Behaviour, stress and welfare of Sprague Dawley rats (*Rattus norvegicus*) on diet board feeding for 24 months

Sakari Laaksonen^a, Timo Nevalainen^b, Jukka Ketola^a, Jann Hau^c, Pentti Nieminen^d, Kristiina Haasio^e, Iris Kasanen^f and Hanna-Marja Voipio^a

^aLaboratory Animal Centre, University of Oulu, Oulu, Finland; forename.surname(at)oulu.fi

^bLaboratory Animal Centre, University of Eastern Finland, Kuopio, Finland;

timo.nevalainen(at)uef.fi

^cDepartment of Experimental Medicine, University of Copenhagen, Copenhagen, Denmark;

jhau(at)sund.ku.dk

^dMedical Informatics and Statistics Group, University of Oulu, Oulu, Finland;

pentti.nieminen(at)oulu.fi

^eOrion Corporation Orion Pharma, Espoo, Finland; kristiina.haasio(at)orionpharma.com

^fCharles River Discovery and Research Services Finland, Kuopio, Finland;

iiris.kasanen(at)crl.com

Corresponding author: Sakari Laaksonen, PO Box 5000, 90014 University of Oulu, Finland.

Email [sakari.laaksonen\(at\)oulu.fi](mailto:sakari.laaksonen(at)oulu.fi)

Tel +358 294 485097

18

19 **Abstract**

20 Diet board (DB) feeding aims to reduce the health hazards associated with *ad libitum* (AL)
21 feeding. Rats have to gnaw wood to detach food from the DB, reducing their food consumption.
22 We studied the welfare effects of DB by measuring faecal corticosterone metabolites (FCM),
23 elevated plus-maze (EPM) behaviour and cage behaviour. In this two-year experiment, 147
24 group housed (n=3) Hsd:Sprague Dawley® male and female rats were subjected to DB or AL
25 feeding. DB feeding in females elevated FCMs and increased eating observations by 85 %. The
26 DB males were observed eating 30 % more often and resting 4.2 % less than their AL
27 counterparts. The DB rats of both sexes had 19 % increased cage exploration during daytime
28 and 20 % reduced grooming during night-time compared to the AL rats. The increased FCMs
29 may indicate slight stress in DB females. The EPM results indicate there was no anxiety due to
30 DB feeding at six months. The cage behaviour could point to mild chronic stress in DB rats, but
31 the lack of effect on escape-related behaviour and agonism suggests that there were no
32 substantial welfare problems. DB feeding did not seem to disturb the circadian rhythm. The
33 smaller food requirements of DB females meant they had to sacrifice less time than males
34 gnawing at the DB to satisfy their appetite.

35 **Keywords**

36 Rat, diet board, welfare, behaviour, refinement

37 **Abbreviations**

38 DB: diet board; AL: *ad libitum*; FCM: faecal corticosterone metabolites; EPM: elevated plus-
39 maze; DR: dietary restriction; ACH: air changes per hour; HPA axis: hypothalamic-pituitary-
40 adrenal axis;

1 Introduction

The common practice of feeding laboratory rats *ad libitum* (AL) predisposes them to health hazards resulting from overfeeding and obesity. The energy overload leads to metabolic disturbances, increases the incidence and advances the emergence of degenerative diseases and tumours, and shortens the animals' life spans compared to restricted fed rats (Christian et al., 1998; Keenan et al., 1999). Furthermore, the presence of overfeeding-related background pathology in experiments decreases the signal–noise ratio and complicates the interpretation of results (Keenan et al., 1999).

The custom of providing continuous unlimited access to food is associated with some unarguable advantages. It ensures adequate nutrition for all of the animals in the cage and is quick and easy for the caretakers to carry out. Importantly, animals can decide when and how much to eat, automatically adjusting their food intake to their light-dark and sleep-wake rhythms i.e. their physiological and biochemical circadian rhythms remain undisturbed by feeding regimes (Nelson, 1988).

