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Effects of sex and time of day on metabolism and excretion of corticosterone in urine and feces of mice

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Abstract

Non-invasive techniques to monitor stress hormones in small animals like mice offer several advantages and are highly demanded in laboratory as well as in field research. Since knowledge about the species-specific metabolism and excretion of glucocorticoids is essential to develop such a technique, we conducted radiometabolism studies in mice (*Mus musculus* f. *domesticus*, strain C57BL/6J). Each mouse was injected intraperitoneally with 740 kBq of ^{3}H-labelled corticosterone and all voided urine and fecal samples were collected for five days. In a first experiment 16 animals (eight of each sex) received the injection at 9 a.m., while eight mice (four of each sex) were injected at 9 p.m. in a second experiment. In both experiments radioactive metabolites were recovered predominantly in the feces, although males excreted significantly higher proportions via the feces (about 73%) than females (about 53%). Peak radioactivity in the urine was detected within about 2 h after injection, while in the feces peak concentrations were observed later (depending on the time of injection: about 10 h postinjection in experiment 1 and about 4 h postinjection in experiment 2, thus proving an effect of the time of day). The number and relative abundance of fecal [^{3}H]corticosterone metabolites was determined by high performance liquid chromatography (HPLC). The HPLC separations revealed that corticosterone was extensively metabolized mainly to more polar substances. Regarding the types of metabolites formed, significant differences were found between males and females, but not between the experiments. Additionally, the immunoreactivity of these metabolites was assessed by screening the HPLC fractions with four enzyme immunoassays (EIA). However, only a newly established EIA for 5α-pregnane-3β,11β,21-triol-20-one (measuring corticosterone metabolites with a 5α-3β,11β-diol structure) detected several peaks of radioactive metabolites with high intensity in both sexes, while the other EIAs showed only minor immunoreactivity. Thus, our study for the first time provides substantial information about metabolism and excretion of corticosterone in urine and feces of mice and is the first demonstrating a significant impact of the animals' sex and the time of day. Based on these data it should be possible to monitor adrenocortical activity non-invasively in this species by measuring fecal corticosterone metabolites with the newly developed EIA. Since mice are extensively used in research world-wide, this could open new perspectives in various fields from ecology to behavioral endocrinology.

Keywords: Mice; Stress; Non-invasive monitoring; Corticosterone metabolism; Urinary and fecal excretion; Sex differences; Time-dependence; Enzyme immunoassay (EIA); Reverse-phase high performance liquid chromatography (RP-HPLC)

1. Introduction

Stress is well known to have a significant impact on a variety of physiological and psychological parameters. Its disruptive effect on immune functions, reproduction, and behavior of mammals has been broadly shown (Harbuz and Lightman, 1992; Riley, 1981; von Holst, 1998). Assessing an animals' stress physiology is therefore essential for the understanding and improvement of animal welfare, health, and reproduction. Stressful situations usually evoke an increased glucocorticoid production and output from the adrenal cortex, which is mediated by the release of adrenocorticotropic hormone (ACTH) from the pituitary gland (Axelrod and Reisine, 1984; Munck et al., 1984). Plasma glucocorticoid concentrations are therefore widely used to diagnose stress responses in various species (cf. Broom and Johnson,

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1993; Munck et al., 1984; Sachser et al., 1998; von Holst, 1998). However, difficulties in collecting blood samples and negative effects of the sampling procedure itself pose serious limitations to this approach, particularly for small animals like rodents. In mice and rats for example capturing, handling, and bleeding are stressful events, which markedly influence the animals' glucocorticoid concentrations (Armario et al., 1986; Gärtner et al., 1980; Haemisch et al., 1999; Hennessy and Levine, 1978; Quirce and Maickel, 1981; Riley, 1981; Tuli et al., 1995). Since mice are widely used in laboratory as well as in field research to investigate for example questions in behavioral endocrinology and ecology, it would be of great interest to have alternative methods to monitor stress hormones in these animals (especially in field endocrinology investigations).

The analysis of corticosterone metabolites (corticosterone is the major glucocorticoid in mice; Spackman and Riley, 1978) excreted into urine and feces of mice could offer such a non-invasive technique to assess adrenocortical function. Especially the collection of fecal samples is very easy and feedback-free, as there is no need to capture and handle the animal. This non-invasive technique to measure steroid metabolites in fecal samples has been established in an increasing number of species and is already used to investigate questions in the fields of 'stress and animal welfare,' 'reproductive physiology,' and 'behavioral ecology' (e.g., Brown et al., 1997; Heistermann et al., 1995; Möstl et al., 1999; Moss et al., 2001; Palme et al., 2000; Schatz and Palme, 2001; Schwarzenberger et al., 1996; Teskey-Gerstl et al., 2000; Wasser, 1996; Wasser et al., 2000). However, due to pronounced interspecies differences concerning the metabolism of glucocorticoids, the excretion of metabolites and therefore the suitability of different immunoassays for their measurement (cf. Macdonald et al., 1983; Palme et al., 1996; Schatz and Palme, 2001; Taylor, 1971; Vylitova et al., 1998; Wasser et al., 2000) it is not possible to draw analogous conclusions from other studies to the specific situation present in the mouse.

Since only limited information about the metabolism of glucocorticoids and their excretion into the urine and feces of mice is available (cf. Han et al., 1983; Kley et al., 1976; Marandici and Monder, 1985; Shire, 1980) we conducted radiometabolism studies with ^{3}H-labelled corticosterone using laboratory mice as experimental animals. Our objectives were: (i) to elucidate the time course of corticosterone metabolite excretion and the proportion of metabolites excreted into urine and feces and (ii) to characterize the excreted fecal corticosterone metabolites to some extend in order to establish enzyme immunoassays for their quantification. Furthermore, the impact of the animals' sex and the time of day on metabolism and excretion of [^{3}H]corticosterone metabolites was investigated, since information about gender-specific effects and influences of the animals' activity rhythm are largely lacking. Such effects could be of high relevance for studies investigating hormone metabolites in fecal samples not only in mice, but also in other species.

2. Methods

2.1. Animals and general housing conditions

To investigate the metabolism and excretion of corticosterone in mice we conducted so-called radiometabolism studies. In total 24 adult C57BL/6J mice (*Mus musculus* f. *domesticus*, 12 males and 12 females) were used (the breeding pairs of our local stock were obtained from Harlan–Winkelmann GmbH, Borchen, Germany). Up to the age of 9 weeks the animals were housed in same sex groups of four individuals in standard Macrolone cages type III ($38 \times 22 \times 15$ cm) with sawdust as bedding material (Allspan, Höveler GmbH KG, Langenfeld, Germany). The animal housing room was maintained under standard laboratory conditions (light–dark cycle: 12:12 h, lights on at 8 a.m.; temperature: 21 ± 1 °C; relative humidity: 50 ± 10%). Commercial mouse diet (Altromin No. 1324, Altromin GmbH, Lage, Germany; content: protein 19%, fat 4%, raw fiber 6%, ash 7%, vitamins and minerals) and bottled tap water were available ad libitum. At 10 weeks of age the groups were separated and the mice were housed individually for the following 3 weeks under the same conditions as described above to habituate to single housing like during the experiment.

