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The diet board – a novel method of dietary restriction for laboratory rats

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ACADEMIC DISSERTATION

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Abstract

Laboratory rodents are routinely fed *ad libitum*, *i.e.* food is available at all times in unlimited quantities. The practice of *ad libitum* feeding has been widely criticized. Concerns include the high incidence of morbidity and the low survival rates associated with *ad libitum* feeding. It has also been claimed to accentuate inter-individual variability in research results. Dietary restriction, *i.e.* restricting caloric intake, has been proposed to resolve many of these problems. Dietary restriction does decrease morbidity and mortality and can also decrease result variation. However, the current methods of dietary restriction can also have negative impacts on both animal welfare and scientific integrity. Dietary restriction often subjects the animals to social isolation and to abnormal feeding schedules. As a consequence, the animals may suffer from unfulfilled behavioural needs and the interpretation of research results might suffer from the disrupted circadian rhythms.

A novel method of dietary restriction, the diet board, was developed as a solution for feeding laboratory rats. The diet board is a wooden board into which food pellets are embedded. In order to obtain food, the rats have to gnaw at the wood, making it more difficult to eat. The diet board can be kept in the cage continuously. The diet board offers the possibility of combining dietary restriction with group-housing and unaltered circadian eating rhythms. In this dissertation, the diet board and its basic characteristics will be presented.

The results are based on one experiment and some pilot studies preceding it. A total of 60 male Wistar rats were used. The rats were seven weeks old at the beginning of the ten week experiment. Half of the animals were fed exclusively with the diet board and the other half *ad libitum,* with the rats housed in groups of three.

The diet board provided mild to moderate dietary restriction. The food intake was 85% of the *ad libitum* level and the diet board rats gained 15% less weight. The diet board rats had significantly less gonadal fat, but their skeletal growth was only minimally hindered.

Serum albumin, alanine aminotransferase and protein levels were not altered by diet board feeding. Serum creatine kinase was slightly elevated in the diet board group.

More differences were observed in variables related to energy metabolism. The diet board group had lower levels of serum triglycerides, free fatty acids and cholesterol; and a higher serum corticosterone. Surprisingly, serum ghrelin was significantly higher in the *ad libitum* rats. In serum leptin, adiponectin and insulin, no differences were detected.

In variables related to stress reactions, the *ad libitum* rats had larger adrenal glands with a higher adrenaline and noradrenaline content, indicating elevated sympathetic tone compared to the diet board rats. In concordance with their elevated corticosterone levels, the diet board rats had a lower secretion rate of faecal immunoglobulin A.

In conclusion, the diet board elicits milder, but qualitatively similar metabolic responses as those reported with other methods of dietary restriction. The variation in growth did not differ between the groups, indicating that the diet board did not result in uneven food accessibility or increased competition for food. The diet board was well suited for group-housing. The welfare implications are not conclusive, but there were no signs of stress-related pathology in the diet board group. The diet board seems a promising refinement alternative to the current methods of dietary restriction.

Tiivistelmä

Koe-eläiminä käytettyjä jyrsijöitä ruokitaan yleensä vapaasti, toisin sanoen niillä on jatkuvasti ruokaa saatavilla. Tätä ruokintamenetelmää on kritisoitu tiedeyhteisössä laajalti. Suurimpina huolenaiheina on esitetty rajoittamattoman ruoansaannin aiheuttama kohonnut sairastavuus ja lyhentynyt elinikä. Lisäksi vapaasti ruokituilla jyrsijöillä tutkimustulosten hajonta on suuri. Rajoitettua ruokintaa on ehdotettu ratkaisuksi näihin ongelmiin. Rajoitettu ruokinta todella vähentää sairastavuutta, pidentää elinikää ja voi pienentää yksilöiden välistä hajontaa. Nykyisin käytetyillä rajoitetun ruokinnan menetelmillä on kuitenkin haittapuolensa niin eläinten hyvinvoinnin kuin myös tutkimuksen laadun kannalta. Rajoitettu ruokinta johtaa usein yksittäishoitoon ja epänormaaleihin ruokailurytmeihin. Eläimet eivät välttämättä pääse toteuttamaan käyttäytymistarpeitaan ja häiriintyneet vuorokausirytmit voivat vaikeuttaa tutkimustulosten tulkintaa.

