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Non-invasive measurement of adrenocortical and gonadal activity in male and female guinea pigs (*Cavia aperea f. porcellus*)

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ABSTRACT

Taking blood samples is a common method in biomedical and biological research using guinea pigs. However, most blood sampling techniques are complicated and highly invasive and may therefore not be appropriate for certain research topics concerning stress and reproduction. Thus, a non-invasive method to measure steroid hormones is critically needed. The aim of this study was the biological validation of corresponding enzyme immunoassays for the measurement of fecal cortisol, progesterone, estrogen, and testosterone metabolites in guinea pigs. We examined the effect of subcutaneous injections of ACTH or saline on fecal cortisol metabolites to investigate the suitability of fecal samples to monitor adrenocortical activity. Furthermore, we investigated whether fecal sex steroid metabolites accurately reflected endocrine changes observed in plasma samples during female estrous cycles and male puberty, respectively. In addition, we compared fecal testosterone metabolites of intact males, castrated males, and females to investigate the reliability of fecal samples in discriminating gonadal status of males. Concentrations of fecal cortisol metabolites were significantly increased following ACTH challenge, indicating that adrenocortical activity can be monitored via fecal samples. Secondly, in females, plasma and fecal gonadal steroids were significantly correlated in most subjects. The assay for testosterone metabolites, on the other hand, could not clearly discriminate between test groups. From these findings we conclude that fecal samples can be used for the non-invasive assessment of adrenocortical and female reproductive status in guinea pigs. Testosterone metabolism seems to be more complex and further investigations are needed to establish a more suitable assay.

1. Introduction

Guinea pigs are commonly used as animal models in biomedical and behavioral research. Scientific knowledge about their physiology and behavior has grown constantly during the last decades. Most of the physiological data have been obtained with the use of blood samples. Apart from bleeding after decapitation (e.g. Fujieda et al., 1982; Rigaudière et al., 1976), blood collection in guinea pigs is often performed by cardiac puncture (e.g. Challis et al., 1971; Feder et al., 1968; Garris, 1986), toenail clipping (e.g. Grace and O'Dell, 1970), puncturing of the ear veins (e.g. Sachser and Pröve, 1984), or implantation of catheters (e.g. Blatchley et al., 1976; Liu et al., 2001). All of these methods are highly invasive and in most cases require the use of anesthesia, which can profoundly confound experimental results. Therefore, for certain recent topics concerning the behavioral endocrinology of guinea pigs these sampling techniques may not be the methods of choice. For example, longitudinal studies using repeated endocrine measurements for the investigation of e.g. HPA- or HPG-axes development may greatly profit from a non-invasive sampling technique. Similarly, investigations of hormone-behavior relationships may be more accurate if a non-invasive approach is used.

When monitoring adrenocortical activity, blood samples may not be suitable because they represent hormone concentrations at only a single point of time. This can be misleading, because cortisol, which is the major adrenal glucocorticoid in guinea pigs (Dalle and Delost, 1974; Malinowska and Nathanielsz, 1974), as well as testosterone, shows diurnal variation (Sachser, 1994). Furthermore, handling, physical restraint, and the blood sampling procedure itself may profoundly affect cortisol concentrations: Sachser (1994) showed that plasma cortisol concentrations of male guinea pigs increased significantly within the first 5 min after catching the subject. Certain types of subjects like pregnant females or juveniles may react even more sensitively to blood sampling. Stressful events during the later stages of pregnancy can have dramatic effects on the offspring's physiology, behavior, and morphology (reviewed in Kaiser and Sachser, 2005; Owen et al., 2005).

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Another serious problem concerning the long-term monitoring of the reproductive status and development in guinea pigs is the blood volume that is required for the simultaneous analysis of both female sex steroids (progesterone and estrogens). Moreover, for the successful investigation of reproductive-endocrine relationships, repeated sampling is necessary. Pregnant guinea pigs, however, should be handled as little as possible in the later stages of pregnancy to avoid preterm labor, which makes repeated blood sampling and large sample volumes problematic. In juveniles, on the other hand, the required sample volume is rarely obtained because of their smaller body size with the consequence that only one sex steroid can be analyzed in most cases. Considering all these problems, there is an urgent need for a practical and non-invasive solution to monitor the HPA- and HPG-axes activity in guinea pigs.

Analyzing hormones in fecal samples can be a useful alternative and has several advantages (for review see: Möstl and Palme, 2002; Palme, 2005; Touma and Palme, 2005). This completely non-invasive technique has already been used and validated successfully to monitor gonadal and adrenocortical activity in numerous species of laboratory, domestic, zoo, and wild animals (e.g. Huber et al., 2003b; Schwarzenberger, 2007; Touma and Palme, 2005). Circulating steroid hormones are metabolized by the liver and excreted via the kidneys into the urine or via the bile into the gut. Depending on the intestinal passage time, fecal steroid metabolites are then excreted with a species-specific time lag (Palme et al., 2005). Consequently, fecal hormone metabolites reflect the production rate of a specific hormone over a certain time period, and unlike blood samples, are less affected by short-term fluctuations of hormone secretion. In this way, steroid metabolites measured from feces may reflect the overall hormonal status of a subject better than plasma samples (Touma and Palme, 2005). Collecting feces is easy and can be done without handling the animal. So, repeated sampling is possible even in pregnant or young subjects and does not interfere with the animal's endocrine status. Additionally, even very small amounts of sample are sufficient for several different hormone assays. Another advantage is that sensitive enzyme immunoassays (EIAs) can measure hormone metabolites in feces even if only a very small percentage is excreted via the feces (Palme et al., 1996).

Although non-invasive methods have been validated and used successfully in many species, no information is available on validated assays measuring fecal steroid metabolites in guinea pigs. As the guinea pig is a commonly used model species in biological research, the aim of this study was the biological validation of corresponding EIAs for the measurement of fecal cortisol, progesterone, estrogen, and testosterone metabolites in guinea pigs. This validation was achieved by examining: (1) the effect of subcutaneous injections of saline or ACTH on fecal cortisol metabolites in order to investigate the suitability of fecal samples to monitor adrenocortical activity, (2) whether fecal metabolites of gonadal steroids accurately reflected hormonal changes during estrous cycles of females and hormonal puberty of males, respectively, and (3) the reliability of the assay system in discriminating gonadal status of males by comparing fecal testosterone metabolites of intact males, castrated males, and females.

2. Methods

Experiments were performed with the permission of the Austrian Federal Ministry for Education, Science, and Culture (GZ. 66.006/0024-BrGT/2005).

