Journal of the American Association for Laboratory Animal Science
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Vol 64, No 4
July 2025
Pages 744–749

Effects of Dietary Restriction on Regulation of Energy Metabolism in Male Wistar Rats (*Rattus norvegicus*)

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Laboratory rats are most often fed ad libitum (AL), but dietary restriction (DR) is commonly used to provide appropriate experimental designs. The current methods of DR have shortcomings; animals are often subjected to social isolation, periods of fasting, and disturbed diurnal eating rhythms. The diet board was developed to solve these problems. The diet board offers the possibility of combining group housing with moderate DR without disturbing diurnal eating rhythms or subjecting animals to periods of fasting. In this study, the diet board's validity as a DR method was investigated by assessing possible endocrine effects associated with the previously observed decreases in weight gain and adiposity. Male Wistar rats (n = 30/group) were housed in groups of 3 and fed either with the diet board or AL over a 10-wk study period. Serum ghrelin, leptin, insulin, and adiponectin concentrations and liver triglyceride content and their variance were measured at the end of the study. The diet board showed no reduction potential in energy metabolism parameters. In the serum levels of the adiposity-related hormones leptin, insulin, and adiponectin or liver triglycerides, no statistically significant differences were found. In contrast, levels of ghrelin were significantly lower in the DR rats compared with the controls. In conclusion, diet board feeding induces mild hormonal compensatory changes, thus offering an alternative method of moderate DR in group-housed rats.

Abbreviations and Acronyms: AL, ad libitum; DR, dietary restriction

DOI: 10.30802/AALAS-JAALAS-24-138

Introduction

The most common way of feeding laboratory rodents is ad libitum (AL). In some cases, dietary restriction (DR) is used. There are several reasons for using DR in laboratory rodents. First, the feeding method may be an integral part of the experimental setup. DR-fed rodents can be used as animal models of the effects of weight reduction and maintenance in obesity-related diseases. There are other fields of research where DR is often used to provide appropriate experimental designs for example in the study of eating disorders and other forms of malnutrition. Second, DR may be implemented when the mortality or morbidity of the experimental animals needs to be decreased. It has been repeatedly shown that AL feeding increases the mortality and morbidity of laboratory rodents compared with DR-fed counterparts.[1–7] In toxicology and regulatory testing, the short lifespan and high incidence of neoplastic diseases in AL-fed rodents are problems, for which DR has been recommended as a solution.[1,8]

A third reason to use DR is to control interindividual variability and to standardize the food intake of the experimental animals. In AL feeding, food intake is highly variable between different individuals, whereas DR can offer the possibility to control the food intake.[9–11] A fourth reason to use DR is to establish a stronger motivation to eat. This is required in some behavioral tests, where food is used as a reward.

Obesity caused by AL may lead to increased numbers of animals needed in the studies either via poor survival or increased interindividual variability. A potential increase in each and any parameter variance leads to an exponential increase in the number of animals required for the same study outcome. This has been shown to be the case with organ weights but not with other parameters.[11–13]

Regardless of the rationale behind the choice of the feeding method, a common way to implement DR in laboratory rodents is to provide them with a precalculated portion of food once a day, during working hours. To avoid aggressive competition for food, the animals are often single housed.[14] This method of DR has shortcomings with respect to both scientific integrity and animal welfare. Social isolation is a potent stressor for rats and can lead to decreased welfare.[15–19] Current European legislation recommends that rats be housed individually only if there is a justification based on veterinary, welfare, or experimental grounds.[20] Another concern is the diurnal rhythms of the experimental animals. When fed AL, rats eat several small meals during the dark period.[21,22] The current methods of DR disrupt the diurnal rhythms of feeding and consequently interfere with many other physiologic variables.[23–25] One might hypothesize that it is very difficult to differentiate the effects of the stress of social isolation and the altered diurnal rhythms from those of the actual caloric restriction. This may confound the interpretation of research results.

Our research group has developed a feeding device, the diet board, to solve some of the problems associated with the current

Submitted: 20 Nov 2024. Revision requested: 27 Dec 2024. Accepted: 02 May 2025.
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methods of DR in laboratory rats. The advantages of the diet board include the possibility of group housing the animals and allowing them to maintain normal diurnal eating rhythms while subjecting them to moderate levels of DR.[26–28]

The purpose of this study was to determine the diet board's effects on the serum levels and variance of the hormones ghrelin, leptin, insulin, and adiponectin or liver triglycerides compared with AL feeding. These hormones reflect the levels of food intake and adiposity of the individual.[29,30] It is not sufficient that the diet board simply produces similar effects on weight gain and thus shows face validity to the current methods of DR. To be an acceptable alternative, the diet board should possess true construct validity; that is, the same mechanisms should be responsible for the similarities in the observed outcomes.[31–33] Our goal was to assess how the diet board would affect the hormone and liver triglyceride levels, and their variance compared with AL feeding.