Olemme kehittäneet uuden ruokintamenetelmän, rehulaudan, ongelman ratkaisemiseksi. Rehulauta on puinen ristikko, jossa ruokapelletit on asetettu puun sisään porattuihin reikiin. Rotan täytyy jyrsiä puuta voidakseen syödä pelletit. Näin syöminen vaikeutuu ja hidastuu. Rehulauta tarjoaa mahdollisuuden yhdistää rajoitettu ruokinta rottien ryhmähoitoon. Lisäksi rehulautaa voidaan pitää häkissä jatkuvasti, mikä mahdollistaa rottien häiriintymättömän vuorokausirytmin. Tässä väitöskirjassa esitellään ensimmäisiä tutkimustuloksia rehulaudan ominaisuuksista ja vaikutuksista.

Kokeellisessa osuudessa käytimme 60 Wistar rottaurosta. Kymmenviikkoinen koe alkoi kun eläimet olivat seitsemän viikkoa vanhoja. Puolet eläimistä söi yksinomaan rehulaudasta ja puolet ruokittiin vapaasti.

Rehulauta rajoitti eläinten kasvua 15%. Rehulautarotat söivät 85% siitä mitä vapaasti ruokitut. Rehulautarotilla oli huomattavasti vähemmän rasvaa, mutta luustonkasvu hidastui vain vähäisesti.

Seerumista tutkittiin useampia kliinisen kemian muuttujia, jotta nähtäisiin, voiko rehulauta häiritä energia-aineenvaihduntaan liittymättömien tutkimustulosten tulkintaa. Neljästä parametrista vain yhdessä, kreatiinikinaasissa, oli eroa ryhmien välillä. Rehulautarotilla kreatiinikinaasi oli merkitsevästi korkeampi.

Energia-aineenvaihduntaan liittyvissä muuttujissa oli enemmän eroja. Rasvahapot, kolesteroli ja triglyseridit olivat matalampia rehulautarotilla, kun taas kortikosteroni oli koholla vapaasti ruokittuihin verrattuna. Muista tutkituista hormoneista greliini oli rehulautarotilla koholla, kun taas insuliinissa, leptiinissä ja adiponektiinissä ei havaittu eroja.

Vapaasti ruokituilla rotilla oli suuremmat lisämunuaiset ja niissä korkeammat adrenaliini ja noradrenaliinipitoisuudet. Rehulautarotilla suoliston immunoglobuliini A:n eritys oli vähäisempää.

Yhteenvetona, rehulaudan vaikutukset aineenvaihduntaan ovat samansuuntaisia, mutta lievempiä, kuin muissa rajoitetun ruokinnan menetelmissä. Rehulauta sopii hyvin rottien ryhmähoitoon; rottien välinen kilpailu ei lisääntynyt, koska kullekin yksilölle riitti ruokailutilaa ristikossa. Rehulaudan vaikutuksia hyvinvointiin ei voitu varmuudella määrittää, mutta mitään viitteitä vakavista hyvinvointiongelmista ei nähty. Rehulauta vaikuttaa lupaavalta vaihtoehdolta rottien ruokintaan.

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Contents

List of original publications

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I Kasanen, I., Inhilä, K., Nevalainen, J., Väisänen, S., Mertanen, A., Mering S., Nevalainen, T. A novel dietary restriction method for group-housed rats: weight gain and clinical chemistry characterization. *Laboratory Animals 2009;43;138–148*

II Kasanen, I., Inhilä, K., Vainio, O., Kiviniemi, V., Hau, J., Scheinin, M., Mering, S., Nevalainen, T. The diet board: welfare impacts of a novel method of dietary restriction in laboratory rats. *Laboratory Animals 2009; 43:215–223*