2.1. Fecal glucocorticoid metabolites (experiments 1a–1c)

2.1.1. Sample collection

Cages were provided with sawdust as bedding material. We collected excreta directly from the bedding material, and always sampled all fecal pellets from each cage. During the whole sampling procedure, the animals stayed in their cages and were not handled. Urine-contaminated feces were not used in the analysis. After sample collection, we renewed urine-contaminated bedding material. During the dark period, we collected samples under dimmed light conditions (~5 lux). Immediately after collection, samples were frozen and stored at −20 °C until further analysis.

2.1.2. Subjects and housing conditions

Six male and 6 female guinea pigs (*Cavia aperea f. porcellus*) from a heterogeneous multicolored stock were used in experiments 1a–1c. Subjects did not differ substantially in age and body weight (age: 18 ± 4 months, body weight: 995 ± 40 g). Females were neither pregnant nor lactating. Before the beginning of the experiment, animals were housed in isosexual groups of two (cage size 90 × 48 × 40 cm). The housing room was maintained under standard conditions (LD 12:12, lights on at 0600 h; 20 ± 2 °C). Each subject received 40 g guinea pig pellets (Altromin 3013, Lage, Germany), supplemented with 40 g fruit and vegetables and a handful of hay daily at 1030 h. Water was available ad libitum. Twenty-four hours before the beginning of experiment 1a, subjects were separated and housed individually for the duration of experiments under the same conditions as described above. During this 24 h-habituation phase, we collected fecal samples in 6 h-intervals to habituate guinea pigs to individual housing and the sampling procedure.

2.1.3. Experimental design

In experiments 1a–1c, we used the same 12 subjects. In this way, each animal acted as its own control. Experiment 1a was performed as a control experiment to analyze diurnal variation of fecal cortisol metabolites. Animals were left undisturbed and did not receive any injections. Experiment 1a lasted 48 h starting at 1200 h. In accordance with experiments 1b and 1c, we collected fecal samples in 2 h-intervals during the first 24 h and in 6 h-intervals during the second 24 h.

Experiment 1b, the second control experiment, investigated the effect of handling and the injection procedure itself, and was performed immediately after experiment 1a. It lasted 72 h: immediately after the final sampling session of experiment 1a at 1200 h, each subject received an s.c. injection of 0.2 ml sterile isotonic saline solution. As in experiment 1a, we collected fecal samples in 2 h-intervals during the first 24 h and in 6 h-intervals during the second 24 h. During the third and last 24 h, we took samples in 12 h-intervals.

Experiment 1c started immediately after experiment 1b. To stimulate adrenocortical activity, we performed an ACTH challenge. Each subject was injected s.c. with 0.2 ml Synacthen® Depot (equivalent to 0.2 mg or 20 IU $ACTH_{1-24}$; Novartis Pharma GmbH, Nürnberg, Germany) at 1200 h. Fecal sampling intervals followed the procedures of experiment 1b. In both experiments 1b and 1c, the procedure of capturing the animal, injection, and returning the subject into its cage lasted a maximum of 3 min.

2.2. Fecal progesterone metabolites and estrogen (experiment 2)

2.2.1. Subjects and housing conditions

The 7 females used in experiment 2 were different individuals than those used in experiments 1a–1c, although part of the same heterogeneous stock. These female subjects did not differ in mean age or body weight (age: 16 ± 3 months, weight: 945 ± 35 g), and were neither pregnant nor lactating during the experiment. They were kept under the same housing and feeding conditions as described in experiment 1. Twenty-four hours before the beginning of the experiment, we separated the 7 females from their isosexual groups. They were held individually for the duration of the experiment (cage size in both situations 90 × 48 × 40 cm).

2.2.2. Experimental design and sample collection

During this experiment, we collected blood and fecal samples once a day for 22 consecutive days. We chose this particular time period to ensure that each subject ran through a full estrous cycle, the mean duration of the guinea pig estrous cycle being 16 days (Selle, 1922). The vaginal membrane of each subject was checked visually between 1100 and 1200 h once a day to determine estrus. During estrus, the membrane is open for 1–6 days (Weir, 1970), and this vaginal opening correlates well with physiological estrus (Young, 1937). We defined estrus as the first day the vagina was fully opened (Day 0 of the cycle).

Blood and fecal samples were collected around midday (1100–1200 h). We took blood samples without anesthetizing the subjects following the protocol of Sachser and Pröve (1984): each sample (500 μl) was collected with heparinized capillaries by puncturing the marginal ear vessels with a sterile lancet. After centrifugation, the plasma was stored at −20 °C until further analysis. The whole sampling procedure including catching the female, taking the sample, and returning the animal to its cage, lasted maximally 3 min. After taking the blood samples, the bedding material in the cages was renewed. We collected feces directly after defecation from the bedding material without handling the animal.

2.3. Fecal testosterone metabolites (experiments 3a and 3b)

2.3.1. Subjects and housing conditions

In experiment 3a, we used 19 juvenile intact males, and in experiment 3b, we used 4 castrated and 5 intact adult males, and 5 non-pregnant, non-lactating adult females. All were descendants of the same heterogeneous stock as the subjects used in the other experiments. In the 4 castrated males, gonadectomy had been performed at least 6 months before the beginning of the experiment. Housing and

feeding conditions were identical to experiments 1 and 2. Age and body weight of the adult subjects were similar to experiments 1 and 2. The 19 juveniles were kept in separate littering cages with their mothers and litter mates (1 adult lactating female + 3–4 pups/cage) until weaning (3 weeks of age). Afterwards, they were held in isosexual pairs.

2.3.2. Experimental design and sample collection

Experiment 3a was designed to investigate whether the hormonal changes that are observed in plasma samples during male puberty (Rigaudière et al., 1976) are also reflected in fecal samples. In this sense, we collected plasma and fecal samples from each juvenile male in weekly intervals (±2 days) starting at the age of 12 days until 133 days of age. We took blood samples (100 μl) using the method of Sachser and Pröve (1984) as described above. For sample collection, we shortly separated each juvenile male from his litter or pair group (for maximally 20 min), respectively, and immediately took the blood sample. The whole sampling procedure including catching the male and taking the blood sample lasted maximally 3 min. After blood sampling, we transferred the subject into a separate cage for fecal sample collection. After defecation, it was immediately returned to its home cage. Sample collection took place between 0900 and 1000 h. We collected fecal samples directly from the cage floor without further handling of the subject.

In experiment 3b, we collected 5 fecal samples/subject in weekly intervals from 4 castrated, 5 intact males, and 5 females to investigate if gonadal status can be evaluated using fecal samples. We also applied the same fecal sampling procedure as in experiment 3a. Animals were shortly separated from their isosexual group for sample collection and returned immediately afterwards.