Ethical review. The study was done in the Laboratory Animal Centre, University of Eastern Finland. The study plan was reviewed and approved by the Finnish National Ethics Committee. The study complies fully with the EU Directive (2010/63/EU) on the protection of animals used for scientific purposes and the corresponding Finnish legislation.

Materials and Methods

Animals and housing. Sixty male HsdBrlHan:WIST rats (NLAC, Kuopio, Finland) were used. The animals were 7 wk old when the study began. The breeding unit and the experimental unit were free of the pathogens listed in the Federation of European Laboratory Animal Science Association recommendations for health monitoring.[34] The animals were group-housed (3 rats per cage) in solid-bottom stainless-steel cages with a wire-grid top (48.5 × 28.5 × 20.0 cm; Franke Finland Ltd) in a cubicle.

The ambient room temperature and relative humidity were 21 ± 1 °C and 55% ± 15%, respectively, and the ventilation provided 15 air changes per hour. The light-dark cycle was 12 h:12 h, and the lights went on at 0700. Tap water in polycarbonate bottles was always freely available for all animals. The animals were fed with autoclaved Lactamin R36 chow (Lantmännen, Stockholm, Sweden). Food availability is described below. Aspen chips (5 × 5 × 1 mm; Tapvei Ltd, Paekna, Estonia) and aspen wool (Tapvei Ltd, Paekna, Estonia) were used as bedding and nesting material; both were changed twice every week.

The diet board. The diet board (Figure 1A and B) consisted of 2 aspen boards (35.0 × 12.2 × 2.7 cm); 2 corners of each board were removed (6.0 × 6.0 cm) to facilitate the rats' movement within the cage. Each board had 20 vertical drill holes (Ø 12.5 mm) with a 2- to 3-mm slot open to the side of the board. The holes were filled with food pellets that were fixed in place by autoclaving the board (121 °C, 20 min, 220 kPa; Finn-Aqua 121821 D; Steris Finn-Aqua). The control animals had similar autoclaved boards of the same size but without the drill holes and food. The boards were placed into the cage in the form of a cross made by intersecting 2 boards providing 4 separate compartments. The boards were made of aspen, which is the same material as the bedding and nesting material, and presumably had the same emission profile but at lower concentrations than the nonautoclaved aspen bedding material.[35]

Study design. The animals were divided into 2 groups (n = 30 in each group). The study group (DR) was fed exclusively with the diet board throughout the experiment. The control group (AL) was fed AL, which means food was always available in unrestricted quantities in the food hopper.

The groups were formed in the following manner. Ten litters with a minimum of 6 male siblings were ordered for the study, and 6 males from each litter were chosen for the experiment. These 6 siblings were housed together from weaning until the beginning of the study. On a Friday, each of the sextets was divided at random into 2 cages; one cage designated for diet board feeding and the other cage for AL feeding. Thus, both the DR and AL groups consisted of 10 cages with 3 males from the same litter in each cage. The animals were ear tattooed. All animals continued to be fed AL during the following weekend to allow them an acclimatization period. The study began on the following Monday when the animals were 7 wk old. The diet boards and plain boards were introduced into the cages, and food was removed from the food hoppers of the DR rats' cages. The boards were replaced with new ones weekly at each Monday cage change. The animals entered the study in 3 cohorts, all following the same week-day routine. The cages were changed twice a week, on Monday afternoon (1200 to 1400) and Friday morning (0900 to 1100). The rats were weighed during each cage change. The study lasted 10 wk.

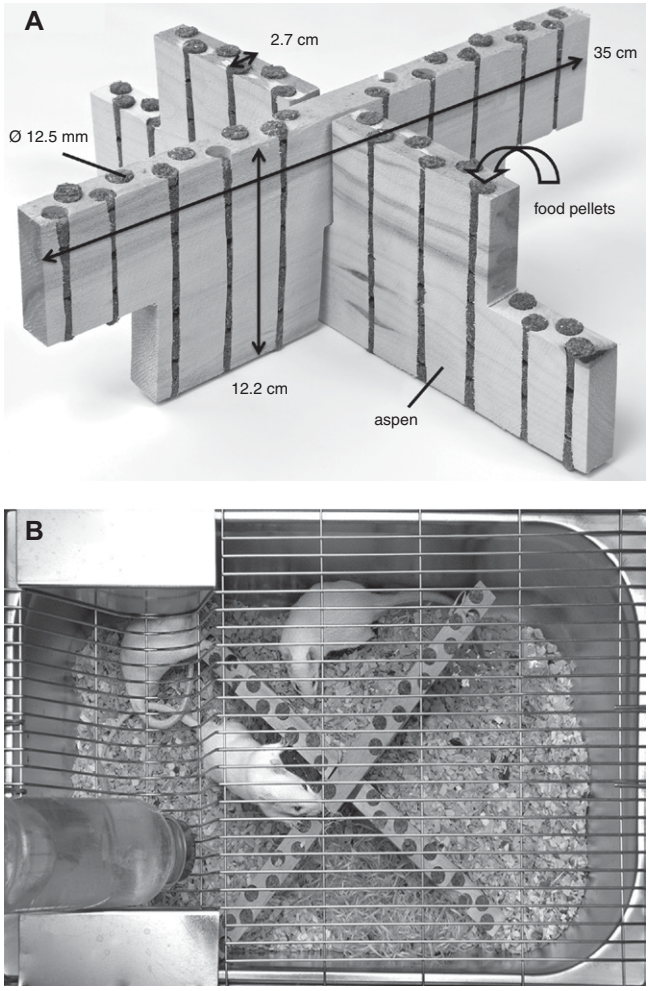

Figure 1. (A) Structure of the diet board with dimensions and (B) its placement in the cage environment.