III Kasanen, I., Inhilä, K., Kiviniemi, V., Nevalainen, T., Scheinin, M. Savontaus, E. The diet board - effects on hormones involved in regulation of energy metabolism. *Laboratory Animals (submitted)*

IV Kasanen, I., Sørensen, D., Forkman, B., Sandøe, P. Ethics of feeding – the omnivore dilemma. *Animal Welfare (in press)*

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Aims of the study

I. To test the general suitability and efficacy of the diet board for dietary restriction in laboratory rats.
II. To investigate whether the diet board alters research results compared to *ad libitum* feeding.
III. To investigate whether the diet board's effects on body weight and adiposity are mediated by physiological processes similar to those observed with other methods of dietary restriction.
IV. To assess the diet board's refinement potential.
V. To estimate the diet board's reduction potential.

Abbreviations

ACTH	corticotropin
ADR	adrenaline
AL	*ad libitum*
ALB	albumin
ALT	alanine aminotransferase
CHOL	total cholesterol
CI	confidence interval
CK	creatine kinase
CORT	corticosterone
CRH	corticotrophin-releasing hormone
DR	dietary restriction
FFA	free fatty acids
HPA	hypothalamus-pituitary-adrenal
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
IgA	immunoglobulin A
NLAC	National Laboratory Animal Center
NOR	noradrenaline
PROT	total protein
SD	standard deviation
SNS	sympathetic nervous system
TRIGLY	triglycerides

1 Introduction

A vast number of laboratory rats are used in experiments each year. There is legislation based on scientific findings regulating how these animals should be housed and treated to ensure their well-being. Many scientific protocols and animal models have been developed with increasing sophistication. Yet there is one aspect in the everyday care of laboratory rodents that has not been optimized, *i.e.* how they are fed.

Most commonly laboratory rodents are fed *ad libitum,* with continuous access to unlimited amounts of food. The practice of *ad libitum* feeding has been subject to a severe critique over the past years. *Ad libitum* feeding has been argued to be the least controlled factor in biomedical research, to produce obese and morbid animals with limited value as disease models and to increase the number of animals needed in safety evaluations and research due to increased mortality and inter-individual variation (Allaben et al. 1996, Duffy et al. 2001, Hart, Turturro 1995, Hubert et al. 2000, Keenan et al. 1999, Keenan, Laroque & Dixit 1998, Keenan et al. 1996, Leakey, Seng & Alleben 2004, Leakey, Seng & Allaben 2003, Masoro 1995, Turturro et al. 1997, Turturro et al. 1996).

Dietary restriction has been proposed to solve these problems and to promote better science with fewer animals. Indeed, dietary restriction reliably decreases morbidity and mortality and has also been shown to decrease result variation (Hubert et al. 2000, Leakey, Seng & Alleben 2004, Carney et al. 2004, Duffy et al. 2004a, Duffy et al. 2004b, Masoro 2006c, Masoro 2005).

Despite these benefits, dietary restriction has not been widely implemented into the routine care of laboratory rats. One reason is practicality; *ad libitum* feeding is by far the least time-consuming way of nourishing rodents. Furthermore, dietary restriction has its drawbacks with respect to both animal welfare and scientific integrity. Dietary restriction is most often implemented by giving individually housed animals one meal per day. This practice can threaten the rats' well-being by subjecting them to social isolation and suffering attributable to hunger and frustration of behavioural needs. The quality of research results can also be diminished by disrupted circadian rhythms, isolation stress and periods of fasting.

Different alternatives and solutions have been developed. The composition of the diet can be altered to provide some health benefits whilst feeding the animals *ad libitum*. The individual food intake can be controlled by sophisticated feeding devices. However, none of these inventions offers the possibility to combine all of the benefits of dietary restriction with the practicality of *ad libitum* feeding without impairing the welfare of the animals.

Finding an optimal way of feeding laboratory rats is a challenge. This study presents one possible solution for the dilemma. The diet board is a

novel method of dietary restriction with promising refinement features. In the first part of this thesis, the existing literature on dietary restriction will be reviewed. The effects of dietary restriction on physiology, behaviour, welfare and the 3Rs are presented. In the following parts, the diet board will be introduced and the research results on the diet board's characteristics and impacts will be described and discussed.