2.4. Endocrine analyses

2.4.1. Fecal steroid analyses

Extraction of fecal samples followed the procedure described by Palme and Möstl (1997): for each sample, 0.5 g of homogenized wet feces was suspended in 5 ml of 80% methanol. The suspended sample was vortexed for about 2 min. After centrifugation (15 min at 2500g), an aliquot of the supernatant was diluted with assay buffer (final dilution: testosterone metabolites 1:50; all other steroids 1:10) and frozen until further analysis.

Cortisol is the major glucocorticoid in guinea pigs (Dalle and Delost, 1974; Malinowska and Nathanielsz, 1974). As cortisol is heavily metabolized in the liver before excretion, we used a group-specific 11-oxoetiocholanolone-EIA measuring 3α-OH-11-one cortisol metabolites in feces (Möstl et al., 2002). The cross-reactions of the EIA with relevant steroids (Steraloids, Wilton, USA) were as follows: 5β-androstane-3α-ol-11,17-dione, 100%; 5β-pregnane-3α-ol-11,20-dione, 37%; 5β-androstane-3α,11β-diol-17-one, 3.3%; 5β-androstane-3,11,17-trione, 1.2%. All other steroids tested had cross-reactions below 1%. The intra- and interassay coefficients of variation were 9.5% and 11.3%. Sensitivity of the assay at 90% binding was 2.0 pg/well.

Progesterone is also almost completely metabolized before its fecal excretion, with the consequence that authentic progesterone is detectable only in very small amounts (reviewed in Schwarzenberger, 2007). Therefore, we used a group-specific assay established for fecal progesterone metabolites (5α-20-one EIA; Schwarzenberger et al., 1996). This assay cross-reacted with relevant steroids as follows: progesterone, 100%; 4-pregnene-3β-ol-20-one, 14%; 4-pregnene-3α-ol-20-one, 390%; 5α-pregnane-3α-ol-20-one, 89%; 5α-pregnane-3β-ol-20-one, 56%; 5α-pregnane-3,20-dione, 168%; 5β-pregnane-3α-ol-20-one, 88%; 5β-pregnane-3β-ol-20-one, 5%; 5β-pregnane-3,20-dione, 26%; 5-pregnene-3β-ol-20-one, 17%. All other steroids tested had cross-reactions below 1%. The intra- and interassay coefficients of variation were 11.3% and 16.2%. Sensitivity of the assay at 90% binding was 2.6 pg/well.

As fecal estrogens in mammals, on the other hand, consist mainly of estrone, estradiol-17α or -17β, an antibody against total estrogens was used for their determination (Palme and Möstl, 1994). Cross-reactions with relevant steroids were as follows: estrone, 100%; estradiol-17α, 19%; estradiol-17β, 70%; estriol, 129%; 1,3,5(10),7-estratetraene-3,17β-diol, 20%; 1,3,5(10),7-estratetraene-3-ol-17-one, 87%; 1,3,5(10),7-estrapentaene-3-ol-17-one, 1%. All other steroids tested had cross-reactions below 1%. The intra- and interassay coefficients of variation were 12.4% and 14.3%. Sensitivity of the assay at 90% binding was 0.1 pg/well.

For testosterone, we used an assay measuring 17β-OH-androgens (Palme and Möstl, 1994). This assay cross-reacted with relevant steroids as follows: testosterone (100%), 5α-androstane-17β-ol-3-one (24%) and 5β-androstane-17β-ol-3-one (12%), 4-androstene-3β,17β-diol (8%), 5α-androstane-3α,17β-diol (6%), 5α-androstane-3β,17β-diol (1%), 5β-androstane-3β,17β-diol (1%). All other steroids tested had cross-reactions below 1%. The intra- and interassay coefficients of variation were 7.9% and 12.9% for testosterone metabolites, respectively. Sensitivity of the assay at 90% binding was 0.3 pg/well.

2.4.2. Plasma steroid analyses

For the analysis of blood samples, we extracted gonadal steroids from plasma (200 μl in females, 20 μl in males) with 5 ml of diethylether. After evaporation of the ether, assay buffer was added (2:1 for estrogens, 1:2 for progesterone, 1:10 for testosterone). For the estrogen and testosterone assays, the same antibodies as for the fecal samples were used (for details see above). For progesterone, we used a specific progesterone EIA (4-ene-20-one-EIA; Schwarzenberger et al., 1996). This assay cross-reacted with relevant steroids as follows: progesterone, 100%; 4-pregnene-3β-ol-20-one, 26%; 4-pregnene-3α-ol-20-one, 8%; 5α-pregnane-3α-ol-20-one, 5%; 5α-pregnane-3β-ol-20-one, 18%; 5α-pregnane-3,20-dione, 40%; 5β-pregnane-3α-ol-20-one, 3%; 5β-pregnane-3β-ol-20-one, 7%; 5β-pregnane-3,20-dione, 71%; 5-pregnene-3β-ol-20-one, 29%. All other steroids tested had cross-reactions below 1%. The intra- and interassay coefficients of variation were 10.4% and 12.8%, respectively. Sensitivity of the assay at 90% binding was 1.5 pg/well.

2.5. Statistical analyses

We carried out all statistical tests with SPSS 13.0 (SPSS Inc., USA). As not all data were normally distributed and sample sizes were relatively small, we consequently used non-parametric tests. For cortisol metabolites: (i) we calculated medians for each time point of an individual over the two sampling days of experiment 1a for the analysis of the diurnal variation of cortisol metabolites, and used Friedman tests for the comparison of these medians. Friedman tests were also used to investigate more than two related time points within experiments 1b and 1c. As this statistical procedure test does not allow missing values in the data set (i.e. the whole subject is eliminated from the analysis when a value is missing in the time series), we used linear interpolation to eliminate gaps in the cortisol data (SPSS 13.0). (ii) As each animal served as its own control, consequently, we also used multiple Wilcoxon- and Friedman tests (Bonferroni-corrected) for comparing related time points between experiments. (iii) Male and female cortisol data were compared using Mann–Whitney *U*-tests.

To investigate whether fecal progesterone, estrogen, and testosterone correlated with plasma gonadal steroids, we used Spearman correlations. We correlated corresponding plasma and fecal data either within the same subject (progesterone and estrogens) during the estrous cycle, or at certain time points (testosterone). Plasma and fecal estrogen values could only be correlated in three subjects, because in the other four subjects we did not obtain enough plasma for both the progesterone and the estrogen assay.