Humane endpoints. Three different age-specific humane endpoints were established. From 7 to 8 wk of age, the limit was preset to 15% loss of body weight. From 8 to 13 wk of age, there were 2 humane endpoints: failure to gain weight during a 2-wk period or a 5% loss of body weight for one week. Thereafter, the limit was set at 10% loss of body weight for one week. Additional clinical signs included in the humane endpoints throughout the study were dehydration, unexpected disease, trauma, or dental problems.

Blood sampling. A terminal blood sample was taken by cardiac puncture at the end of week 10 between 0900 to 1300. The animals were anesthetized with a mixture of O_2 and CO_2 (1:1). The blood was allowed to coagulate at room temperature for 10 to 15 min, and the samples were then centrifuged at 3,600 rpm for 15 min at 4 °C (Megafuge 1.0R; Heraeus Instruments, Hanau, Germany). The serum was cooled on dry ice and stored at –80 °C.

Euthanasia and postmortem examination. The animals were euthanized at the end of study week 10 with carbon dioxide immediately after the cardiac puncture. A postmortem examination was performed without delay. The liver was removed, washed in 0.9% NaCl, divided into 3 plastic tubes, and frozen in liquid nitrogen. The other organs were checked for gross abnormalities.

Leptin, adiponectin, ghrelin, and insulin. The concentrations of serum insulin (Rat Ultrasensitive ELISA Kit; Mercodia AB, Uppsala, Sweden), ghrelin (Total Ghrelin RIA Kit; Linco Research, St. Charles, MO), leptin (Rat Leptin RIA Kit), and adiponectin (Rat Adiponectin ELISA Kit; Linco Research, St. Charles, MO) were measured using commercial kits.

Liver triglycerides. Liver triglycerides were isolated and measured as previously described[36] by using the Folch method for extraction and Free Glycerol Reagent (F6428) and Triglyceride Reagent (T2449; Sigma Diagnostics) for quantitation.

Data processing and statistical analyses. The appropriate size of the experiment was estimated with the Resource equation method.[37] The differences between the DR and AL groups were analyzed with linear mixed models using all collected data. A random litter effect was included in the models. All the variables were log transformed before the statistical analysis. The results are presented as model-based estimates of the geometric means, their ratios, and their 95% CI. Box plots from the raw data are used for the graphic presentation of the measured variables. The equality of variances was investigated with likelihood ratio tests performed on residual variance terms. The statistical software package used to process and analyze the data was SPSS 14.0 for Windows. The graphs were drawn with SigmaPlot 10.0 (Systat Software).

Results

Detailed results on growth and adiposity have been published elsewhere. They showed that at the end of the 10-wk experiment with the diet board (15% DR as compared with AL), the rat weights (mean ± SD) were 321 ± 38 g for the DR group and 360 ± 35 g for the AL group, a difference with $P < 0.0001$. Moreover, the weight of gonadal fat, postmortem, was 4.47 ± 0.90 g in DR rats and 5.66 ± 1.06 g in AL rats, a difference with $P < 0.0001$.[26]

Leptin, adiponectin, ghrelin, and insulin. The serum hormone concentrations were analyzed from terminal blood samples taken at the end of test week 10. The serum ghrelin concentrations were lower in the DR animals. The model-based estimates of the geometric means for the groups were 1,700 pg/mL (95% CI [1,380 to 2,100]) in DR rats and 2,400 pg/mL (95% CI [1,940 to 2,970]) in AL rats. The ratio of the geometric means was 0.71 (95% CI [0.607 to 0.83], $P < 0.001$). No statistically significant differences were found between the DR and AL groups in the serum levels of leptin, adiponectin, or insulin ($P > 0.05$) (Figure 2; Table 1).

Liver triglycerides. The liver triglyceride content was analyzed from the liver samples taken at necropsy at the end of test week 10. No difference was found between the DR and AL groups ($P > 0.05$) (Figure 2).

Result variation. The equality of variances was investigated for serum concentrations of ghrelin, leptin, insulin, and adiponectin and liver triglycerides. No statistically significant differences ($P > 0.05$) were detected between the DR and AL groups in the variances of any of these variables (Table 1).

Humane endpoints. None of the animals reached any of the preset humane endpoints.

Discussion

The diet board is a method of restricting the weight gain of laboratory rats. It allows the rats to be group-housed and does not expose the animals to periods of fasting. The diurnal rhythms of physical activity, blood pressure, and heart rate remain undisturbed.[28] The purpose of this study was to validate the diet board as an alternative method of DR in studies on energy metabolism.