With a moderate dietary restriction (DR) to 75-85 % of the AL food intake, health hazards can be considerably limited and longevity increased (Keenan et al., 1999; Nevalainen et al., 2011). Nonetheless, great care must be exercised not to restrict feeding excessively, as this could inhibit growth and cause suffering through starvation (Nolen, 1972). Severe DR may also suppress treatment-related pathological changes, introducing bias into the assessment of the health effects of test substances (Seng et al., 1998).

Unfortunately there are some downsides to DR. Traditionally, it has been carried out by giving solitary rats restricted rations or by limiting their daily access to food to a short period of time (Toth and Gardiner, 2000). Usually the meals have been supplied during the inactive light period (Nelson, 1988). With this kind of meal-feeding, several physiological circadian rhythms

regulated by the light-dark rhythm become readjusted to the feeding schedule and locomotor activity is partly shifted to the normal rest period (Duffy et al., 1990; Nelson, 1988). However, many other rhythms remain regulated by the light-dark-cycle with the result being that the animals suffer from an internal desynchronization which may modify substantially several experimental variables (Duffy et al., 1990; Nelson, 1988).

DR in group housing introduces additional problems. Providing a group of rats with a limited amount of food easily leads to an uneven food intake among cagemates due to the group hierarchy (Christian et al., 1998). Individually adjusted rationing is possible with software controlled devices that identify rats *e.g.* by microchips, but this is costly and impractical for large scale routine feeding. Rats can be separated during mealtimes but that adds considerably to the workload of caretakers, which is also true for meal feeding in general (Moraal et al., 2012). Until now, no inexpensive, easy to use and reliable large scale DR method for group housed rats has been available.

In an effort to find a practical means of DR in group housing, the diet board (DB) (Figure 1) was developed (Kasanen, 2009; Kemppinen et al., 2008; Laaksonen et al., 2013). With DB, food pellets are tightly embedded in grooves cut into aspen board, and rats have to gnaw at the wood to detach the food. The objective is to increase the workload sufficiently that the rats will only eat enough to meet their basic sustenance requirements. As the DB is available continuously, rats can control when they want to eat and thus their circadian rhythms are not disturbed, unless the work demand is too high. Feeding from the DB is in essence a laborious form of AL, its goal being to combine the benefits of AL feeding and DR.

In addition to preventing overfeeding, DB carries with it a potential enrichment value. By partitioning the space, DB increases the cage complexity and provides rats with an opportunity to withdraw from the others. Working for food by gnawing wood, a natural function for rodents, may serve as foraging enrichment. Furthermore, the possibility to feed whenever at will gives

90 rats some control over the feeding restriction, which sets DB feeding apart from meal-feeding
91 regimens.

92 This two-year study with Sprague Dawley rats compared the effects of DB to AL feeding. As
93 we reported earlier, the DR effect of DB was relatively mild, yet it reduced the obesity of DB
94 fed rats significantly (Laaksonen et al., 2013). The food consumption of DB males was on
95 average 7 % smaller and body weights were 11-19 % lower compared to the controls throughout
96 the course of the experiment. The DB females weighed 6-11 % less than the controls even
97 though no difference was detected in their food consumption, indicating a higher energy
98 expenditure with DBs.

99 Here we report the welfare effects of DB feeding. Our hypothesis was that DB feeding would
100 not cause major distress to the rats or impair their welfare, which was tested by measurements
101 of faecal corticosterone metabolites (FCM), open arm parameters in elevated plus-maze (EPM)
102 and cage behaviour analysis.

2 Material and methods

2.1 Ethical approval

The study protocol was approved by the National Animal Experiment Board of Finland. The animal care and experimental procedures were in line with Finnish and European legislation and recommendations (Council of Europe, 1986; European Parliament and the Council of the European Union, 2010; Ministry of Agriculture and Forestry of Finland, 2006; Parliament of Finland, 2006)

2.2 Animals

The study included 147 outbred Hsd:Sprague Dawley[®] male and female rats, nine weeks old at the beginning of the experiment, born in the Laboratory Animal Centre, University of Oulu, Finland. The initial mean (SD) body weight was 303 g (24 g) for males and 202 g (11 g) for females. Their microbiological health was monitored according to FELASA recommendations (Nicklas et al., 2002), with the only finding being serum antibodies of rat minute virus.