For the comparison of fecal testosterone metabolites in intact males, castrated males, and females, we calculated the median for each subject from repeated measurements and performed a Kruskal–Wallis test afterwards.

Differences were considered significant when $P \leqslant 0.05$.

3. Results

3.1. Fecal cortisol metabolites (experiments 1a–1c)

As male and female data did not differ significantly at any sample point (Mann–Whitney *U*-test, $P > 0.05$ for each sample point), we combined male ($n = 6$) and female ($n = 6$) datasets in the analysis. In experiment 1a, excretion of fecal cortisol metabolites showed a significant diurnal variation (Friedman ANOVA, $n = 12$, $\chi^2 = 21.19$, df = 11, $P = 0.031$) with maximal values measured during the first third of the dark phase, i.e. the sampling period between 2000 and 2200 h (median_{2000h}: 639.97 ng/g, range_{2000h}: 220.31–1000.14 ng/g; median_{2200h}: 516.50 ng/g, range_{2200h}: 277.31–1210.64 ng/g). Afterwards concentrations decreased and reached minimal values around the beginning of the light phase (median_{0600h}: 333.96 ng/g, range_{0600h}: 206.14–796.4 ng/g).

In contrast to the control experiment, a significant diurnal variation of fecal cortisol metabolites was absent in experiment 1b (median_{2000h}: 362.90 ng/g, range_{2000h}: 199.89–793.56 ng/g; median_{0600h}: 288.12 ng/g, range_{0600h}: 213.55–692.12 ng/g; Friedman ANOVA, $n = 12$, $\chi^2 = 20.85$, df = 18, $P = 0.287$). Furthermore, we did not find a significant effect of saline injection on concentrations of cortisol metabolites compared to experiment 1a (Wilcoxon signed ranks test, Bonferroni-corrected $^*P < 0.0029$, all sampling points $P > 0.0029$; Fig. 1).

In the ACTH challenge (experiment 1c), we found a highly significant variation of fecal cortisol metabolites (Friedman ANOVA, $n = 12$, $\chi^2 = 177.81$, df = 18, $P < 0.001$; Fig. 1). A significant increase in concentrations was measured 8 h after the injection (median_{8hpi}: 971.545 ng/g, range_{8hpi}: 259.47–2634.28 ng/g). Peak concentrations were observed 18 h (median; range: 14–20 h) after the injection (median_{18hpi}: 2646.56 ng/g, range_{18hpi}: 1394.25–5213.23 ng/g).

Concentrations of cortisol metabolites between ACTH treatment and control/saline treatments were significantly different between 8 and 48 h after the injection (Friedman ANOVA (0–48 h)/Wilcoxon

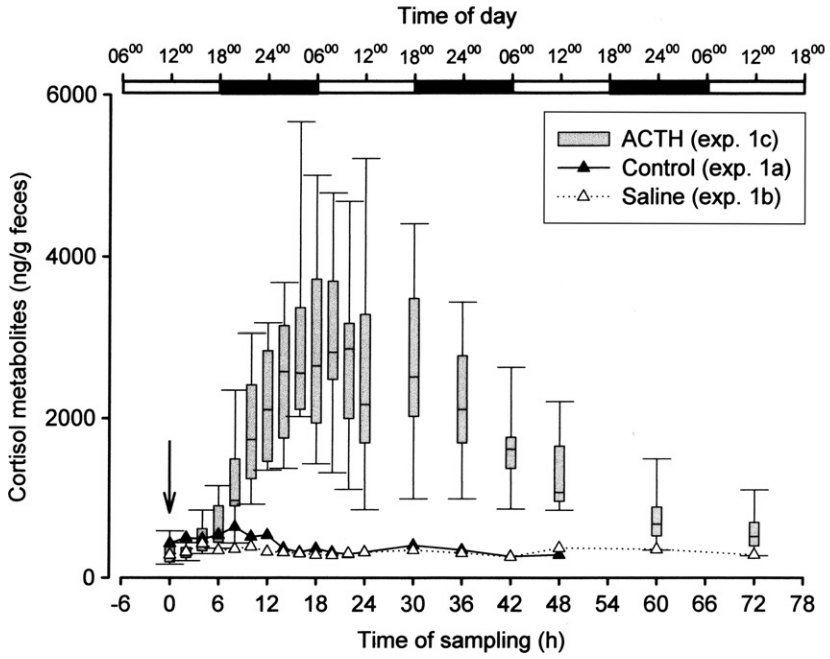

Fig. 1. Excretion pattern of cortisol metabolites in fecal samples of untreated (control, solid triangles, experiment 1a)-, saline injected (open triangles, experiment 1b)-, or ACTH injected (box–whisker-plots, experiment 1c) guinea pigs. The arrow marks the time of injection. Symbols (open/solid triangles) represent medians. Box–whisker-plots show medians (lines in the boxes), 25% and 75% quartiles (boxes), and 10% and 90% ranges (whiskers). After Bonferroni-correction, significant differences between treatments were found between 8 and 48 h after injection. Light (white horizontal bars) and dark (black horizontal bar) phase periods are indicated at the top. Sample size (individuals): $N = 12$.

test (60–72 h), Bonferroni-corrected $^{*}P < 0.0026$; 0–6 h: $P > 0.0026$, 8–48 h: all $P < 0.002$, 60–72 h: $P > 0.0026$; Fig. 1).

3.2. Progesterone and estrogen in plasma and feces (experiment 2)

Progesterone and estrogen concentrations in plasma and fecal samples were strongly correlated. In 5 out of 7 female subjects, the positive correlation found between plasma and fecal progesterone concentrations was significant and in another female marginally significant (Table 1). Estrogen concentrations in plasma and fecal samples were significantly positively correlated in each of the three analyzed subjects (Table 1). In plasma and fecal samples, progesterone concentrations were low around estrus (between Days −3 and 2; e.g. Day 0: $\text{median}_{\text{plasma}}$: 0.36 ng/ml, $\text{range}_{\text{plasma}}$: 0.10–1.20 ng/ml; $\text{median}_{\text{feces}}$: 73.70 ng/g, $\text{range}_{\text{feces}}$: 1.00–275.00 ng/g), and increased thereafter, with the highest peak found at Day −7 in plasma and at Day −5 in feces (Fig. 2a; Day −7: $\text{median}_{\text{plasma}}$: 1.65 ng/ml, $\text{range}_{\text{plasma}}$: 0.19–6.30 ng/ml; Day −5: $\text{median}_{\text{feces}}$: 349.00 ng/g, $\text{range}_{\text{feces}}$: 63.00–635.00 ng/g). Estrogen values peaked at Day −5 ($\text{median}_{\text{plasma}}$: 0.054 ng/ml, $\text{range}_{\text{plasma}}$: 0.002–0.106 ng/ml; $\text{median}_{\text{feces}}$: 9.10 ng/g, $\text{range}_{\text{feces}}$: 0.60–17.60 ng/g) and at Days 0 and 1 of the estrus cycle in both plasma and fecal samples (Fig. 2b; Day 0: $\text{median}_{\text{plasma}}$: 0.053 ng/ml, $\text{range}_{\text{plasma}}$: 0.011–0.144 ng/ml; $\text{median}_{\text{feces}}$: 9.20 ng/g, $\text{range}_{\text{feces}}$: 1.80–28.90 ng/g; Day 1: $\text{median}_{\text{plasma}}$: 0.051 ng/ml, $\text{range}_{\text{plasma}}$: 0.014–0.088 ng/ml; $\text{median}_{\text{feces}}$: 8.85 ng/g, $\text{range}_{\text{feces}}$: 2.30–15.40 ng/g).