The diet board significantly reduces the food consumption of rats.[26] The difference in absolute food consumption is approximately 15% during the sixth week of diet board feeding. When related to body weight, diet board and AL animals consume an equal amount of food per gram of body weight. The caloric intake is roughly estimated as 0.2 kcal/g body weight/day in both groups. This phenomenon is well known in DR research; the energy intake/body weight ratio remains the same on different levels of food intake.[35] These results suggest that the effects of the diet board are a result of true DR and not attributable to increased caloric expenditure due to physical exertion (that is gnawing).

The 15% difference in food intake corresponds with the 15% difference in body weight after 10 wk of diet board feeding. The diet board-fed animals also have 30% less gonadal fat and 3% shorter tibiae.[26] Thus, the diet board decreases fat accumulation quite effectively but disturbs skeletal growth only minimally. This is in accordance with other studies, where the lean body mass[38] and musculoskeletal system[39] were only marginally affected by DR.

Obesity in rats is typically a problem of outbred rats, and the majority of DR studies are done with Wistar or Sprague–Dawley stocks. DR procedures applied to rats are variable, as duration ranges from 10 d to 6 mo, severity ranges from 20% to 75% reduction from AL amounts,[40–43] and food presentation requires either permanent or temporary solitary housing. None of these come without consequences. Feeding time may derail diurnal rhythm,[25] and DR severity and duration may have a major impact on results and their interpretation.

The current methods of DR have extensive effects on lipid metabolism in rodents; DR has been repeatedly shown to decrease serum cholesterol, triglyceride, and free fatty acids (FFA) levels.[4,40,42,44,45] The diet board-fed animals also have lower levels of serum triglycerides, FFA, and cholesterol.[26] Liver triglycerides have been reported to be unaffected by DR,[42] which was also the case of diet board.[26]

Leptin and adiponectin are produced and secreted from fat tissue, insulin from the pancreas, and ghrelin from the gastric mucosa.[30] The circulating levels of leptin, adiponectin, and insulin reflect the fat content of the body.[29,46–49] The current methods of DR have been associated with decreased serum levels of leptin and insulin and increased levels of adiponectin.[40,42,48,50–53] In this study, however, no statistically significant differences were found between the DR and AL animals in their serum levels of leptin, insulin, or adiponectin ($P > 0.05$). The degree of DR (85% of AL) achieved with the diet board was relatively mild, thus possibly not eliciting as prominent changes as more severe regimes of DR. Furthermore, in the current methods of DR, the animals are often food deprived for long periods every day, which is not the case in diet board feeding.

