Body Weight and Faecal Corticosterone Metabolite Excretion in Male Sprague-Dawley Rats Following Short Transportation and Transfer from Group-housing to Single-housing

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Summary

Body weight and faecal excretion of corticosterone metabolites (CM) were recorded daily in 18 young male Sprague-Dawley rats from the day they arrived at the animal facility from the breeder. The animals were group-housed (n=3) and divided in two groups after 7 days. One group (n=9 animals in 3 cages) was moved to another room in the facility and the other group remained in the original holding room. After an additional 7 days the animals which had been moved were separated and single housed for an additional 7 days. The body weights developed normally in all rats during the three-week period. Faecal CM excretion appeared high immediately after the rats arrived from the breeder, and decreased to reach significantly lower levels 6 days after arrival. This was likely related to natural fluctuations in faecal CM output rather than substantial stress. None of the husbandry procedures performed during the study had any effect on faecal corticosterone metabolite secretion compared to control. The results suggest that neither transportation from the breeder, moving within the facility, nor being transferred from group housing to single housing are events stressful enough to be reflected by the parameters analysed in the present study. However, the faecal CM excretions clearly fluctuate over several days, which must be considered when using faecal samples for non-invasive stress assessment.

Introduction

It is common practice to allow laboratory animals to adapt to the new environment after arrival from the breeder (*Kohler et al., 1978*) and acclimatise for at least one week prior to experimentation (*Waynforth et al., 2002*). In connection with experimentation, animals are often moved within the facility/institution into a new environment, and during a postoperative period animals are frequently housed separate from other animals in a recovery room. Little is known, however, about the stress response caused by the translocation of the animals from breeder to user facility, and within the user facility, and few data are available on the time required for the animals to adapt to a new environment. The fact that most animals used in laboratories are social animals adds yet another source of possible stress stimulus when they are separated from their peers, which is often the case, and which may affect the experimental data. As a concrete example, in our research group we use rats that are allowed to acclimatise for seven days before they are translocated into another laboratory for experimentation, during which they are kept single-housed (*Goldkuhl et al., 2008*).

Aguila and co-workers (*Aguila et al., 1988*) studied the stress response in mice subjected to long-distance transportation (18-20 h by air or 36-42 h

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by truck) and reported that plasma corticosterone levels were significantly increased after transportation, but returned to normal within 24 h. A recent study by Capdevila and co-workers (*Capdevila et al., 2007*) of heart rate, body temperature and activity levels in rats before and after transport using radio-telemetry transmitters suggests that rats takes three days to acclimatise to a new environment after transportation. Tuli and co-workers (*Tuli et al., 1995*) demonstrated that simply moving mice from one room or floor to another was stressful and resulted in increased plasma corticosterone levels, which, however, also returned to basal levels within 24 h of the move both in single-housed and group-housed mice. Sharp and co-workers reported that single-housed rats displayed higher heart rate and mean arterial blood pressure than group-housed rats both during resting and after experimental procedures such as cage change, restraint, and injections (*Sharp et al., 2002*).

Stress is a significant source of experimental errors and a major cause of suffering in laboratory animals, and reducing stressful conditions in normal husbandry as well as during and after experimental procedures is essential. Corticosteroids maintain cardiovascular homeostasis (*Cairncross et al., 1979; Mangos et al., 2000; Rascher et al., 1979; Tajima et al., 1983*), mobilise energy stores (*Desborough, 2000*) and mediate immune suppression (*Whitten et al., 1998*). Elevated corticosteroid release significantly alters the normal physiology and metabolism of the animal and thereby increases variation within and between individual animals (*Hau et al., 2001; Morton & Hau, 2002*). Persisting substantial stress results in pathological lesions (*Suleman et al., 2000*) and various adverse effects such as impaired fecundity and immunosuppression (*Glaser et al., 1987; Klein et al., 1992; Moberg & Mench, 2000*).