Table 1
Spearman correlation between sex steroids measured in plasma and fecal samples of 7 adult female guinea pigs during the estrous cycle

Subject	Progesterone			Estrogen		
	n[a]	r_S	P	n[a]	r_S	P
1	19	0.856	<0.001	19	0.794	<0.001
2	19	0.857	<0.001	19	0.996	<0.001
3	20	0.899	<0.001	20	0.958	<0.001
4	19	0.273	0.258			
5	19	0.815	<0.001			
6	19	0.482	0.036			
7	19	0.439	0.060			

[a] n represents the number of fecal samples from each subject.

3.3. Fecal testosterone metabolites (experiments 3a and 3b)

In both fecal and plasma samples of juvenile males, we observed a fluctuating pattern in testosterone concentrations during development, with high peaks during the early pubertal period and regular smaller peaks in adulthood (Fig. 3). However, fecal and plasma testosterone levels did not correlate significantly at any point of time (Spearman correlation, Bonferroni-corrected $^{*}P < 0.0028$, all $P > 0.0028$). When we compared testosterone metabolites of adult intact males, castrated males, and females (experiment 3b), we found overall significant differences among the three groups (Kruskal–Wallis test, $n_1 = 5$, $n_2 = 4$, $n_3 = 5$, $\chi^2 = 8.431$, df = 2, $P = 0.015$; $\text{median}_{\text{intact males}}$: 306.50 ng/g, $\text{range}_{\text{intact males}}$: 234.54–481.15 ng/g; $\text{median}_{\text{castrated males}}$: 210.37 ng/g, $\text{range}_{\text{castrated males}}$: 130.62–373.91 ng/g; $\text{median}_{\text{females}}$: 86.94 ng/g, $\text{range}_{\text{females}}$: 83.13–232.40 ng/g; Fig. 4). However, although intact and castrated males had significantly higher testosterone metabolite concentrations than females (Mann–Whitney U-test, intact males vs. females: $Z = -2.611$, $P = 0.009$; castrated males vs. females: $Z = -1.960$, $P = 0.050$; Fig. 4), castrated and intact males did not differ significantly (Mann–Whitney U-test, $Z = -1.225$, $P = 0.221$; Fig. 4).

4. Discussion

The ACTH challenge clearly demonstrated that in guinea pigs, adrenal activity is reflected in concentrations of fecal cortisol metabolites. Compared to the two control experiments, we found peak values of cortisol metabolites around 18 h after the ACTH-injection. This delay between the hormonal events in the blood

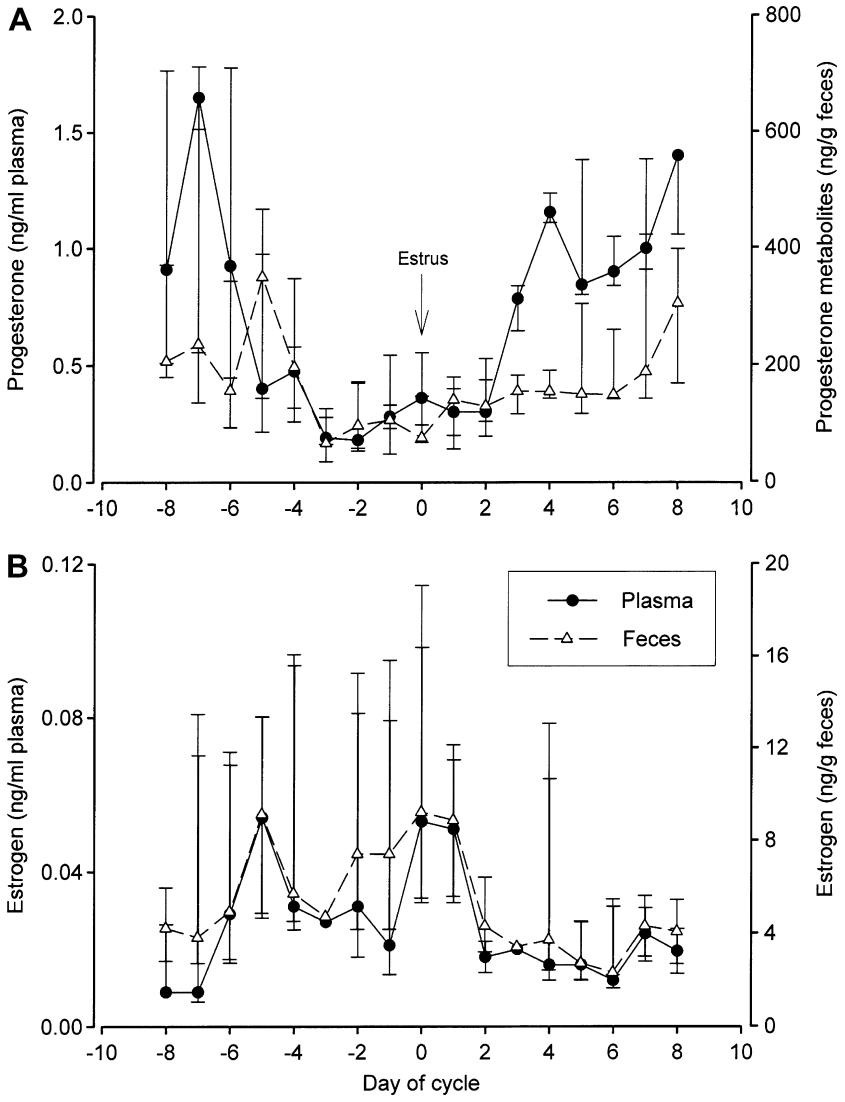

Fig. 2. Median progesterone (A) and estrogen concentrations (B) in plasma (solid circles) and feces (open triangles) during the guinea pig estrous cycle. Error bars represent 25% and 75% quartiles, respectively. The arrow indicates the time of estrus (Day 0); progesterone: sample sizes (individuals) at Day −8/−7/−6/−5/−4/−3/−2/−1/0/1/2/3/4/5/6/7/8 are 5/6/6/5/6/4/7/7/7/6/7/4/6/6/5/6/6; estrogen: sample sizes (individuals) on Day −8/−7/−6/−5/−4/−3/−2/−1/0/1/2/3/4/5/6/7/8 are 3/3/3/2/3/1/3/3/3/2/3/1/3/3/3/2/2.

