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Analyzing corticosterone metabolites in fecal samples of mice: a noninvasive technique to monitor stress hormones

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Abstract

In small animals like mice, the monitoring of endocrine functions over time is constrained seriously by the adverse effects of blood sampling. Therefore, noninvasive techniques to monitor, for example, stress hormones in these animals are highly demanded in laboratory as well as in field research. The aim of our study was to evaluate the biological relevance of a recently developed technique to monitor stress hormone metabolites in fecal samples of laboratory mice. In total, six experiments were performed using six male and six female mice each. Two adrenocorticotropic hormone (ACTH) challenge tests, two dexamethasone (Dex) suppression tests and two control experiments [investigating effects of the injection procedure itself and the diurnal variation (DV) of glucocorticoids (GCs), respectively] were conducted. The experiments clearly demonstrated that pharmacological stimulation and suppression of adrenocortical activity was reflected accurately by means of corticosterone metabolite (CM) measurements in the feces of males and females. Furthermore, the technique proved sensitive enough to detect dosage-dependent effects of the ACTH/Dex treatment and facilitated to reveal profound effects of the injection procedure itself. Even the naturally occurring DV of GCs could be monitored reliably. Thus, our results confirm that measurement of fecal CM with the recently established 5α-pregnane-3β,11β,21-triol-20-one enzyme immunoassay is a very powerful tool to monitor adrenocortical activity in laboratory mice. Since mice represent the vast majority of all rodents used for research worldwide and the number of transgenic and knockout mice utilized as animal models is still increasing, this noninvasive technique can open new perspectives in biomedical and behavioral science.

Keywords: Mouse; Corticosterone; Noninvasive monitoring; Feces; Steroid metabolites; Adrenocortical activity; ACTH challenge; Dexamethasone suppression; Circadian rhythm; Behavioral science

Introduction

Rodents represent the vast majority of all vertebrates used for research worldwide (accounting for about 80% within the EU; survey of 1996). Especially mice are extensively utilized in biomedical and behavioral sciences, as they are relatively easy to handle and breed and can be easily kept in large numbers (more than 25 million mice are used in laboratories each year; Malakoff, 2001). Additionally, recently developed techniques allow genetic manipulations of these animals bringing about transgenic and knockout mice used as animal models in a diverse array of research fields (Bürki and Ledermann, 1995; Hanahan, 1989; Nelson, 1997; Picciotto and Wickman, 1998). These genetically modified mice are extremely valuable, for example, to investigate molecular mechanisms underlying human disorders (Philipps et al., 2002; Price et al., 1998).

However, due to their small body size, physiological data, like endocrine or immunological parameters, which are mainly measured form blood samples, are difficult to obtain. Blood collection in mice is usually performed by incision of the tail vein, puncturing the orbital sinus or by bleeding after decapitation (Morton et al., 1993). Besides animal welfare issues, this approach raises serious problems in obtaining frequent samples from individuals, for example, to monitor endocrine changes during the course of a disease or a social encounter. A further limitation of invasive sampling techniques is that circulating hormone levels are affected rapidly in response to the stress of

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handling, physical restraint and the blood sampling procedure itself (Armario et al., 1986; Gärtner et al., 1980; Haemisch et al., 1999; Hennessy and Levine, 1978), which can seriously interfere with the experimental results (Halberg et al., 1960; Quirce and Maickel, 1981; Riley, 1981). An additional drawback of measurements in blood is that blood samples represent concentrations at only a single point of time. Since steroid hormones and especially glucocorticoids (GCs) may exhibit regular as well as episodic changes over time (i.e., circadian variations and pulsatile secretion patterns; Axelrod and Reisine, 1984; Halberg et al., 1960), hormone levels representing a very narrow time frame might be unrepresentative and possibly misleading.

Alternative techniques of measuring steroids or their metabolites in saliva, urine or feces might overcome these problems (Whitten et al., 1998). However, although sampling of saliva or urine is less invasive than blood collection in mice, their practical application remains limited due to the very small sample volume obtained and the need to handle the animals during sampling. Analyzing steroid metabolites in fecal samples, however, could offer a completely noninvasive alternative to monitor, for example, adrenocortical activity in mice.

Using fecal samples offers several advantages (Möstl and Palme, 2002; Schwarzenberger et al., 1996; Whitten et al., 1998). Feces are very easy to collect and sampling can be done without disturbing the animal, as there is no need to capture and handle it. Therefore, repeated sampling of the same individual is possible without affecting the animal's endocrine status. This allows monitoring short-term hormonal changes in reaction to specific situations or treatments as well as assessing long-term endocrine profiles during longitudinal studies, investigating, for example, seasonal variations or patterns during the course of a disease. Additionally, in fecal samples, circulating hormone levels are integrated over a certain period of time and thus do not represent only a single sample point like blood samples. Fecal samples are therefore less affected by episodic fluctuations of hormone secretion and might represent the animal's hormonal status more accurately.

In the last years, this noninvasive technique of measuring fecal steroid metabolites has been established in an increasing number of species (domestic, farm, zoo and feral animals) and is now widely used to investigate hormone–behavior relationships as well as questions in the fields of 'stress and animal welfare', 'reproductive physiology' and 'behavioral ecology' (e.g., Brown et al., 1997; Moss et al., 2001; Palme et al., 2000; Schwarzenberger et al., 1996; Wasser et al., 2000). However, studies applying this technique successfully to monitor endocrine functions in small rodents are rare. There are a few publications on fecal GC measurements in voles, deer mice and rats (Bamberg et al., 2001; Harper and Austad, 2000, 2001; Hayssen et al., 2002) and some studies measuring sex steroids in fecal samples of house mice (Billitti et al., 1998; Muir et al., 2001), but convincing data on corticosterone metabolite (CM) analysis in feces of mice are largely lacking (corticosterone is the major GC in mice; Spackman and Riley, 1978).

Since there are pronounced inter-species differences concerning the metabolism of GCs, the excretion of metabolites and therefore the suitability of different immunoassays for their measurement (cf. Palme et al., 1996; Schatz and Palme, 2001; Schwarzenberger et al., 1996; 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. Therefore, we recently conducted radiometabolism studies with ^{3}H-labeled corticosterone (Touma et al., 2003) providing detailed information about the route and delay of excretion as well as about the biochemical characteristics of metabolites excreted into the feces of mice. On the basis of these data, we were able to develop and establish a new enzyme immunoassay (EIA) using a group specific antibody (detecting steroids with a 5α-3β,11β-diol structure) that proved best suited to measure CM in fecal samples of male and female mice (Touma et al., 2003). However, an extensive biological validation of the technique was still needed.