2.2. Administration of radiolabelled corticosterone and sample collection

On day 0 of the experiment each mouse was injected intraperitoneally with 740 kBq ($= 20$ μCi) of [^{3}H]corticosterone (1,2,6,7-[^{3}H]corticosterone, specific activity: 2.74 TBq/mmol, Amersham Pharmacia Biotech Europe GmbH, Freiburg i. Br., Germany) diluted in 1 ml of sterile isotonic saline solution containing 5% (v/v) ethanol. The whole procedure of catching, fixation, injection, and returning the mouse into the cage lasted a maximum time of 3 min.

In order to investigate possible effects of the animals' sex and the time of day on the excretion pattern of steroid metabolites, the 24 experimental animals were divided into two groups (referred to as experiment 1 and experiment 2). A total of 16 mice (eight of each sex) received the injection at 9 a.m. (1 h after the beginning of the light phase; experiment 1) and eight animals (four of each sex) were injected at 9 p.m. (1 h after the beginning of the dark phase; experiment 2). For the following 5 days all fecal and urine samples were collected quantitatively and stored at −30 °C until analysis. During the

first 24 h after the injection sampling was done according to the following time schedule: 0, 2, 4, 6, 8, 10, 12, 14, 16, 20, 24 h postinjection; afterwards all excreta were sampled in intervals of 12 h until day 5 of the experiment.

To enable individual sampling and quantitative collection of all voided urine and feces without the need to handle the animal, the mice were housed singly in stainless steel wire cages type III ($38 \times 22 \times 15$ cm), which were placed in standard Macrolone cages type III. All excreta dropped through the bars of the wire cage and could be easily collected from the floor of the lower Macrolone cage, which was completely covered with filter paper (Art. No. 10334368, Schleicher and Schuell GmbH, Dassel, Germany) to immediately adsorb the urine. During each sampling the filter paper was renewed and the spots, where urine has dropped, were marked before freezing the whole paper in a plastic vial. Additionally, the amount of feces voided during the sampling interval was measured and possible crosscontaminations between urine and feces were estimated (i.e., when fecal boli were lying on urine spots). To habituate the mice to this sampling procedure and to the housing in wire cages the animals were already placed into this housing system 3 days prior to the injection and samples were collected in 12 h intervals during this time.

As mice are nocturnal animals and their steroid secretion pattern is known to show a circadian variation (Halberg et al., 1960; Spackman and Riley, 1978), all sample collections performed during the dark phase of the light–dark cycle were done under dimmed light conditions (about 3–4 lux) to avoid disturbing the animals' natural activity rhythm.

2.3. Extraction and determination of steroid metabolites

2.3.1. Urine samples

To determine the amount of excreted radioactivity in the urine samples, the spots where urine has dropped on the filter paper were cut out and dissected into pieces of about 2–3 cm^2. These strips of each sample were filled into scintillation vials (Art. No. 6008117, Packard Instruments, Meriden, CT, USA) and were mixed with 2 ml of 80% methanol. Then 12 ml of scintillation fluid (Quicksafe A, No. 100800, Zinsser Analytic, Maidenhead, UK) were added to each vial 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 program. Validation experiments proved a high reliability of this technique (measurements as described above revealed a recovery of $97.2 \pm 1.4\%$, when certain amounts of [^{3}H]corticosterone were dropped on filter papers).

2.3.2. Fecal samples

To measure the radioactive steroid metabolites excreted into the feces, each fecal sample was well homogenized (with mortar and pestle) and an aliquot of 0.05 g was extracted with 1 ml of 80% methanol for 30 min on a vortex (Vortex Evaporator, Labconco, Kansas City, USA). After centrifugation (15 min at 2500*g*) a 100 µl aliquot of the supernatant (in duplicates) was mixed with 6 ml scintillation fluid and was measured in the liquid scintillation counter as described above. The amount of radioactivity in the fecal sample was then calculated by adjustment to the total weight of the sample.

The total recovery of radiolabelled metabolites was calculated as the ratio of the combined sums of the recovered radioactivity in urine and feces divided by the total amount of administered radioactivity.

2.4. High performance liquid chromatography (HPLC)

To characterize the fecal [^{3}H]corticosterone metabolites, HPLC separations were performed with those fecal samples containing peak concentrations of radioactivity. These samples (about 0.8 ml of the methanol supernatant—see above—were evaporated completely and reconstituted in 100 µl of 20% methanol) were subjected to a reversed-phase HPLC using a Novapak C_{18} column (3.9×150 mm, Millipore Corporation, Milford, MA, USA) with a Mini-Guard column (C_{18}) and a solvent system of methanol:water at a flow rate of 1 ml per min (cf. Teskey-Gerstl et al., 2000). Steroids were separated in a linear gradient of 20–100% methanol. The percentage of methanol remained constant at 20% for 5 min and increased linearly up to 100% within the next 30 min. Three fractions per minute were collected. Additionally, elution positions of 17β-oestradiol-disulfate ($E_2\beta$-diSO_4), oestrone-glucuronide (E_1G), oestrone-sulfate (E_1S), cortisol, corticosterone (CC), and 5α-pregnane-3β,11β,21-triol-20-one were determined in the HPLC system to allow comparison with the elution profile of the [^{3}H]corticosterone metabolites (all steroids were obtained from Steraloids, Wilton, NH, USA). The presence of radioactivity in the different HPLC fractions was measured by counting an aliquot of each fraction (50 µl of an 1:5 dilution with assay buffer) in a liquid scintillation counter (TopCount, Packard Instruments, Meriden, CT, USA) after adding 200 µl of scintillation fluid (Microscint PS, Art. No. 6013631, Packard Instruments, Meriden, CT, USA) to each well.