and the manifestation of the respective signal in the feces was shown to be related to the intestinal transit time (Palme et al., 1996, 2005). The gut passage time in the guinea pig is approximately 20 h (Harkness and Wagner, 1995). Concentrations of fecal cortisol metabolites did not differ significantly between males and females at any time point, pointing to the possibility that fecal cortisol metabolites might be similar in male and female guinea pigs. A similar result was reported for red deer (Huber et al., 2003a), whereas in rats and mice, fecal metabolite patterns differ largely between the sexes leading to different binding affinities of the group-specific antibody (Lepschy et al., 2007; Touma et al., 2003).

Interestingly, the injection procedure itself, which was investigated in experiment 1b, had no significant impact on fecal cortisol metabolites compared to the first control experiment (1a). A similar result was also reported in rats (Lepschy et al., 2007). Lepschy et al. (2007) pointed out that, although handling and injection were likely to be stressful for the subjects, a relatively short manipulation time (less than 3 min per subject in our study) might not have caused a significant elevation of cortisol concentrations. Furthermore, fecal samples integrate a time period of several hours, in which such short-term elevations in plasma cortisol concentrations might be dampened again to basal levels. Both assumptions are likely to be true for this study.

In experiment 1b, a diurnal variation of fecal cortisol metabolite concentrations was completely absent. It is therefore possible that a diurnal variation in fecal cortisol metabolites is absent in guinea pigs which would be advantageous as the sampling method would not be affected by the time of day. However, in experiment 1a, we found a significant diurnal variation with peak values during the first third of the dark period and nadirs at the beginning of the light phase. In experiment 1a, which was performed first, fecal cortisol metabolite concentrations were elevated during the first 12 h compared to experiment 1b. Possibly, this was the result of stress caused by the new housing situation. The same phenomenon was also reported previously in rats (Lepschy et al., 2007). It is therefore possible that a 24 h-period was not sufficient for the subjects to habituate to the new housing- and sampling procedure. In the light of future research using fecal samples in guinea pigs, this should be taken into account.

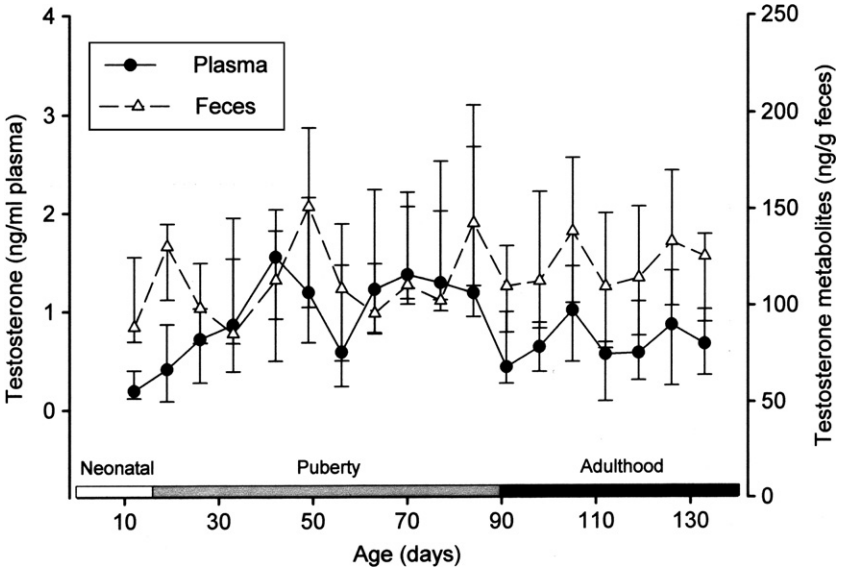

Fig. 3. Median testosterone concentrations in plasma (solid circles) and feces (open triangles) during pubertal development of guinea pig males. Error bars represent 25% and 75% quartiles, respectively. The developmental stages (after Rigaudière et al., 1976) are indicated at the bottom (neonatal period from 0 to 16 days of age: white bar, pubertal period from days 16 to 90: grey bar, adulthood from day 90: black bar). Sample sizes at Day 12/19/26/33/42/49/56/63/70/77/84/91/98/105/112/126/133 are 11/17/19/16/18/18/18/19/18/16/17/15/14/8/14/16/14.

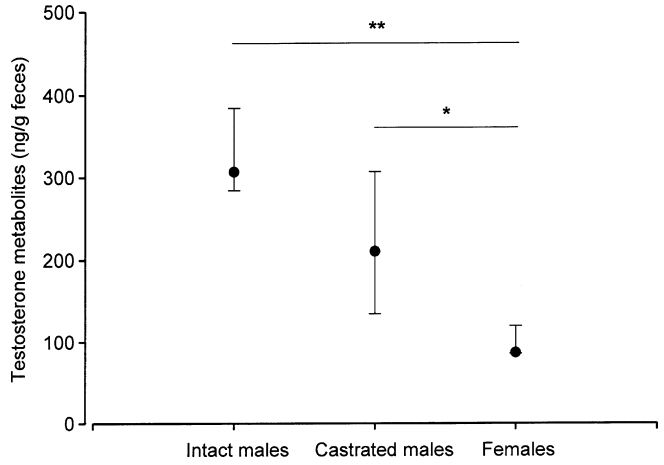

Fig. 4. Median fecal testosterone metabolite concentrations of adult intact males, castrated males, and females. Symbols (solid circles) represent medians with 25% and 75% quartiles. Significant differences between groups are marked with asterisks ($^{**}P < 0.01$, $^{*}P < 0.05$).

