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Quantitative Effects of Diet on Fecal Corticosterone Metabolites in Two Strains of Laboratory Mice

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Abstract. *The analysis of glucocorticoids excreted in feces is becoming a widespread technique for determining animal wellbeing in a wide variety of settings. In the present study an extraction protocol and an ELISA assay for quantifying fecal corticosterone metabolites (FCM) in BALB/c and C57bl/6 mice were validated. Lower ratios of solvent (ethanol) to mass of fecal sample were found to be sufficient in extracting FCM compared to what has been reported previously. Feeding mice a high energy diet, high in fat content (60% of calories from fat), significantly lowered the FCM excretion, approximately halving the FCM output. This diet also reduced the fecal mass voided to approximately a third of that of the regular diet. The two reductions were not correlated. A difference in defecation pattern was seen between the two strains, with the BALB/c mice having a more pronounced diurnal rhythm compared to the C57bl/6 mice. Furthermore, throughout the experiment, the C57bl/6 mice excreted significantly higher levels of FCM compared to the BALB/c mice. The mice were also challenged with synthetic adrenocorticotropic hormone (ACTH) and dexamethasone (DEX). The effect of the challenges could readily be detected, but had a considerably lesser impact on data than did the difference in diet. The study demonstrates some problematic consequences of expressing FCM excretion as a measure of fecal dry mass. The study also serves to emphasize the caution that must be exercised when interpreting FCM excretion in conjunction with an uncontrolled or varied diet, or perturbations of gastro-intestinal functioning.*

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Assessing stress through quantitative studies of fecal glucocorticoids is a method that has found much usage in laboratory animal research as well as in wildlife and conservation biology studies. Widely lauded for its non-invasiveness (1), integrative measurements (2), unbiased results (3) and ease of execution (4), the technique has a large base of proponents. In laboratory rodents, its use as a biomarker of stress and post-surgical pain has increased during the last decades (5-8). However, in mice, measurements of fecal glucocorticoids and glucocorticoid metabolites vary greatly depending on the sampling interval, the quantification method and the experimental setup (6, 8-10). There are therefore still several aspects of fecal glucocorticoid interpretation that need to be investigated in the laboratory mouse. A possible confounding variable is the effect of diet, and consequently fecal mass, on the glucocorticoid clearance through the gastro-intestinal route. As the fecal excretion of glucocorticoid metabolites appear to be proportional to the preceding levels in the circulation, it has been pointed out that an increased intake of feed should dilute the deposited metabolites of glucocorticoids (11, 12). A competing theory has been proposed, where the quantity of glucocorticoid metabolites excreted is influenced by the amount of dry mass passing through the GI tract, possibly due to the altered blood flow through the portal vein (10, 13). If the fecal mass regulates the clearance of glucocorticoids from the blood stream this would have a profound effect on, for example, fecal corticosterone metabolite (FCM) measurements. Furthermore, a reduced clearance from the blood would have a considerable effect on the functioning of the hypothalamic-pituitary-adrenal (HPA) axis as high levels of circulating glucocorticoids potentially inhibit further release of stress hormones. If the level of fecal glucocorticoids is affected by either food intake or dry mass passing through the intestines, their use as biomarkers of, for example, post-surgical pain and stress could be called into question, since altered food consumption and transient ileus are common phenomena in relation to surgery (14-17). Using fecal glucocorticoid measurements as indicators of stress or pain could therefore be biased by the surgically induced changes in

food consumption and defecation rate (8). Further research is therefore needed to scrutinize the factors that may affect the metabolism, excretion and interpretability of FCM in the laboratory mouse.

A poorly investigated aspect of FCM is the effect of different types of diet on the HPA-axis and FCM. A recent study by Dantzer *et al*. of red squirrels demonstrated that fecal hormone metabolites (FHM) vary depending on the diet (18). Similar influences on FHM have been demonstrated in the past, in other wildlife species with naturally varying diets, *e.g*. primates (19), bears (20) and birds (21). Little is however known about the effects of diet on FCM in laboratory mice. The effect of diet on the HPA-axis and excretion of FCM was tested in two strains of mice. In a controlled environment, half of the mice were fed a high-energy diet and the other half received a regular mouse chow diet. The high fat diet was expected to result in a highly reduced fecal output, testing whether or not the diet affected the measured FCM output or levels of circulating corticosterone (CORT) and adrenocorticotropic hormone (ACTH) in serum.

Materials and Methods

The animal experiments performed in this study were approved by the Animal Experiments Inspectorate under the Danish Ministry of Justice (license number 2011/561-1980). All the procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (22) in a fully AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) accredited facility.

