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Research paper

Excretion and measurement of corticosterone and testosterone metabolites in bank voles (*Myodes glareolus*)

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ABSTRACT

The bank vole is a commonly used model species in behavioral and ecophysiological studies. Thus, presenting a validated method for noninvasive monitoring of corticosterone and testosterone secretion is of high relevance. Here, we evaluated the effect of time of day and an ACTH challenge test on measured fecal corticosterone (FCM) and testosterone (FTM) metabolites in both sexes. Furthermore, we performed radiometabolism experiments for both steroids and sexes to study metabolism and excretion of ^{3}H-corticosterone and ^{3}H-testosterone. FCM and FTM were analysed with a 5α-pregnane-3β,11β,21-triol-20-one enzyme immunoassay (EIA) and a testosterone (measuring 17β-hydroxyandrostanes) EIA, respectively. Males had significantly higher FCM levels than females and their main excretion route was via the feces (~72%), whereas females excreted nearly equal portions in both feces and urine. For testosterone the main excretion route was via the feces in both sexes (~80%). The time course of excretion was similar in both sexes, but for the first time a significant difference between injected steroids was found: Corticosterone was excreted faster than testosterone, both in urine (median of peak levels: 4 h vs 6 h) and feces (6 h vs 8 h). Several metabolites were present in the feces and the tested EIAs reacted with some of them. Time of day had a significant effect on measured fecal steroid metabolites. As expected, males had significantly higher FTM levels than females. ACTH administration significantly increased FCM values; peaks were observed 4–8 h after injection. In conclusion, both tested EIAs proved suited for a non-invasive measurement of glucocorticoids and androgens in bank voles.

1. Introduction

The endocrine system is responsible for numerous essential bodily functions in animals, including development from a fetus to an adult, as well as everyday maintenance of physiological homeostasis (Campbell and Reece, 2005). Hormones also play a major role in the behavior of animals and thus are important for research linking animal behavior and physiology. However, endocrinological studies can be problematic, as they often require invasive procedures to collect blood samples. Thus, the sampling procedure itself can significantly alter the results, leading to incorrect conclusions (Gärtner et al., 1980; Haemisch et al., 1999). Also, some of the traditional sampling methods are considered unethical. In order to avoid these problems, researchers have shown increasing interest in noninvasive methods (Möstl and Palme, 2002; Sheriff et al., 2011). For many species fecal samples have been shown to be a practical and well suited method for this purpose (Touma and Palme, 2005; Sheriff et al., 2011).

Besides practical and ethical issues, using fecal samples for endocrinological studies has also other advantages compared to blood sampling. Hormone concentration measured from blood samples provides only a snapshot of the hormonal status of the animal, which is problematic particularly in the case of hormones with strong diel and episodic fluctuations and fast reaction to stimuli, like glucocorticoids. Fecal samples, on the other hand, reflect the hormone secretion for a longer period of time and thus dampen minor and short-term fluctuations in hormone secretion (Palme, 2005, 2012; Palme et al., 2005). However, using samples other than blood for measuring given hormones in an animal is not straightforward. The intestinal gut passage time in a certain species determines the time gap between hormone secretion and the excretion of its metabolites in feces. Thus, in order to correctly interpret results based on fecal analyses one should always know the average gut passage time of the given species (Palme et al., 2005). Also sex, age, time of the day and even diet may affect the metabolism (Touma et al., 2003; Dantzer et al., 2011a; Goymann, 2012).

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Another important matter is the excretion route (urine vs feces) of the hormone or its metabolites, which can vary significantly between species and sometimes even sexes (Touma et al., 2003; Palme et al., 2005).

There can be some steroid hormone- and species-specific differences in the ratio of hormone metabolites and actual hormone present in the feces (Palme et al., 1997, 2005). However, in the case of many groups of hormones, like glucocorticoids, it is safe to say that only hormone metabolites are available in the excreta (Palme and Möstl, 1997; Touma et al., 2003; Palme et al., 2005). Thus, immunoassays designed for blood samples are often unsuited for analyzing fecal samples, as they target actual hormone molecules rather than their metabolites. Using immunoassays especially designed for hormone metabolites is advisable in order to get reliable results (Möstl et al., 2005; Fanson et al., 2016). However, hormone metabolites are a highly diverse group of molecules, as the types of metabolites can vary between species and even sexes (Palme et al., 2005). Hence, finding a suitable group specific enzyme immunoassay (EIA) for quantification is of great importance.

Here, we performed a multiphase validation experiment in order to test the applicability of two EIAs for measuring fecal corticosterone and testosterone metabolites in bank voles (*Myodes glareolus*). For measuring fecal corticosterone metabolites (FCM) we used a 5α-pregnane-3β,11β,21-triol-20-one EIA, which measures steroid metabolites with 5α-3β,11β-diol structure and was first developed and validated for mice (*Mus musculus f. domesticus*) (Touma et al., 2003, 2004). It has been shown to be also suited for rabbits (*Oryctolagus cuniculus*; Monclús et al., 2006), rats (*Rattus norvegicus*; Lepschy et al., 2007), hamsters (*Mesocricetus auratus*; Chelini et al., 2010) and several squirrel species (Bosson et al., 2009, 2013; Dantzer et al., 2010; Hammond et al., 2015). For fecal testosterone metabolites (FTM) we used a testosterone EIA (measuring 17β-hydroxyandrogens) first described by Palme and Möstl (1994). This latter EIA has also been validated for measuring androgen metabolites in female and male red squirrels (*Tamiasciurus hudsonicus*; Dantzer et al., 2011a,b). These particular steroids were chosen for their significant role in behavioral and physiological functions in animals. Stress reactions have an important role in the survival of all animals but in case of prolonged stress it can also strongly affect the reproduction and immune functions of the individual. Corticosterone acts as the main stress hormone especially in some small rodents (Boonstra, 2004). Testosterone, on the other hand, is the main reproductive hormone of males in most vertebrate species, including the bank vole. Testosterone levels often indicate the reproductive status (immature vs mature), but it can also strongly affect the behavior of the individual, making it a very interesting variable for physiological, ethological and ecological studies (Campbell and Reece, 2005).