In order to assess and recognise stress in laboratory animals, evaluation of various clinical signs and behavioural parameters can be undertaken, such as body weight gain, food and water consumption, urination, defecation, activity, posture, vocalisation etc. (*Martini et al., 2000; Morton & Hau, 2002*). Reduction in body weight gain has been observed in several studies where rats have been subjected to different acute or chronic stressful conditions, such as metabolic cage housing (*Eriksson et al., 2004*) and electric foot shock, immobilisation or immersion in cold water (*Retana-Marquez et al., 2003*). To complement such studies, stress can also be assessed by quantifying endogenous stress markers, such as corticosterone. The level of corticosterone can be investigated in several ways. Non-invasive measures may be obtained by quantifying corticosterone excreted in faeces. Since corticosterone is metabolised in the liver before excretion in the faeces, a more accurate denomination of the measured products is immunoreactive corticosterone metabolites (*Bamberg et al., 2001; Eriksson et al., 2004; Lepschy et al., 2007; Royo et al., 2004*). Thus, the term corticosterone metabolites (CM) is used in the present investigation.

Faecal CM excretion has been shown to be useful to assess preceding stress in several species including rats (*Lepschy et al., 2007; Royo et al., 2004; Siswanto et al., 2008*) and mice (*Harper & Austad, 2000; Touma et al., 2004*). This methodology is associated with complicating factors due to uncertainty in the delay of the response and large intra-experimental variation (*Carlsson et al., 2007; Lepschy et al., 2007; Pihl & Hau, 2003; Royo et al., 2004*). However, it has advantages over blood sampling, since no human intervention is necessary during the time period to be investigated and it is possible to analyse total CM output eliminating the uncertainty of integrating areas below graphs to obtain reliable measures. Thus, changes in body weight gain and excreted CM could be useful measures of preceding stress. It has been shown, though, that the increase in corticosterone must be substantial to be detected in faeces (*Siswanto et al., 2008*), and surgical procedures are an example of such a stressor (*Royo et al., 2004*). However, it is not fully understood how these measures are applicable for detecting possible stressors such as transportation and single-housing in male Sprague-Dawley rats.

The aim of the present study was to investigate whether rats are affected during their adaptation to a new environment and separation from other rats. Based on the studies above, changes in daily body weight gain and daily faecal CM excretion were chosen as measures to assess the possible effects on the animals. The order of manipulations was based on the experimental situation described above (*Goldkuhl et al., 2008*). It was hypothesised that if the procedures were substantially stressful to the animals, this would be displayed as a significant effect on body weight gain after the interventions, as well as a significant increase in excreted CM.

Materials and Methods

Animals

All animal experiments in the present study were approved by the Animal Ethics Committee in Uppsala, Sweden. Eighteen Sprague-Dawley rats (transported by truck from Scanbur B & K, Sollentuna, Sweden), were used. Male rats were used to reduce the variations related to oestrus cycle hormonal fluctuations. The average body (bw) weight of the animals on the day of arrival was 242 ± 2 g, ranging from 232 to 254 g. During the first seven days after arrival in the local animal facility they were kept in Macrolone size IV cages in groups of three rats per cage, which were housed in standard animal rooms under standard conditions; 12 hours of light (from 6 am to 6 pm) and 12 hours of darkness, relative humidity 30-60 %, temperature 21±1°C, and air was changed approximately 15 times per hour. Cages were cleaned daily in connection with collection of all faecal pellets. Aspen chips (Finn Tapvei, Kortteinen, Finland) were used as bedding material. The animals had free access to food pellets (R36 Laktamin, Stockholm, Sweden) and tap water at all times. Each cage contained a wooden tunnel and paper sheets as environmental enrichment.

Experimental design

All rats arrived from the breeder at 2 pm. The breeder is located approximately 50 kilometres from the Biomedical Centre, and the duration of the transport was approximately 45 minutes. The day of arrival is referred to as Day 0. Body weight was recorded and the rats were put in clean cages in groups of three as described above. Faecal sampling started Day 1 at 9 am, followed by sampling at 9 am every 24 hours until Day 21. Thus, faecal samples Day 1 correspond to all faecal pellets produced from arrival Day 0 until collection Day 1, and similarly for the subsequent days. They were picked up one by one, weighed and placed into a new clean cage containing new aspen chips, the same wooden tunnel and a new sheet of paper. Faecal pellets from each cage were pooled and each cage treated as one experimental unit, resulting in six experimental units in total for the faecal samples. Cages were handled in the same consecutive order every day. Faecal pellets were stored at -20°C until analysis. Body weight registration and faecal pellet sampling from all cages was continued daily at 9 am throughout the study.