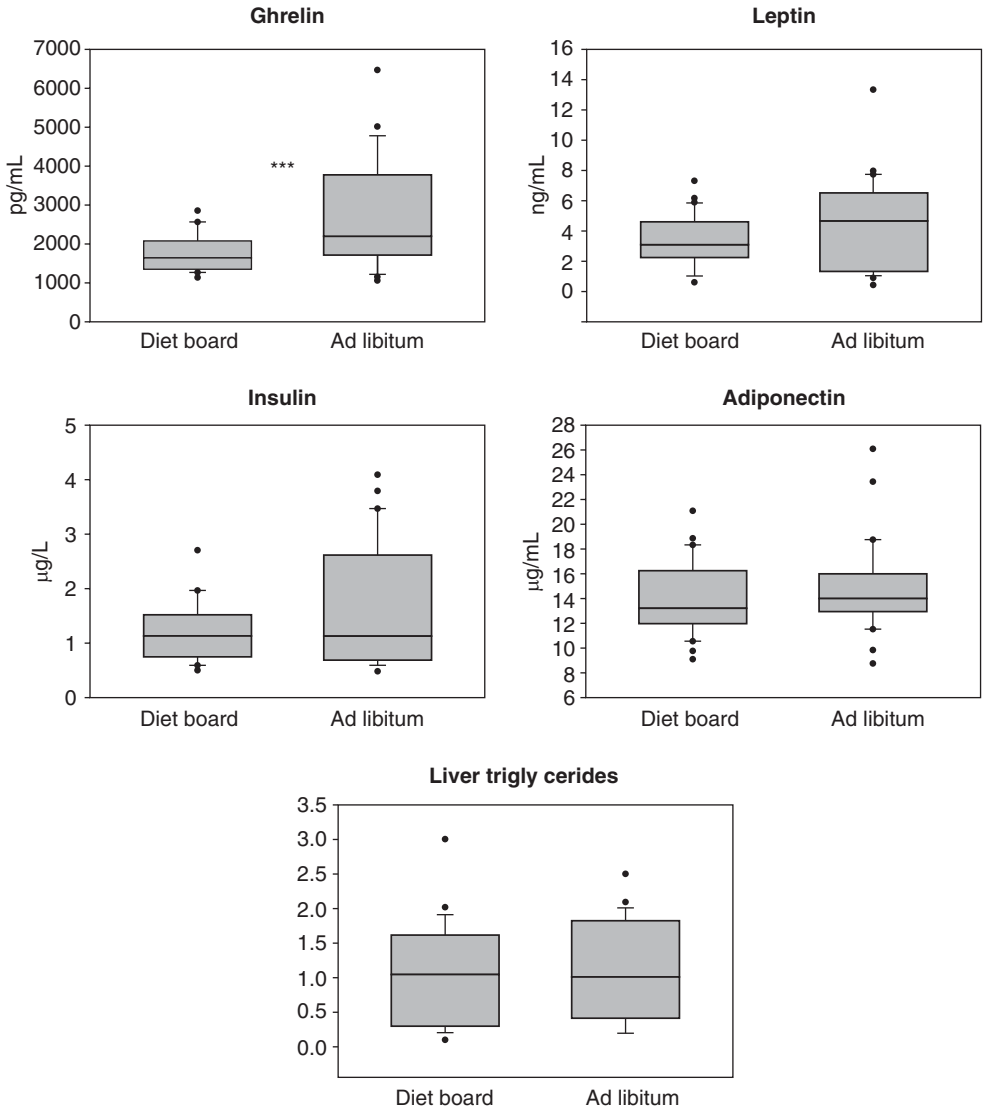

Figure 2. Box plots of serum ghrelin, leptin, insulin, and adiponectin concentrations and liver triglyceride content at the end of the 10-wk diet restriction experiment with the diet board. ***, $P < 0.001$, statistical significance of the differences between the diet board ($n = 30$) and the ad libitum ($n = 30$) groups tested with a linear mixed model.

The serum levels of ghrelin were significantly ($P < 0.001$) lower in DR rats compared with AL rats, and the decrease from 2,400 to 1,700 pg/mL is so large that it cannot be considered a false positive result (Figure 2; Table 1). This result is at odds with the known functions of ghrelin. Ghrelin is an orexigenic hormone, and decreased food intake and low body weight are associated with elevated levels of ghrelin.[29,54–56] Increased levels of both plasma and gastric ghrelin have been observed in rats subjected to DR ranging in severity from 30% to 75% of AL food intake.[41,42,54] There are, however, examples where DR has failed to increase serum ghrelin[43] or even resulted in decreased ghrelin levels.[40] A possible explanation behind the decreased ghrelin levels in DR rats observed in this study could be the timing of the meals. Even though diet-board-fed rats show similar diurnal rhythms of activity compared with the AL rats,[28] it could be that the diet board rats distribute their food intake in a more prolonged and scattered manner throughout the night, thus having eaten more recently before the blood sampling than the AL animals. The decreased levels of FFA in the terminal blood samples collected in the morning provide support for the notion that the diet board rats' eating activity is more pronounced during the end of the dark phase compared with the AL-fed rats.[26]

Increased secretion of corticosterone is an integral part of the organism's metabolic response to decreased energy intake, and

Table 1. Values and distributions of hormones and adiponectin at the end of the 10-wk study.

	Diet board				Ad libitum			
Group	Mean	Quartile 1	Median	Quartile 3	Mean	Quartile 1	Median	Quartile 3
Ghrelin (pg/mL)	1,764	1,405	1,741	2,147	2,705	1,624	2,175	3,680
Leptin (ng/mL)	3.5	2.4	3.2	4.7	4.6	1.4	4.6	6.6
Insulin (µg/L)	1.2	0.8	1.2	1.5	1.6	0.7	1.2	2.7
Adiponectin (µg/mL)	13.9	11.9	13.3	16.8	14.9	12.6	13.7	16.1

elevated levels of corticosterone have been repeatedly observed in rodents subjected to DR.[45,56–61] Serum corticosterone concentrations are likewise elevated in the diet board rats.[27] In line with our results, DR has been reported to increase corticosterone levels in the absence of significant effects on adiponectin or ghrelin levels.[43]

DR has been reported to decrease result variation[11,13,62–64] and has been named a "powerful Reduction tool".[65] Diet board feeding, however, does not seem to possess this kind of reduction potential. No differences were found in the variation of the investigated variables, neither in this study nor in our previous studies[26] (Table 1). Perhaps the most striking difference to the earlier studies is having rats group housed all the time. In the study of Moraal et al.,[11] the DR rats were denied access to food during the day and received a restricted amount of food during the night while single housed. Furthermore, they[11] were able to feed the animals with precalculated doses while in our study the rats were able to eat at will provided they work (gnaw) for food. The likely explanation is that the diet board feeding does not offer any more control over the animals' food intake than AL feeding.

Moraal et al.,[11] showed that food restriction feeding leads to a significantly reduced variation in body weight, growth, and lung weight, thereby indicating the potential for reduction. With respect to the 9 blood parameters tested, they found no differences in the variation, indicating that a 25% reduction in food intake may not affect the variation of clinical chemistry values.[11] These findings are in line with our results although diet board leads to only a 15% reduction in food intake.