Bank voles are widely used for laboratory experiments as well as field studies in Northern Europe (review by Bujalska and Hansson (2000), recent combined laboratory and field experiments eg. by Ylönen et al. (2006), Klemme et al. (2008), Eccard et al. (2011b), Haapakoski et al. (2012), Sipari et al. (2014), (2016a,b)). Monitoring the endocrine functions in small rodents is often performed using blood samples, collected from the tail vein or from the orbital sinus (Bradshaw, 2003). Few studies have been exploiting fecal samples for glucocorticoid measurements in bank voles, but to our knowledge, a proper analytical and physiological validation of a method to measure fecal steroid metabolites (FCM or FTM) is still lacking. Harper and Austad (2000) performed a validation experiment for measuring FCM in red-backed voles (*Myodes gapperi*) with a corticosterone radioimmunoassay (RIA), which has been used as a reference for experiments using fecal samples for monitoring stress in bank voles (Ylönen et al., 2006; Eccard et al., 2011a). However, the RIA used in Harper and Austad (2000) was designed to detect the actual hormone rather than its metabolites. Its ability to measure FCM is based on cross-reactions with the metabolites of the parent hormone (Harper and Austad, 2000; Möstl et al., 2005). Thus, a validation of a method designed to measure particularly fecal corticosterone metabolites seems favorable. The experimental design of our study was based on the design and procedures described by Touma et al. (2003, 2004), in which the authors validated the suitability of a 5α-pregnane-3β,11β,21-triol-20-one EIA for FCM measurements in mice and tested the effects of sex and time of day on metabolism and excretion route of the metabolites using radiometabolism experiments. Additionally, we applied the same principles for testosterone metabolites as well. To our knowledge, only one radiometabolism experiment with testosterone has been described in a small rodent species so far (Billitti et al., 1998). As the bank vole is a commonly used model species in behavioral and ecophysiological studies (e.g. Bujalska and Hansson (2000), Ylönen et al. (2006), Klemme et al. (2008), Eccard et al. (2011), Haapakoski et al. (2012), Sipari et al. (2014), (2016a,b)) this kind of a thorough validation is of high relevance for a noninvasive measurement of glucocorticoids and androgens.

2. Material and methods

2.1. Experimental animals

All 48 animals used in this experiment (24 females and 24 males) were laboratory born (August 2012), adult individuals, descending from a colony of wild captured bank voles from Central-Finland, close to the Konnevesi Research station. After weaning (ca. at the age of 20 days) all animals were housed individually in standard mouse cages (43 × 26 × 15 cm) with wood shavings and hay as bedding, until the experiment started (see below; Section 2.2). Light/dark cycle in the laboratory (18L:6D, lights on 6:00 and off 24:00) was a standard long day cycle resembling natural light cycle in the north during summer. The laboratory was maintained at 22 ± 1 °C. Standard mice pellets (Labfor R36, Lantmännen) and bottled tap water were provided *ad libitum*. Animals were maintained in the laboratory at the Konnevesi Research station in Finland.

2.2. Experimental design

The experiments were performed during June–July 2013. All procedures conducted during the experiment were approved by the Finnish State Committee for Animal Experimentation (Licence Code: ESLH-2008-05258/Ym-23). Five days before the experiments started all animals were moved to the experimental cages for habituation (Table 1). To facilitate the collection of feces and urine, and to avoid unnecessary handling of the animals during the

Table 1
The time course of the experiments and distribution of the test animals. (Note that there was one week time between the ACTH experiment and the radiometabolism study).

1. Habituation to the experimental cages (5 days)	All 48 animals (24 males and 24 females)	
2. Diurnal fluctuation study (72 h)	*Corticosterone* 12 ♂ and 12 ♀	*Testosterone* 12 ♂ and 12 ♀
3. ACTH challenge (72 h)	12 ♂ and 12 ♀ (same individuals as above)	–
4. Radiometabolism study (72 h)	6 ♂ and 6 ♀ (randomly selected from the individuals used above)	6 ♂ and 6 ♀ (randomly selected from the individuals used above)

experiment, the voles were housed in steel wire cages which enabled the excreta to drop through the bottom of the cage. A plastic container covered with paper towels was placed below each wire cage to collect feces and urine. To provide some shelter for the animals, some hay was added in one corner of the wire cages. The hay was sparse and coarse enough not to hinder the excreta dropping through the bottom of the cage.

2.3. Evaluation of diurnal fluctuations of the measured steroids

First, the normal diurnal fluctuations of the excreted steroids were evaluated. Animals received no handling or other physical disturbances 5 days before the experiment. Only fecal samples were collected during 72 h according to the following time schedule: 09:00, 11:00, 13:00, 15:00, 17:00, 19:00, 21:00, 01:00, 05:00 during the first 24 h, 09:00, 21:00 on the next day, and only at 09:00 the following two days. All fecal samples were collected from the paper covered containers under each cage into plastic Eppendorf-tubes (1.5 ml) using tweezers, and stored in freezer at −20 °C. Feces clearly contaminated with urine (e.g. feces lying in urine spots) were not collected. In order to not disturb the animals' normal activity rhythms by turning the lights on during night (during the dark phase of their L:D regime) we used head lamps with low light intensity to collect the night samples.

2.4. ACTH challenge

To stimulate adrenocortical activity an ACTH challenge test was performed. Twelve males and twelve females were injected with synthetic ACTH (ACTH Depot, Defiante Farmaceutica S.A), each with a dosage of 60 μg/100 g body weight. The ACTH was mixed with sterile isotonic saline solution and the total volume of each injection was 250 μL. The injections were performed at 09:00 am intraperitoneally. This procedure took mostly only 1–2 (max 4) minutes per animal. Again, feces were collected as described above. However, for the animals in the testosterone test group (12 males and 12 females) no ACTH injections or artificial increases of testosterone levels were performed. To evaluate the testosterone EIA used, we relied on a biological validation comparing males and non-pregnant females, as the latter are expected to have notably lower testosterone levels (reported for example in mice: Dreau et al., 1997; Tiwari et al., 2010).

2.5. Radiometabolism study; route, time course and characterization of excreted metabolites

To study the metabolism and excretion of corticosterone and testosterone in bank voles we performed a radiometabolism study. We selected six males and six females for each steroid from the original individuals used for the diurnal fluctuation study and ACTH challenge (Table 1). There was 1 week between the end of the ACTH experiment and the beginning of the metabolism study, during which the animals remained in their experimental cages. These voles were injected intraperitoneally (at 09:00 am) with 740 kBq (20 μCi) ^{3}H-corticosterone (1,2,6,7-^{3}H-corticosterone) or ^{3}H-testosterone (1,2,6,7-^{3}H-testosterone, specific activity of both: 3.1–3.9 TBq/mmol, BIOTREND Chemikalien GmbH, Köln, Germany). The radiolabeled hormone was mixed with sterile isotonic saline solution containing 5% ethanol. The total volume of each injection was 250 μL. Feces sampling was performed as described above. To study the excretion route of the steroids also urine samples were collected. At each sampling time the paper towels were renewed and papers with urine spots were collected into small plastic bags and stored at −20 °C. Before freezing, the urine spots on the paper were circled with a drawing pen to facilitate analysis later on.