Thus, the aim of our study was to test whether adrenocortical function can be monitored reliably by measuring CM in fecal samples of mice using the mentioned recently developed EIA. Therefore, pharmacological stimulation [adrenocorticotropic hormone (ACTH) challenge] and suppression [dexamethasone (Dex) suppression] of adrenocortical activity was used, respectively, to induce specific changes in circulating stress hormone concentrations that should be detectable in the feces afterwards. Additionally, two control experiments were performed investigating effects of the injection procedure itself and if the naturally occurring diurnal variation (DV) of hormones is also reflected in the feces of mice.

Methods

Animals and general housing conditions

To investigate if adrenocortical activity is reflected by GC metabolites excreted in fecal samples of mice, six experiments were performed using a total of 72 adult C57BL/6J mice (*Mus musculus* f. *domesticus*) from our local stock. In each experiment, six males and six females were used. Up to the age of 9 weeks, the animals were housed in unisex groups of four individuals in transparent polycarbonate cages (standard Macrolon cages type III, 38 × 22 × 15 cm) with sawdust as bedding material (Allspan, Höveler GmbH & Co. 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) 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 during the experiments.

Experimental design

ACTH challenge

To stimulate adrenocortical activity of the animals, we performed two ACTH challenge experiments using different concentrations of ACTH (referred to as 'low dosage' and 'high dosage' ACTH experiment). At the beginning of the experiments, each mouse was injected at 9 a.m. intraperitoneally with a defined amount of synthetic ACTH (Synacthen, Ciba-Geigy AG, Basel, Switzerland) dissolved in 1 ml of sterile isotonic saline solution. In the low dosage ACTH experiment, 20 μg/100 g body weight was injected, while 60 μg/100 g body weight was administered in the high dosage ACTH experiment. In all experiments, the whole handling procedure of catching, fixation, injection and returning the mouse into the cage lasted a maximum time of 3 min. For the following 4 days, all fecal samples were collected quantitatively and stored at −30 °C until analysis. Sampling times were: 0, 2, 4, 6, 8, 10, 12, 16, 20, 24, 36, 48, 72 and 96 h post injection.

Dex suppression

To suppress the animals' adrenocortical activity, two Dex suppression experiments were carried out using the same protocol as described above [Dex is an artificial steroid that mimics endogenous GCs and reduces circulating corticosterone levels via the negative feedback mechanism of the hypothalamic–pituitary–adrenal (HPA) system; Axelrod and Reisine, 1984; von Holst, 1998]. As in the ACTH challenge experiments, two different concentrations of Dex (Fortecortin No. 1353, Merck KGaA, Darmstadt, Germany) were used to investigate dosage-dependent effects on the pattern of excreted fecal steroid metabolites. In the low dosage Dex experiment, 100 μg/100 g body weight was injected, while in the high dosage Dex experiment, 300 μg/100 g body weight was administered to each mouse.

Effects of the injection procedure

Since the handling procedure of capture, fixation and injection of ACTH or Dex is a relatively stressful event for the mouse and therefore is likely to influence the animals' GC concentrations, a control experiment was conducted to investigate effects of the injection procedure itself on the pattern of excreted fecal steroid metabolites. The experimental design was identical to that of the ACTH challenge and the Dex suppression experiments. However, this time the mice received only an injection of 1 ml sterile isotonic saline solution.

Evaluation of DVs

To avoid confounding effects of the well-known DV of stress hormones in mice (see introduction), a further control experiment was set up, allowing to distinguish between effects of the pharmacological stimulation/suppression of adrenocortical activity from naturally occurring circadian rhythms of hormone concentrations. Additionally, possible adverse effects of the technique used to collect the fecal samples (see below) should be detected. In contrast to the previously described experiments, the mice were now left undisturbed in their cages and did not receive any injections or handling. However, fecal samples were collected according to the same sampling regime as in all other experiments.

Sample collection

To facilitate individual sampling and quantitative collection of all voided feces without handling the animal, the method described by Touma et al. (2003) was used. Briefly, the mice were housed individually in stainless steel wire cages (38 × 22 × 15 cm), which were placed in standard Macrolon cages of the same size. All excreta dropped through the bars of the wire cage and could be easily collected from the floor of the lower cage, which was completely covered with filter paper to immediately adsorb the urine. During each sampling, the filter paper was renewed and the amount of feces voided during the sampling interval was measured (since mice defecated quite frequently, in about 95% of all sampling intervals enough fecal material could be collected for the hormone metabolite analysis). 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 before the injection and samples were collected in 12-h intervals during this time.

Since mice are nocturnal animals and their steroid excretion pattern is known to be influenced by their activity (Touma et al., 2003), all sample collections performed during the dark phase of the light–dark cycle were conducted under dimmed light conditions (about 3–4 lx) to avoid disturbing the animals' natural activity rhythm.

The presented work complies with current regulations covering animal experimentation in Germany. The experiments were approved by the competent local authority as well as by the 'Animal Welfare Officer' of the University of Muenster.

Extraction and determination of fecal steroid metabolites

Extraction procedure

Fecal steroid metabolites were extracted according to the method described by Palme and Möstl (1997) (adapted and validated for mice by Touma et al., 2003). Briefly, each fecal sample was homogenized and an aliquot of 0.05 g was shaken with 1 ml of 80% methanol for 30 min on a multi-vortex. After centrifugation (10 min at 2500 × *g*), an

aliquot of the supernatant was diluted (1:10) with assay buffer (Tris/HCl 20 mM, pH 7.5) and frozen at −20°C until analysis.

Enzyme immunoassay

To determine the amount of fecal CM, we used a recently established 5α-pregnane-3β,11β,21-triol-20-one EIA, which proved well suited to assess CM in fecal samples of mice (Touma et al., 2003). This EIA utilizes a group-specific antibody measuring steroids with a 5α-3β,11β-diol structure. The antibody cross-reacted 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 cross-reactivities less than 1%. If more positions differed, the cross-reactivity was even less (<0.1%). Cross-reaction with Dex was also very low (<0.01%) and 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) cross-reacted less than 1%. The intra- and inter-assay coefficients of variation were 9.1% and 14.0%, respectively.