The diet board does have shortcomings. It is not possible to regulate or register the food intake of individual animals. Rats younger than 7 wk are unable to get enough food from the diet board, and the animals require at least a one-week period of learning and adjustment with the diet board before the food intake and behavior has stabilized. The diet board cannot be used on animals with dental problems or other difficulties in eating.

In conclusion, the diet board offers a method of achieving moderate DR. The weight gain and adiposity are moderately decreased. The levels of the adiposity-related hormones leptin, insulin, and adiponectin are not significantly decreased. The decreased serum levels of the hunger signal ghrelin and FFA in the morning suggest that the diet board rats' eating activity is more pronounced during the end of the dark phase compared with the AL-fed rats. Diet board feeding offers a low-tech and pragmatic method for DR with group housing in rats, which does not cause disruption to the animals' diurnal rhythms. The diet board does not possess reduction potential for tested energy metabolism parameters.

Conflict of Interest

The authors have no conflicts of interest to declare.

Funding

This study was financially supported by the Finnish Ministry of Education, the Academy of Finland, the European College of Laboratory Animal Medicine and European Society of Laboratory Animal Veterinarian Foundations, Universities Federation for Animal Welfare, the Research and Science Foundation of Farmos, the Finnish Foundation of Veterinary Sciences, and the Oskar Öflund Foundation.

References

1. Allaben WT, Turturro A, Leakey JEA, Seng JE, Hart RW. FDA points-to-consider documents: the need for dietary control for the reduction of experimental variability within animal assays and the use of dietary restriction to achieve dietary control. *Toxicol Pathol.* 1996;24(6):776–781.
2. Duffy PH, Lewis SM, Mayhugh MA, et al. The effects of different levels of dietary restriction on non-neoplastic diseases in male Sprague-Dawley rats. *Aging Clin Exp Res.* 2004;16(1):68–78.
3. Duffy PH, Lewis SM, Mayhugh MA, et al. The effects of different levels of dietary restriction on neoplastic pathology in the male Sprague-Dawley rat. *Aging Clin Exp Res.* 2004;16(6):448–456.
4. Hubert MF, Laroque P, Gillet JP, Keenan KP. The effects of diet, ad libitum feeding, and moderate and severe dietary restriction on body weight, survival, clinical pathology parameters, and cause of death in control sprague-dawley rats. *Toxicol Sci.* 2000;58(1):195–207.
5. Keenan KP, Ballam GC, Soper KA, Laroque P, Coleman JB, Dixit R. Diet, caloric restriction, and the rodent bioassay. *Toxicol Sci.* 1999;52(2 suppl):24–34.
6. Leakey JEA, Seng JE, Alleben WT. Influence of body weight, diet, and stress on aging, survival and pathological endpoints in rodents: implications for toxicity testing and risk assessment. *Regul Res Perspect.* 2004;4:1–29.
7. Masoro EJ. Dietary restriction-induced life extension: a broadly based biological phenomenon. *Biogerontology.* 2006;7(3): 153–155.
8. Hart RW, Keenan K, Turturro A, et al. Caloric restriction and toxicity. *Toxicol Sci.* 1995;25(2):184–195.
9. Keenan KP, Laroque P, Dixit R. Need for dietary control by caloric restriction in rodent toxicology and carcinogenicity studies. *J Toxicol Environ Health B Crit Rev.* 1998;1(2):135–148.
10. Turturro A, Duffy P, Hart R, Allaben WT. Rationale for the use of dietary control in toxicity studies—B6C3F1 mouse. *Toxicol Pathol.* 1996;24(6):769–775.
11. Moraal M, Leenaars PPAM, Arnts H, et al. The influence of food restriction versus ad libitum feeding of chow and purified diets on variation in body weight, growth and physiology of female Wistar rats. *Lab Anim.* 2012;46(2):101–107.
12. Keenan KP, Soper KA, Smith PF, Ballam GC, Clark RL. Diet, overfeeding, and moderate dietary restriction in control Sprague-Dawley rats: I. Effects on spontaneous neoplasms. *Toxicol Pathol.* 1995;23(3):269–286.
13. Duffy PH, Seng JE, Lewis SM, et al. The effects of different levels of dietary restriction on aging and survival in the Sprague-Dawley rat: implications for chronic studies. *Aging (Milano).* 2001;13(4):263–272.
14. Pugh TD, Klopp RG, Weindruch R. Controlling caloric consumption: protocols for rodents and rhesus monkeys. *Neurobiol Aging.* 1999;20(2):157–165.
15. Brenes JC, Rodríguez O, Fornaguera J. Differential effect of environment enrichment and social isolation on depressive-like behavior, spontaneous activity and serotonin and norepinephrine concentration in prefrontal cortex and ventral striatum. *Pharmacol Biochem Behav.* 2008;89(1):85–93.
16. Hall FS. Social deprivation of neonatal, adolescent, and adult rats has distinct neurochemical and behavioral consequences. *Crit Rev Neurobiol.* 1998;12(1–2):129–162.
17. Karim A, Arslan MI. Isolation modifies the behavioural response in rats. *Bangladesh Med Res Counc Bull.* 2000;26(1):27–32.
18. Krohn T, Sørensen D, Ottesen J, Hansen AK. The effects of individual housing on mice and rats: a review. *Anim Welf.* 2006;15(4):343–352.
19. Serra M, Pisu MG, Floris I, Biggio G. Social isolation-induced changes in the hypothalamic–pituitary–adrenal axis in the rat. *Stress.* 2005;8(4):259–264.
20. *Commission Recommendation on 18 June 2007 on Guidelines for the Accommodation and Care of Animals Used for Experimental and Other Scientific Purposes.* 2007/526/EC. European Commission. 2007. [Cited 26 May 2015]. Available at: http://eur-lex.europa.eu/JOHtml.do?uri=OJ:L:2007:197:SOM:EN:HTML.
21. Spiteri NJ. Circadian patterning of feeding, drinking and activity during diurnal food access in rats. *Physiol Behav.* 1982;28(1):139–147.
22. Strubbe JH, Woods SC. The timing of meals. *Psychol Rev.* 2004;111(1):128–141.
23. Claassen V. *Neglected Factors in Pharmacology and Neuroscience Research: Biopharmaceutics, Animal Characteristics, Maintenance, Testing Conditions.* Elsevier; 1994.
24. Damiola F, Le Minh N, Preitner N, Kornmann B, Fleury-Olela F, Schibler U. Restricted feeding uncouples circadian oscillators in

peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. *Genes Dev.* 2000;14(23):2950–2961.
25. Nelson W. Food restriction, circadian disorder and longevity of rats and mice. *J Nutr.* 1988;118(3):286–289.
26. Kasanen IHE, Inhilä KJ, Nevalainen JI, et al. A novel dietary restriction method for group-housed rats: weight gain and clinical chemistry characterization. *Lab Anim.* 2009;43(2):138–148.
27. Kasanen IHE, Inhilä KJ, Vainio OM, et al. The diet board: welfare impacts of a novel method of dietary restriction in laboratory rats. *Lab Anim.* 2009;43(3):215–223.
28. Kemppinen N, Meller A, Mauranen K, et al. Work for food – a solution to restricting food intake in group housed rats? *Scand J Lab Anim Sci.* 2008;35:81–90.
29. Popovic V, Duntas LH. Brain somatic cross-talk: ghrelin, leptin and ultimate challengers of obesity. *Nutr Neurosci.* 2005;8(1):1–5.
30. Valassi E, Scacchi M, Cavagnini F. Neuroendocrine control of food intake. *Nutr Metab Cardiovasc Dis.* 2008;18(2):158–168.
31. Carmines EG, Zeller RA. *Reliability and Validity Assessment.* Sage Publications Inc; 1979.
32. Guion RM. On Trinitarian doctrines of validity. *Prof Psychol.* 1980;11(3):385–398.
33. Trochim W, Donnelly JP. *The Research Methods Knowledge Base.* 3rd ed. Cengage Learning; 2006.
34. Nicklas W, Baneux P, Boot R, et al. Recommendations for the health monitoring of rodent and rabbit colonies in breeding and experimental units. *Lab Anim.* 2002;36(1):20–42.
35. Nevalainen T, Vartiainen T. Volatile organic compounds in commonly used beddings before and after autoclaving. *Scand J Lab Anim Sci.* 1996;23:2.
36. Ruohonen ST, Pesonen U, Moritz N, et al. Transgenic mice overexpressing neuropeptide Y in noradrenergic neurons. *Diabetes.* 2008;57(6):1517–1525.
37. Festing MFW, Overend P, Das RG, et al. *The Design of Animal Experiments: Reducing the Use of Animals in Research through Better Experimental Design.* The Royal Society of Medicine Press Ltd. 2002.
38. Yu BP, Masoro EJ, Murata I, Bertrand HA, Lynd FT. Life-span study of SPF Fischer 344 male rats fed ad libitum or restricted diets: longevity, growth, lean body mass and disease. *Sci Aging Knowl Environ.* 2002;2002(37):cp18.
39. Keenan KP, Hoe C-M, Mixson L, et al. Diabesity: a polygenic model of dietary-induced obesity from ad libitum overfeeding of Sprague–Dawley rats and its modulation by moderate and marked dietary restriction. *Toxicol Pathol.* 2005;33(6):650–674.
40. Martin B, Pearson M, Kebejian L, et al. Sex-dependent metabolic, neuroendocrine, and cognitive responses to dietary energy restriction and excess. *Endocrinology.* 2007;148(9):4318–4333.
41. Barazzoni R, Zanetti M, Stebel M, Biolo G, Cattin L, Guarnieri G. Hyperleptinemia prevents increased plasma ghrelin concentration during short-term moderate caloric restriction in rats. *Gastroenterology.* 2003;124(5):1188–1192.
42. Barazzoni R, Zanetti M, Bosutti A, et al. Moderate caloric restriction, but not physiological hyperleptinemia per se, enhances mitochondrial oxidative capacity in rat liver and skeletal muscle—tissue-specific impact on tissue triglyceride content and AKT activation. *Endocrinology.* 2005;146(4):2098–2106.
43. Johansson A, Fredriksson R, Winnergren S, Hulting AL, Schiöth HB, Lindblom J. The relative impact of chronic food restriction and acute food deprivation on plasma hormone levels and hypothalamic neuropeptide expression. *Peptides.* 2008;29(9):1588–1595.
44. Curi R, Hell NS. Metabolic changes of twenty weeks food-restriction schedule in rats. *Physiol Behav.* 1986;36(2):239–243.