2.6. Analysis of samples

Extraction of steroids was conducted according to the method described by Palme et al. (2013). Briefly, each fecal sample was homogenized and an aliquot of 0.05 g was mixed with 80% methanol (1 ml) and shaken in a multi-vortex. After this, the suspension was centrifuged for 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 stored in freezer at −20 °C until analysis. To determine the amount of corticosterone metabolites a 5α-pregnane-3β,11β,21-triol-20-one EIA was used, which utilizes a group-specific antibody measuring steroids with a 5α-3β,11β-diol structure. To measure testosterone metabolites, a testosterone EIA measuring 17β-hydroxyandrostanes was selected. Details of both EIAs included cross-reactions are given in Touma et al. (2003) and Palme and Möstl (1994), respectively. To determine the route and time course of the steroid excretion, tested by the radiometabolism experiments, the analysis procedure followed the methods described in Touma et al. (2003). Briefly, after extraction (urine: cut out and dissected spots dissolved in 2 ml 80% methanol; feces: see above), the radioactivity of the methanolic extracts of each sample (urine and feces) was measured using a liquid scintillation counter (Tri-Carb 2100TR, Packard Instruments, Meriden, CT USA). Following this, peak excretion samples and thus delay times of urinary and fecal excretion were calculated. Comparing the ratio of recovered radioactivity in feces and urine indicates the excretion route. To determine the type and relative abundance of fecal ^{3}H-corticosterone and -testosterone metabolites a reversed-phase high performance liquid chromatography (HPLC) was performed. Radioactive steroid metabolites were separated from eight peak concentration fecal samples (four per each steroid; two samples from males and two from females) according to their polarity by using HPLC. Radioactivity of the fractions was measured in a liquid scintillation counter and immunoreactivity with the above mentioned EIAs. For characterizing ^{3}H-corticosterone metabolites, also a corticosterone EIA (Palme and Möstl, 1997), also used in the HPLC immunograms of mice (Touma et al., 2003), was utilized (Fig. 4).

2.7. Statistical analysis

Statistical analyses were performed using R 3.0.3 and IBM SPSS Statistics 20. All data were tested for normality using Kolmogorov-Smirnov and Shapiro-Wilk tests of normality. Steroid metabolite levels in circadian fluctuation studies and ACTH challenge were log-transformed for normality and analysed using linear mixed-effect models fitted by REML. For FCM levels (circadian fluctuation and ACTH) data covered twelve males and twelve females. However, during the laboratory analyses we noticed that the samples originally collected for analyzing diurnal fluctuations in corticosterone were large enough enabling the analysis with the testosterone EIA as well. Thus, the final sample size for statistical analyses of diurnal fluctuations in testosterone was doubled (24 males and 24 females). For model selection we used the Akaike information criterion (AIC) and the model with the lowest AIC value was selected for the analyses. For diurnal fluctuation studies, sex and time were entered as fixed factors, and individual as random factor. When comparing FCM levels between the diurnal fluctuation study (baseline level) and the ACTH challenge experiment, the model was extended by adding treatment (diurnal fluctuation i.e. baseline level vs ACTH) as fixed factor. Additionally, paired *t*-test were performed when comparing FCM levels between diurnal fluctuation and the ACTH challenge within the individual time points. Route of excretion

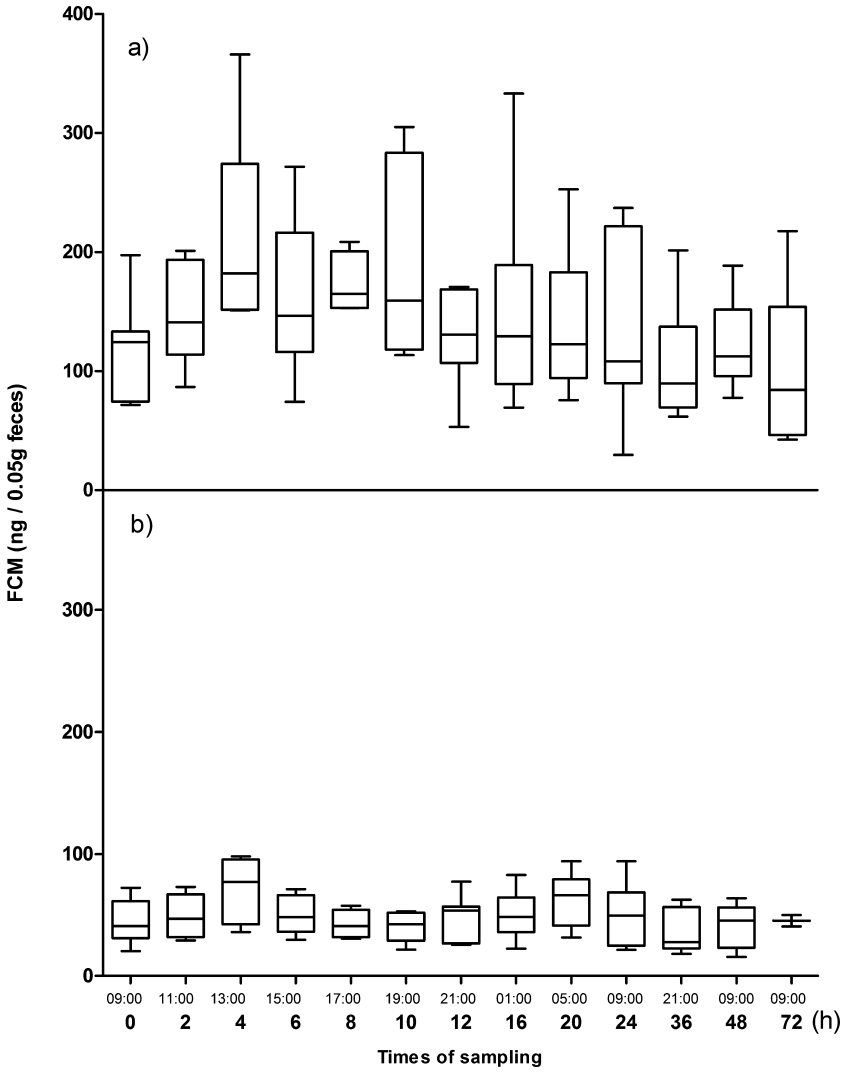

Fig. 1. Undisturbed diurnal variations of fecal corticosterone metabolites (FCM) in males (a) and females (b). Note the discontinuity in time in the x-axis due to the changes in the sampling interval after 12 h. Light/dark cycle in the laboratory during the experiment was 18L:6D with dark phase from 24:00 till 06:00.

was analysed by comparing the ratios of recovered radioactivity in urine and fecal samples between males and females by using Welch two sample *t*-test for corticosterone metabolites (data normally distributed) and Wilcoxon rank sum test for testosterone metabolites (data not normally distributed). For comparing the excretion rate between females and males and the time course of excretion between urine and feces, we used Pearson correlation for normally distributed data and Spearman's correlation for not normally distributed data. A paired *t*-test was used to compare delay times of peak excretion samples for both steroids (in urine and feces, respectively).

3. Results

3.1. Corticosterone

3.1.1. Evaluation of diurnal fluctuations

Time of day had a significant effect on FCM levels ($T_{169} = -3.385$, $p < 0.001$). The highest concentrations in both females and males occurred in the afternoon (at 13:00) and the lowest levels were observed in the morning (at 9:00) and evening (at 21:00; Fig. 1). Males had significantly higher FCM levels than females ($T_{22} = 6.259$, $p < 0.001$).