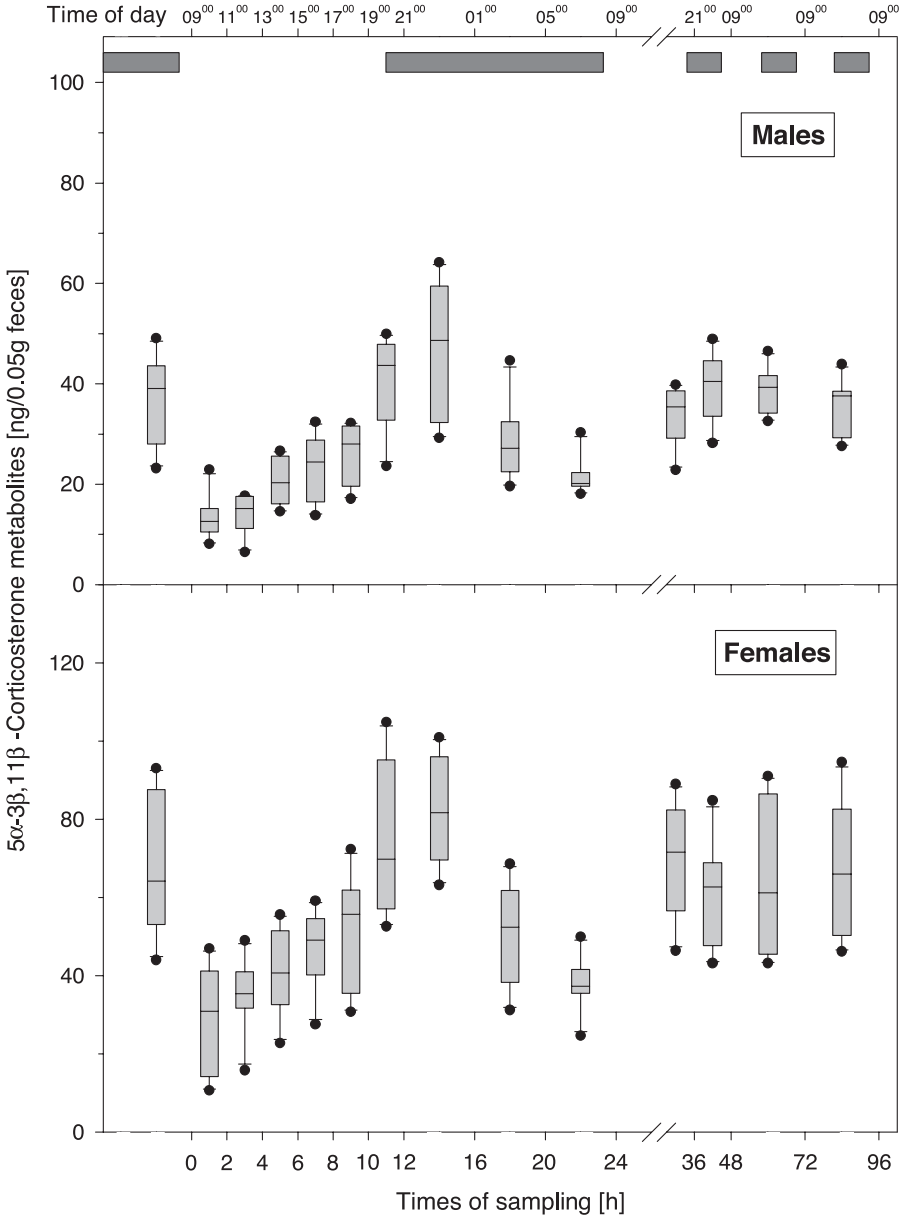

Fig. 1. Excretion profile of immunoreactive CM in fecal samples of male (top) and female (bottom) mice (note different *y*-axis scales) to evaluate DVs. Data are given as box–whisker plots showing medians (lines in the boxes), 25% and 75% quartiles (boxes), 10% and 90% ranges (whiskers) and outliers (dots). Time of day and dark phase periods (horizontal bars) are indicated in the top panel.

Statistical analysis

Since parts of the obtained data were not normally distributed, the data were analyzed by non-parametrical statistics (Siegel and Castellan, 1988). All tests were calculated using the software package SPSS (version 10.0). 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-test, two-tailed), while differences between more than two independent samples were calculated with the Kruskal–Wallis *H*-test (KWH-test). In case of significant variation proved by the KWH-test, post hoc comparisons between the groups were done using multiple MWU-tests. Here, significance levels were corrected according to the sequential Bonferonni technique (Rice, 1989). Differences were considered significant if their probability of occurring by chance was less than 5%.