2.5. Determination of immunoreactivity

The immunoreactivity of the HPLC fractions was determined in four different enzyme immunoassays (EIA): two previously described EIAs for corticosterone (for details see Palme and Möstl, 1997 and Goymann et al., 1999, respectively; the latter EIA utilizes a commercially available antibody—ICN Biomedicals, Costa Mesa, CA, USA—in connection with a biotinylated label), an 11-xoaetiocholanolone EIA measuring gluco-

corticoid metabolites with a 5β,3α-ol-11-one structure (for details see Möstl et al., 2002) and a newly established 5α-pregnane-3β,11β,21-triol-20-one EIA. This new EIA also used a double-antibody technique and was performed on anti-rabbit-IgG-coated microtiter plates. The antiserum was raised in rabbits against 5α-pregnane-3β,11β,21-triol-20-one (linked at position C_{20} to carboxymethyloxim) and coupled with bovine serum albumin. The same steroid was used as label, linked at C_{20} to biotinyl-3,6,9-trioxaundecanediamin (EZ-Link Biotin-LC-PEO-Amine, Pierce, Rockford, IL, USA), and standard (range: 0.8–200 pg/well). Details of the performed assay procedure are described by Palme and Möstl (1997). Briefly, standards (50 μl) or samples (50 μl) were incubated in duplicate with label (100 μl) and antibody (100 μl) overnight at 4 °C. Following incubation, the plates were washed four times with 0.02% Tween 20 washing solution (Art. No. 822184, Merck, Darmstadt, Germany) and blotted dry before 250 μl streptavidin horseradish peroxidase conjugate (= 4.2 mU, Art. No. 1089153, Boehringer, Mannheim, Germany) were added into each well. Plates were then left at 4 °C in the dark on stirring tables for 45 min. After another washing step 250 μl tetramethylbenzidine (= 69.4 nmol/well; Art. No. 87748, Fluka Chemika, Vienna, Austria) was added and the plates were incubated for an additional 45 min at 4 °C before the enzymatic reaction was stopped by the addition of 50 μl/well of 2 mol/L sulphuric acid. Absorbance was measured at a wavelength of 450 nm (reference filter: 620 nm) with an automatic plate reader (DigiScan, Asys Hitech GmbH, Eugendorf, Austria). Crossreactivities of the two corticosterone EIAs and the 11-oxoaetiocholanolone EIA are given by Palme and Möstl (1997), Goymann et al. (1999) , and Möstl et al. (2002), respectively. The antibody used in the newly established 5α-pregnane-3β,11β,21-triol-20-one EIA crossreacted with various steroids (Steraloids, Wilton, NH, USA) as follows: 5α-pregnane-3β,11β,21-triol-20-one (100%); 5α-pregnane-3β,11β,20β,21-tetrol (110%); 5α-pregnane-3β,11β,17α,21-tetrol-20-one (45%); 5α-androstane-3β,11β-diol-17-one (230%). Cortisol, corticosterone or metabolites, which differed at one of the three recognized positions (5α,3β- or 11β-ol) showed crossreactivities less than 1%. If more positions differed, the crossreactivity was even less (<0.1%). All tested gonadal steroids such as progesterone, androstenedione, and dehydroepiandrosterone or their reduced metabolites (e.g., 5α/β-pregnane-3β-ol-20-one; 5α/β-androstane-3β,17β-diol; 5α/β-androstane-3β-ol-17-one) crossreacted less than 1%. The inter- and intraassay coefficients of variation were 9.1 and 14.0%, respectively.

2.6. Statistical analyses

Since some of the obtained data were not normally distributed and the sample size was relatively small, the data were analyzed by non-parametrical statistics (Siegel and Castellan, 1988). All tests were calculated using the software package SPSS 10.0. Two independent samples were compared using the Mann–Whitney U test. Correlation between more than two independent samples were calculated with the Kendall W test (both two-tailed). ANOVA on ranks was used to evaluate differences between more than two dependent samples. Recoveries of radioactive metabolites in urine and feces are given as means with standard deviation. Differences were considered significant if their probability of occurring by chance was less than 5%.

3. Results

3.1. Time course and route of steroid excretion

3.1.1. Experiment 1

The mean total recovery of the administered radioactivity (urine and feces combined) was 94.8 ± 5.0% for all animals. Concerning the total amount of recovered radioactive metabolites no differences were detected between sexes (Mann–Whitney U test, $n_{males} = n_{females} = 8$, $U = 29$, $p < 0.793$). But with respect to the route of steroid excretion significant differences were found between males and females (see Fig. 1). Males excreted significantly more [^{3}H]corticosterone metabolites via the feces than females (males: 71.7 ± 4.0%, females: 56.2 ± 4.4%; Mann–Whitney U test, $n_{males} = n_{females} = 8$, $U = 0$, $p < 0.001$; see Fig. 1), while higher proportions of radioactivity were found in the urine of females (males: 28.3 ± 4.0%, females: 43.8 ± 4.4%; Mann–Whitney U test, $n_{males} = n_{females} = 8$, $U = 0$, $p < 0.001$; see Fig. 1). In

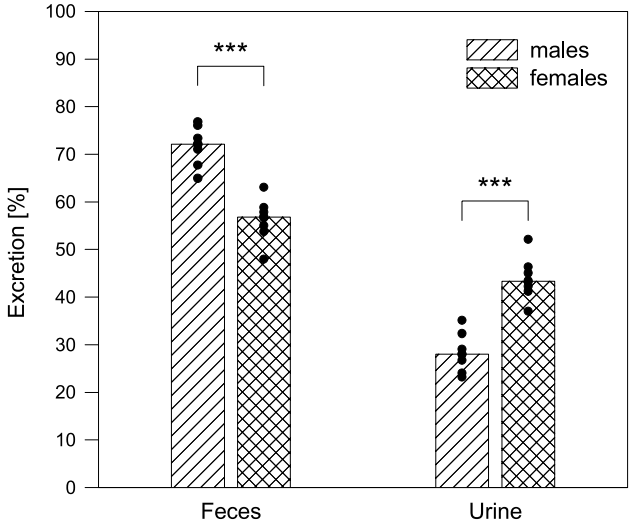

Fig. 1. Amount of [^{3}H]corticosterone metabolites (%) excreted via urine and feces in male and female mice used in experiment 1 (injection at 9 a.m.). Medians and individual values are given. Statistics: Mann–Whitney U test (two-tailed), $n_{males} = n_{females} = 8$, $U = 0$, ***$p < 0.001$.

contrast, the time course of excretion of radioactivity via urine and feces was similar for both sexes (excretion profiles in urine: Kendall W test, $n_{\text{males}} = n_{\text{females}} = 8$, $W = 0.72$, df $= 18$, $p < 0.004$; excretion profiles in feces: Kendall W test, $n_{\text{males}} = n_{\text{females}} = 8$, $W = 0.94$, df $= 18$, $p < 0.001$) and showed a significant variation, i.e., distinct peaks of excretion were recorded (excretion profiles in urine: ANOVA on ranks, $n_{\text{males+females}} = 16$, $\chi^2_r = 36.5$, df $= 18$, $p < 0.004$, see Fig. 2; excretion profiles in feces: ANOVA on ranks, $n_{\text{males+females}} = 16$, $\chi^2_r = 169.2$, df $= 18$, $p < 0.001$, see Fig. 3). In males and females peak radioactivity was recovered in the first urinary samples collected after the administration of [^{3}H]corticosterone (median: 2 h, range: 2–6 h) and subsequently decreased rapidly (see Fig. 2). In the feces maximal radioactivity was reached later (see Fig. 3). In both sexes peak concentrations of [^{3}H]corticosterone metabolites were observed about 10 h after the injection (median: 10 h, range: 8–12 h) and declined quickly afterwards (see Fig. 3). Thus, the vast majority of radioactive metabolites ($98.7 \pm 1.1\%$) was excreted via the urine and the feces within the first 24 h after steroid administration. Background levels, however, were reached within the next few days.