3.1.2. ACTH challenge

ACTH administration significantly increased concentrations of corticosterone metabolites in both males and females ($T_{333} = -2.465$, $p = 0.014$). A clear peak in the FCM levels was observed 6–8 h after injection in males and 6 h after injection in females (Fig. 2). The ACTH increased mean FCM concentrations three-fold compared to normal diurnal levels at the same time point. In males the FCM levels were significantly elevated by the ACTH injection from 6 to 20 h after injection (diurnal fluctuation vs ACTH challenge: Time point 6: $T_{11} = -5.288$, $p < 0.001$; time point 8: $T_{11} = -6.862$, $p < 0.001$; time point 10: $T_{11} = -2.3433$, $p = 0.039$; time point 12: $T_{11} = -3.257$, $p = 0.008$; time point 16: $T_{11} = -2.727$, $p = 0.020$; time point 20: $T_{11} = -2.631$, $p = 0.023$, and in females from 4 to 12 h after injection (time point 4: $T_{11} = -2.618$, $p = 0.024$; time point 6: $T_{11} = -4.373$, $p = 0.001$; time point 8: $T_{11} = -5.098$, $p < 0.001$; time point 10: $T_{11} = -6.491$, $p < 0.001$; time point 12: $T_{11} = -2.890$, $p = 0.015$). Males had significantly higher FCM concentrations than females ($T_{22} = 3.490$, $p = 0.002$). There were no significant interactions.

3.1.3. Route and time course of excreted ^{3}H-corticosterone

There were significant sex-specific differences in the excretion route of ^{3}H-corticosterone. Males excreted on average 69.7 ± 5.1%

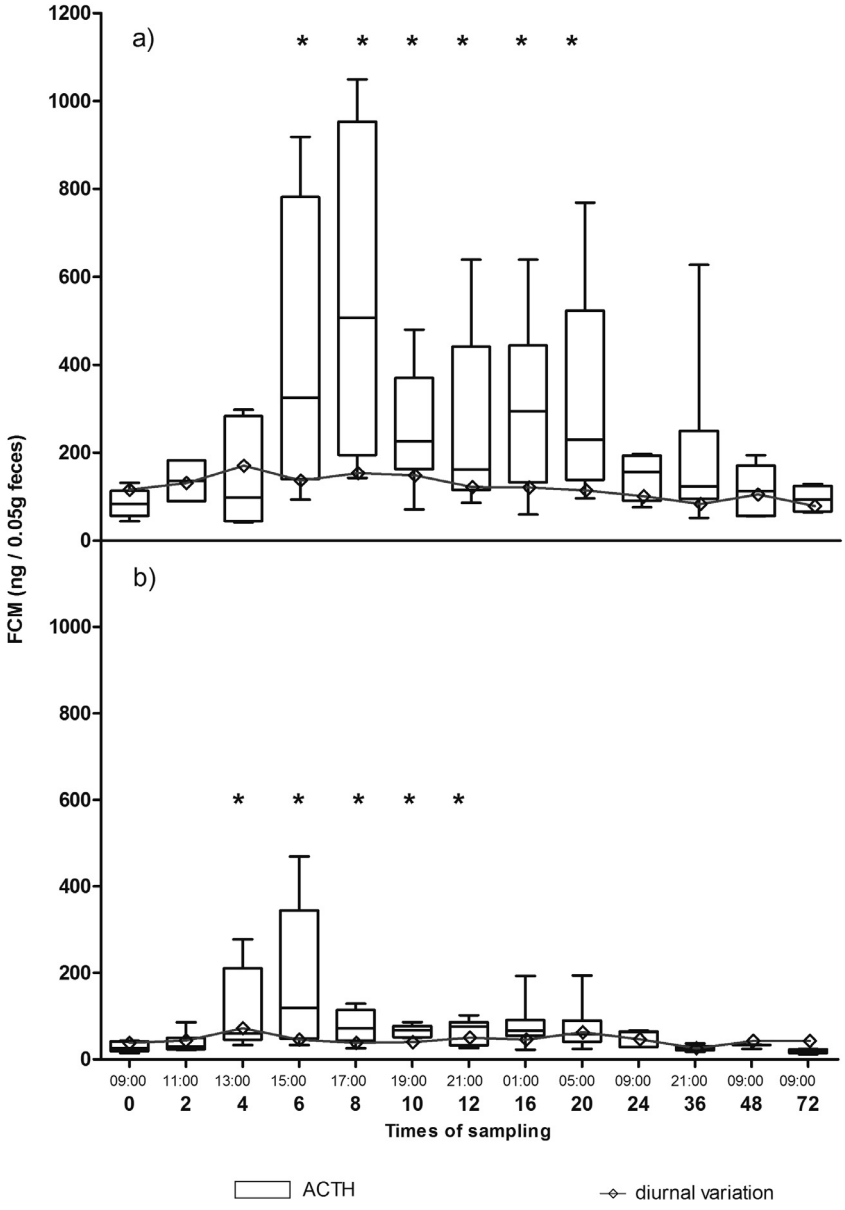

Fig. 2. Concentrations of fecal corticosterone metabolites (FCM) in males (a) and females (b) after ACTH injection. Significant differences ($P < 0.05$) between the ACTH challenge and diurnal variation (ACTH > diurnal variation) are marked with asterisks. Medians of the diurnal variation are given as line plot. Note the discontinuity in time in the x-axis due to the changes in the sampling interval after 12 h. Light/dark cycle in the laboratory during the experiment was 18L:6D with dark phase from 24:00 till 06:00.

of the recovered radioactivity via the feces, whereas females excreted only 51.5 ± 3.9% ($T_{9.353} = -2.810$, $p = 0.020$). The time course of excretion followed similar trends in males and females (excretion via feces: Pearson correlation $r = 0.77$, $p = 0.003$, excretion via urine: Spearman's correlation $r = 0.923$, $p < 0.01$). However, there was a clear difference between feces and urine (Pearson correlation $r = -0.068$, $p = 0.834$). In urine the notably highest amount of radioactivity was recovered in the first sample (mostly 2 h) after the injection (Fig. 3a). In the feces peak concentrations were reached 4–6 h after the injection of ^{3}H-corticosterone. A second peak was observed in some individuals around 16 h after injection (Fig. 3b). This pattern was similar in both sexes.

3.1.4. Characterization of fecal ^{3}H-corticosterone metabolites

Injected ^{3}H-corticosterone was heavily metabolized. There were sex differences regarding the formed metabolites. Females produced a wider range (fractions 10–75) of metabolites than males (fractions 10–50; Fig. 4), some being even less polar than corticosterone. The 5α-pregnane-3β,11β,21-triol-20-one EIA detected several metabolites in both sexes. In contrast, the corticosterone EIA showed only very minor immunoreactivity.

3.2. Testosterone

3.2.1. Evaluation of diurnal fluctuations

Testosterone metabolite levels were significantly affected by sex ($T_{367} = 12.084$, $p < 0.001$) and time of the day ($T_{367} = 2.476$, $p = 0.014$). Males had on average six times higher concentrations than females. There was a significant interaction between sex and time of the day (L-ratio$_6$ = 6.564, $p = 0.01$). Unlike in FCM, the diurnal variation in TM was less pronounced (Fig. 5). However, highest concentrations seemed to be excreted in the evening and at night.