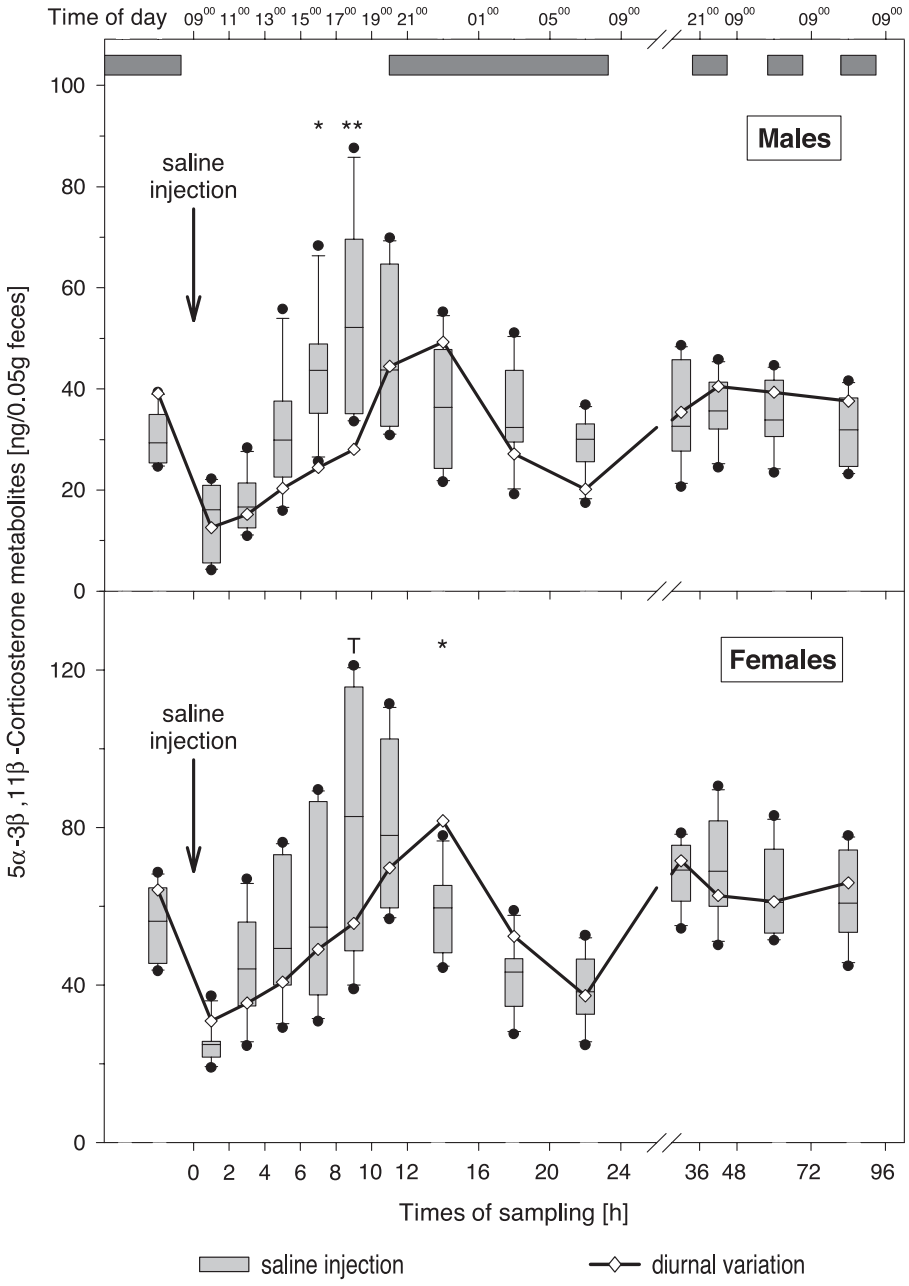

Fig. 2. Excretion profile of immunoreactive CM in fecal samples of male (top) and female (bottom) mice to evaluate effects of the injection procedure itself (saline injection). Data are given as box–whisker plots (for a description, see legend of Fig. 1). Significant differences to the 'diurnal variation' (medians are given as line plot) are marked with asterisks [MWU-test (two-tailed), $n_1 = n_2 = 6$, $U = 0–7$, ** $P < 0.01$, * $P < 0.05$, T $P < 0.1$]. Time of day and dark phase periods (horizontal bars) are indicated in the top panel. The arrows mark the time of injection.

Results

Control experiments

Evaluation of DVs

The experiment to evaluate diurnal changes of corticosterone concentrations revealed a significant variation of excreted fecal CM in males and females (ANOVA on ranks, $n_{males} = n_{females} = 6$, $\chi_r^2 = 59.7$ and 42.3, $df = 13$, both $P < 0.001$; see Fig. 1). A clear pattern of increasing and decreasing concentrations was observed reaching maximal values in the first third of the dark phase (16 h after start of the experiment, i.e., in the sampling interval between 2100 and 0100 h) and minimal values at the beginning of the light phase (2 h after start of the experiment, i.e., in the sampling interval between 0900 and 1100 h). Relatively high concentrations of CM were also found in samples representing larger sampling intervals, that is, in samples collected over periods of 12 h (sample points at $T = 0$, 36 and 48) and 24 h (sample points at $T = 72$ and 96), respectively. This pattern

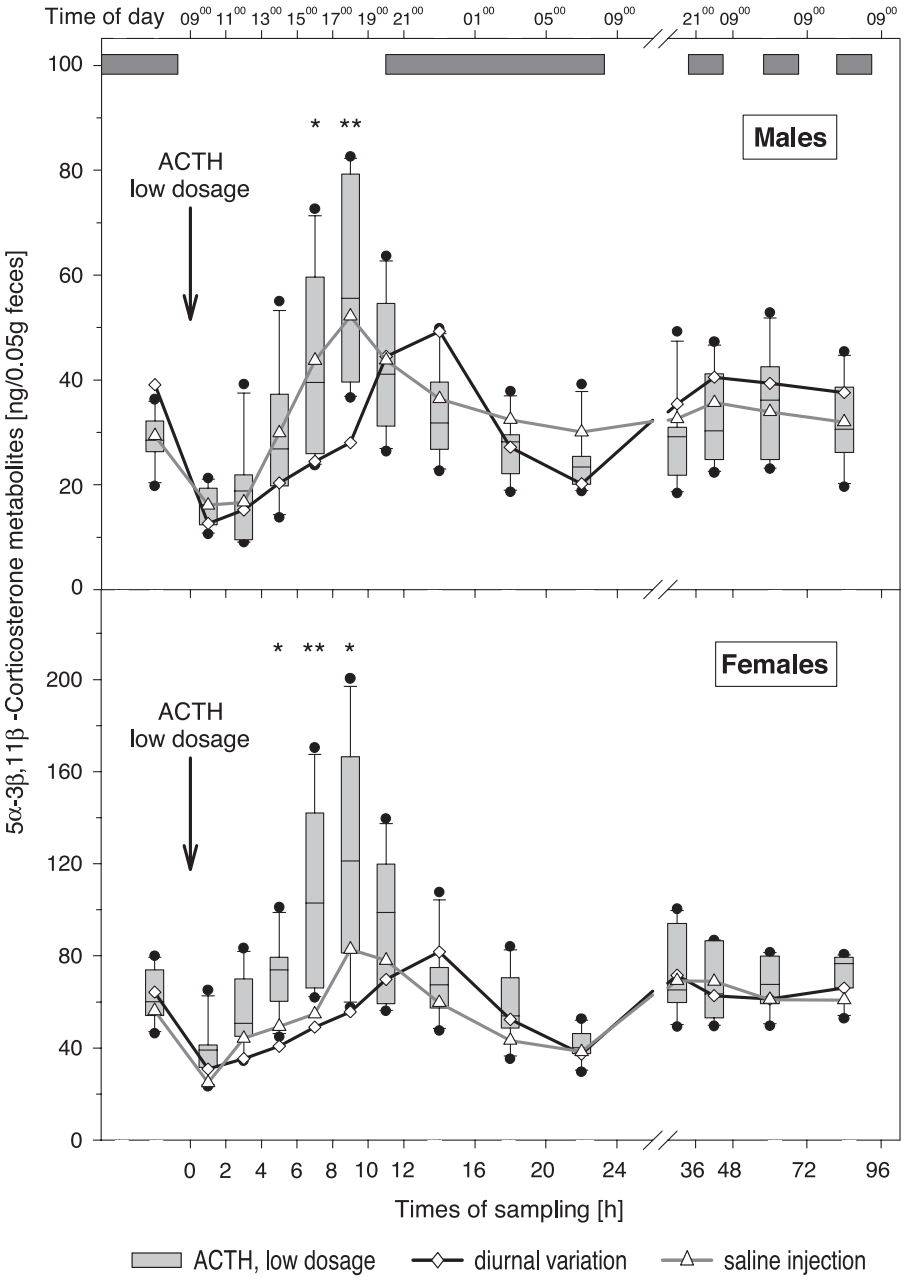

Fig. 3. Excretion profile of immunoreactive CM in fecal samples of male (top) and female (bottom) mice treated with a 'low dosage' of ACTH. Data are given as box–whisker plots (for a description, see legend of Fig. 1). Significant differences between the ACTH challenges and the two control experiments 'diurnal variation' and 'saline injection' (medians are given as line plots) are marked with asterisks (KWH-test, $n_1 = n_2 = n_3 = 6$, $H = 6.5–11.5$, $df = 2$, ** $P < 0.01$, * $P < 0.05$). Time of day and dark phase periods (horizontal bars) are indicated in the top panel. The arrows mark the time of injection.

of diurnally varying CM concentrations was in principle identical for both sexes (see Fig. 1). However, in females, absolute concentrations of basal and peak values were higher than in males (MWU-test, $n_1 = n_2 = 6$, for all sample points, $U = 0–4$, $0.001 < P < 0.05$).