3.1.2. Experiment 2

To investigate possible effects of the time of day on the excretion pattern of steroid metabolites, a second group of eight animals received the injection at the beginning of the dark phase with the same amount of [^{3}H]corticosterone as the mice in experiment 1.

Concerning the total amount of recovered [^{3}H]corticosterone metabolites and the proportions excreted via the urine and the feces no statistical differences were found between the animals of experiment 1 and the mice used in experiment 2 (total recovery: $96.4 \pm 2.0\%$; excretion into the feces: males $74.7 \pm 7.3\%$, females $49.8 \pm 4.5\%$). However, a significant influence of the time of injection on the excretion pattern and thereby on the lag time between administration of radiolabelled corticosterone and peak concentrations in the feces was found (see Fig. 4). Compared to experiment 1 the maximal concentrations of radioactivity in the feces were detected significantly earlier in experiment 2 (Mann–Whitney U test, $n_{\text{Exp1}} = 16$, $n_{\text{Exp2}} = 8$, $U = 0$, $p < 0.001$; see Fig. 4). Here, peak excretion already occurred 4 h after administration (median: 4 h, range: 4–6 h). This difference was not found for the excretion

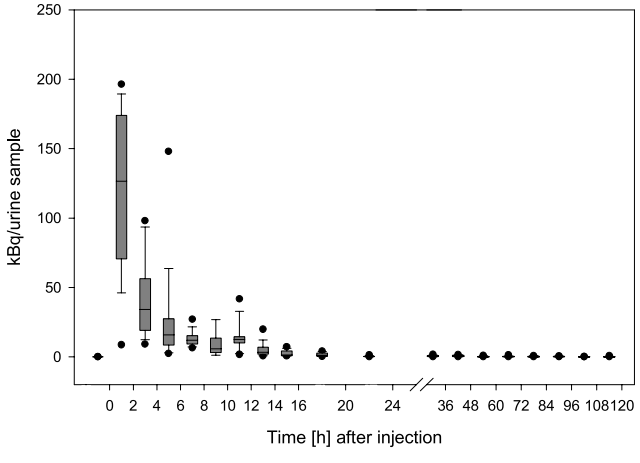

Fig. 2. Time course of excretion of [^{3}H]corticosterone metabolites (kBq) in the urine of males and females used in experiment 1 (injection at 9 a.m.). Data are given as boxplot diagrams showing medians (lines in the boxes), 25 and 75% quartiles (boxes), 10 and 90% ranges (whiskers) and outliners (dots).

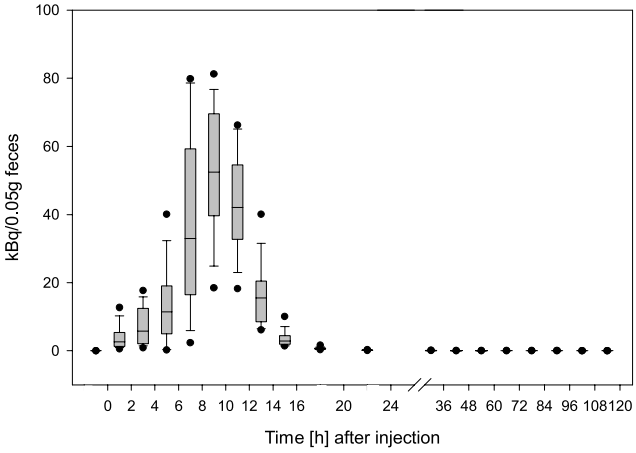

Fig. 3. Time course of excretion of [^{3}H]corticosterone metabolites (kBq) in the feces of males and females used in experiment 1 (injection at 9 a.m.). Data are given as boxplot diagrams showing medians (lines in the boxes), 25 and 75% quartiles (boxes), 10 and 90% ranges (whiskers) and outliners (dots).

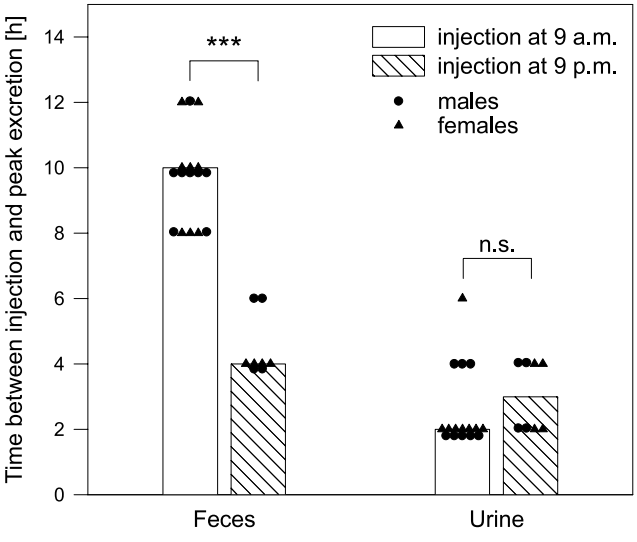

Fig. 4. Delay time (h) between administration and peak excretion of [^{3}H]corticosterone metabolites in urine and feces of males and females used in experiment 1 (injection at 9 a.m.) and experiment 2 (injection at 9 p.m.). Medians and individual values are given. Statistics: Mann–Whitney U test (two-tailed), $n_{\text{Exp1}} = 16$, $n_{\text{Exp2}} = 8$; feces: $U = 0$, $^{***}p < 0.001$; urine: $U = 50$, n.s. $p < 0.299$.

pattern of [^{3}H]corticosterone metabolites in the urine (maximal concentrations were reached within 2–4 h in both groups; Mann–Whitney U test, $n_{Exp1} = 16$, $n_{Exp2} = 8$, $U = 50$, $p < 0.299$; see Fig. 4).