2.3 Housing

Two weeks prior to starting the experiment, rats were moved from the breeding barrier to the research unit, ear marked and regrouped into three rat cages. If two of the rats died in the course of the experiment, a companion rat (not included in the analyses) was added into the cage. The cages were solid bottom type IV (59.5 x 38 x 20 cm) polycarbonate cages with wire lids (Tecniplast 1354G, Italy). Aspen shavings (PM90L) were supplied as nesting material and aspen chips (4HP) as bedding (Tapvei, Kiili, Estonia). Untreated municipal tap water was offered *ad libitum* in polycarbonate bottles. Cages and bottles were changed twice a week at 09:00 - 10:00.

Room temperature was 21 ± 1 °C, relative humidity 40-60 %, ventilation rate 15 ACH and illuminance 350 lx at 1 m height. Full fluorescent tube lighting was on 07:00 - 18:00, and lights came on gradually at 06:00 – 07:00 and dimmed at 18:00 – 19:00.

2.4 The diet board

Two 2.7 x 14 x 36 cm aspen boards (Tapvei, Kortteinen, Finland) were joined in the middle to form a cross-shaped wall structure (Figure 1a). There were 10 vertical grooves (diameter 12 mm, 6 mm wide gap) parallel to the 14 cm edge in both sides of each board, filled with tightly fitting food pellets. The 6 x 6 cm rectangular passages at the ends of the boards and the space between the DB and the lid allowed the rats to move between compartments. The control cages were supplied with similarly structured plain boards without food pellets.

2.5 Feeding

Autoclaved natural ingredient food (Lab For R 36, Lantmännen, Kimstad, Sweden) was available continuously either in cage lid hoppers or via the DBs. Filled DBs, control group food and control cage boards were autoclaved simultaneously (45 min, 121 °C). Both AL food and DBs were changed once a week. DBs (and the control cage boards) were turned upside down on the fourth day to make the eating workload more constant.

2.6 Study design and randomization

Originally, 144 rats were divided into four equal study groups: DB males and females (experimental groups), and AL males and females (control groups). Four birth cohorts comprised five litters each: four litters of four males and four females, and one litter of two males and two females. Littermates were divided randomly but evenly into DB and AL groups. Within the study groups, rats were randomized into the cages, separating siblings from each other. Every cohort was housed in its own cage rack, with groups randomized evenly on the shelves.

An extra cage of DB males was added after the removal of a DB male due to an injury two weeks after the beginning, making the total number of rats 147.

2.7 Sample size

The resource equation was used to determine the required sample as the SDs of many outcome variables could not be estimated reliably, prohibiting the valid use of power analysis (Festing et al., 2002). The experimental unit was either a cage or a rat, depending on the response variable. In this study, $E = 47$ (*number of experimental units - 1*) - 3 (*number of treatment combinations - 1*) - 3 (*number of blocks - 1*) = 41, with cage as the experimental unit, exceeding the recommended minimum value (40) for complex experiments.

2.8 Faecal corticosterone metabolites

The levels of immunoreactive FCMs were analysed from cage samples of faeces of a period of 72 hours, between two cage changes. The sampling time points were 0, 1 wk, and 3, 6, 12, 18 and 24 mo from the beginning of the DB feeding. The zero sample was collected 1 wk after moving the animals from the breeding barrier to the research unit and regrouping them. The 1 wk time point was included to estimate the stress effect of starting the DB feeding.

All faecal pellets were collected from the cage. The pellets were weighed, tap water added, the mixture weighed and blended with a rod-mixer, and the homogenate sampled. Samples were stored at ≤ -20 °C until the end of the experiment, and transported to the laboratory in EPS boxes with dry ice. The analyses were conducted in the University of Copenhagen, Department of Experimental Medicine, with the Corticosterone ELISA kit (DRG EIA-4164), as previously described. (Kalliokoski et al., 2012) For the statistical analysis, total cage FCMs were divided by the combined body weights of all rats in the cage.