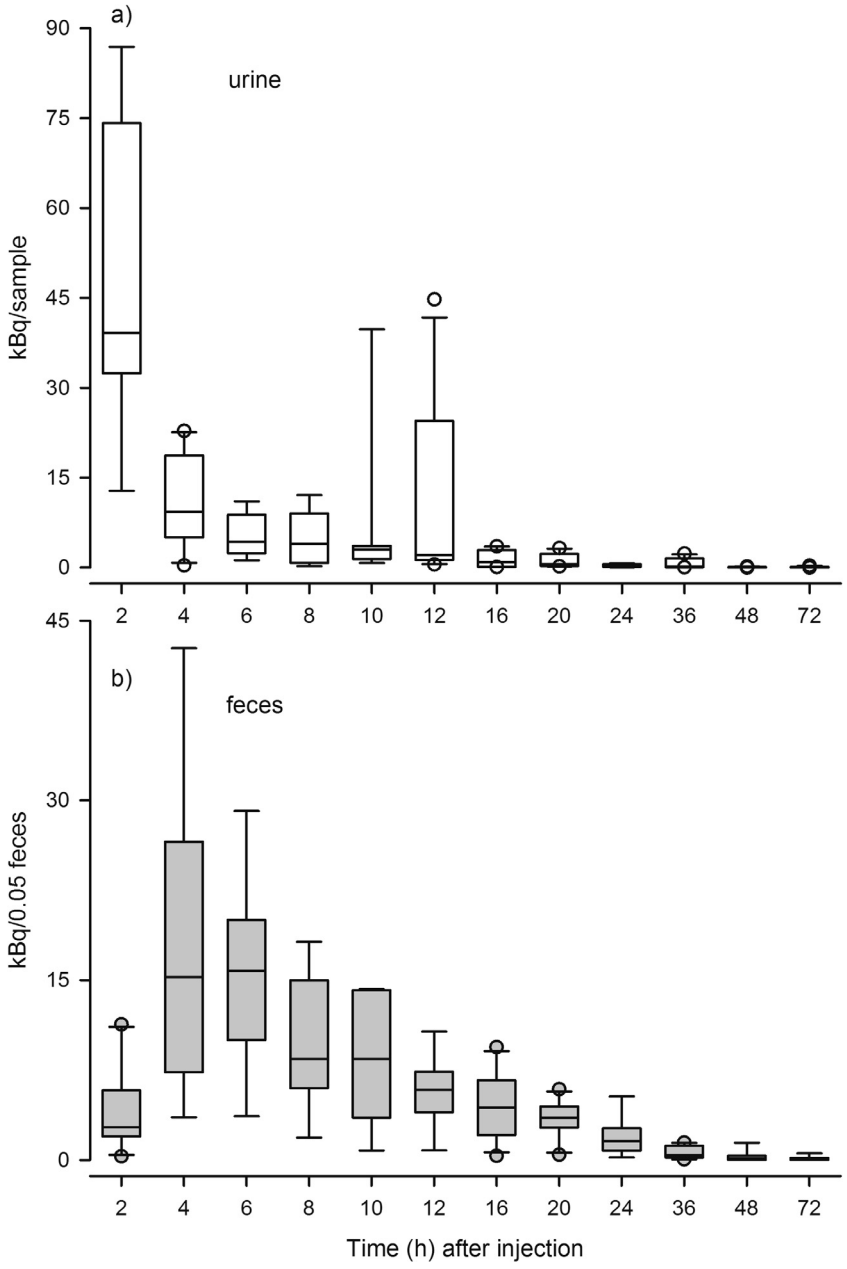

Fig. 3. Time course of the excretion of ^{3}H-corticosterone metabolites (kBq) in urine (a) and feces (b) in males and females grouped together.

3.2.2. Time course and route of excreted ^{3}H-testosterone

Males excreted more ^{3}H-testosterone metabolites via feces than females, but the difference was not significant (males: 83.1 ± 3.7%, females: 70.7 ± 9.0%, W = 12, p = 0.394). The time course of excretion was similar in both sexes (excretion via feces: Spearman's correlation r = 0.559, p = 0.059, excretion via urine: Spearman's correlation r = 0.783, p = 0.003). A larger portion of testosterone metabolites was excreted via the feces compared to CM ($T_{21.18} = -2.5023$, p = 0.021). The excretion rate in testosterone differed between feces and urine (Spearman's correlation r = 0.378, p = 0.226). Similar to corticosterone, excretion via the urine was faster than via the feces. Highest concentrations of ^{3}H-FTM were observed 4–16 h (median: 8 h) after the administration of the hormone, whereas peak radioactivity in urine was recovered 2–10 (median: 6) hours after the injection (Fig. 6). Interestingly, excretion of ^{3}H-testosterone was significantly (p = 0.003 and 0.002, respectively) slower than that of ^{3}H-corticosterone (Fig. 7), both via urine (median peak levels: 6 h vs 4 h) and feces (median: 8 h vs 6 h).

3.2.3. Characterization of fecal ^{3}H-testosterone metabolites

Similar to corticosterone, testosterone was heavily metabolized and with clear sex specific differences. There was one prominent peak of radioactivity found in both sexes, accompanied with several small ones (Fig. 8). In females, the main peak eluted between fractions 40–45 and in males between fractions 32–36. Unfortunately the testosterone EIA was not able to pick up the main metabolite, but it detected many of the less prominent ones.

4. Discussion

Our multiphase validation experiment for measuring glucocorticoids and androgens noninvasively in bank voles revealed interesting results. We observed sex differences in the route of