Animals and housing conditions. Male C57bl/6 (n=24) and BALB/c mice (n=36), aged 10-12 weeks, weighing approximately 20-25 grams on arrival, were obtained from Taconic, Ry, Denmark. Short term single housing of mice is generally not considered stressful, and the mice were housed individually one week prior to the study for habituation in Macrolon cages (Tecniplast, Varese, Italy) with food pellets (Altromin 1319; Brogaarden, Gentofte, Denmark) and acidified tap water provided *ad libitum*. Wooden chips (Tapvei Oy., Kortteinen, Finland) were used as bedding and bite bricks (Tapvet®; Tapvei), Enviro-dri® nesting material (Shepherd Specialty Papers, Watertown, TN, USA) and cardboard houses (Brogaarden) were provided as environmental enrichment. Room temperature was maintained at 20 ± 2˚C, air humidity was 30 - 60% and the light regimen was a 12/12 hour dark/artificial light cycle with lighting period starting at 6:30 am.

Prior to the experiment, fecal samples were collected from group housed male C57bl/6 mice not undergoing any experiments, in order to determine the extraction efficiency. These mice were housed under similar conditions, receiving the same diet.

Experimental design. Four days prior to the experiment, half of the mice were randomly assigned to receive a high energy diet (D12492; Research Diets Inc., New Brunswick, NJ, USA). This diet had a caloric density of 21.1 MJ/kg supplied mainly in the form of fat (60% of caloric content), compared to the 12.5 MJ/kg of the regular diet, where calories were supplied mainly in the form of carbohydrates (only 13% of caloric content from fat). At 8:00 am the morning of the experiment, one third of the mice received an injection of synthetic ACTH (1.5 mg/kg bodyweight $ACTH_{1-24}$ (human); Polypeptide Laboratories France SAS, Strasbourg, France), one third of the mice received an injection of dexamethasone (DEX; 2.5 mg/kg bodyweight Dexafort®; Intervet UK Ltd., Milton Keynes, UK), and the remaining mice received no injections serving as controls. Both injection solutions were prepared in isotonic saline and sterile filtered prior to the injections. All the mice were placed on new bedding at the start of the experiment. As interactions with researchers and multiple changes of bedding will potentially trigger a stress response that would bias data, each individual mouse was sampled only once per experiment. The cage beddings were collected at 3, 6, 9, 12, 18, or 24 hours following the injections, as dictated by a cross-over design, and frozen awaiting sorting. The study was designed so that no mouse received the same treatment or was sampled at the same time twice. In total, the experiment was repeated three times for the C57bl/6 and twice for the BALB/c mice. A small repeat experiment with twelve of the BALB/c mice had to be conducted as a few samples in the second run had to be excluded. Samples were excluded prior to FCM analysis when it was deemed that outside stressors (unrelated to the experiment) were likely to have had a significant impact on these individuals during or prior to the experiment.

Data collection. The FCM were quantified as described previously (23, 24). Briefly, corticosterone metabolites were extracted by incubating feces in 96% ethanol overnight at a ratio of 5 ml ethanol per gram faeces. Corticosterone levels were analyzed in duplicate using DRG-Diagnostics corticosterone ELISA (EIA-4164; DRG Instruments GmbH, Marburg, Germany) in accordance with the manufacturer's instructions. Standards included in the kit were replaced with a custom nine-point standard curve, prepared in 96% ethanol from analytical grade corticosterone (46148; Sigma-Aldrich, St. Louis MO, USA) in concentrations spanning a range between 50 and 0.19 ng/ml. The kit has been verified to have a cross-reactivity equivalent to 7.4% with progesterone, 3.4% with deoxycorticosterone, 1.6 % with 11-dehydrocorticosterone, 0.3% with cortisol and pregnenolone and <0.1% with other steroids. The FCM are expressed in nanogram-equivalents of CORT throughout, as the exact composition of the FCM is beyond the scope of this study.

Determination of extraction efficiency. An appropriate ratio of the extraction medium to a given quantity of feces had to be established before the experiment was conducted; referred to as the solvent ratio. Since *a priori* knowledge of the FCM contained in a sample cannot be obtained, no simple method exists for determining an appropriate solvent ratio. Moreover, samples cannot easily be spiked with a known concentration of analyte prior to extraction. This is due to the unknown composition of metabolites that make up the FCM, as well as to practical issues regarding the spiking of solid samples. Instead, a set of samples were collected, which were representative of the sample sizes (in mass) that could be expected from the sampling regimen. Approximated weights: 0.04, 0.08, 0.4, 0.7, and 1 grams (n= 3 for each). These samples were then extracted at three different solvent ratios 3:1, 5:1 and 10:1 (milliliters of 96% ethanol to gram feces). By plotting the extracted FCM against the solvent ratios, solvent ratios insufficient for a proper extraction should be detectable. Insufficient extractions should be evident as the data being skewed towards the baseline. With multiple solvent ratios, a systematic departure in average extracted analyte towards the lower ratios was tested for using linear regressions.