A cage was excluded from the dataset if two of the rats had already been removed, if there was some health problem or a rat had needed to be euthanized shortly before, during or shortly after the collection period, or if a water bottle had leaked making the faeces substantially wet.

2.9 Elevated plus-maze

EPM testing was performed at six months from the beginning. The EPM was made of grey, smooth-surfaced PVC. It was 50 cm high, with 10 x 50 cm arms, 30 cm walls around the closed arms, and a 10 x 10 cm central square. A camera (Creative Live! Cam Optia, Creative labs inc., China) was mounted 1.5 m above the center, connected to a computer with maze tracking software (ANY-maze Video Tracking System, Stoelting Co., Wood Dale, IL, USA). Halogen light was reflected obliquely from the ceiling, with the illuminance 90 lx on the central square, 110 and 150 lx on the open arm ends, and 10 and 45 lx on the closed arm ends.

Each cohort was tested over a three day period from Saturday to Monday at 20:00 - 23:00. One rat per cage was tested every night. A cage was brought into the testing room, and after 1 min adaptation to the light, a rat was positioned on the central square facing an open arm. The operator monitored the 5 min test from a computer outside the test room. Immediately after the test, the maze was sprayed with 70 % ethanol and wiped dry. The ethanol was allowed to evaporate for a minimum of 2 min before the next rat was introduced to the maze.

The relative open arm entries and time (the proportion of open arm to combined open and closed arm entries, and the corresponding proportion of time spent in these arms) were calculated for the statistical analysis. The criterion for entering an arm was that all of the rat's paws were over the borderline.

2.10 Cage behaviour recording

A total of 24 rats were observed. Rats from an experimental and control group cage from the same cohort were recorded simultaneously, four days round-the-clock from Friday 18:00 to

Tuesday 18:00. Pairs of female cages were recorded on study weeks 24 and 82 and pairs of male cages on weeks 81 and 90. On week 24, there was only two days of material, from Sunday 18:00 to Tuesday 18:00. Due to a failure in operation of the recorder, two days of week 24 female cage pair footage was lost, as well as the entire recordings of male cages for week 21 and 74, and female cages for week 75 and 93.

The cages were situated on a table in the animal room below cameras with infrared LEDs (WZ16PV408-0, Bosch Security Systems B.V., Eindhoven, The Netherlands). The videos were stored with a digital recorder (EST 4120, EnviroTek Co., Ltd, Seoul, Korea). Rats were ID-marked with a black permanent marker pen (Textmark, Japan) on their backs.

The behavioural categories used are described in Table 1. Rats were observed individually with the instantaneous sampling method, with 1 min intervals for eating and 5 min intervals for other categories (Martin and Bateson, 2007). The numbers of observations in each behavioural category were compared statistically between DB and AL groups in 24 h, 12 h dark and 12 h light periods (Figure 4). The circadian distributions of eating and total activity were visually compared between DB and AL groups from actograms of rats in male and female cage pairs (Figure 5).

2.11 Health, welfare and humane endpoints

Health problems discovered in the daily monitoring were documented and a clinical examination was performed if needed. The study-specific humane end points included tooth problems and excessive reduction or inadequate increase in body weight with age-specific weight limits, as described earlier (Laaksonen et al., 2013). In addition, any other welfare disturbance that could not be quickly ameliorated was an indication for euthanasia.

Rats were euthanized by heart puncture blood collection under isoflurane anaesthesia, and death was ensured with CO₂ in a prefilled chamber.

220 2.12 Statistics

221 The data were analysed with IBM® SPSS® Statistics 20 (IBM Corporation, Somers, NY, USA).

222 The main outcome variables were FCM, EPM open arm parameters and numbers of behavioural
223 observations in the home cage. The main explanatory variable was the feeding method. Males
224 and females were first analysed separately to evaluate if there was any interaction between sex
225 and feeding method. If there was no interaction, their data were combined.