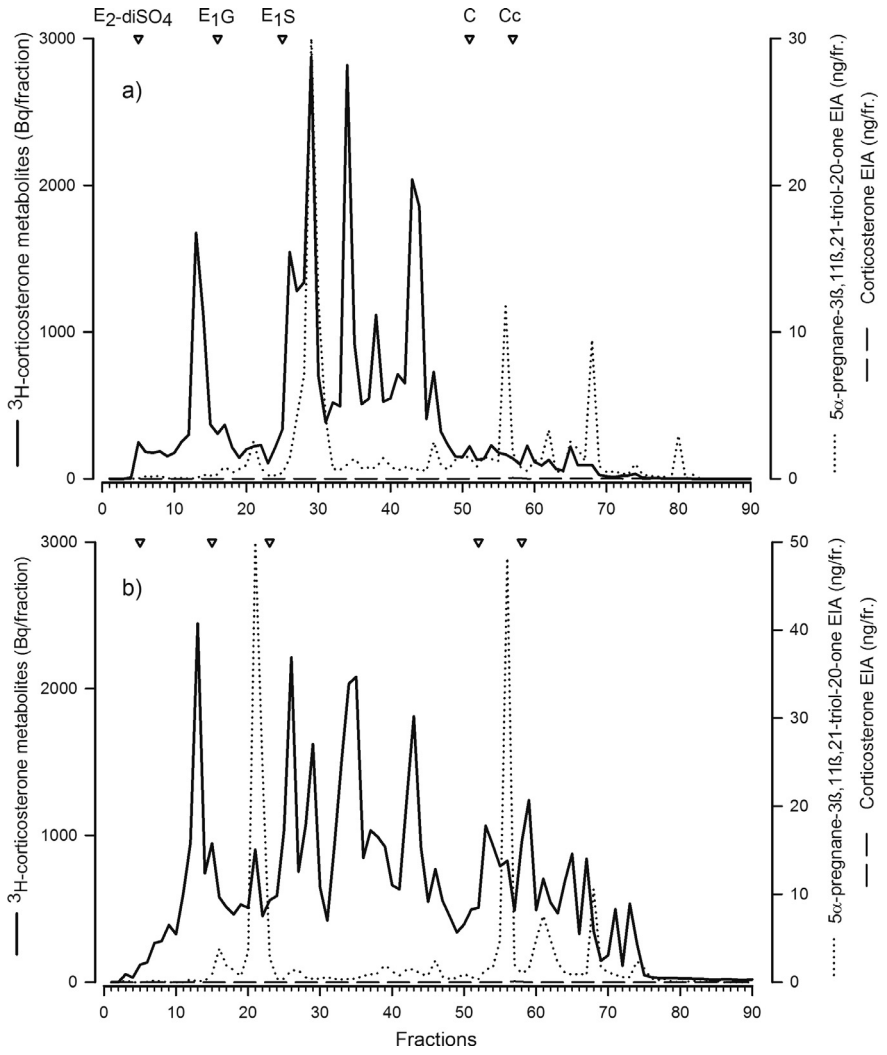

Fig. 4. High performance liquid chromatographic (HPLC) separation of fecal ^{3}H-corticosterone metabolites in one male (a) and one female (b). Immunoreactivity was tested with two different enzyme immunoassays. Open triangles mark the approximate elution positions of respective standards (E_2-diSO_4 = 17β-estradiol-disulfate, E_1G = estrone-glucuronide, E_1S = estrone-sulfate, C = cortisol, Cc = corticosterone).

excretion (only ^{3}H-corticosterone) and formed fecal metabolites (^{3}H-corticosterone and ^{3}H-testosterone). Most notable, for the first time a significant difference in the time course of excretion between steroids was found (CM were excreted faster than TM). Time of day had a significant effect on measured fecal steroid metabolites (more pronounced in FCM). Our result demonstrate that a 5α-pregnane-3β,11β,21-triol-20-one EIA (measuring corticosterone metabolites with a 5α-3β,11β-diol structure and first established by Touma et al. (2003)), can also be used for reliable measurements of fecal corticosterone metabolites in bank voles. The EIA was able to detect the ACTH induced increase in FCM concentrations in both sexes, as well as several metabolites in our radiometabolism study. Also, a testosterone EIA (measuring 17β-hydroxyandrostanes), first described by Palme and Möstl (1994), showed to be suitable for assessing fecal testosterone metabolites in this species.

We observed strong diurnal fluctuations in FCM concentrations in bank voles. We kept the animals under a light regime resembling the natural light cycle in the north during summer. Variation in light/dark cycle length will most likely affect adrenocortical activity (both in plasma corticosterone and in FCM levels), but its influence needs to be further evaluated. Anyway, based on the observed variations which were also found in other small rodents (Touma et al., 2004; Lepschy et al., 2007) it is critical to take time of day (and day length) into account when planning experiments. However, the highest concentrations in both sexes tended to occur during afternoon hours, but females also expressed high levels in the early morning. Opposite to mice (Touma et al., 2003) the amounts of measured FCM were significantly higher in males than in females. A similar dimorphism has been observed in Syrian hamsters and common hamsters and is likely caused by the larger adrenal glands and greater steroid production in males (Huhman et al., 2003; Franceschini et al., 2007; Chelini et al., 2010). However, sometimes the chosen EIA detects more FCM formed by one sex, which can cause sex differences in the results (Touma et al., 2003). The ACTH challenge experiment confirms the physiological sensitivity of the selected EIA, as there was a clear and significant increase in the FCM concentrations in both sexes. Peak FCM values were detected around 6–8 h after the administration of the ACTH in the feces. The radiometabolism study confirmed this time lag. Additional peaks (after 12–16 h) probably reflected an enterohepatic recirculation of some metabolites (Palme et al., 2005). A similar time lag has been observed in mice (Touma et al., 2003), spiny mice (*Acomys cahirinus*; Nováková et al., 2008) and even in red squirrels (Dantzer et al., 2010) whereas in syrian hamster, member of the same family as bank voles, the gut passage time is 3–4 times

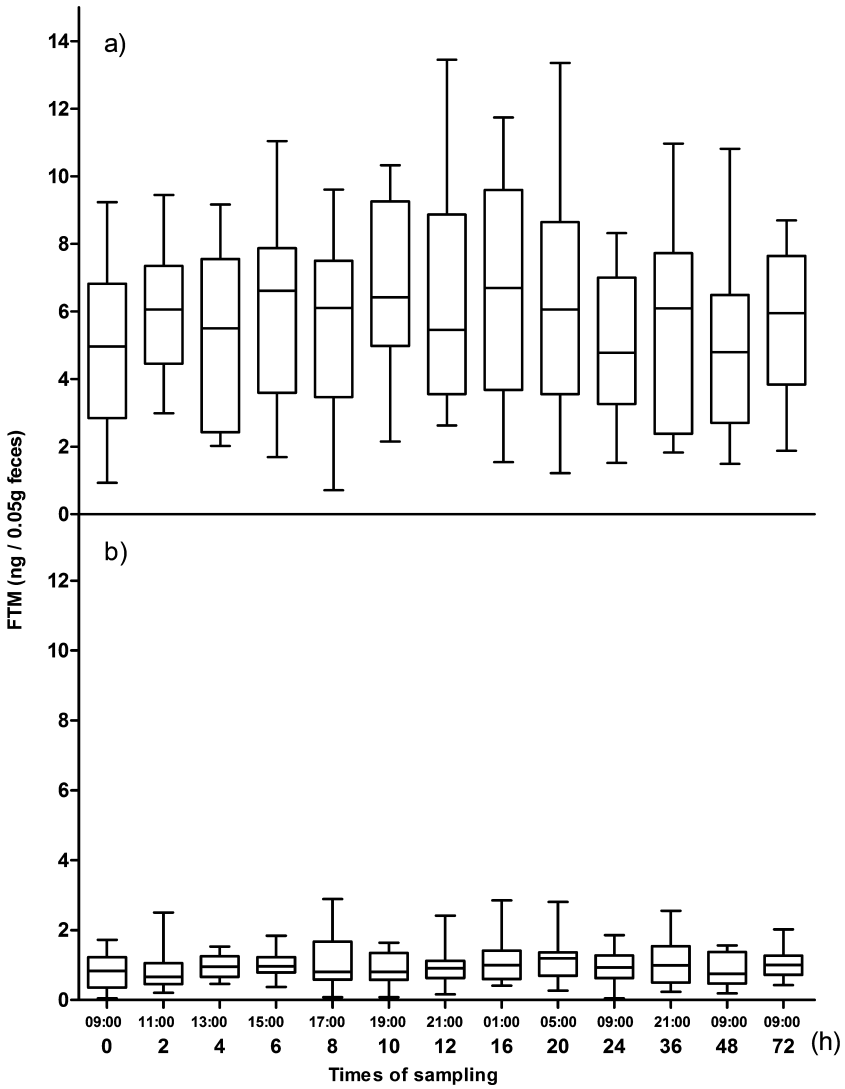

Fig. 5. Undisturbed diurnal variations of fecal testosterone metabolites (FTM) in males (a) and in females (b). Light/dark cycle in the laboratory during the experiment was 18L:6D with dark phase from 24:00 till 06:00.

longer (Chelini et al., 2010). This time indicates the average gut passage time of corticosterone metabolites in bank voles, which is a highly important piece of information when examining the endocrine status of animals based on excreta (Palme et al., 2005). As found in almost all studies dealing with FCM (Palme et al., 2005; Touma and Palme, 2005) individual differences in secretion and excretion of corticosterone were also present in our study. Besides other reasons (Goymann, 2012), this could be a consequence of a varying activity rhythm in bank voles. Bank voles are largely nocturnal but they have a polyphasic activity pattern consisting of several activity bouts also during the day (Ylönen, 1988; Halle, 2000). The occurrence of these activity bouts can vary between individuals which may appear as inconsistency in the results. Nevertheless, adding up the diel variation in FCM concentrations and the observed gut passage time it seems that on average the strongest corticosterone production occurs during the early hours at dawn.