2 Review of the literature

2.1. The rat as an eater in the wild

The laboratory rat originates from the wild brown rat (*Rattus norwegicus*). The rat is a crepuscular, gregarious, omnivorous species. The versatility and adaptability of the rat are essential features of the species; therefore it is difficult to describe the ecology or ethology of wild rats in any detail. It does seem that these animals can adapt their diet, social structure, behaviour and circadian rhythms to a variety of different environmental conditions.

The rat is a highly social animal with complex interactions with its conspecifics. Rats live in colonies in self-dug burrows (or in manmade sheltered locations), where the animals are safe from predators and where the females have their nesting sites. The burrows can be as much as 50 cm deep and have several entries and nests within the same network of burrows. The colony can be polygynous with one dominant male, several females and their offspring. Probably a more common scenario is a mixed colony with several females and males. The males have an evident hierarchy, where the subordinate males may be driven to the periphery of the territory. Females show territorial aggression usually only in relation to the nesting site, whereas the male will defend the whole burrow system from foreign males. The home range is usually rather small, around 30 m in diameter, though this will depend on the availability of food and nesting sites. (Barnett 1963, Calhoun 1962, Lore, Flannelly 1977)

Knowledge about the time-budgets of wild rats is scarce. Wild rats spend a large part of their active time ambulating and exploring their surroundings at regular intervals. It is not clear how great a proportion of the overall locomotor activity is actual foraging behaviour, *i.e.* searching for food. The vast majority of the activity and eating occurs during the time between dusk and dawn. Both the general and the feeding activity show a bimodal pattern with peaks close to the beginning and the end of the dark period. (Calhoun 1962, Takahashi, Lore 1980) Rats are commonly observed to hoard food, *i.e.* transport food particles to a safer location before consumption. However, true storage behaviour, *i.e.* saving food to be consumed later, is very seldom encountered. (Takahashi, Lore 1980, Barnett 1951)

The feeding behaviour is characterized by a balance between neophilia and neophobia. Rats are extremely curious and explorative animals and prefer diversity in their nutrition. They are very flexible and will thrive on almost any kind of food provided their nutritional needs are met. Nonetheless, rats also display an avoidance of novel food. Novel foods are introduced into the menu of the colony by a gradual process. First one rat (typically a young adult male low in the hierarchy) samples a small quantity

of the novel food. If post-ingestive illness occurs, a conditioned aversion for that food is acquired which occurs not only in the individual consuming the unpalatable food, but also in the rest of the animals in the colony. It has been proposed that rats can also acquire their preferences for novel foods from other rats. The mechanisms behind this information transfer are not known. (Berdoy, Macdonald 1991)

Factors influencing feeding behaviour include dominance, reproductive status, predator threat and the availability of food. Dominant animals eat at the most popular times, after dusk and before dawn. If there are spatially limited resources, the subordinate animals will eat at less popular times, even during the light hours. Both predator (or human) threat and the availability of food can alter the rats' circadian feeding rhythms. Wild rats are flexible in this matter and will accommodate their schedules. For example, if there are fox hunting during the night-time, this will cause the rats to become active during the daytime. (Berdoy, Macdonald 1991)

Rats will establish a feeding order where some of the animals have better access to food. However, direct aggression or fighting with respect to feeding is seldom observed within the colony. Moreover, it is not invariably the most dominant animal that gains control over the food. In cases of food scarcity, the females are not left without food even though the dominant animals are the oldest and largest males. (Lore, Flannelly 1977)

The wild rat reaches a body weight of 500 g in males and 400 g in females by the age of one year with respective body lengths of 26 cm and 25 cm (Calhoun 1962). The fat content of the body in wild-captured individuals has reported to be on average less than 10 % (Toates, Rowland 1987).