After seven days, which is the time rats are allowed to acclimatise, a group of nine rats in three randomly selected cages were moved (approximately 50 m) from the animal room into a laboratory with similar environmental conditions. This group is referred to as the test group. The translocation was performed in the morning at 9 am, immediately after finishing body weight registration and faecal sampling. The remaining nine rats were maintained in the three cages in the original room as a control group throughout the remainder of the experiment.

After another seven days, rats in the test group were separated from each other and housed individually in Macrolone size III cages, yet in the same laboratory. The duration of seven days was chosen to allow possible effects to decline as during the first week. The separation was followed by another seven days of sampling. The faecal samples from single-housed animals were extracted and analysed separately. When comparing with pre-single-housing values and with the control group, the values were pooled to represent the original cage and the mean faecal CM level µg/24h kg bw was presented. The experiment was terminated at Day 21, when the

rats were injected with a lethal dose of 2.5 ml i.p. pentobarbital 100 mg/ml (Pentobarbital, Apoteket, Sweden) or used in unrelated studies.

Extraction and quantification procedures

All faecal pellets from each cage were dried in a heat cabinet at 30°C for 2 h. MilliQ H_2O (4 g per 1 g faeces) was added and the samples were thoroughly homogenised with a Severin Profi-mix hand blender (Severin Elektrogenerate GmBH, Sundern, Germany). Faecal steroids were extracted from the homogenates using dichloromethane (CH_2Cl_2) as previously described by Eriksson et al (*Eriksson et al., 2004*) modified from Pihl and Hau (*Pihl & Hau, 2003*). Corticosterone and immunoreactive corticosterone metabolites were quantified using Correlate-EIA (Assay Designs Inc., Ann Arbor, MI, USA) according to the manufacturer's manual. The kit has been verified to have a cross reactivity equivalent to 28.6 % against deoxycorticosterone, 1.7 % against progesterone, 0.13 % against testosterone, 0.28 % against tetrahydrocorticosterone, 0.18 % against aldosterone and less than 0.05 % against cortisol, pregnenolone, beta-estradiol, cortisone and 11-dehydrocorticosterone acetate. The intra-assay and the inter-assay coefficients of variation were about 8.2 % and 8.4 %, respectively. The obtained CM values (µg/g faeces) from the ELISA was multiplied by faecal weight (g), and divided by time interval and body weight. The CM value in µg/24h kg bw thus represents the total CM excretion during the time period of interest.

Statistical analysis

Based on data on CM levels previously obtained in our laboratory (*Eriksson et al., 2004; Pihl & Hau, 2003; Royo et al., 2004*), group sizes used in the present study were determined by a power analysis as described in the literature (*Festing & Weigler, 2003; Svendsen & Hau, 1986*). A statistical power of 80% was considered satisfactory. Body weight changes were analysed with linear regression using GraphPad® Prism 5.01. Differences in daily faecal CM excretion were determined with analysis of variance (ANOVA) with Dunnett's post-hoc test, using SPSS® version 14.0, and with linear regression using GraphPad® Prism 5.01. Data are presented as mean values ± s.e.m. Statistics are presented as $F_{(df1, df2)} = x$; p = significance, where df1 and df2 are degrees of freedom between groups and within groups respectively. For linear regression, the goodness of fit (r^2) is also presented. P-values <0.05 were considered significant.

Results

Body weight changes

The body weight on the day of arrival was 238 ± 2 g in the control group and 242 ± 2 g in the test group. There were no significant differences in body weight gain between groups during any time period of the study (Fig 1).