The radiometabolism studies also revealed that there are clear sex-specific differences in the excretion route of FCM in bank voles. Males excreted around 70% of FCM via feces and 30% via urine, whereas in females the ratio is nearly half-and-half (51.5% vs 48.5%). Interestingly these percentages were almost identical to those found in mice (Touma et al., 2003). HPLC immunograms showed that there were also some sex-specific differences in the corticosterone metabolites excreted in feces. Females produced a wider range of metabolites, some being even less polar than corticosterone. The HPLC also shows that plasma corticosterone is heavily metabolized, which indicates that there are almost no measurable amounts of corticosterone left in the feces, only its metabolites. This strongly supports the use of group specific EIAs particularly designed for detecting metabolites, rather than the actual steroid when using fecal samples (Möstl et al., 2005; Fanson et al., 2016).

For testing the biological sensitivity of the selected EIA for testosterone metabolites, we relied on a biological validation by comparing the natural FTM concentrations of males and non-pregnant females. The EIA was able to detect even the low concentrations of FTM in females, and to show the expected differences in FTM concentrations between males and females. There were diurnal fluctuations in FTM levels (indicated by a significant effect of "time of day"), but with no clear pattern. However, the highest FTM concentrations in males occurred at night. Based on the radiometabolism study, testosterone is slower metabolized and excreted than corticosterone. The highest values of FTM in feces

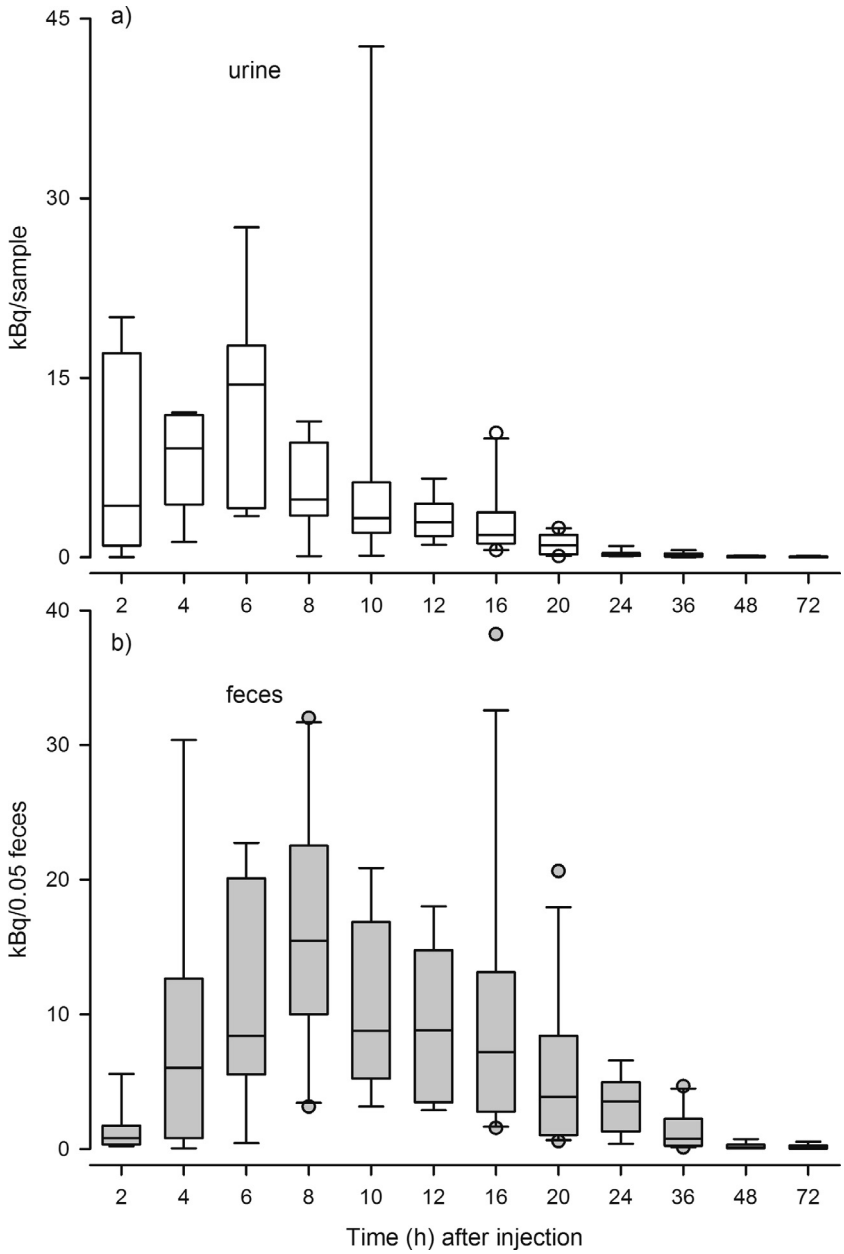

Fig. 6. Time course of the excretion of ^{3}H-testosterone metabolites (kBq) in urine (a) and feces (b) of males and females grouped together.

were observed 8 h after injection, whereas the peak values in urine occurred after 6 h. These time lags are similar to the results reported with house mice and deer mice (*Peromyscus maniculatus*; Billitti et al., 1998). Adding up the gut passage time, the highest testosterone production seems to occur around morning and before noon. With the excretion route there was no significant difference between the sexes. The main excretion route for FTM was via the feces, as was also found in house mice and deer mice (Billitti et al., 1998). The HPLC showed that also the injected testosterone was heavily metabolized. One prominent, although not identical peak of radioactivity accompanied with some smaller ones was present in both sexes. Unfortunately the testosterone EIA was not able to detect the main metabolite peak, but several minor ones. However, the physiological validation showed that the EIA detected the expected difference (Dreau et al., 1997; Tiwari et al., 2010) in the FTM concentration between males and non-pregnant females based on those less prominent metabolites formed and excreted. Billitti et al. (1998) also found a comparable testosterone EIA suited for measuring FTM in house and deer mice. However, more studies are needed to determine the influence of the season or reproductive status (for example pregnant females were found to have elevated FTM levels in red squirrels; Dantzer et al., 2011b) on FTM concentrations in bank voles and evaluating the stimulatory effect of GnRH or LH injections on FTM levels might also be advantageous to further validate the method.