226 Repeated measures (FCM and home cage behaviour) were examined using multivariable linear
227 regression analysis with generalized estimating equations (GEE) to take account of the
228 correlation among results at different time points (Vittinghoff et al., 2005). In the FCM-
229 analyses, birth cohort and time point were included in the models. In the cage behaviour
230 analyses, the number of observations during a 12 or 24 h period was used as the data sample in
231 each time point, with sex, feeding method and time point being included in the models.

232 The EPM open arm parameters were analysed using independent samples Mann-Whitney U
233 test. A nonparametric test was chosen due to the pronounced skewness in the outcome variable
234 distributions.

235 In the parametric tests, the homogeneity of variances was determined with the Levene's test,
236 and the normality of residuals was evaluated graphically with histograms and normality plots.

237 P-values < 0.05 were considered statistically significant.

3 Results

3.1 Faecal corticosterone metabolites

In the repeated measures analysis including all time points, DB females had higher FCM values than all of the other study groups (Figure 2). The time of measurement had a significant effect, i.e. there was a general declining trend. Significant differences between study groups were: DB females – AL females (model-based effect 1.6 $\mu\text{g/kg/72h}$, 95% CI [0.4, 2.8], $P = 0.009$), DB females – DB males (model-based effect 2.9 $\mu\text{g/kg/72h}$, 95% CI [1.8, 4.1], $P < 0.001$) and DB females – AL males (model-based effect 2.4 $\mu\text{g/kg/72h}$, 95% CI [1.0, 3.7], $P < 0.001$).

3.2 Elevated plus-maze

Females displayed higher relative open arm times than males (females: median 11.5 %, interquartile range [2.6, 19.8]; males: median 4.7 %, interquartile range [0.0, 12.9]; $P = 0.01$), but the effect of feeding method was not significant ($P=0.4$) (Figure 3(a)).

Neither feeding method ($P=0.7$) nor sex ($P=0.1$) exerted any statistically significant effect on the relative open arm entries (Figure 3(b)).

3.3 Cage behaviour

3.3.1 Eating. DB males were observed to be eating on average 30 % more often than AL males in the 24 h cycles (model-based effect 24 observations, 95% CI [3.4, 45.2], $P=0.02$) (Figures 4 and 5). In the 12 h dark periods, the average difference was 20 % and in the 12 h light periods 34 %, and although rather large, these differences were not statistically significant.

DB females were observed eating 85 % more often than AL females in the 24 h cycles (model-based effect 45 observations, 95% CI [22.1, 68.4], $P<0.001$), and 100 % more often in the 12 h

dark periods (model-based effect 41 observations, 95% CI [18.7, 63.9], $P < 0.001$) (Figure 4). In the 12 h light periods, the observed 16.5 % difference was statistically non-significant.

3.3.2 Rest. There were fewer rest observations in DB groups of both sexes compared to AL groups in all time periods (Figure 4). However, the differences were statistically significant only in males, in the 24 h cycles (4.3 % difference, model-based effect 9 observations, 95% CI [3.0, 14.2], $P = 0.003$) and 12 h light periods (6.1 % difference, model-based effect 7 observations, 95% CI [2.8, 11.2], $P = 0.001$).

3.3.3 Cage exploration. There was no interaction between sex and feeding method, and thus the data of males and females were combined (Figure 4). The DB rats were observed exploring the cage more often than AL rats at all time periods, but the difference was significant only when it was estimated for the 24 h cycles (19 % difference, model-based effect 7 observations, 95% CI [1.6, 11.6], $P = 0.009$) and 12 h light periods (52 % difference, model-based effect 6 observations, 95% CI [3.1, 8.2], $P < 0.001$).

3.3.4 Grooming. There was no interaction between sex and feeding method, and the data of males and females were combined (Figure 4). The DB rats groomed themselves significantly less than the AL rats in the 24 h cycles (20 % difference, model-based effect 3 observations, 95% CI [0.5, 5.9], $P = 0.019$) and 12 h dark periods (28 % difference, model-based effect 4 observations, 95% CI [0.8, 6.9], $P = 0.014$).