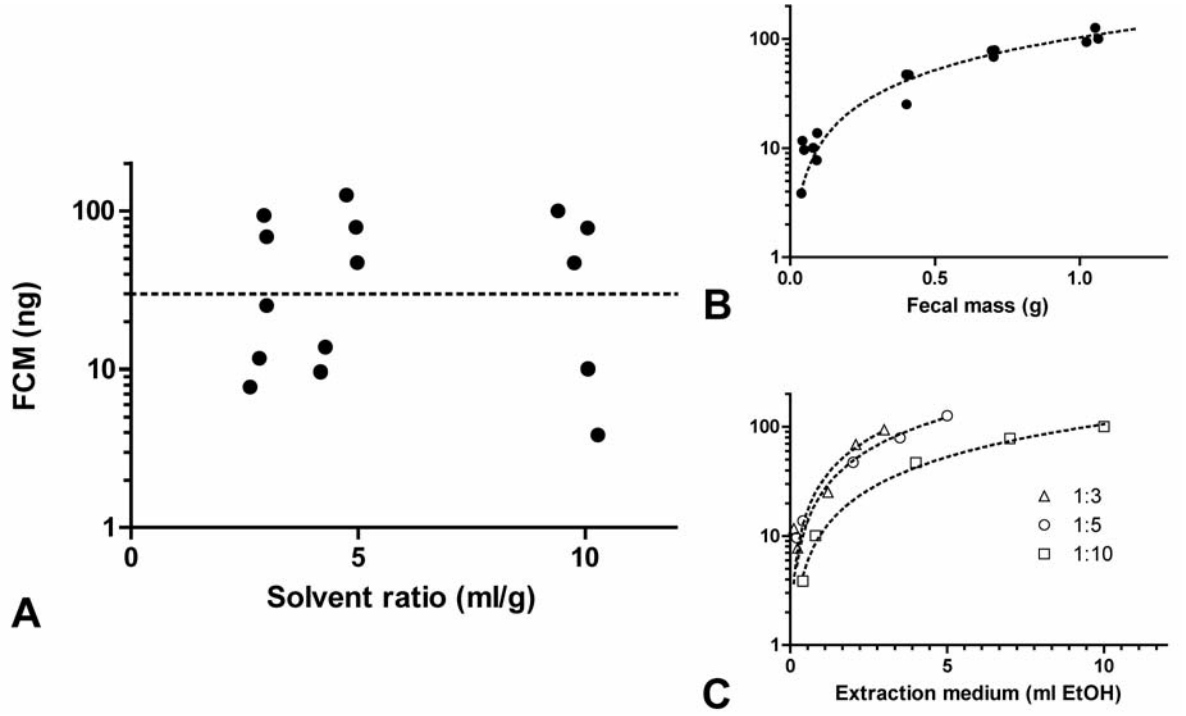

Figure 1. *Extraction efficiency of a random selection of 15 fecal samples from group housed C57bl/6 mice extracted with three different solvent ratios (ml ethanol to g feces). A. Since a best fit regression of FCM as a function of the solvent ratio was found to have a slope not significantly different from zero, the dashed line marks the log-normal average FCM extracted (29.8 ng). B. Linear dependence of the extracted FCM on the sample mass (refer to Table III) and consequently, C., the volume of extraction solvent (for the three solvent ratios).*

Assay validation. Assay validation has been emphasized to be essential when quantifying FCM, due to the unknown composition of metabolites detected in these assays (25, 26). Well-established methods for assessing assay validity (27) were employed. As an analyte-free sample matrix could not be obtained, the assay recovery was determined by standard additions to a random selection of samples (n= 10). Standard additions of 5.4 ng/ml or 10.4 ng/ml CORT were made to all the samples and the degree of recovery was estimated as the slope in a regression of the expected concentration to the recovered concentration. Parallelity was similarly determined through serial dilutions of samples (n= 10) which were found to have a high starting concentration. Parallelity was determined in a log-log plot of concentration as a function of dilution factor (dilutions 1:1, 1:2, 1:4 and 1:8 were used). All the final samples, and a subset analyzed in multiple assays, were used to determine the within and between sample coefficients of variation respectively.