Effects of the injection procedure

Similar to the variation of CM concentrations reported in the first control experiment, significant changes were also observed in fecal samples of males and females used in the second experiment to evaluate effects of the injection procedure itself (ANOVA on ranks, $n_{\text{males}} = n_{\text{females}} = 6$, $\chi_r^2 = 33.8$ and 48.4, $df = 13$, both $P < 0.001$; see Fig. 2). However, the time course of excretion differed significantly from the observed DV in both sexes (MWU-test, $n_1 = n_2 = 6$; males: sample points at $T = 8$ and 10, $U = 0$ and 3, $0.001 < P < 0.05$; females: sample point at $T = 16$, $U = 5$, $P < 0.05$). Peak values were measured about 10 h after the saline

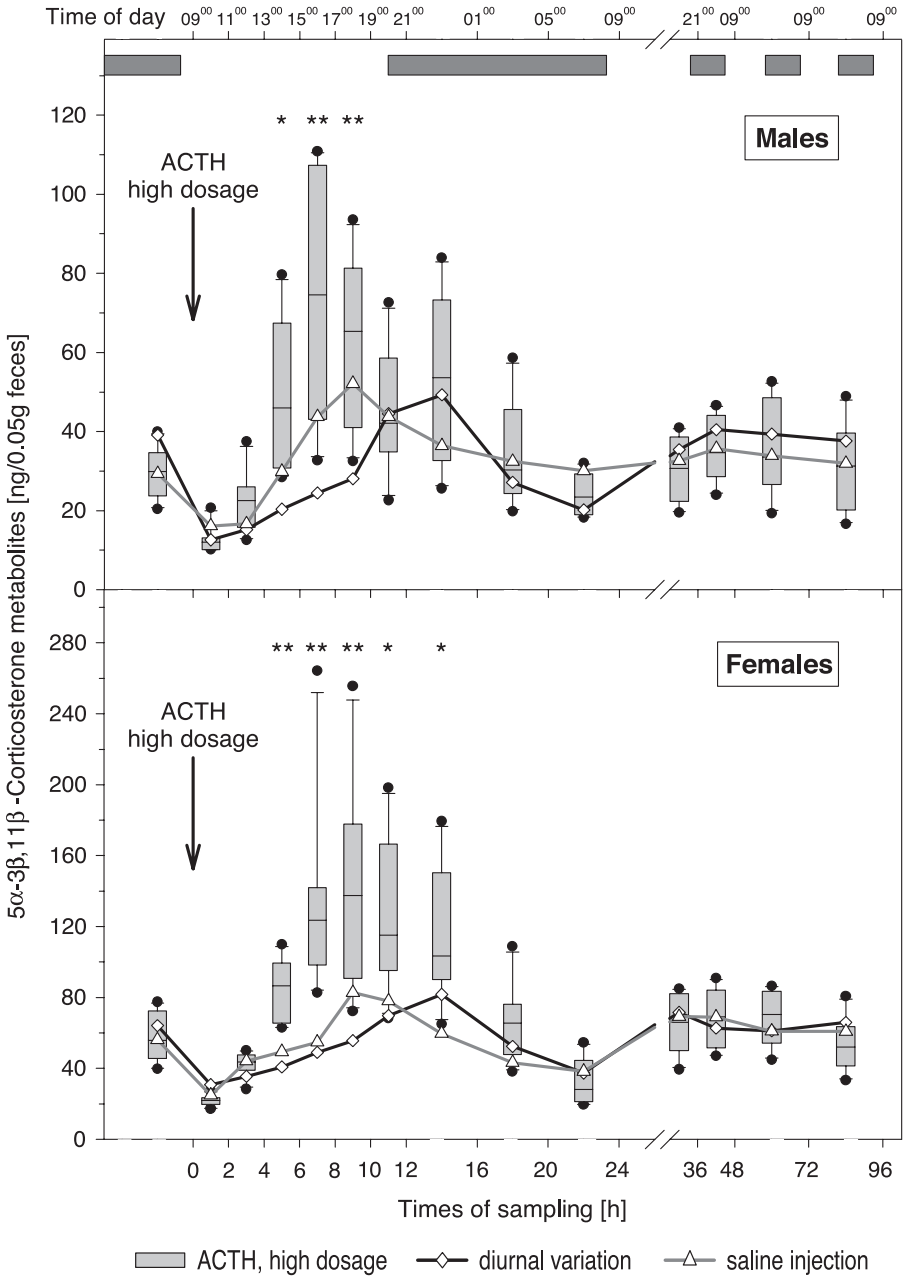

Fig. 4. Excretion profile of immunoreactive CM in fecal samples of male (top) and female (bottom) mice treated with a 'high dosage' of ACTH. Data are given as box–whisker plots (for a description, see legend of Fig. 1). Significant differences between the ACTH challenges and the two control experiments 'diurnal variation' and 'saline injection' (medians are given as line plots) are marked with asterisks (KWH-test, $n_1 = n_2 = n_3 = 6$, $H = 8.7–11.4$, $df = 2$, ** $P < 0.01$, * $P < 0.05$). Time of day and dark phase periods (horizontal bars) are indicated in the top panel. The arrows mark the time of injection.

injection (SI) and decreased again afterwards bringing about a clearly different pattern of CM excretion as a reaction to the injection procedure (see Fig. 2). This effect was evident in the period between 6 and 16 h after the injection. Comparison between the sexes revealed the same differences, as described above. Regarding absolute concentrations of CM, females showed higher values than males (note the different *y*-axis scales in Fig. 2).

Pharmacological stimulation and suppression of adrenocortical activity

ACTH challenge

In both ACTH challenge experiments, significant variations of CM were found for both sexes (low and high dosage ACTH, males and females: ANOVA on ranks, each $n = 6$, $\chi^2_r = 36.2–53.9$, $df = 13$, all $P < 0.001$). As expected, the injection of ACTH caused a sharp rise of