3.3.5 Outside directed interest. There was no interaction between sex and feeding method, and the data of males and females were combined (Figure 4). The feeding method did not affect outside directed interest significantly at any of the time periods.

3.3.6 Drinking, social grooming and agonism. Short bouts relative to the sampling interval and the low total amount of observations rendered the results of drinking (2.8 observations per 24

282 h), social grooming (1.6 observations per 24 h) and agonism (0.4 observations per 24 h) too
283 unreliable for statistical comparisons (Saibaba et al., 1996).

284 *3.3.7 Circadian distributions of activity and eating.* In a visual inspection of the actograms, the
285 circadian distributions of all activity and eating did not differ substantially between DB and AL
286 rats (Figure 5).

4 Discussion

The primary issue investigated in this study was whether DB feeding would affect experimental results or impede animal well-being compared to the standard AL feeding, as these questions need to be answered before DB can be used to feed rats in experiments. We evaluated the potential stress and welfare effects of long-term DB feeding by analysing the FCMs, EPM open arm parameters and home cage behaviour. No clear signs of distress or impaired welfare were found, and the circadian distribution of behaviours was not substantially affected.

In addition to estimating the possible negative effects of DB, its potential as an enrichment item should be considered. Rats have been shown to prefer partitioned cages to non-partitioned ones, although nest-boxes that protect from light and cold are even more preferred (Manser et al., 1998). The four compartments created by the DB increase cage complexity and offer an opportunity to avoid contact with other group members if needed. Having to detach food from the board may satisfy inherent needs for gnawing and foraging, thereby reducing the behavioural deprivation created by the barren cage conditions (Balcombe, 2006). As opposed to meal-feeding DR, rats can eat from the DB whenever they want to, thus being able to satisfy their hunger at will and having a control over the stressful effect of DR.

To compare the stress levels between groups, FCMs over a three day period were used instead of plasma corticosterone, in order to avoid invasive procedures and to reduce the effect of short-term fluctuations on the results. In a repeated measures analysis, the FCMs were higher in DB females than in all other groups (Figure 2).

The mean values diagram shows an apparent difference from six to 18 months between the female groups, and also from three to 18 months between DB females and the male groups. The FCM curve of AL females seems to follow the curves of both male groups, except for the three month time point where AL and DB females are on the same level.

Stress and DR activate different glucocorticoid regulatory pathways. In acute stress without DR, corticosterone levels rise through the activation of the HPA axis (Mormède et al., 2007). With long-term stress, the trough values remain elevated but the maximum decreases, and the 24 h mean value returns to the normal level (Dallman et al., 2000). In contrast, DR elevates glucocorticoid levels independently of the HPA axis (Han et al., 1995; Toth and Gardiner, 2000). The corticosterone level depends on the severity of DR and meal timing relative to the circadian cycle (Belda et al., 2005; Chacón et al., 2005; Levay et al., 2010). In fact with mild DR, corticosterone concentrations do not necessarily increase at all, especially if feeding takes place at the beginning of the dark period, making the circadian rhythm similar as in AL feeding (Levay et al., 2010).

As the food consumption of DB females did not differ from the AL group (Laaksonen et al., 2013), and the circadian distributions of eating and rest were very similar between feeding groups (Figure 5), it seems unlikely that the elevated FCM levels in DB females would have resulted from a DR effect or disrupted circadian rhythm. Although female rats have generally higher plasma corticosterone levels than males (Woodward et al., 1991), Pihl and Hau (Pihl and Hau, 2003) did not detect any difference in FCM levels relative to body weights between sexes in 7- and 10-week-old rats. Their results coincide our findings in the AL male and female groups, with the exception of the higher three month levels of females compared to the males. It appears possible that the elevated FCM levels of DB females, observed from six to 18 months, reflect real stress instead of a sex distinction.