Serum chemistry. In order to test whether the high energy diet had an effect on serum CORT and ACTH, blood samples were collected from all the BALB/c mice the day after the last experiment (between 13:30 pm and 15:00 pm). The mice were moved, one at a time, to an adjacent room where they were immediately concussed, decapitated, and exsanguinated; the whole process carried out in less than one minute. Serum concentrations of CORT and ACTH were analyzed using ELISAs, EIA4164 (DRG Instruments) and ACTH (rat) EIA (Phoenix Pharmaceuticals Inc., Burlingame, CA, USA), respectively, in accordance with the manufacturers' instructions. Serum was not collected from the C57bl/6 mice.

Statistical analysis. Q-Q plots were constructed to test for normal distribution. The FCM were logarithmically transformed after which they exhibited a normal distribution. Linear regressions were used to test for the effect of fecal mass on FCM excretion. Analysis of variance was used for testing the effects of diet, differences in strains, and the effect of ACTH/DEX injections. The interaction of treatments with time was accounted for as *e.g.* the effect of a stressor can be seen in elevated FCM levels with an average retention time of 9 hours (28) – the effect of ACTH would thus be expected at 9 hours, but not at 3 or 24 hours. Similarly the other treatment effects can be expected to differ over time. *P*-values < 0.05 were considered significant. All the statistical tests were performed using PASW Statistics v.18 (SPSS Inc., Chicago, IL, USA).

Results

Extraction efficiency. The extracted CORT was linearly related to the mass of the sample, and consequently in linear relation to the quantity of solvent used forming a linear regression for each solvent to mass ratio (Figure 1). A best fit regression for the quantity of CORT extracted as a function

Table I. *Assay validation statistics. Parallelism is judged by regressing the measured concentrations of a sample against their dilution factor on a log-log scale. Ideal coefficients for the spike recovery and parallelism experiments are 1 and -1 respectively.*

	Coefficients of variance		Spike recovery		Parallelism	
	Within assays (Intra)	Between assays (Inter)	Slope of standard additions		Slope of serial dilutions	
Statistic	11.1%	10.4%	0.96	95% CI: 0.56-1.36	–1.06	95% CI: 1.67-(-0.44)
n	135	37	10		10	

Table II. *Serum ACTH and CORT in BALB/c mice, which were fed two types of diet. Normality was tested using the Shapiro-Wilk test. The CORT data followed a log-normal distribution and have hence been log-transformed. As the ACTH data was not normally distributed the non-parametric Mann-Whitney U-test was employed for the ACTH data whereas the CORT data was tested using a regular T-test. One outlier for the CORT data has been removed due to its disproportionate effect on the data. The significance level for the test of normality with this data point present is however shown in brackets.*

		Descriptive			Test of normality			Mann-Whitney *U*-test		
		n	Average	95% CI	W	df	Significance	U	n	Significance
ACTH (ng/ml)	Regular diet	16	3.83	1.45-6.21	0.66	16	$p< 0.001$	138	34	$p= 0.84$
	High energy diet	18	2.31	1.77-2.85	0.92	18	$p= 0.15$			
								T test		
								t	df	Significance
CORT (log_{10}(ng/ml))	Regular diet	16	1.94	1.79-2.09	0.97	16	$p= 0.87$	0.25	31	$p= 0.81$
	High energy diet	17 (18)	1.91	1.74-2.09	0.99	17 (18)	$p= 1.00$ ($p= 0.052$)			

Table III. *FCM regressed on the mass of sample for the two types of diets (gathering both strains and all treatment groups). Linear regressions through the origin ($y= \beta x$) were used to describe the relation. FCM data were log-transformed prior to the analysis. Both regressions were significant at a level of $p< 0.001$, individually. The difference in slopes was tested using analysis of variance (the interaction of Mass × Diet on FCM).*

	Diet	Slope (β)	95% CI	n	ANOVA
Mass *vs*. FCM	Regular	1.48	1.36-1.60	74	$p< 0.001$
	High energy	2.85	2.51-3.19	79	

Table IV. *Effects influencing the excreted FCM as determined through analysis of variance. Significant effects are marked in bold type.*

Factors	df	F	Significance	Partial η^2
Strain	1	48.9	***p< 0.001***	0.296
Diet × time	6	8.92	***p< 0.001***	0.283
ACHT × time	6	2.91	***p< 0.001***	0.131
DEX × time	6	3.17	***p< 0.05***	0.141
Strain × time	1	3.03	$p= 0.084$	0.025
Strain × diet × time	10	1.39	$p= 0.192$	0.107
Covariates	**df**	**F**	**Significance**	**Partial η^2**
Mass	1	67.9	***p< 0.001***	0.369
	Model df	Error df	R^2	Adjusted R^2
	37	116	0.949	0.932

of the extraction media ratio was found to have a slope that was not significantly different from null (95% CI: -7.2-8.3; $p= 0.89$). Similarly, the intra-sample variance was not found to relate to the solvent ratio (data not shown). A solvent ratio of 5 mg/ml was considered appropriate for the study. For very small samples, where this ratio was deemed impractical, slightly higher ratios were employed on a case-by-case basis.