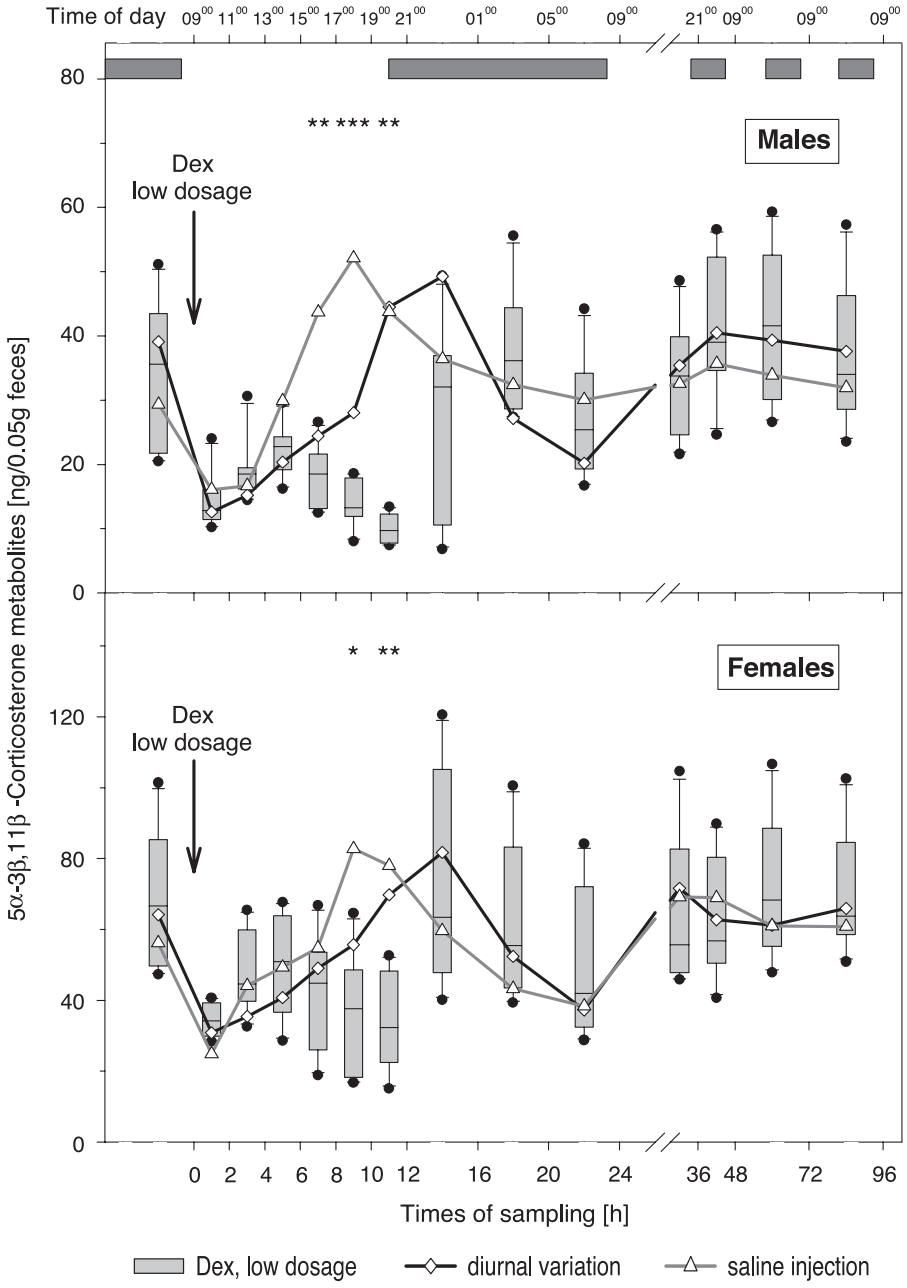

Fig. 5. Excretion profile of immunoreactive CM in fecal samples of male (top) and female (bottom) mice treated with a 'low dosage' of Dex. Data are given as box–whisker plots (for a description, see legend of Fig. 1). Significant differences between the Dex suppressions and the two control experiments 'diurnal variation' and 'saline injection' (medians are given as line plots) are marked with asterisks (KWH-test, $n_1 = n_2 = n_3 = 6$, $H = 8.9–14.4$, $df = 2$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$). Time of day and dark phase periods (horizontal bars) are indicated in the top panel. The arrows mark the time of injection.

circulating corticosterone concentrations in all four groups, which was reflected in clearly increased CM concentrations in the feces 8–10 h later (see Figs. 3 and 4). Significant differences between the CM excretion patterns observed in the ACTH challenges and the two control experiments were revealed extending over the period of 6–10 h after injection in the low dosage experiment (KWH-test, $n_1 = n_2 = n_3 = 6$, $df = 2$; males: sample points at $T = 8$ and 10, $H = 6.6$ and 11.5, $0.001 < P < 0.05$; females: sample points at $T = 6$, 8 and 10, $H = 6.5–9.6$, $0.001 < P < 0.05$) and 6–16 h in the high dosage experiment (KWH-test, $n_1 = n_2 = n_3 = 6$, $df = 2$; males: sample points at $T = 6$, 8 and 10, $H = 8.9–11.4$, $0.001 < P < 0.05$; females: sample points at $T = 6$, 8, 10, 12 and 16, $H = 8.7–11.1$, $0.001 < P < 0.05$), respectively. Additionally, a clear dosage dependency was recorded regarding the increase of excreted CM and peak levels reached in reaction to the ACTH injection (see Figs. 3 and 4). CM concentrations increased from initial to peak values by about 425% in the low dosage experiment ($\text{median}_{\text{males}} = 338.8$, $\text{range}_{\text{males}} =$