3.2. Characterization of steroid metabolites by HPLC and antibody screening

To characterize some of the excreted [^{3}H]corticosterone metabolites in the feces of mice, fecal samples containing peak radioactivity were subjected to an HPLC and the fractions were analyzed with different enzyme immunoassays (cf. Section 2). The analyses were performed with samples of all 24 animals to elucidate—besides individual variation—impacts of sex and time of injection on the metabolism of corticosterone in mice. One representative male and female were chosen from each experimental group to visualize the obtained results, which are plotted in so-called HPLC-immunograms (see Figs. 5 and 6).

3.2.1. Experiment 1

The HPLC separations revealed several peaks of radioactivity (>20) indicating a large number of different

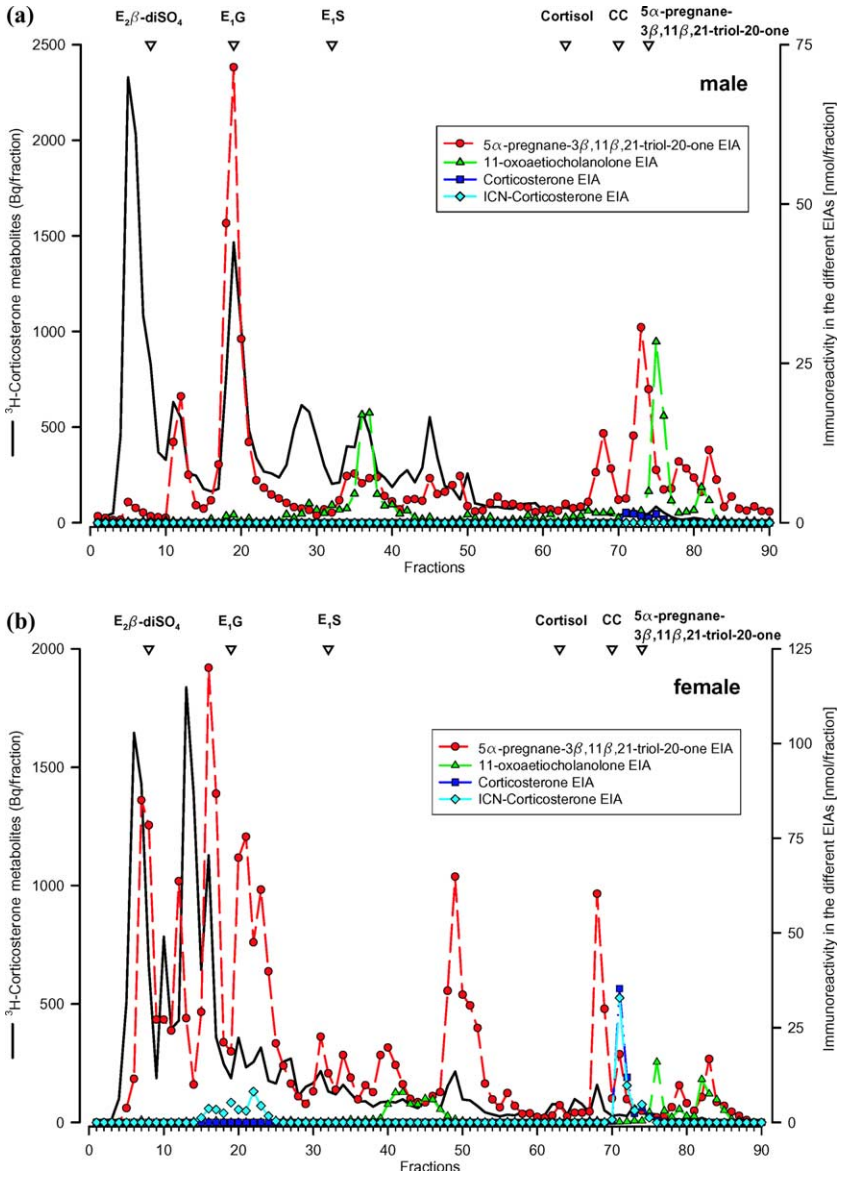

Fig. 5. Reverse-phase high performance liquid chromatographic (RP-HPLC) separation of [^{3}H]corticosterone metabolites in the feces of a male (a) and a female (b) mouse used in experiment 1 (injection at 9 a.m.). Radioactivity of each fraction was determined by liquid scintillation counting and immunoreactivity was measured in four different enzyme immunoassays (EIA): two corticosterone EIAs, an 11-oxoaetiocholanolone EIA and a 5α-pregnane-3β,11β,21-triol-20-one EIA. For details of the performed EIAs see Section 2. Open triangles mark the approximate elution positions of respective standards (17βoestradiol-disulfate = $E_2\beta$-diSO$_4$, oestrone-glucuronide = E_1G, oestrone-sulfate = E_1S, cortisol, corticosterone = CC, 5α-pregnane-3β,11β,21-triol-20-one).