FCM assay validation. The assay validation results are presented in Table I. The detection limit of the assay, calculated as the lowest concentration significantly higher (α=0.05, one-sided confidence interval) than the negative standards (zero concentrations), was found to be approximately 0.22 ng/ml. No samples in this study had to be excluded due to falling below the detection limit. All the samples could be analyzed without being further diluted.

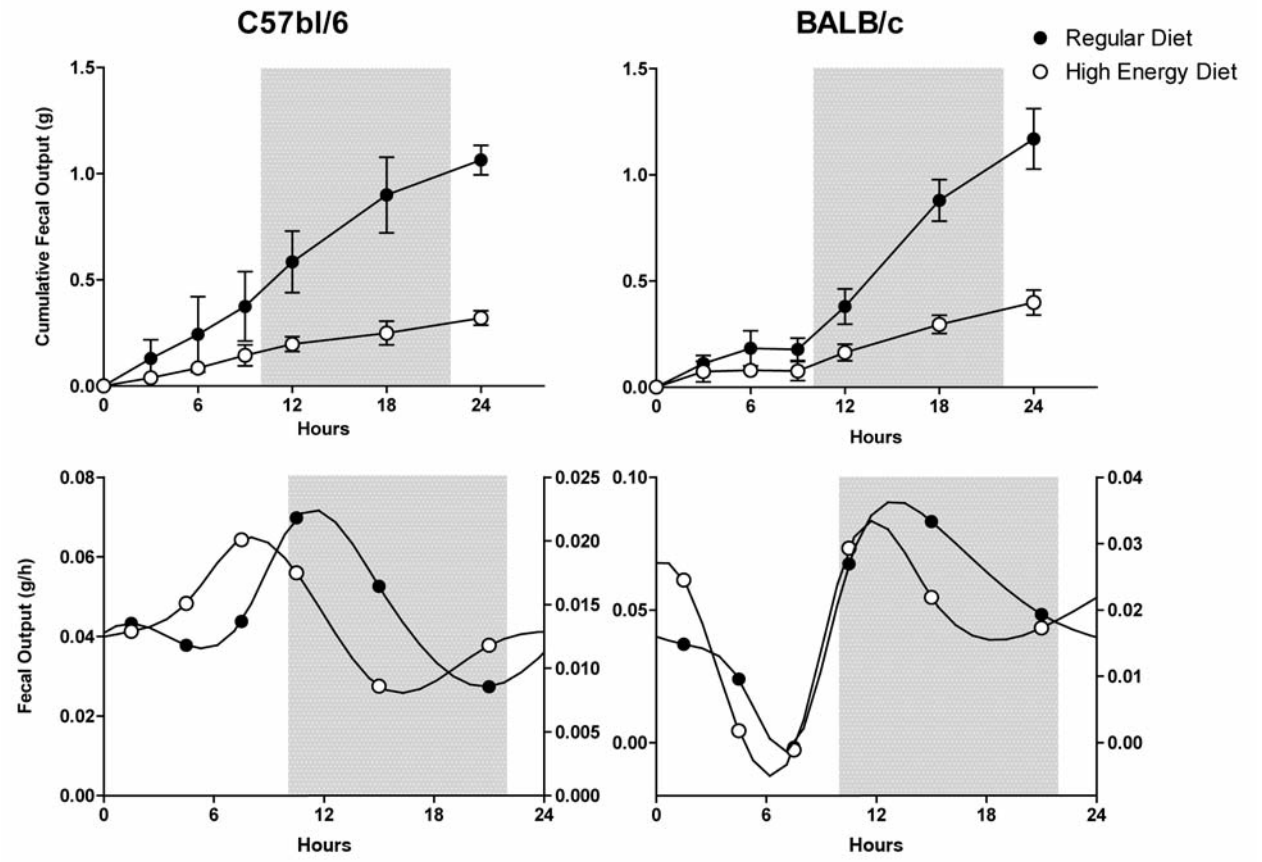

Figure 2. *Diurnal defecation rhythm of two strains of male mice. The grey box represents nighttime, i.e. lights off. Error bars in the cumulative graphs represent 95% confidence intervals (n= 6). The average fecal outputs per hour are calculated population averages with cubic splines fitted through the data. The high energy diet mice are plotted on the right hand y-axis to facilitate side-by-side comparison.*