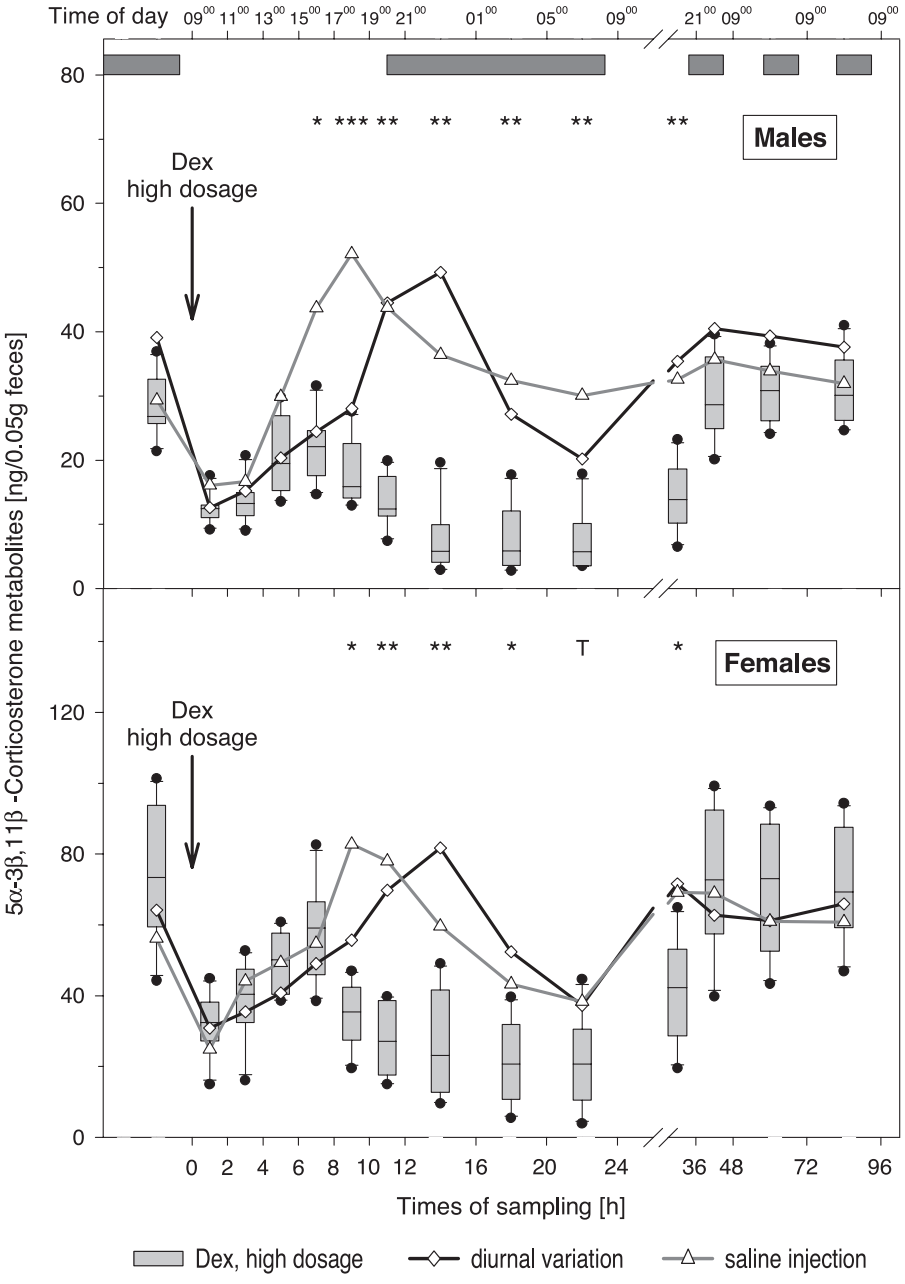

Fig. 6. Excretion profile of immunoreactive CM in fecal samples of male (top) and female (bottom) mice treated with a 'high dosage' of Dex. Data are given as box–whisker plots (for a description, see legend of Fig. 1). Significant differences between the Dex suppressions and the two control experiments 'diurnal variation' and 'saline injection' (medians are given as line plots) are marked with asterisks (KWH-test, $n_1 = n_2 = n_3 = 6$, $H = 8.2–13.1$, $df = 2$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, T $P < 0.1$). Time of day and dark phase periods (horizontal bars) are indicated in the top panel. The arrows mark the time of injection.

233.8–565.4; $median_{females}$ = 467.5, $range_{females}$ = 320.4–527.4) and about 665% in the high dosage ACTH experiment ($median_{males}$ = 588.7, $range_{males}$ = 402.8–880.5; $median_{females}$ = 718.1, $range_{females}$ = 586.1–970.3), bringing about a significant ACTH dosage effect (MWU-test, $n_1 = n_2 = 6$; males: $U = 5$, $P < 0.05$; females: $U = 0$, $P < 0.01$). Although females again showed generally higher absolute concentrations of excreted CM (note the different scales of the *y*-axis in the respective panels of Figs. 3 and 4), the percentage increase did not differ significantly between the sexes in both ACTH experiments.

Post hoc comparison of the different experimental groups (ACTH, DV and SI, see Figs. 3 and 4) revealed in males significant effects of the ACTH injection only between the ACTH and the DV group, but not between ACTH and SI treatment (MWU-test, $n_1 = n_2 = 6$; low dosage ACTH vs. DV: sample point at $T = 10$, $U = 0$, $P < 0.05$; high dosage ACTH vs. DV: sample points at $T = 6$, 8 and 10, all $U = 0$, all $P < 0.05$). In females, however, significant differences were confirmed between the ACTH groups and both control experiments (MWU-test, $n_1 = n_2 = 6$; low dosage ACTH vs. DV: sample points at $T = 6$, 8 and 10, $U = 0–2$, all $P < 0.05$; low dosage ACTH vs. SI: sample point at $T = 8$, $U = 4$, $P < 0.05$; high dosage ACTH vs. DV: sample points at $T = 6$, 8, 10 and 12, $U = 0–2$, all $P < 0.05$; high dosage ACTH vs. SI: sample points at $T = 8$ and 16, $U = 1$ and 2, both $P < 0.05$).

Dex suppression

In both Dex suppression experiments, the time course of excreted CM also showed a significant variation in males and females (low and high dosage Dex, males and females: ANOVA on ranks, each $n = 6$, $\chi_r^2 = 36.2–53.9$, $df = 13$, all $P < 0.001$). Within the first hours after the injection of Dex (sample points at $T = 2$, 4 and 6), CM concentrations resembled the pattern observed in the 'diurnal variation' and 'saline injection' experiments (see Figs. 5 and 6). From sample point $T = 8$ onwards, however, clearly decreased concentrations were found over extended periods of time in animals injected with Dex. Significantly lower CM concentrations were detected between 8 and 12 h after injection in the low dosage Dex experiment (KWH-test, $n_1 = n_2 = n_3 = 6$, $df = 2$; males: sample points at $T = 8$, 10 and 12, $H = 10.1–14.4$, $0.001 \leq P < 0.01$; females: sample points at $T = 10$ and 12, $H = 8.9$ and 11.2, $0.001 < P < 0.05$) and between 8 and 36 h after injection in the high dosage experiment (KWH-test, $n_1 = n_2 = n_3 = 6$, $df = 2$; males: sample points at $T = 8$, 10, 12, 16, 20, 24 and 36, $H = 9.1–13.1$, $0.001 \leq P < 0.05$; females: sample points at $T = 10$, 12, 16, 20 and 36, $H = 8.2–12.4$, $0.001 < P < 0.05$). Thus, suppression of adrenocortical activity lasted for about 6 and 30 h, respectively. Afterwards, CM excretion returned to levels measured in the control groups and stayed on this level until the end of the experiment.

Post hoc comparisons of the experimental groups confirmed these differences between the two Dex experiments and both control experiments for both sexes (MWU-test, $n_1 = n_2 = 6$; males, low dosage Dex vs. DV: sample points at $T = 10$ and 12, $U = 0$ and 2, both $P < 0.05$; males, low dosage Dex vs. SI: sample point at $T = 8$, 10 and 12, $U = 0–1$, all $P < 0.05$; males, high dosage Dex vs. DV: sample points at $T = 12$, 16, 20, 24 and 36, all $U = 0$, all $P < 0.05$; males, high dosage Dex vs. SI: sample points at $T = 8$, 10, 12, 16, 20, 24 and 36, $U = 0–1$, all $P < 0.05$; females, low dosage Dex vs. DV: sample points at $T = 12$, $U = 0.5$, $P < 0.05$; females, low dosage Dex vs. SI: sample points at $T = 10$ and 12, $U = 0$ and 3, both $P < 0.05$; females, high dosage Dex vs. DV: sample points at $T = 12$, 16, 20 and 36, all $U = 0–4$, all $P < 0.05$; females, high dosage Dex vs. SI: sample points at $T = 10$, 12, 16 and 36, $U = 0–3$, all $P < 0.05$).