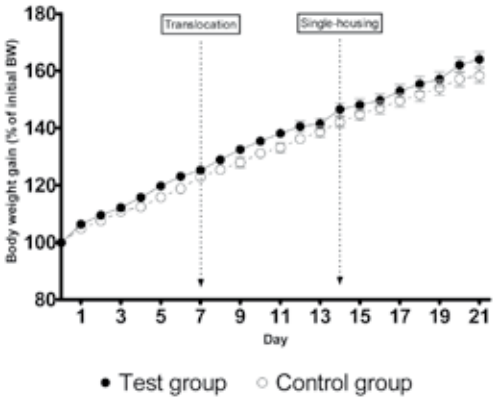

Figure 1. Body weight changes for rats in the control group (n=9) and the test group (n=9) expressed as percent increase from initial body weight.
after arrival for the control group (n=3) and the test group (n=3), expressed as percent change from initial value.

Faecal corticosterone metabolite excretion

All statistics on CM levels were performed on data shown in the main graphs in Fig 2 and 3, where the CM levels are expressed as µg/24h kg bw (further discussed below). Since all animals were subjected to equal treatment during the first seven days, the

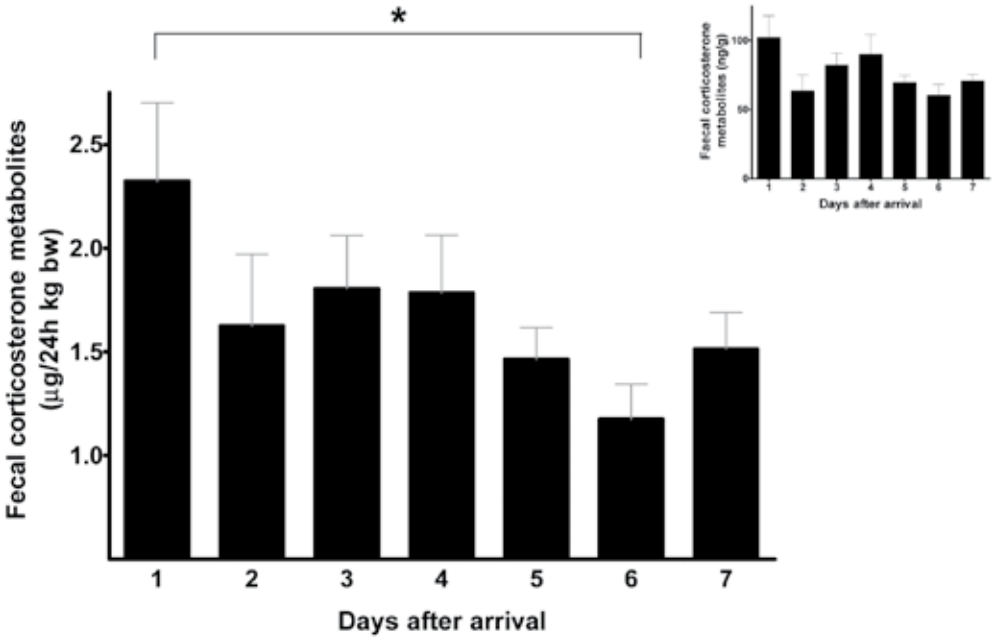

Figure 2. Faecal corticosterone metabolite levels for all experimental units (n=6) during the first seven days after arrival, expressed as µg/24h kg bw. Inset: The levels expressed as ng/g.