However, most strikingly our radiometabolism experiments revealed for the first time significant differences between steroids in the time course of their excretion. Corticosterone was excreted faster than testosterone, both in urine (median of peak levels: 4 h vs 6 h) and feces (6 h vs 8 h). In general, steroids are quickly removed from the circulation by the liver and excreted via the kidney into the urine or via the bile into the duodenum. This species-specific delay between injection (and thus peak concentrations in the plasma) and the appearance of the respective signal in the feces

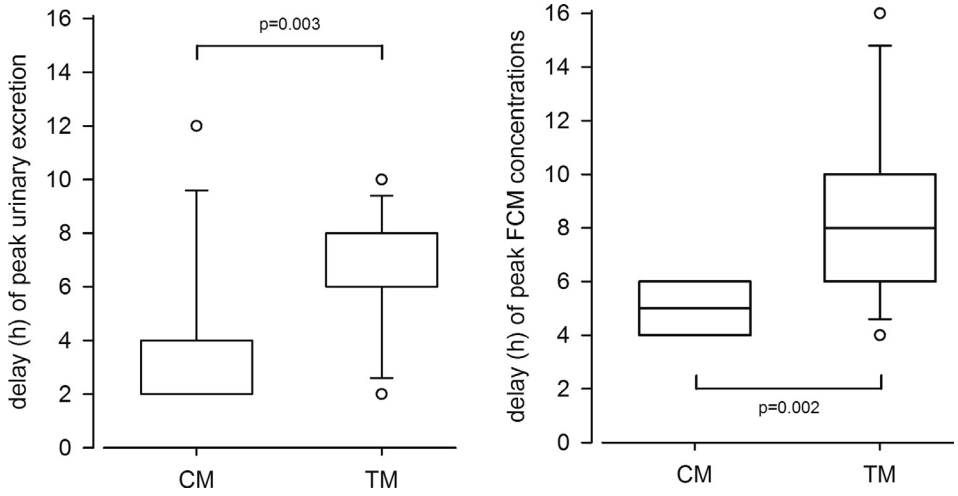

Fig. 7. Boxplots of delay (h) of peak excretion of ^{3}H-corticosterone (CM) and ^{3}H-testosterone metabolites (TM) in urine (left panel) and feces (right panel) of females and males grouped together.

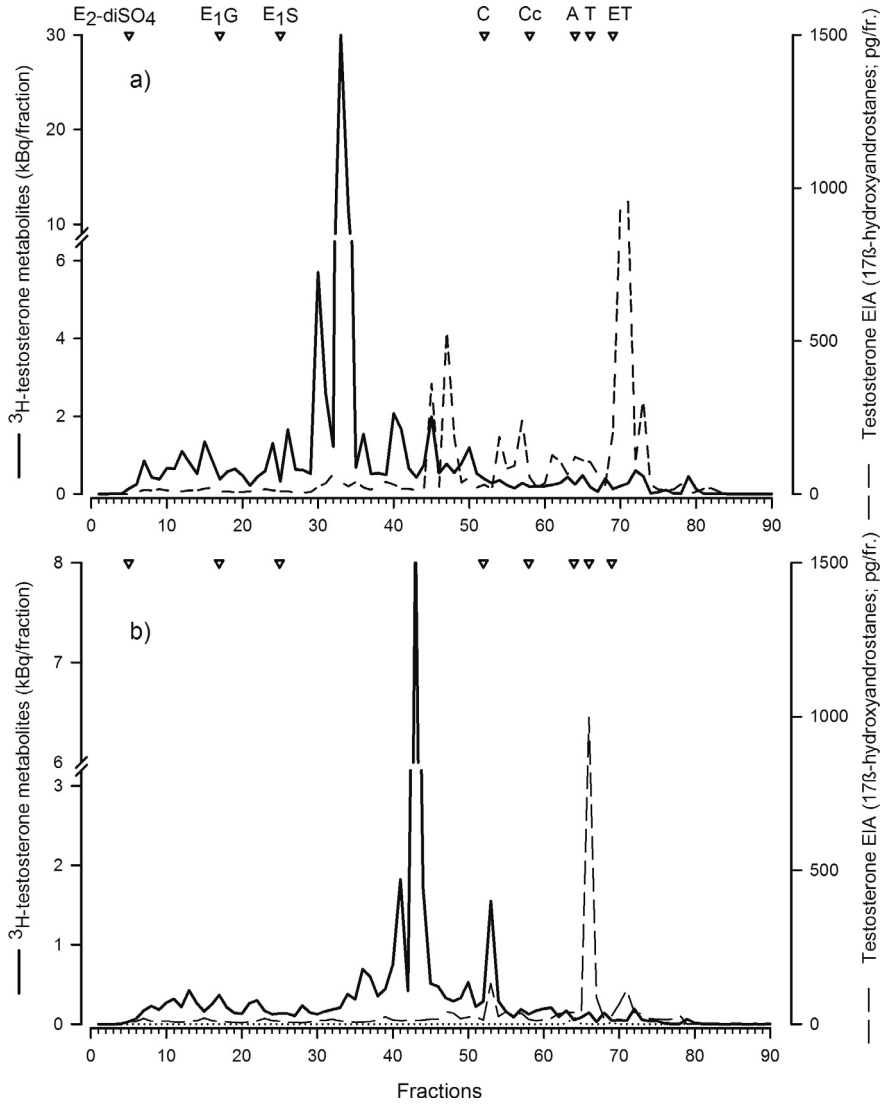

Fig. 8. High performance liquid chromatographic (HPLC) separation of fecal ^{3}H-testosterone metabolites of one male (a) and one female (b). Immunoreactivity was tested with the testosterone enzyme immunoassays. Open triangles mark the approximate elution positions of respective standards (E_2-diSO$_4$ = 17β-estradiol-disulfate, E_1-G = estrone-glucuronide, E_1S = estrone-sulfate, C = cortisol, Cc = corticosterone, A = androstenedione, T = testosterone, ET = epitestosterone).

was found to be closely related to the animals' intestinal transit time from duodenum to rectum (Palme et al., 2005). As animals were kept under the same conditions the delayed excretion of testosterone might most likely be attributed to its slower removal from the circulation. Plasma binding proteins are known to be important modulators of steroid action in mammals (Hammond, 2011). Steroids bound to such proteins are less prone to metabolism. Although knowledge in bank voles is lacking, higher amounts of sex hormone binding globulin could be a reason for such a delayed excretion.

With these validation experiments we were able to provide essential background knowledge of the endocrine physiology in bank vole, and to validate a 5α-pregnane-3β,11β,21-triol-20-one EIA for measuring fecal corticosterone metabolites and preliminary a testosterone EIA for measuring fecal androgen metabolites (17β-hydroxyandrostanes) in this species. Further studies, regarding the influence of season and reproductive status on FCM and especially FTM levels are needed. Those methods for noninvasive monitoring of adrenocortical activity and testosterone secretion in this model species will be of great use for further endocrinological studies.

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