2.2. Feeding rats in the laboratory environment

The wild rat was domesticated about 150 years ago. The laboratory rat has retained the complete behavioural repertoire of the wild rat. The domestic rat is, however, less emotional, less aggressive, more curious and performs better in cognitive tasks compared to wild-derived rats kept and bred in captivity. (Boice 1981)

The standard way of housing laboratory rats is to keep them in plastic or steel boxes (*e.g*. 30 x 50 x 20 cm) with a wire-grid cover. The animals are provided with bedding material and possibly nesting material or some other type of enrichment such as tubes or chewing blocks. A water bottle and food pellets are placed in a concave part of the grid cover *(i.e.* food hopper). The animals can be housed singly or in same-sex groups of two to four animals.

When laboratory rats have continuous access to food, they display similar circadian feeding patterns as the wild rat. The laboratory rat consumes the major part of its daily food intake soon after the onset of the dark phase. The other peak in eating activity occurs just before the beginning of the

dark phase. (Spiteri 1982, Strubbe, Woods 2004) Quite obviously, almost no time is spent in foraging and looking for food.

2.2.1. *Ad libitum* feeding

Ad libitum (AL) feeding is defined as food being freely available in unlimited quantities at all times. This is the most common way of feeding laboratory rats. The animals have access to a complete pelleted feed from the food hopper. The rationale behind AL feeding is purely practical; AL feeding is the least time-consuming way of providing food for laboratory rodents and allows the animals to be group-housed without provoking inter-individual aggression.

Even though food is available AL, it is not consumed boundlessly by the animals. Very little eating occurs during the daytime. Food intake is also affected by the energy requirements of the animals. For example, low ambient temperatures and lactation will increase the food intake of AL fed animals. (Ritskes-Hoitinga, Strubbe 2004)

The body fat content of AL fed laboratory rats depends greatly on the strain or stock, sex and age of the animals. In different studies, the fat content has ranged between 12 % to 25 % of total body weight. (Chengelis et al. 2006, Escriva et al. 2007, Roth et al. 2007)

Despite its practicality, AL feeding has its drawbacks. During the past decade, the practice of routinely feeding laboratory rats AL has raised serious criticism. AL feeding has been alleged to be the least controlled factor in biomedical research. It can even be considered as a form of malnutrition. AL fed rats have higher morbidity and mortality compared to animals subjected to dietary restriction. The validity of overfed, obese animals as research models has also been questioned. (Keenan et al. 1999)

2.2.2. Dietary restriction

The term dietary restriction (DR) is used here to describe any method of restricting the food intake leading to decreased caloric intake. It generally agreed that a DR regime should provide the essential amounts of vitamins, trace elements and macronutrients. The animals' food intake per body weight ratio is similar in DR and AL due to the fact that the body weight decreases in proportion to the degree of DR. Thus, the animal will receive a similar amount of nutrients per body weight and avoid any specific nutrient deficiencies. This makes DR completely different from undernutrition seen in people, where nutritional deficits cause serious health problems. DR is most often used in experimental animals when investigating certain phenomena such as obesity, type II diabetes, ageing and eating disorders. Figure 1. illustrates the number of scientific publications dealing with DR during the last decades.