Another possible factor elevating glucocorticoid levels is physical activity. Short-term exercise increases basal and circadian corticosterone in rats (Chen et al., 2017), whereas in long-term studies with moderate exercise intensity, corticosterone has attenuated back to non-training levels (Campbell et al., 2009; Lanza et al., 2012; Lanza et al., 2015). DB feeding increased eating time relatively more in females than males suggesting that the physical effort of gnawing wood, together with the higher cage exploration activity compared to the AL fed rats, could

have contributed to their higher FCMs. However, since there is no apparent difference between female groups in 1 wk and 3 mo samples, it remains open whether an exercise effect could have emerged at a later stage of the experiment.

It was expected that the FCM curves of the DB groups would spike at the 1 wk time point, as the beginning of DB feeding is quite demanding for young rats. There indeed is a spike in the DB males' curve at 1 wk, but considering the large variation typical of FCMs and the lower mean value of DB males in the zero samples compared to other groups, this could be more an apparent than a real effect. On the other hand, moving rats from the breeding barrier to the research unit and regrouping them one week before zero sampling may have stressed them enough to raise the zero sample levels, thus blurring the effect of starting the DB feeding.

The relative open arm entries and open arm time in the EPM test are reduced by anxiety (Cruz et al., 1994; Hogg, 1996). In the EPM test at six months, no statistically significant difference between feeding groups was found in either sex, indicating that the extra workload involved in DB feeding did not provoke anxiety (Figure 3).

The cage behaviour analysis was aimed at assessing whether the DB fed rats had to spend a disproportionate time eating, thereby disrupting their normal rest periods and circadian rhythms, and whether this feeding method would affect their welfare. Of the behavioural categories included especially agonism and outside directed interest, along with a reduced sleep time, have been linked to an impaired welfare (Abou-Ismaïl et al., 2007; Burman et al., 2008). Grooming and social grooming can be seen as positive welfare indicators. Play behaviour would be another excellent sign of well-being, but due to its scant occurrence in middle-aged and old rats, it was excluded from the behavioural category list after an initial scanning of the video material.

The person observing behaviour could not be blinded to the feeding method, as it was obvious in the videos. Another possible source of bias was food in the hopper restricting visibility in the

AL cages. In addition, due to a recorder failure a smaller number of cage pairs was analysed than was originally intended.

DB males were observed to be eating 30 % and DB females 85 % more often than the AL rats (Figure 4). DB males were seen resting 4.2 % less than the AL males, but the smaller difference in the rest observations of females was not statistically significant; understandable in view of their notably smaller food requirements and therefore shorter feeding time than that of males. Furthermore, males spent generally more time resting than females regardless of the feeding method. Thereby DB females had more non-sleeping spare time to allocate to the increased eating workload.

The DB fed rats explored the cage 19 % more often than the AL fed in the light periods, and the AL fed rats groomed themselves in the dark periods 20 % more often than the DB rats (Figure 4). There was no significant difference in the outside directed interest between feeding groups, implying that the DB did not exert any effect on escape behaviour.

Apart from eating, the observed differences in behaviour were relatively small and only part of them were statistically significant, yet they might reflect some degree of mild chronic stress due to DB feeding. The fewer rest observations of the DB males could be linked to reduced sleep time and HPA hyperactivity (Abou-Ismaïl et al., 2007). The reduction in self-grooming of the DB rats could be interpreted as a sign of stress, although it is plausible that the interest in grooming is simply reduced by the increased time needed to be allocated to eating (Spruijt et al., 1992). On the other hand, no difference in escape-related behaviour, together with the minuscule incidence of agonism, indicate that there was no substantial welfare problem linked with DB feeding. Moreover, the more frequent cage exploration observations in the DB groups could result from their better physical condition and the more slowly evolving aging-related changes, achieved by DR and increased physical exercise (Altun et al., 2007). This possibility is

supported by the significantly lower body weights and body mass indexes in both male and female DB groups compared to AL groups (Laaksonen et al., 2013).

The distribution of rest observations between the light and dark periods was very similar in the AL and DB groups (Figure 5). Even though the daytime difference in rest observations between male groups was statistically significant, it was not likely to disrupt the physiological circadian rhythms to the extent of restricted meal feeding during the light period.