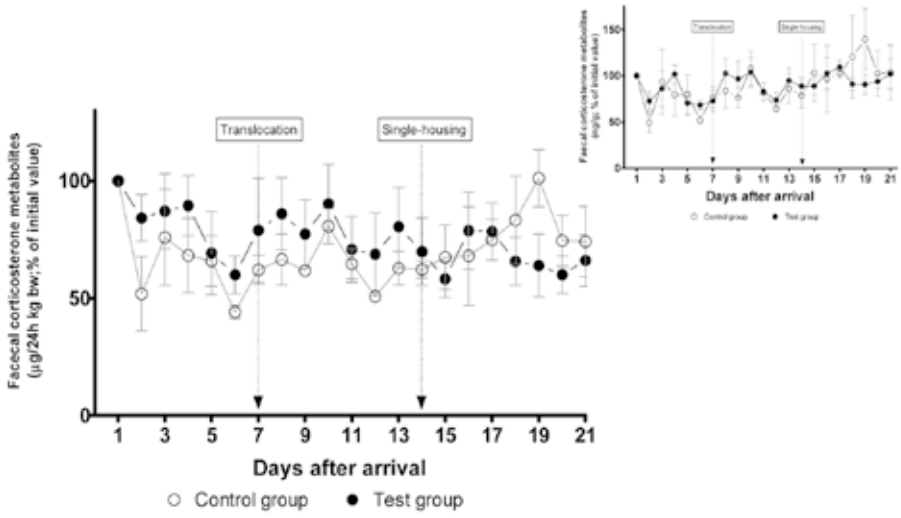

Figure 3. Excretion pattern of faecal corticosterone metabolites in µg/24h kg bw, during 21 days after arrival for the control group (n=3) and the test group (n=3), expressed as percent change from initial value. Inset: The percent change based on levels expressed in ng/g.

two groups were merged and analysed as one for this period (Fig 2). The CM values Day 1 were not significantly different between groups ($F_{(1,4)}$= 0.91; p = 0.39). The CM excretion (µg excreted per 24h and per kg rat) in all animals was high Day 1, representing the levels just after the rats had arrived in the animal facility (Fig 2). The excretion declined significantly during the first week (-0.13 ± 0.05 units/day; $F_{(1,40)}$ = 7.0; r^2 = 0.15; p = 0.01) and was at the lowest six days after arrival. At this time, the CM excretion was significantly lower than during Day 1 (Dunnett's post-hoc $F_{(20,105)}$ = 1.2; p = 0.005). The CM excretion for each group on Days 1-21 is shown in Fig 3. The daily CM levels were compared between the groups by ANOVA. It was found that the two groups were statistically different from each other Day 6, 7 and 8 ($F_{(1,4)} \geq 12.69$; $p \leq 0.024$). As the two groups differed Day 7, which was the reference point for days 8-14, relative changes and the excretion patterns of CM were compared between groups, instead of comparing absolute values. The excretion pattern was similar for both groups. During Days 1-7, the levels declined with a slope of -0.15 ± 0.06 units/day in the test group ($F_{(1,19)}$ = 7.27; r^2 = 0.28; p = 0.01) , and with -0.11 ± 0.06 units/day in the control group, although this decline was not significant ($F_{(1,19)}$ = 3.45; r^2 = 0.15; p = 0.08). The slopes were not statistically different from each other ($F_{(1,38)}$ = 0.26; p = 0.61). After translocation, there were no differences in CM excretion between Days 8-14 and the reference point Day 7 in any of the two groups (Dunnett's post-hoc $F_{(7,16)} \leq 1.14$; $p \geq 0.39$), and no trends were observed (slopes -0.036 ± 0.03 and -0.007 ± 0.03 for test and control group, respectively). After separation, there were no significant differences in CM excretion between Days 15-21 and the reference point Day 14 in any of the two groups (Dunnett's post-hoc $F_{(7,16)} \leq 1.08$; $p \geq 0.42$), and no trends were observed (slopes -0.019 ± 0.03 and0.046 ± 0.04 test and contol group, repectivly.

Discussion

Body weight loss or a reduction in body weight gain in growing rats may be an indication of disease, pain, stress or discomfort in an animal (*Morton & Hau, 2002*). A slightly reduced body weight gain has been demonstrated in rats housed in metabolic cages, followed by a normal weight gain after returning the animals to their home cages (*Eriksson et al., 2004*). In the present study, the environmental changes that the rats were exposed to had no significant impact on their body weight gain.

Since the excreted amounts of CM fluctuate in a diurnal way (*Pihl & Hau, 2003*) the total faecal excretion of CM was calculated in the present study as a mean value in micrograms excreted CM per 24 hours and per kilogram body weight. This is in contrast to the method used in many other studies where just the concentration has been used as a measure of faecal CM. We consider the latter less reliable since the concentration in different faecal pellets has been demonstrated to differ by as much as 40% (*Carlsson et al., 2007; Pihl & Hau, 2003*), which could bias the results if single pellets are picked for spot-checking. The measures may also be affected by food intake and faecal productivity (*Goymann et al., 2006*), which could bias the results when using concentrations even if all pellets are collected. However, the present study does not provide support either of the two methods, since relative changes of faecal CM during the 21 days were comparable between the two measures, as shown in the insets of Figure 2 and 3