Distribution of metabolites. The FCM data for both strains of mice, accounting for differences in diet, were found incompatible with a normal distribution. FCM, whether expressed in absolute quantities (ng), per time (ng/h), or per mass (ng/g feces), were found to conform well to a log-normal distribution. Only the C57bl/6 mice on a regular diet deviated significantly from the log-normal distribution (Shapiro-Wilk test for data expressed in absolute quantities: W_{36}=0.93, p=0.025). By further separating this dataset into the different treatments (ACTH/DEX/control) the inconsistency with a log-normal distribution was eliminated; the ACTH group (expectedly) skewing the data slightly towards the high end. Q-Q plots (not shown) did not reveal systematic deviations from the expected log-normal distribution.

Serum CORT data was also found to conform well to a log-normal distribution (W_{33}=0.98, p= 0.89), but not to a normal distribution (W_{33}=0.80, p< 0.001). Serum ACTH conformed well to neither, although the mice fed on a high energy diet did pass the Shapiro-Wilk test (Table II).

Diurnal rhythms. For both strains of mice, defecation and FCM excretion followed a distinct diurnal rhythm (Figure 2). The diurnal rhythm was more pronounced in the BALB/c mice, hardly defecating at all during the midday (hours 3-9). When looking at the fecal output per hours the C57bl/6 mice, fed on a high energy diet, had a slightly perturbed diurnal rhythm.

Effect of treatments. Linear regressions (Table III) revealed different slopes for the two diets when expressing the FCM output as a function of fecal mass. The major effects influencing the excretion of FCM in male C57bl/6 and BALB/c mice are summarized in Table IV. The strain of mouse and the weight of the sample had a significant effect on the FCM contents. The C57bl/6 mice excreted, on average, higher levels of FCM compared to the BALB/c mice. The time from initiating the experiment until collection of feces also had a strong influence. There was, however, a strong interaction between time and both the treatment and diet (Figure 3); these variables must therefore be considered inextricable. The levels of this interaction are presented separately in Figure 4, as they are difficult to discern in

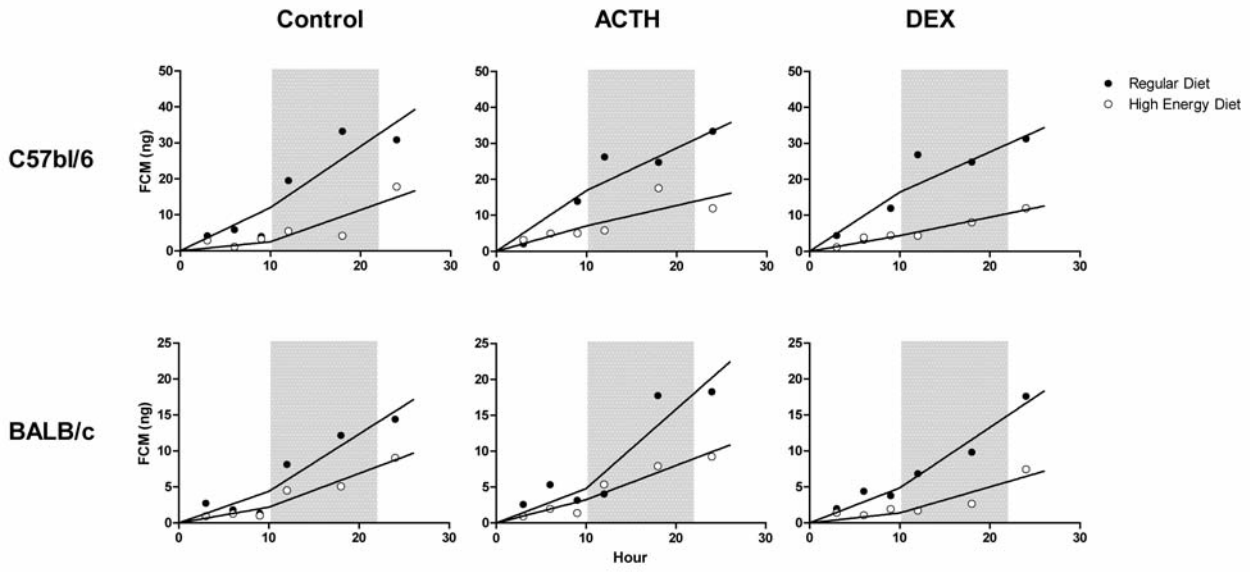

Figure 3. *Cumulative FCM excretion over 24 hours for both strains, diets and treatments. The points correspond to the log-normal average of two mice. The interpolated lines consist of two linear regressions, one for daytime, passing through the origin, and one for the nighttime (t> 10).*