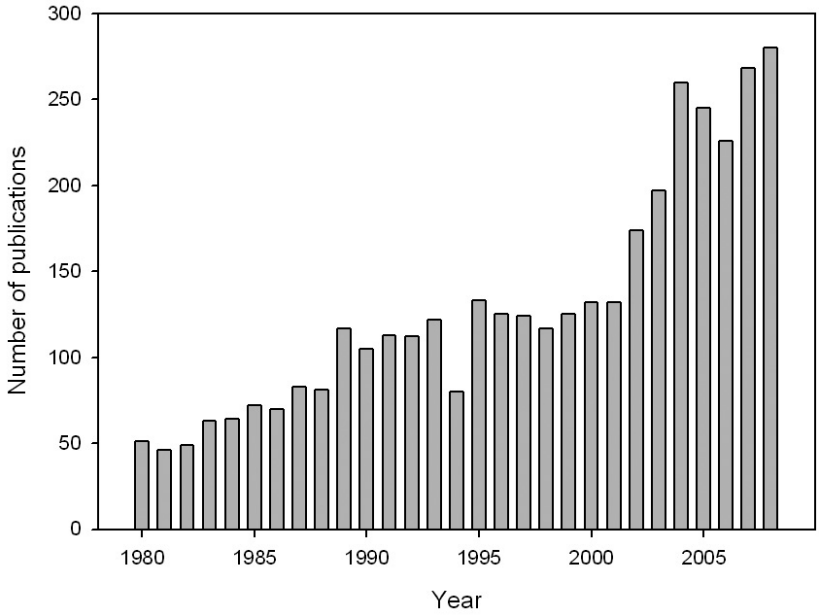

Figure 1. The number of publications in which dietary restriction was used or reviewed in rodents between 1980 and 2008. The search was done in PubMed using the phrase: ("dietary restriction" OR "caloric restriction" OR "food restriction" OR "restricted feeding") AND (rodent OR rat OR mouse)

DR can help resolve the problems associated with AL feeding. DR has been shown to promote health and longevity in a variety of species. It has been suggested that DR could lead to a reduction in the numbers of animals needed in experiments by decreasing inter-individual variation or by promoting longevity. Animals subjected to DR are also healthier, which could be viewed as a benefit from both a research and a welfare point of view. (Allaben et al. 1996, Hubert et al. 2000, Keenan et al. 1999, Leakey, Seng & Alleben 2004, Turturro et al. 1997, Masoro 2005)

Despite these benefits, DR is seldom used when caloric restriction *per se* is not being studied. DR is more labour-intensive than AL. Most of the methods of DR require the animals to be housed alone, which can confound research results due to isolation stress (Hall 1998, Karim, Arslan 2000, Krohn et al. 2006) and does not comply with the recommendations of the European legislation (2007/526/EC). The traditional methods of DR can also disrupt circadian rhythms, causing problems when comparing the results of DR animals to those of their AL fed controls (Claassen 1994, Damiola et al. 2000, Nelson 1988).

DR can be implemented in several ways. The most common way of restricting the food intake of laboratory rodents is to house them singly and give them one, pre-calculated meal per day. We will designate this protocol as the "traditional method of DR". In this method, the important variables are the degree of DR, *i.e.* how much food is actually given to the animals,

and the timing of the meal in relation to the light-dark rhythm. The severity of DR varies so that the amount of food offered ranges from only 20 % to 90 % of the food intake of the AL controls. Most publications describe the use of moderate dietary restriction, *i.e.* 60 % to 80 % of the AL food intake. (Pugh, Klopp & Weindruch 1999) Caloric intake can also be restricted by allowing the animals to eat only when the lights are on or by providing food only every other day. The palatability or energy content of the food can be altered. Examples of DR protocols in rats are shown in Table 1.

Table 1. Examples of DR regimes from the literature. Abbreviations: animals per cage (/cage); body weight (BW); day (d); homozygous Brattleboro (DI); every day (ED); every other day (EOD); Fischer 344 (F344); hours after lights on (HALO); Long-Evans (LE); male or female (M/F); months (mo); weeks (w); weight loss (WL); Sprague-Dawley (SD); years (y); Zivic-Miller (Z-M)