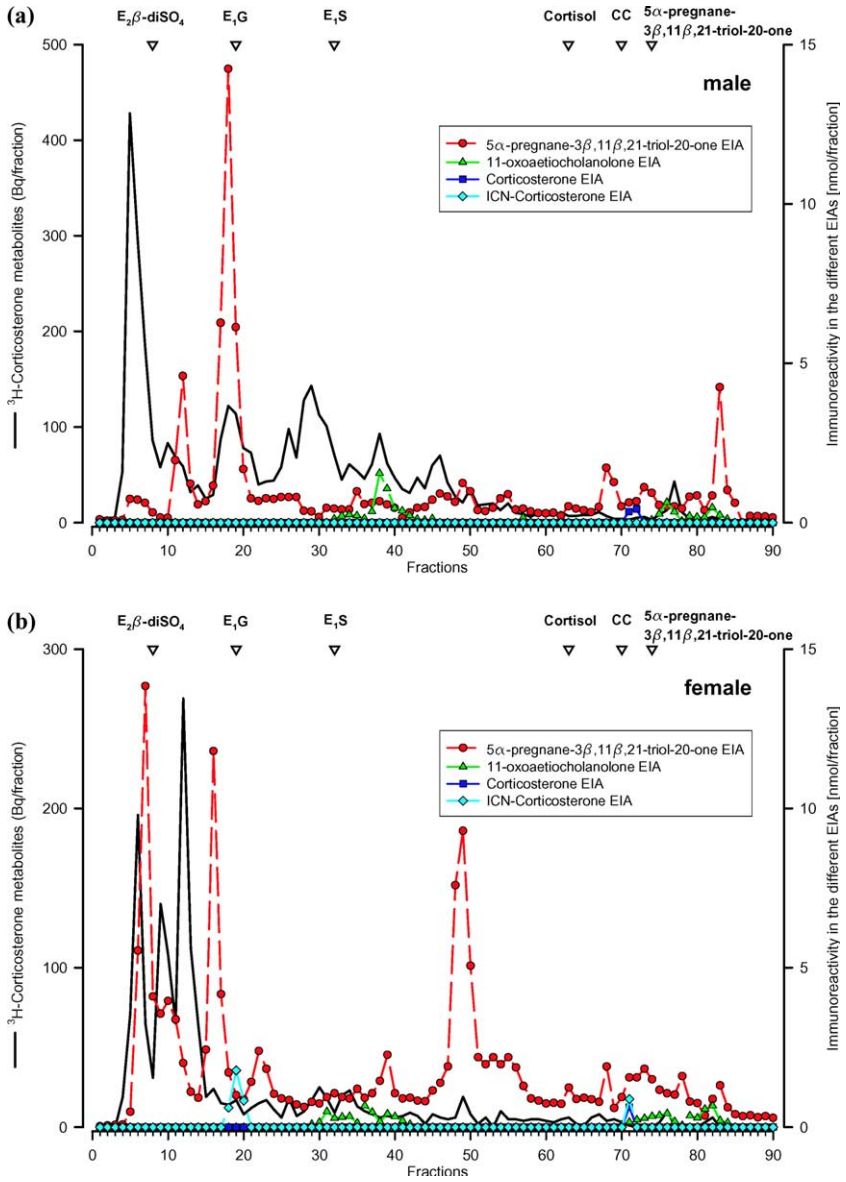

Fig. 6. Reverse-phase high performance liquid chromatographic (RP-HPLC) separation of [^{3}H]corticosterone metabolites in the feces of a male (a) and a female (b) mouse used in experiment 2 (injection at 9 p.m.). For details see legend of Fig. 5.

metabolites being formed by males and females (see Figs. 5a and b). Almost all metabolites were found to be more polar than the injected corticosterone (more than 80% of the radioactivity was recovered within the first 50 fractions) while non-metabolized corticosterone itself was present only in very small amounts if at all (cf. Figs. 5a and b and Table 1). Although some individual differences concerning the relative proportions of excreted metabolites were found within animals of the same sex, the patterns of radioactive metabolites were consistent within males and females, respectively. All males showed one very polar dominant peak of corticosterone metabolites with its maximum in fraction five followed by another large peak eluting around the position of E_1G (cf. Fig. 5a and Table 1). Additionally, a number of less prominent peaks was found within the first 50 fractions (e.g., in fractions 10–13, 26–30, 35–37, and 44–46), while only very small peaks were seen in the later fractions (cf. Fig. 5a and Table 1). In all females, however, there was also one very polar peak eluting around fraction 6 or 7, but in contrast to the males this peak was immediately followed by two to three other prominent peaks eluting between the positions of $E_2\beta$-diSO$_4$ and E_1G, respectively (cf. Fig. 5b and Table 1). Thus, in females more than 65% of the total amount of radioactivity in each of the HPLC separations was recovered within the first 25 fractions, which was about 10% more than in males (see Table 1). Furthermore, in all females one distinct peak was found around fraction 49 and some noticeable peaks of more apolar metabolites were observed eluting

Table 1
Radioactivity (%) recorded in different fractions of the RP-HPLC separations

Fractions	Experiment 1					Experiment 2				
	Males	Females	Statistics			Males	Females	Statistics		
	Mean ± SD	Mean ± SD	Difference between the sexes			Mean ± SD	Mean ± SD	Difference between the sexes		
01–05	10.6 ± 1.7	4.4 ± 2.1	**	$p < 0.003$	$U = 1$	10.8 ± 4.0	4.8 ± 1.3	*	$p < 0.021$	$U = 0$
06–10	17.3 ± 2.7	20.5 ± 5.0	n.s.	$p < 0.225$	$U = 15$	21.8 ± 4.1	24.8 ± 6.7	n.s.	$p < 0.149$	$U = 3$
11–15	8.9 ± 4.6	21.1 ± 9.8	*	$p < 0.025$	$U = 7$	7.2 ± 3.6	22.3 ± 9.5	*	$p < 0.021$	$U = 0$
16–20	12.5 ± 3.7	13.6 ± 4.6	n.s.	$p < 0.701$	$U = 21,5$	10.2 ± 2.3	12.6 ± 3.3	n.s.	$p < 0.386$	$U = 5$
21–25	8.2 ± 4.1	8.8 ± 4.6	n.s.	$p < 0.749$	$U = 22$	7.1 ± 1.8	4.8 ± 1.1	n.s.	$p < 0.083$	$U = 2$
26–30	9.9 ± 3.4	6.2 ± 1.8	*	$p < 0.035$	$U = 8$	9.4 ± 2.9	5.7 ± 1.8	*	$p < 0.043$	$U = 1$
31–35	6.4 ± 0.3	5.2 ± 1.3	n.s.	$p < 0.084$	$U = 11$	6.8 ± 1.3	7.5 ± 5.0	n.s.	$p < 0.999$	$U = 8$
36–40	7.5 ± 3.4	3.2 ± 0.7	**	$p < 0.006$	$U = 3$	7.1 ± 1.5	2.6 ± 1.4	*	$p < 0.021$	$U = 0$
41–45	6.1 ± 0.8	2.5 ± 0.4	**	$p < 0.002$	$U = 0$	5.7 ± 1.0	1.9 ± 0.5	*	$p < 0.021$	$U = 0$
46–50	4.5 ± 1.0	3.9 ± 1.1	n.s.	$p < 0.277$	$U = 16$	4.8 ± 0.6	3.6 ± 1.4	n.s.	$p < 0.386$	$U = 5$
51–55	1.9 ± 0.4	1.6 ± 0.3	*	$p < 0.045$	$U = 9$	2.3 ± 0.8	1.8 ± 1.0	n.s.	$p < 0.386$	$U = 5$
56–60	1.5 ± 0.2	1.3 ± 0.4	n.s.	$p < 0.159$	$U = 13,5$	1.2 ± 0.5	1.6 ± 0.7	n.s.	$p < 0.386$	$U = 5$
61–65	1.2 ± 0.4	2.2 ± 0.8	*	$p < 0.018$	$U = 6$	0.9 ± 0.2	1.5 ± 1.0	n.s.	$p < 0.248$	$U = 4$
66–70	1.0 ± 0.2	3.1 ± 1.9	**	$p < 0.009$	$U = 4$	0.8 ± 0.3	2.8 ± 1.2	*	$p < 0.043$	$U = 1$
71–75	1.0 ± 0.3	1.4 ± 0.8	n.s.	$p < 0.225$	$U = 15$	1.2 ± 0.3	0.8 ± 0.1	*	$p < 0.043$	$U = 1$
76–80	0.8 ± 0.2	0.6 ± 0.2	n.s.	$p < 0.055$	$U = 9,5$	1.6 ± 1.0	0.6 ± 0.4	n.s.	$p < 0.149$	$U = 3$
81–85	0.5 ± 0.4	0.4 ± 0.3	n.s.	$p < 0.701$	$U = 21,5$	1.0 ± 1.1	0.2 ± 0.2	n.s.	$p < 0.248$	$U = 4$
86–90	0.2 ± 0.1	0.1 ± 0.1	*	$p < 0.045$	$U = 9$	0.1 ± 0.1	0.0 ± 0.1	n.s.	$p < 0.083$	$U = 2$