Discussion

The major objective of our study was to prove the suitability of a recently developed technique (cf. Touma et al., 2003) to monitor stress hormones noninvasively in fecal samples of laboratory mice. Therefore, we tested whether adrenocortical function can be monitored accurately by analyzing fecal CM in male and female mice. To evaluate the biological relevance of the technique for both sexes in a statistically meaningful manner, different validation experiments were performed using dosage-dependent pharmacological suppression and stimulation of the adrenal cortex, respectively. Additionally, two control experiments were performed investigating effects of the injection procedure itself and the naturally occurring DV of hormones.

Suppression of adrenocortical activity was achieved by injection of Dex. In the Dex experiments, a clear dosage-dependent suppression of adrenocortical activity was confirmed for both sexes (see Figs. 5 and 6). Compared to the control groups (SI experiment and DV experiment), significantly decreased CM concentrations were found about 8–10 h after the injection of Dex lasting for about 6 h in the low dosage experiment and for about 30 h in the high dosage experiment. This is in accordance with other studies, describing a time delay of about 10 h in the mouse (Billitti et al., 1998; Harper and Austad, 2000; Touma et al., 2003). This species-specific lag time between hormonal events in the blood and the appearance of the respective signal in the feces was found to be well related to the animals' intestinal transit time (Palme et al., 1996). 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, Touma et al. (2003) showed by comparing two radiometabolism experiments starting at different times (9 a.m. and 9 p.m., respectively) that in mice, this delay also depends on the time of day at which the event of interest occurred (lag times of about 10 and 4 h were observed, respectively). Obviously, CM excretion patterns may change with the animals' activity. This fact should be

kept in mind for future studies investigating fecal CM in mice.

In the ACTH challenge tests, adrenocortical activity was stimulated by application of ACTH, inducing the adrenal cortex to synthesize and release corticosterone into the blood (Axelrod and Reisine, 1984; von Holst, 1998). In both experiments, again, after a lag time of about 8–10 h, peak concentrations of CM (in a dosage-dependent manner) were measured in the feces of males and females (see Figs. 3 and 4). In the females, significant effects of the ACTH application were confirmed at several sampling points against both control groups. Surprisingly, in the males, statistical differences could only be proven between the two ACTH treatment groups and the DV group, but not between the animals injected with ACTH and saline, respectively.

The SI experiment served to evaluate possible effects of the injection procedure itself, since capture, fixation and injection are likely to be stressful events for a mouse and therefore could influence the animals' GC concentrations (cf. Armario et al., 1986; Gärtner et al., 1980; Haemisch et al., 1999; Hennessy and Levine, 1978; Riley, 1981). Obviously, these adverse effects could be monitored here, as males and females showed CM excretion patterns similar to those observed in the ACTH experiments (see Figs. 3 and 4) and differed significantly from the DV group (see Fig. 2). However, peak values reached, at least in the females, were higher in the animals injected with ACTH than in the saline-treated ones. Nevertheless, the profound adrenocortical reaction to a SI observed in both sexes (in males it was even statistically undistinguishable from effects of an ACTH injection—in the low as well as in the high dosage experiment) underlines the fact that the injection procedure itself poses a serious stressor, which should not be disregarded. Although a SI can be regarded as a short-term stressor, a pronounced adrenocortical reaction was detected in the feces of males and females. Since fecal samples represent a more integrated measure of stress response (due to the pooling effects in the digestive tract), it becomes clear that routine laboratory procedures like blood sampling or intraperitoneal injections can have a distinct impact on the animals' stress physiology and might produce undesired side effects, for example, in pharmacological experiments, since modulating effects of GC on immune functions are well known (Harbuz and Lightman, 1992; Munck et al., 1984; Riley, 1981).

Another difference between the sexes was found in all six experiments. Although time courses of excreted CM were quite similar for males and females, absolute concentrations differed significantly between the sexes. Females generally showed values about twice as high as males, indicating females to have also higher plasma corticosterone concentrations as described in other studies (Handa et al., 1994; Laviola et al., 2002). However, since circulating corticosterone levels might vary significantly over the females' estrous cycle, direct comparisons of absolute GC concentrations are difficult. Although we have no specific data about the estrous cycles of the experimental females, it is likely that in our experiments, all cyclic states were represented randomly, as the animals were housed singly and thereby synchronization of cyclic activity was prohibited. Therefore, the observed differences in CM concentrations between males and females are probably related to differences in blood levels, which are in this case probably influenced only to a minor degree by the females' estrous cycle. An additional factor bringing about this effect of females showing higher fecal CM concentrations than males is probably that the group-specific antibody used in our EIA detects more of the CM formed by females, as reported by Touma et al. (2003). Since, at least in mice, fecal metabolite patterns are known to differ significantly between the sexes (Stalvey, 2002; Touma et al., 2003), it seems likely that such a phenomenon proves relevant for other species, too and therefore should be taken into account in studies investigating fecal steroid metabolites.

Taken together, we showed for the first time that stimulation and suppression of the adrenal cortex is reflected accurately by means of CM measurements in fecal samples of male and female mice. Furthermore, the technique proved sensitive enough to detect dosage-dependent effects of the ACTH/Dex treatment and facilitated to reveal profound effects of the injection procedure itself. Even the naturally occurring DV of GC concentrations could be monitored reliably, which is a prerequisite to interpret endocrine profiles with frequent sample collection regimes. In conclusion, our results confirm that measurement of fecal CM with the recently developed 5α-pregnane-3β,11β,21-triol-20-one EIA is a very powerful tool to monitor adrenocortical activity in laboratory mice.

This technique can be very valuable, as it allows frequent sampling of individual animals, even over long periods, and avoids blood sampling-related stress effects, which can adversely affect the experiments and might produce misleading results. In genetically designed mouse models, which are extensively used in biomedical research (Malakoff, 2001; Price et al., 1998) and behavioral endocrinology (Nelson, 1997; Picciotto and Wickman, 1998), hormonal and behavioral patterns can be monitored simultaneously, for example, during the course of a disease or the establishment of a dominance hierarchy. Since changes in the activity of the HPA system are known to be linked to some neurological disorders like Parkinson's or Alzheimer's disease (Hartmann et al., 1997; Höschl and Hajek, 2001), the new technique allows to investigate these endocrine changes in the respective mouse model in more detail and thereby could help to understand the underlying mechanisms. Furthermore, utilizing fecal CM measurements could markedly reduce the total number of animals used for research, as individuals can 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 analysis. Another

advantage of this technique is that the DV of GC can be monitored repeatedly in individual animals. Since circadian rhythms are likely to be disturbed well before other symptoms of a disease appear (Hartmann et al., 1997; Magri et al., 1997), it might help to recognize the onset of these changes much earlier and thereby bring forward an early therapeutic treatment, which could then be much more effective. Additionally, field researchers could investigate various questions on the relation between adrenocortical function and, for example, specific behavioral patterns, reproduction or population density by means of analyzing fecal samples of feral mice.

Thus, our successfully established and extensively validated non-invasive technique to monitor stress hormones in fecal samples of mice can open new perspectives in biomedical and behavioral science.

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