Figure 3. In essence, all other factors being equal, an ACTH injection of 1.5 mg/kg bodyweight would, six hours into the experiment, result in an increased FCM excretion of, on average, 2 ng (in Figure 4: $10^{0.3} \approx 2$). Similarly, a DEX injection of 2.5 mg/kg bodyweight would reduce the FCM excretion at the 24 hour mark by, on average 2 ng. Despite the difference in diurnal rhythm between the two strains when looking at defecation, no interaction could be found between strain and diet when studying FCM.

Serum chemistry. Although the high energy diet fed BALB/c mice had, on average, lower serum ACTH levels and variance compared to the BALB/c mice fed on regular diet, the difference was not statistically significant (Table II).

Discussion

The solvent ratios were equally efficient at extracting CORT from the fecal samples. Although the lowest ratio was impractical it did not seem to reduce the extraction efficiency. In contrast, approximately fifty times higher ratios have been reported as being necessary for a proper extraction of FCM (29, 30). These findings were, however, partly extrapolated from studies on thyroid hormones T_3 and T_4, which are not steroid hormones, and may also partly be attributed to species differences. In addition, the extracted concentrations of hormones were judged regressed on the solvent ratios (30), which violates the underlying assumption of independence (both quantities are dependent on the extraction medium volume) resulting in a potentially

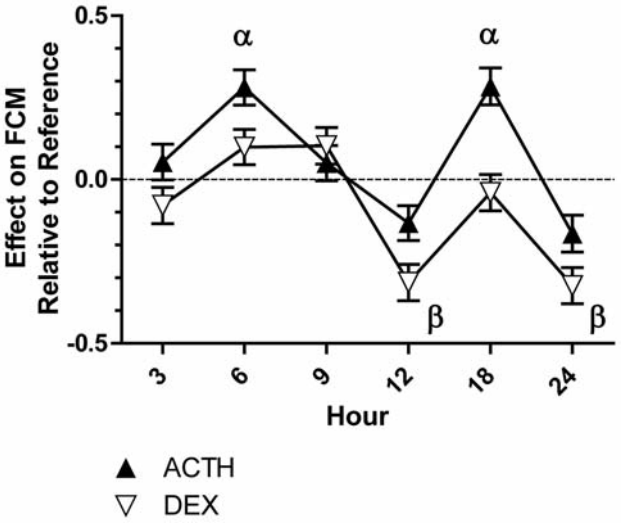

Figure 4. *The effect on the cumulative (log-transformed) FCM by the ACTH/DEX injections isolated from the analysis of variance model. Values are relative to the reference, set at zero ("no ACTH" and "no DEX", respectively). Error bars represent ±SEM; α signifies that the ACTH has a significant (elevating, p< 0.05) effect relative to the reference; β signifies that the DEX has a significant (lowering, p< 0.05) effect relative to the reference.*

"spurious" correlation (31). It has been recommended that the extraction medium, be fully evaporated following the liquid phase extraction (29). We have, however, encountered problems of lowered concentrations of FCM following the subsequent resuspension in aqueous assay buffers (data not

published), possibly due to adherence to glass/plastic ware or aggregation of the hydrophobic analytes. Reduced (intra-sample) precision was accepted in favor of fidelity of the measured concentrations to the original samples. Most samples in the present study distributed themselves on the lower end of the standard curve, while the inter-assay coefficient of variation was gauged from samples evenly distributed across the full range, explaining why the intra- and inter-assay coefficients of variation were, uncommonly (30), of comparable size. The assay has a high degree of accuracy, as shown by the serial dilutions and standard additions, but the precision could be improved upon.

As has been described for various species before both CORT and FCM data were log-normally distributed (23, 32-34). The adrenal production of corticosterone (and cortisol) is induced by ACTH through a G protein-coupled receptor, initiating a multi-stage signaling cascade (35). Multiplicative processes, such as the release of CORT by ACTH, give rise to log-normal distributions (36). With small sample sizes the differences between the normal and log-normal distribution may be difficult to distinguish. The long tail of the log-normal distribution will, however, greatly inflate the type I (false positive) error rate of any erroneously fitted normal distribution. Therefore, when analyzing glucocorticoid data, log-transformations should be the default.