Percentage of radioactivity recorded in different fractions of the RP-HPLC separations of fecal samples from males and females used in experiment 1 (injection at 9 a.m.) and experiment 2 (injection at 9 p.m.). Data are given as means with standard deviation (SD). Statistical differences between the sexes were calculated using the Mann–Whitney U test (two-tailed, Exp. 1: $n_{\text{males}} = n_{\text{females}} = 8$; Exp. 2: $n = n_{\text{males}} = n_{\text{females}} = 4$).

later than cortisol (cf. Fig. 5b and Table 1). These differences in the patterns of excreted corticosterone metabolites between males and females were proven significant by comparing the percentage of radioactivity recorded in the different fractions of the HPLC separations (see Table 1).

Concerning the immunoreactivity of these corticosterone metabolites the newly established EIA for 5α-pregnane-3β,11β,21-triol-20-one showed the best results, as several peaks of radioactive metabolites were clearly detected by this assay in both sexes (males: e.g., fractions 11–12, 18–20, 34–35, 37–38, 45–46, 47–48; females: e.g., fractions 7, 15–17, 20–23, 49; cf. Figs. 5a and b). Additionally, about four peaks of metabolites were detected that were less polar than cortisol (cf. Figs. 5a and b). However, the metabolites formed by females cross-reacted even more with the antibody used in this assay (e.g., metabolites peaking in fractions 7 or 49; cf. Fig. 5b). 5α-pregnane-3β,11β,21-triol-20-one, which was separately chromatographed, eluted around fraction 74. At this position only small amounts of radioactivity and immunoreactivity were detected in all HPLC immunograms.

On the other hand, the other three tested enzyme immunoassays showed only little crossreactivities with the corticosterone metabolites found in the feces of males and females. The 11-oxoaetiocholanolone EIA for example registered about four peaks, while only one peak in fractions 71–72 was detected with the corticosterone EIA described by Palme and Möstl (1997) (cf. Figs. 5a and b). The EIA using the ICN corticosterone antibody reacted even less: some additional peaks of corticosterone metabolites were detected with little intensity between fractions 15 and 23 in females (cf. Fig. 5b), while no crossreactivity (detection limit: 0.6 nmol/fraction) could be recognized in the HPLC separations of males (cf. Fig. 5a).

3.2.2. Experiment 2

Although the total amounts of radioactivity were generally lower in the performed HPLC separations from samples of experiment 2 the same patterns of corticosterone metabolites were observed for males and females, respectively, as described for experiment 1 (cf. Figs. 6a and b and Table 1). Comparing the percentages of recovered radioactive metabolites in the different fractions of the HPLC separations revealed for both sexes no statistical differences between the metabolite patterns found in the two experiments (for males and females: Mann–Whitney U test, $n_{Exp1} = 8$, $n_{Exp2} = 4$, $U = 4$–13, $0.059 < p < 0.850$) proving no effect of the time of injection on the metabolism of [^{3}H]corticosterone. As in experiment 1, however, the same significant differences were found between males and females in experiment 2 concerning the pattern of excreted corticosterone metabolites (see Table 1 and Figs. 6a and b).

The immunoreactivity of the radioactive metabolites as revealed in the tested EIAs were also comparable to the results mentioned for experiment 1 (cf. Figs. 6a and b).

4. Discussion

As known from other studies investigating fecal glucocorticoids, the route and delay of excretion as well as the metabolites formed differ largely between species (cf. Bahr et al., 2000; Möstl et al., 1999; Palme et al., 1996; Schatz and Palme, 2001). Therefore, the aim of our study was to gain information about the metabolism and excretion of corticosterone in urine and feces of mice, since this knowledge is essential to develop, establish, and successfully apply techniques to monitor stress hormones non-invasively in this species. Additionally, we investigated possible impacts of the animals' sex and the time of day on metabolism and excretion of corticosterone metabolites, since information about gender-specific effects and influences of the animals' activity rhythm could be of high relevance for studies investigating fecal hormone metabolites in mice as well as in other species.

In the performed radiometabolism studies almost all of the administered radioactivity was recovered (total recovery >95%), probably due to the rigorous sampling regime and the techniques used to collect even very small amounts of excreta. Thus, the developed sampling procedure, using wire mesh cages with filter paper underneath, is well suited for collecting urine and feces quantitatively in mice. It also indicates that the relatively simple extraction procedure for fecal samples (suspending the feces in 80% methanol) is as efficient for mice as proved for other species (e.g., Bahr et al., 2000; Bamberg et al., 2001; Möstl et al., 1999; Palme and Möstl, 1997; Schatz and Palme, 2001).

In addition, a major advantage of our study was the relatively large number of animals used. As already assumed for other species like ponies or cats (Palme et al., 1996; Schatz and Palme, 2001), here we prove for the first time *significant* differences between males and females concerning the route of excretion of glucocorticoid metabolites. Although most of the corticosterone metabolites were excreted via the feces in both sexes, the amount of radioactivity recovered in the feces of males (about 73%, mean of Exps. 1 and 2) was clearly higher than in females (about 53%, mean of Exps. 1 and 2). The reasons for this difference, however, are unclear, but this fact should be kept in mind for other studies investigating stress hormone metabolites in fecal samples, as similar effects are likely to be found in other species, too.

On the other hand, the time courses of [^{3}H]corticosterone metabolite excretion in urine and feces did not differ between the sexes. In agreement with former

studies investigating mice, rats or other species (Bahr et al., 2000; Bamberg et al., 2001; Billitti et al., 1998; Eriksson and Gustafsson, 1970; Han et al., 1983; Palme et al., 1996; Schatz and Palme, 2001; Teskey-Gerstl et al., 2000) elimination of radioactivity via the urine was very quick, as maxima were already found within a few hours after administration, i.e., in the first urinary samples voided. In the feces, however, peak concentrations were observed after a lag time of about 10 h in experiment 1 (see Fig. 3). This species-specific delay time between injection (and thus peak concentrations in the plasma) and the appearance of the respective signal in the feces was found to be well related to the animals' intestinal transit time from duodenum to rectum (Palme et al., 1996), as the metabolites are quickly excreted via the bile into the gut (Cronholm et al., 1972; Eriksson and Gustafsson, 1970; Eriksson, 1971; Han et al., 1983; Shire, 1980; Taylor, 1971). Our data also support this finding, as the gut passage time is described to be approximately 9–10 h in the mouse (Koopman and Kennis, 1977; Warner, 1981).