In this validation experiment (1a–1c), we kept the subjects separately for the duration of the experiment to facilitate fecal sample collection. As samples were collected in hourly intervals, social isolation of subjects might have been the most practical solution. However, as guinea pigs are social animals, isolated housing may not be suitable for many experiments investigating their behavioral endocrinology. Alternatively, as guinea pigs defecate frequently, animals can be separated from their social groups for short periods of time until defecation, and then returned immediately. This method was used in the testosterone validation experiment and it usually took only a few minutes to obtain a sample. Another practical solution may also be to observe an individual in its social group until it defecates.

In experiment 2, female gonadal steroids, i.e. progesterone and estrogens, measured from plasma and feces were significantly correlated in almost each analyzed subject. In both plasma and fecal samples, progesterone concentrations were low around the time of estrus, increased thereafter, peaked around the middle of the cycle, and decreased again a few days before estrus. Previous guinea pig studies reported similar results for plasma progesterone, reflecting the growth and regression of the progesterone-secreting corpora lutea over the estrous cycle (Blatchley et al., 1976; Challis et al., 1971; Feder et al., 1968). However, as indicated in Fig. 2a, plasma progesterone peaked on Day −7 and Day 0, whereas in fecal progesterone metabolites, we found peaks on Day −5 and Day 1, respectively, indicating a time lag between plasma and fecal hormonal signals. However, on Day 4, plasma progesterone peaked again, whereas there was no corresponding peak in fecal progesterone metabolites. We can only speculate what might have caused these discrepancies shown in Fig. 2a. For instance, the gut passage time may differ across the estrous cycle, maybe due to endocrine changes (Chen et al., 1995). The time lag also seems to differ between females, as indicated by the variance in correlation coefficients in Table 1. When we additionally correlated plasma progesterone concentrations of one day with fecal steroid concentrations on the following day (data not shown), correlation coefficients and *P*-values improved in 3 out of 7 females, which supports the hypothesis of individual differences in gut passage time. However, additional studies are needed to clarify progesterone metabolism and excretion during the estrous cycle in guinea pigs.

Plasma and fecal estrogen concentrations were highly significantly correlated in each analyzed female. Estrogen concentrations in plasma as well as feces showed two distinct peaks reflecting the biphasic follicular phase of the guinea pig estrous cycle (Bland, 1980; Hutz et al., 1990): the first peak was measured 5 days before estrus indicating the first wave of follicular development, and the second peak around estrus preceding ovulation of the second cohort of follicles (Bland, 1980; Hutz et al., 1990). In contrast to progesterone, the hormonal events in the plasma accurately paralleled endocrine changes in feces. However, as we were able to analyze estrogen in only 3 females due to lack of plasma, we cannot draw strong conclusions from these findings, because the small data set may not be representative. In summary, although the correlational results between plasma and fecal gonadal steroids indicate that fecal samples can be used to

assess gonadal status in females, it is necessary to perform consecutive studies to fully understand progesterone/estrogen metabolization and excretion in the guinea pig.

The attempted biological validation of the fecal testosterone assay revealed some difficulties. Plasma and fecal testosterone of pubertal males did not correlate significantly at any point of time. Moreover, although intact males, castrated males, and females overall differed significantly in their concentrations of fecal testosterone metabolites, there was considerable overlapping of values in the castrated and intact male groups. In plasma samples, on the other hand, differences between the same castrated and intact males were highly significant as plasma testosterone of castrated males was in most cases undetectable (data not shown). We can therefore be certain that endogenous testosterone of testicular origin was completely absent in castrated subjects at the time of the investigation. In light of these results, it is likely that the assay for 17β-OH-androgens also measured androgen metabolites of non-testicular origin such as the adrenals. If this was the case, the assay might not be suitable to discriminate gonadal status in fecal samples of male guinea pigs. A similar problem with the same assay system was also reported in old world monkeys, which still produce a considerable amount of dehydroepiandrosterone after castration (Möhle et al., 2002). In guinea pigs, 11β-hydroxyandrostenedione, a C19-steroid produced by the adrenals, is measured in comparable concentrations to intact males in the plasma after castration, while all other major androgens become undetectable (Belanger et al., 1989, 1990, 1993). It is possible that the metabolic products of 11β-hydroxyandrostenedione may be detected by the testosterone antibody, and, consequently, co-measurement may be unavoidable with the current assay. For further investigation, it is important to identify the major androgen metabolites in guinea pig feces for the development of a more suitable assay system.

In conclusion, our study demonstrated for the first time the suitability of fecal steroid metabolites to investigate HPA- and HPG-axes activity in the guinea pig. We showed that adrenal activity can be monitored using fecal samples. Further, although additional investigations are needed, our results suggested that assays for fecal progesterone metabolites and total estrogens appear to be useful tools to investigate gonadal status in female guinea pigs. In this way, we present an important non-invasive alternative to blood samples especially for the long-term monitoring of gonadal or adrenal steroids even in pregnant or juvenile subjects, where blood sampling may be problematic. This not only opens new perspectives for biomedical research, but also for the welfare of this commonly used laboratory rodent. However, the possible co-measurement of non-testicular androgens in fecal samples of guinea pig males should be taken into consideration for future research.

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References

Belanger, B., Belanger, A., Labrie, F., Dupont, A., Cusan, L., Monfette, G., 1989. Comparison of residual C-19 steroids in plasma and prostatic tissue of human, rat and guinea pig after castration: unique importance of extratesticular androgens in men. J. Steroid Biochem. 32, 695–698.

Belanger, B., Couture, J., Caron, S., Bodou, P., Fiet, J., Belanger, A., 1990. Production and secretion of C-19 steroids by rat and guinea pig adrenals. Steroids 55, 360–365.

Belanger, B., Fiet, J., Belanger, A., 1993. Effects of adrenocorticotropin on adrenal and plasma 11-beta-hydroxyandrostenedione in the guinea pig and determination of its relative androgen potency. Steroids 58, 29–34.

Bland, K.P., 1980. Biphasic follicular growth in the guinea-pig estrous cycle. J. Reprod. Fertil. 60, 73–76.

Blatchley, F.R., Donovan, B.T., Ter Haar, M.B., 1976. Plasma progesterone and gonadotrophin levels during the estrous cycle of the guinea pig. Biol. Reprod. 15, 29–38.

Challis, J.R.G., Heap, R.B., Illingworth, D.V., 1971. Concentrations of oestrogen and progesterone in the plasma of non-pregnant, pregnant and lactating guinea-pigs. J. Endocrinol. 51, 333–348.

Chen, T.S., Doong, M.L., Chang, F.Y., Lee, S.D., Wang, P.S., 1995. Effects of sex steroid hormones on gastric emptying and gastrointestinal transit in rats. Am. J. Physiol. Gastrointest. Liver Physiol. 268, G171–G176.