Although FCM output was reduced with the high energy diet, the decreases in fecal mass and FCM output were not correlated. The linear regressions (Table III) revealed that the feces voided on a high energy diet had, on average, a higher concentration of FCM than those of the regular diet. Expressing FCM as output per mass of sample would only confound the data, and is, in laboratory studies, unnecessary and potentially misleading, as pointed out before by a number of researchers (12, 21, 37-39) including our group (9). Expressing data per sample mass is unfortunately often the only approach available in wildlife and conservation studies. The fecal mass still improves the analysis of variance model (Table III) when used as a covariate, by accounting for the variability in intestinal passage time.

A difference in the defecation pattern was observed between the two strains, with the BALB/c mice having a more pronounced diurnal rhythm (Figure 2). This difference was further accentuated as the high energy diet sideways displaced this rhythm in the C57bl/6, but not the BALB/c mice. The effect of diet on diurnal rhythm has been described previously: C57bl/6 mice on a high fat diet consume more of their daily food intake during the light period (40). The change in feed consumption may consequently change the defecation rhythm as seen in the present study. This interaction between strain and diet did not seem to carry over from defecation to FCM output (Table III), underscoring the confounding effect of expressing FCM per sample mass. Although both ACTH and DEX independently had an effect on FCM (Figure 4), it was lower than other effects in the study; for future studies, higher doses should be thus included.

The effect of diet on FHM/FCM has been discussed for a number of species (2, 18-21, 41). The present study demonstrated that mice on a high fat diet showed lower levels of FCM compared to mice fed on standard high fiber diet, which is a fairly well-established phenomenon in rats (39, 42). The physiological basis for this reduction is, however, still unknown. The reduction was not mirrored in circulating serum CORT in the present study or in existing literature, with the possible exception of a study on rats (42). In the latter study however, the introduction of a stressor reduced, rather than increased, the circulating serum CORT, suggesting that the effect of the blood sampling itself may have been measured. In contrast, many studies on rats report unchanged (43), or even elevated (44-47) serum CORT in response to a high fat diet. Male NIH mice have increased plasma CORT on one high fat diet, and not on another ("cafeteria diet"), indicating that the increase in serum CORT is co-dependent on something other than just the fat content (48). The underlying assumption for using FCM as biomarkers is that they reflect an integrative measure of circulating CORT over time (2). That a high energy diet will lead to increased or unperturbed serum CORT while FCM is significantly decreased therefore presents difficulties. Possible explanations of this discrepancy include: Increase in urinary CORT metabolite excretion; a difference in the profile of excreted metabolites; or decrease of turnover time of CORT molecules. Previously no significant variability in the CORT metabolite excretion ratio between urine and feces in mice has been found (28), but differing diets have not been accounted for. Moreover it has been shown that peripheral inactivation of serum CORT is increased in response to a high fat diet (45, 49), which has been proposed as a short-term adaptive mechanism limiting the adverse effects of high fat feeding and high levels of circulating corticosterone. The suggested increase in 11-keto metabolites, due to lowered 11β-HSD-1 activity, would probably go undetected in the present FCM assay. The antibodies employed are highly selective towards the 11-position of the steroid structure, as evident from the less than 2% cross-reactivity to 11-dehydrocorticosterone. Additionally, in the present study only free serum CORT was measured. The expression and affinity of CBG (corticosterone binding globulin) has, in a number of species, been shown to relate intimately to diet (50-52) and an increase in circulating free fatty acids reduces the affinity of CORT to CBG (53). Thus a reduction in total circulating CORT could go undetected, as the free hormones increase. A slower metabolism of CORT would also explain how less FCM can be excreted while serum CORT remains unperturbed. A lower CORT turnover should, in turn, be evident as a reduction in serum ACTH. While the BALB/c mice on a high energy diet did not have

significantly lower levels of serum ACTH the variance was significantly lowered. Similarly Foster *et al*. (54) did not find differences in baseline CORT/ACTH values for rats, but found a blunted ACTH response to a stressor, with the control group having a much larger dynamic range. Thus, the possibility that a high fat diet modulates the CORT turnover in mice cannot currently be confirmed or rejected.

Conclusion

A significant difference in the excretion of FCM between a high fat and a regular diet occurs in two commonly used strains of laboratory mice. Expressing FCM per mass of sample exacerbates, rather than compensates, for this altered FCM excretion. The relationship between the diet and the HPA-axis is complicated and is, at this point, not fully understood. The diet is however important to consider when evaluating FCM data, as it may greatly impact the results, and thus the inferred conclusions.

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