However, in our experiments this retention time was significantly influenced by the time of day of administration, as in experiment 2 maximal concentrations of [^{3}H]corticosterone metabolites were already recorded 4 h after the injection (see Fig. 4). Thus, our data for the first time clearly show, that the time of day, i.e., the animals activity rhythm, plays an important role in affecting the excretion of metabolites via the feces. This fact should be seriously taken into account in studies investigating fecal glucocorticoids, as the gut passage time is obviously influenced by the animals activity pattern. Although mice show phases of activity scattered all around the day, their main activity takes place during nighttime (Tankersley et al., 2002; Valentinuzzi et al., 1997). Therefore, more feces were excreted during the first hours after administration of [^{3}H]corticosterone in experiment 2 (injection at the beginning of the active phase) compared to experiment 1 (injection at the beginning of the passive phase) (data not shown). This could explain the earlier appearance of radioactive metabolites in those samples collected in experiment 2. To our knowledge, the relationship between the animals' activity pattern and its gut passage time has not been considered so far, even not in studies investigating intestinal functions. Therefore, most probably similar findings will be revealed for other species too, when this point is addressed.

Concerning the metabolism of corticosterone, we focussed our analyses on the metabolites excreted via the feces. Since mouse-seized rodents usually void only very small amounts of urine which can hardly be collected, sampling of feces is much easier and more reliable, especially under field conditions. As expected, the HPLC separations of fecal samples containing peak radioactivity revealed a large number (>20) of different metabolites in the feces of male and female mice (see Figs. 5 and 6). Most metabolites were found to be more polar than corticosterone, (see Figs. 5 and 6), which is in accordance with other studies investigating steroid metabolism in mice and rats (Bamberg et al., 2001; Cronholm et al., 1972; Eriksson and Gustafsson, 1970; Eriksson, 1971; Han et al., 1983; Marandici and Monder, 1985; Shire, 1980; Vylitova et al., 1998). Additionally, as found in almost all other species investigated (e.g., Bamberg et al., 2001; Macdonald et al., 1983; Möstl et al., 1999; Palme and Möstl, 1997; Schatz and Palme, 2001; Taylor, 1971; Teskey-Gerstl et al., 2000), the main glucocorticoid present in the blood, which is corticosterone in mice (Spackman and Riley, 1978), was virtually absent in the feces, indicating an intensive steroid metabolism in the liver and in the gut (by the microbial flora).

However, here we present for the first time *significant* differences between the sexes concerning the types of excreted fecal corticosterone metabolites (see Table 1). Females for example formed more very polar, probably (di)-conjugated metabolites and showed higher immunoreactivities in the HPLC fractions, as revealed by the newly established 5α-pregnane-3β,11β,21-triol-20-one EIA (cf. Figs. 5b and 6b). This is even more remarkable, as males in general excreted higher amounts of corticosterone metabolites via the feces than females (see Fig. 1). Furthermore, our experiments proved that the metabolism of corticosterone was not affected by the time of injection, i.e., by the animals' activity rhythm, as there were no significant differences between the metabolite patterns found in the two experiments (cf. Figs. 5, 6 and Table 1). Thus, the shortened time between injection and excretion via the feces obviously had no effect on the types of corticosterone metabolites formed in mice. However, the HPLC separations of the two experiments differed largely concerning the total amount of radioactivity detected (see Figs. 5 and 6). This was probably due to the higher dilution of [^{3}H]corticosterone metabolites, as the total weight of the samples containing peak radioactivity was much higher in experiment 2 than in experiment 1 (see above).

Additionally, the HPLC-immunograms tested the suitability of different EIAs for the quantification of fecal corticosterone metabolites in the mouse. Obviously, the newly developed 5α-pregnane-3β,11β,21-triol-20-one EIA was best suited to measure fecal corticosterone metabolites in males and females. Using this assay several peaks of radioactive metabolites were detected with high intensity in the HPLC fractions of both sexes and for both experiments (cf. Figs. 5 and 6). On the other hand, only a few metabolites were detected by the 11-oxoaetiocholanolone EIA and almost no immunoreactivity was observed using the two corticosterone EIAs (cf. Figs. 5 and 6). Our data thereby confirm previous investigations in other species (e.g., Bamberg et al.,

2001; Goymann et al., 1999; Möstl et al., 1999; Palme et al., 1996; Palme and Möstl, 1997; Teskey-Gerstl et al., 2000) indicating that EIAs using group-specific antibodies (like our newly established one, measuring metabolites with a 5α-3β,11β-diol structure) are better suited for measuring the wide range of glucocorticoid metabolites found in the feces of mammals than highly specific ones.

However, some recent studies (e.g., Harper and Austad, 2000, 2001) already utilized the ICN-corticosterone antibody in a radioimmunoassay for measuring fecal glucocorticoid metabolites in different species of mice (*M. musculus*, *Peromyscus maniculatus*, *Clethrionomys gapperi*). Their reported values are very low, which could be explained by our results showing that authentic corticosterone is virtually absent in fecal samples of mice. Obviously, their test system measured some metabolites, structurally related to corticosterone, although the crossreactivities of the ICN antibody with reduced compounds are still not completely evaluated (cf. Goymann et al., 1999).

In conclusion, our study for the first time provides substantial information about metabolism and excretion of corticosterone in urine and feces of mice and is the first demonstrating a significant impact of the animals' sex and the time of day. Based on these data it should be possible to monitor adrenocortical activity non-invasively in this species by measuring fecal corticosterone metabolites with the newly developed EIA.

Such a technique could be a very valuable tool, as it enables frequent sampling of individual animals—even over long periods—and avoids blood sampling related stress effects. Thereby, it will be possible to monitor for example endocrine changes in rodent populations during the course of different seasons or even over several years. Furthermore, utilizing this technique could markedly reduce the total number of animals used for research, as individuals could be used as their own controls and mice do not need to be sacrificed at different times of the experiment to obtain blood samples for hormone measurements. Additionally, field researchers could then investigate various questions on the relation between adrenocortical function and, e.g., specific behavioral patterns, reproduction or population density by means of analyzing fecal samples of feral mice.

Thus, a non-invasive technique to monitor stress hormones in fecal samples of mice could open new perspectives in various fields from ecology to behavioral endocrinology.

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