Animals	M/F	/cage	Start	Length	Feeding protocol	Lights on	Reference
SD	M	4	50d	35d	65% AL at 1000	0700-1900	(Armario, Montero & Jolin 1987)
F344	M	1	6mo	3w	74% AL 2h before dark phase	12:12	(Barazzoni et al. 2005)
SD	M	1	300-330g	25d	food available during light phase	12:12	(Bodosi et al. 2004)
Wistar	M	1	5w	4w	66% AL at 0900-1000	0800-2000	(Chacon et al. 2004)
SD	F	1	90d	5d	66% AL at 1200	0000-1200	(Chandler-Laney et al. 2007)
BN	M	1	14w	→34mo	14-15w 90% 15-16w 75% 16w -60% AL	14:10	(Chen et al. 2005)
Wistar	M	1	160g	20w	food available 0730-0930	0500-1900	(Curi, Hell 1986)
SD	M	1	6w	→110w	90%, 75% and 60% AL at 1000	0600-1800	(Duffy et al. 2004)
Wistar	M	1	75d	35d	60% and 40% AL	12:12	(Faine et al. 2002)
F344	M	1	14w	→22mo	60% AL at 1100	0600-1800	(Feuers et al. 1989)
Wistar	M	?	150g	2w	food available 0800-1200	0700-1700	(Fuller, Diller 1970)
Wistar	M	1	5/ 21mo	3mo	75-80% AL, until BW 85% AL	?	(Gallardo et al. 2005)
SD	M	1	275-325g	2w	food available 1300-1700	0700-1900	(Gooley, Schomer & Saper 2006)
SD	F	1	?	21d	30% AL	12:12	(Gualillo et al. 2002)
SD	M	1	200-240g	8w	70% AL at 1100	0700-1900	(Gursoy et al. 2001)
F344	M	1	6w	6w	60% AL at 1630	0530-1730	(Han et al. 1995)
SD	M	1	300-414g	37d	BW 80% AL, fed at 1500-1700	0650-1850	(Heiderstadt et al. 2000)
SD	?	1	28d	4w	30% AL	12:12	(Heresi, Chandra 1980)
F344	M	1	8w	8d	food available 2-10 HALO	12:12	(Hirao et al. 2006)
Wistar	M	2	250g	3w	food available 1000-1200	0700-1900	(Holmes, French & Seckl 1997)
Wistar	M	group	300-350g	2w	food available 0900-1100	2100-0900	(Inoue et al. 2004)
CD	M	1	?	10d	food available 1700-1900	0500-1700	(Ip et al. 1977)
Wistar	F+M	?	8w	2w	food available 1100-1300	0700-1900	(Itoh, Katsuura & Hirota 1980)
SD	M	1	28d	5w	50% AL at 1800	0700-1900	(Jahng et al. 2007)
SD	M	2	223g	12d	45% AL at 3 HALO	12:12	(Johansson et al. 2008)
SD	M	2	12w	3d	75%, 50%, 25% and 0% AL at 1700-1800	12:12	(Johnson et al. 2006)
SD	F+M	1	7w	2y	75%, 70% and 48% AL at 0730-0830	0700-1900	(Keenan et al. 2005)
Z-M	M	1	?	2w	food available 0930-1130	0800-2000	(Krieger et al. 1980)
F344	M	?	14w	→22mo	60% AL at 1100	0600-1800	(Leakey et al. 1989)
F344	?	?	14w	→22mo	60% AL at 1100	0600-1800	(Manjgaladze et al. 1993)
Wistar	M	?	250-300g	→20% WL	50%,25% and 12,5%AL at 1700 ED or EOD	0700-1900	(Marinkovic et al. 2007)
F344	M	1	14w	→6-24mo	60% AL at 1100	0600-1800	(Markowska 1999)
SD	F+M	1	4mo	→10mo	80% ,60%AL ED and 100% AL EOD at 1000	0700-1900	(Martin et al. 2007)
SD	M	?	3mo	→24mo	60% AL ED and 100% AL EOD	?	(Martini et al. 2007)
F344	M	1	6w	→6-36mo	60% AL	?	(McCarter, Masoro & Yu 1982)
Wistar	F+M	1 and 5	?	?	maintained at 85% of AL BW	0700-1900	(Molina-Hernandez et al. 2004)
LE and DI	M	1	5w	3d	food available 1-2 HALO	12:12	(Murphy, Wideman 1992)
SD	M	1	5mo	→23mo	92% and 65-70% AL	12:12	(Novelli et al. 2004)
Wistar	M	6	130g	1mo	food available for 4h ED at different times	12:12	(Philippens et al. 1977)
Wistar	F+M	?	5w	5w	various regimes from 65% to 92% AL	12:12	(Pickering, Pickering 1984)
CD	M	2	?	→24mo	65% of AL kcal/g in food, available AL	12:12	(Pitsikas et al. 1990)
SD	M	1	6-12w	2-6w	severity increased from 80% to 64% AL	12:12	(Rehm et al. 2008)
Wistar	F+M	5	weaning	→30mo	80% AL at 0900/food available 0900-1500	0600-1800	(Roe et al. 1995)
F344	M	1	6w	→death	60% AL at 11 HALO	12:12	(Sabatino et al. 1991)
Wistar	M	?	6w	→death	70% AL	?	(Schmucker et al. 1991)
F344	M	1	6w	→24mo	70% AL	12:12	(Shimokawa, Higami 1999)
LE	M	1	300-350g	10-15d	maintained at 90% AL BW fed at 0900	2100-0900	(Stamp et al.)
F344	M	?	?	→30mo	60% AL	12:12	(Stewart, Mitchell & Kalant 1989)
CD	F+M	?	?	3w	75% AL	?	(Stott et al. 2004/6)
SD	M	1	250-270g	4w	food available 1100-1500	0500-1900	(Xu et al. 1999)
F344	M	1	6w	→death	60% AL	12:12	(Yu et al. 1982)
F344	M	1	28d	→25mo	60% AL	12:12	(Zhu et al. 2004)