DB feeding had a clearly weaker effect to restrict food intake and to reduce the body weight in females than males (Laaksonen et al., 2013). If one considers this in terms of time-budgeting, it appears that females were able to allocate more time than males to the increased work demand due to their smaller food requirements. Moreover, it has been found that in female rats, fasting induces a greater increase in the plasma levels of the orexigenic hormone, ghrelin, a greater reduction in the anorexigenic hormone, leptin, and a greater compensatory increase in food intake after fasting, in comparison to males (Gayle et al., 2006). This difference in the control of hunger and satiety could partly explain the greater resistance of females to the DR effect of DB. The higher FCM levels in DB fed females could indicate that they were more easily stressed than males when the free supply of food was limited and they were prepared to work harder to avoid feelings of hunger.

Another approach to the females' relatively higher investment of time on feeding from DBs is that they might actually experience it as rewarding. Female rats are generally more inclined to exercise voluntarily than males, and combined to the foraging opportunity, DB could be a positive stimulus (Rosenfeld, 2017). Supporting this interpretation, there are incidental reports of increased corticosteroid levels in animals with enriched compared to standard housing (Marashi et al., 2003; Moncek et al., 2004). However, it is not clear whether the corticosteroid rise in the reported cases was actually linked to the positive mental effect of the enrichment or

409 rather to an increased levels of physical activity, which could also be the case with DB fed
410 females.

411 **5 Conclusion**

412 DB feeding did not cause any signs of distress or impaired welfare, and it did not change the
413 circadian distribution of behaviours to any significant extent. Although the dietary restrictive
414 effect of the DB feeding regimen was milder than expected, these experiments demonstrate that
415 this laborious feeding method for the rats provides a feasible alternative to traditional *ad libitum*
416 feeding. The time budget analysis shows there is room for increasing the work demand by
417 adjusting the DB structure, and thereby enhancing its DR effect.

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421 **Declaration of conflicting interest**

422 The Authors declare that there is no conflict of interest.

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556

557 **Tables**

558 **Table 1.** Behavioural categories recorded in the instantaneous cage behaviour sampling.

Behavioural category	Description
Eating	Gnaws food from the diet board or food hopper or a detached food pellet
Drinking	Drinks from the water bottle
Rest	Lies immobile, eyes closed or open
Agonism	Aggressive grooming, hair pulling, tugging; threatening gestures, chasing, kicking, boxing, fighting
Outside directed interest	Rearing and sniffing cage walls and outside environment, rearing and listening to outside noise
Grooming	Grooming or scratching itself
Social grooming	Grooming another rat
Cage exploration	Other activity directed inside the cage, not fitting in the other categories

Figure captions

Figure 1. (a) DB in a rat cage. (b) DB before and after use.

DB: diet board

Figure 2. Mean (with SD) cage FCM of the DB and AL rat groups. FCMs are expressed relative to body weights.

FCM: faecal corticosterone metabolites; DB: diet board; AL: *ad libitum*; BW: body weight

Figure 3. The ratio of open to total arm time (a) and the ratio of open to total arm entries (b) by sex and feeding method in the elevated plus-maze.

Figure 4. Mean numbers of observations of different cage behaviour categories by sex and feeding method, in the 12 h dark and light periods. Observation interval was 5 min. Eating was observed with 1 min intervals for the statistical analyses, but for the illustration the mean numbers were divided by five to show them in proportion to the other categories. For males, N=24 (six rats, four 12 h periods), and for females, N=18 (six rats, three 12 h periods).

Figure 5. Circadian distributions of total activity and eating observations of rats in four cages through a period of four days (Friday 18:00 to Tuesday 18:00), observed by instantaneous sampling with 5 min intervals. Black streaks stand for activity, and the dark background represents dark phases (18:00 - 06:00). Lights came on gradually at 06:00 – 07:00 and dimmed at 18:00 – 19:00.

DB: diet board; AL: *ad libitum*

Figure 1a
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Figure 1b
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Figure 2

Figure 3a

Figure 3b

Figure 4

Figure 5

Supplementary file _ Cage behaviour table

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