Dalle, M., Delost, P., 1974. Changes in the concentrations of cortisol and corticosterone in the plasma and adrenal glands of the guinea-pig from birth to weaning. J. Endocrinol. 63, 483–488.

Feder, H.H., Resko, J.A., Goy, W., 1968. Progesterone concentrations in the arterial plasma of guinea-pigs during the oestrous cycle. J. Endocrinol. 40, 505–513.

Fujieda, K., Goff, A.K., Pugeat, M., Strott, C.A., 1982. Regulation of the pituitary–adrenal axis and corticosteroid-binding globulin–cortisol interaction in the guinea pig. Endocrinology 111, 1944–1950.

Garris, D.R., 1986. The ovarian–adrenal axis in the guinea pig: effects of photoperiod, cyclic state and ovarian steroids on cortisol levels. Horm. Metab. Res. 18, 34–37.

Grace, N.D., O'Dell, B.L., 1970. Interrelationship of dietary magnesium and potassium in the guinea pig. J. Nutr. 100, 37–44.

Harkness, J.E., Wagner, J.E., 1995. The Biology and Medicine of Rabbits and Rodents, fourth ed. Williams and Wilkins, Baltimore.

Huber, S., Palme, R., Arnold, W., 2003a. Effects of season, sex, and sample collection on concentrations of fecal cortisol metabolites in red deer (*Cervus elaphus*). Gen. Comp. Endocrinol. 130, 48–54.

Huber, S., Palme, R., Zenker, W., Möstl, E., 2003b. Non-invasive monitoring of the adrenocortical response in red deer. J. Wildl. Manage. 67, 258–266.

Hutz, R.J., Bejvan, S.M., Durning, M., Dierschke, D.J., Fisher, C.L., Zachow, R.J., 1990. Changes in follicular populations, in serum estrogen and progesterone, and in ovarian steroid secretion in vitro during the guinea pig estrous cycle. Biol. Reprod. 42, 266–272.

Kaiser, S., Sachser, N., 2005. The effects of prenatal social stress on behaviour: mechanisms and function. Neurosci. Biobehav. Rev. 29, 283–294.

Lepschy, M., Touma, C., Hruby, R., Palme, R., 2007. Non-invasive measurement of adrenocortical activity in male and female rats. Lab. Anim. 41, 372–387.

Liu, L., Li, A., Matthews, S.G., 2001. Maternal glucocorticoid treatment programs HPA regulation in adult offspring: sex-specific effects. Am. J. Physiol. Endocrinol. Metab. 280, E729–E739.

Malinowska, K.W., Nathanielsz, P.W., 1974. Plasma aldosterone, cortisol and corticosterone in the new-born guinea pig. J. Physiol. 236, 83–93.

Möhle, U., Heistermann, M., Palme, R., Hodges, J.K., 2002. Characterization of urinary and fecal metabolites of testosterone and their measurement for assessing gonadal endocrine function in male nonhuman primates. Gen. Comp. Endocrinol. 129, 135–145.

Möstl, E., Maggs, J.L., Schrötter, G., Besenfelder, U., Palme, R., 2002. Measurement of cortisol metabolites in faeces of ruminants. Vet. Res. Commun. 26, 127–139.

Möstl, E., Palme, R., 2002. Hormones as indicators of stress. Domest. Anim. Endocrinol. 23, 67–74.

Owen, D., Andrews, M.H., Matthews, S.G., 2005. Maternal adversity, glucocorticoids and programming of neuroendocrine function and behaviour. Neurosci. Biobehav. Rev. 29, 209–226.

Palme, R., 2005. Measuring fecal steroids: guidelines for practical application. Ann. NY Acad. Sci. 1046, 75–80.

Palme, R., Fischer, P., Schildorfer, H., Ismail, M.N., 1996. Excretion of infused ^{14}C-steroid hormones via feces and urine in domestic livestock. Anim. Reprod. Sci. 43, 43–63.

Palme, R., Möstl, E., 1994. Biotin-streptavidin enzyme immunoassay for the determination of oestrogens and androgens in boar faeces. In: Görög, S. (Ed.), Advances of Steroid Analysis. Akadémiai Kiadó, Budapest, pp. 111–117.

Palme, R., Möstl, E., 1997. Measurement of cortisol metabolites in faeces of sheep as a parameter of cortisol concentrations. Z. Säugetierkd.—Int. J. Mamm. Biol. 62 (Suppl. II), 192–197.

Palme, R., Rettenbacher, S., Touma, C., El-Bahr, S.M., Möstl, E., 2005. Stress hormones in mammals and birds: comparative aspects regarding metabolism, excretion, and noninvasive measurement in fecal samples. Ann. NY Acad. Sci. 1040, 162–171.

Rigaudière, N., Pelardy, G., Robert, A., Delost, P., 1976. Changes in the concentrations of testosterone and androstenedione in the plasma and testis of the guinea-pig from birth to death. J. Reprod. Fertil. 48, 291–300.

Sachser, N., 1994. Sozialphysiologische Untersuchungen an Hausmeerschweinchen. Paul Parey, Berlin, Hamburg. pp. 21–23.

Sachser, N., Pröve, E., 1984. Short term effects of residence on testosterone responses to fighting in alpha male guinea pigs. Aggr. Behav. 10, 285–292.

Schwarzenberger, F., 2007. The many uses of non-invasive faecal steroid monitoring in zoo and wildlife species. Int. Zoo Yearb. 41, 52–74.

Schwarzenberger, F., Tomasova, K., Holeckova, D., Matern, B., Möstl, E., 1996. Measurement of fecal steroids in the black rhinoceros (*Diceros bicornis*) using group-specific enzyme immunoassays for 20-oxo-pregnanes. Zoo Biol. 15, 159–171.

Selle, R.M., 1922. Changes in the vaginal epithelium of the guinea-pig during the oestrous cycle. Am. J. Physiol. 30, 429–449.

Touma, C., Palme, R., 2005. Measuring fecal glucocorticoids metabolites in mammals and birds: the importance of validation. Ann. NY Acad. Sci. 1046, 54–74.

Touma, C., Sachser, N., Möstl, E., Palme, R., 2003. Effects of sex and time of day on metabolism and excretion of corticosterone in urine and feces of mice. Gen. Comp. Endocrinol. 130, 267–278.

Weir, B.J., 1970. The management and breeding of some more hystricomorph rodents. Lab. Anim. 4, 83–97.

Young, W.C., 1937. The vaginal smear picture, sexual receptivity and the time of ovulation in the guinea pig. Anat. Rec. 67, 305–325.