, our study is the first to describe the influence of the animal's sex and the route of administration in rats. We found differences concerning the excretion patterns, the types of excreted faecal CMs and their reactivity with the 5$\alpha$-pregnane-3$\beta$,11$\beta$,21-triol-20-one EIA. Thus, we add important information to already published studies, describing sexual dimorphism regarding GC secretion and metabolism in rats. The pharmaceutical industry, medical and veterinary research still rely mainly on results obtained by experiments performed exclusively on male rats. Since our experiments confirm that at least CM is strongly influenced by the

animal's sex, our results highlight the need to use both sexes in order to gain valid results. Furthermore, our study demonstrated that stimulation and suppression of the adrenal cortex are accurately reflected by means of CM measurements in faecal samples of male and female rats. By this successful physiological validation, we proved for the first time the suitability of an immunoassay to non-invasively monitor adrenocortical activity in rats of both sexes. This opens up new perspectives for biomedical and pharmacological investigations as well as for animal welfare related issues.

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