45. Harris SB, Gunion MW, Rosenthal MJ, Walford RL. Serum glucose, glucose tolerance, corticosterone and free fatty acids during aging in energy restricted mice. *Mech Ageing Dev.* 1994;73(3):209–221.
46. Lafontan M, Viguerie N. Role of adipokines in the control of energy metabolism: focus on adiponectin. *Curr Opin Pharmacol.* 2006;6(6):580–585.
47. Mao X, Hong J, Dong L. The adiponectin signaling pathway as a novel pharmacological target. *Mini Rev Med Chem.* 2006;6(12):1331–1340.
48. Shimokawa I, Higami Y. A role for leptin in the antiaging action of dietary restriction: a hypothesis. *Aging (Milano).* 1999;11(6): 380–382.
49. Sinha MK, Ohannesian JP, Heiman ML, et al. Nocturnal rise of leptin in lean, obese, and non-insulin-dependent diabetes mellitus subjects. *J Clin Invest.* 1996;97(5):1344–1347.
50. Escrivá F, Gavete ML, Fermín Y, et al. Effect of age and moderate food restriction on insulin sensitivity in Wistar rats: role of adiposity. *J Endocrinol.* 2007;194(1):131–141.
51. Feuers R. The relationship of dietary restriction to circadian variation in physiologic parameters and regulation of metabolism. *Aging (Milano).* 1991;3(4):399–401.
52. Gallardo N, Arribas C, Villar M, et al. ObRa and ObRe are differentially expressed in adipose tissue in aged food-restricted rats: effects on circulating soluble leptin receptor levels. *Endocrinology.* 2005;146(11):4934–4942.
53. Maffei M, Halaas J, Ravussin E, et al. Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. *Nat Med.* 1995;1(11):1155–1161.
54. Gil-Campos M, Aguilera CM, Cañete R, Gil A. Ghrelin: a hormone regulating food intake and energy homeostasis. *Br J Nutr.* 2006;96(2):201–226.
55. Hosoda H, Kojima M, Kangawa K. Biological, physiological, and pharmacological aspects of ghrelin. *J Pharmacol Sci.* 2006;100(5):398–410.
56. Klok MD, Jakobsdottir S, Drent ML. The role of leptin and ghrelin in the regulation of food intake and body weight in humans: a review. *Obes Rev.* 2007;8(1):21–34.
57. Armario A, Montero JL, Jolin T. Chronic food restriction and the circadian rhythms of pituitary-adrenal hormones, growth hormone and thyroid-stimulating hormone. *Ann Nutr Metab.* 1987;31(2):81–87.
58. Han E-S, Levin N, Bengani N, et al. Hyperadrenocorticism and food restriction-induced life extension in the rat: evidence for divergent regulation of pituitary proopiomelanocortin RNA and adrenocorticotropic hormone biosynthesis. *J Gerontol A Biol Sci Med Sci.* 1995;50(5):B288–B294.
59. Heiderstadt KM, McLaughlin RM, Wright DC, Walker SE, Gomez-Sanchez CE. The effect of chronic food and water restriction on open-field behaviour and serum corticosterone levels in rats. *Lab Anim.* 2000;34(1):20–28.
60. Patel N, Finch C. The glucocorticoid paradox of caloric restriction in slowing brain aging. *Neurobiol Aging.* 2002;23(5):707–717.
61. Sabatino F, Masoro EJ, McMahan CA, Kuhn RW. Assessment of the role of the glucocorticoid system in aging processes and in the action of food restriction. *J Gerontol.* 1991;46(5):B171–B179.
62. Leakey JEA, Seng JE, Allaben WT. Body weight considerations in the B6C3F1 mouse and the use of dietary control to standardize background tumor incidence in chronic bioassays. *Toxicol Appl Pharmacol.* 2003;193(2):237–265.
63. Carney EW, Zablotny CL, Marty MS, et al. The effects of feed restriction during in utero and postnatal development in rats. *Toxicol Sci.* 2004;82(1):237–249.
64. Leakey JEA, Seng JE, Latendresse JR, Hussain N, Allen LJ, Allaben WT. Dietary controlled carcinogenicity study of chloral hydrate in male B6C3F1 mice. *Toxicol Appl Pharmacol.* 2003;193(2):266–280.
65. Ritskes-Hoitinga M, Savenije B. *The Power of Moderate Food Restriction. Abstract*, FELASA-ICLAS Joint Symposium, Cernobbio, Italy, 2007.