The faecal samples Day 1 represent those faeces produced after arrival at 2 pm on day 0 and so contained the amount CM excreted during 19 h (2 pm – 9 am), which were therefore extrapolated to 24 h to facilitate comparison. This may have affected the amount of CM in these samples. However, the possible bias should be minimal and result in rather too high than too low levels and thus not affecting the general conclusions. Since faecal CM represents preceding corticosterone elevations, the Day 1 samples should likely be indicating any stress that might have occurred during the transport and in connection with the arrival. The CM excretion appeared high in all animals after their arrival to the animal facility, and declined during the following

days and reached significantly lower values on Day 6. The CM measures Day 6 represent all CM excreted during Day 5. Thus, one could interpret the data as if the animals acclimatised to the new environment within five to six days, as judged by the decrease in CM excretion. However, as discussed below, when considering the entire curve from Day 1 to Day 21, it is clear that the CM levels increase and decline at several occasions in both groups. After the low level on Day 6, an increasing trend between Day 6 and Day 10 was observed in both of the groups, although Day 10 was not significantly different from the reference point Day 7. Since the increasing trend, as well as the overall pattern of excretion, was similar in both groups, this may be part of some possible natural fluctuation over several days. This is supported by the apparent increasing trend in the control group between Day 14 and 19, which cannot be related to any possible stressful treatment. Thus, the increases and declines of CM levels are most likely related to neither stress nor recovery, but simply natural fluctuations of CM excretion. Consequently, neither transportation, separation nor single housing had any effect on CM excretion in the present experimental setup.

The goodness of fit (r^2) was low for the linear regressions performed. Therefore, it should be pointed out that the regressions were performed primarily to compare trends between the two groups rather than to determine statistical differences. The number of experimental units was rather low in the present investigation, and it is possible that an increase of the number of units would show statistical significance at certain time points where no significances are shown at present. On the other hand, since the excretion pattern were similar in both groups regardless of treatment, it is unlikely that increased group sizes would alter the overall outcome of the experiments. Therefore it was considered irrelevant to increase the number of experimental units.

The sampling interval of 24 hours was chosen in order to detect substantial changes in CM, with minimal bias from potential disturbance to the animals in connection with the weighing and sampling procedures. Small occasional changes in corticosterone levels will pass unnoticed if the sampling interval is long. Data from radio telemetry measures have shown that acute stressors such as exposure to a novel environment (a new cage) and handling cause a significant increase in blood pressure and heart rate, but that these physiological parameters return to normal levels within half an hour (*Okva et al., 2006; van den Buuse et al., 2001*). Similar duration of increased serum corticosterone levels has been demonstrated after acute stress (*Atkinson et al., 2006*). Such small changes are unlikely to significantly affect the magnitude of faecal CM excreted over 24 hours. On the other hand, significant stressors such as surgical procedures (*Abelson et al., 2005*) have been shown to be clearly detectable in faeces 24 hours after operation (*Royo et al., 2004*). Another aspect of the sampling interval of 24 h is the possible metabolite degradation in the faecal samples, which could affect the results. However, this has been shown to have only little impact. Royo and co-workers showed that the variation in CM levels between samples kept at room temperature between one and 24 h before freezing was less than 10% (*Royo et al., 2004*). Thus, the sampling interval of 24 hours was considered appropriate for detecting substantial changes in faecal CM levels. However, as shown by the data, the treatments were apparently not stressful enough to show any effect on the faecal CM excretion. This suggests that a 24 h sampling interval, followed by the CM extraction and quantification used in the present study, is not a method sensitive enough to detect other than substantial stress. This is supported by the data obtained in a recent study (*Siswanto et al., 2008*), where it was shown that the preceding increase in blood corticosterone must be substantial to be detected in faecal samples.

In conclusion, the present study suggests that neither truck transportation from a breeder to a new environment, nor moving animals within an animal facility nor separation and single housing are events that stress the animals to such an extent that it affects body weight gain and CM excretion with

the sampling interval and quantification used in the present investigation. On the other hand, the study shows that CM excretion clearly fluctuates over several days, which must be considered when using faecal CM for assessment of preceding stress. Otherwise, excreted CM levels may easily be misinterpreted.

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