There is no widely accepted classification of the severity of DR. The degree of DR is usually described by stating how large a percentage of the AL control animals' food intake is given to the DR animals. It should be noted that these percentages are by no means comparable from one experiment to another. The base-line AL food intake varies greatly between different rodent stocks, strains, sexes and age-groups. Interestingly, even when these variables are standardized, there remain considerable differences in the AL food intake between different laboratories. (Keenan, Laroque & Dixit 1998)

In this paper the degree of DR is not always stated, when referring to the results of other authors. The regimes do not only vary in the severity of DR, but also in the AL animals' food intake, in the composition of the diet, in the length of the experiment and in the accuracy that the methods are described. Some authors state only the amount of food given without mentioning the size of the animals or the AL animals' food intake. Others mention only the percentage of DR without any reference to actual food consumption. Food intake can be restricted by limiting the time when food is available, again often without mentioning the food consumption. In some studies DR is begun mildly and the severity of the restriction is increased every week. Sometimes the DR and AL groups are even fed different diets. Thus, there is no simple way of comparing, categorizing or reporting the severity of DR in different studies. However, in Table 1. an attempt has been made to describe the DR regimes of the majority of the original studies referred to in this text. Table 1 includes only studies done with rats. Studies using other rodent species or very complicated DR protocols and review articles are excluded from the table. Similar DR regimes repeatedly used by the same author are included only once into the table.

2.3. Effects of dietary restriction

In this section, the effects of dietary restriction will be reviewed. The final effects of DR on metabolism are notably uniform despite the considerable methodological variation in the implementation of DR in different studies. The physiological consequences of restricting the caloric intake of animals fall roughly into three categories: the primary physiological reactions to decreased energy intake, the promotion of health and longevity and the timing and phase-setting of the circadian rhythms. The effects of DR on behaviour and welfare are more open to interpretations.

2.3.1. Body weight and composition

Quite obviously decreased energy intake results in decreased body weight. When DR is first introduced, the animals lose weight in direct relation to the severity of the restriction. In growing animals, it is retarded weight gain

which is usually seen instead of actual weight loss. If DR is implemented for longer periods, the organism adapts to the new level of energy intake and sustains a lower but stable body weight. After a period of adaptation, DR rats in fact consume the same amount or more food per gram of body weight than their AL counterparts (Masoro 2005). The metabolic rate of DR animals is higher than that of AL animals when calculated per total body weight but when proportioned to the lean body mass, there is no difference. (McCarter, Palmer 1992)

Most of the weight loss is explained by decreased fat mass. Organ weights, skeletal size and muscle mass have been reported to be very little affected even when the DR regime is rather severe (Yu et al. 1982, McCarter, Masoro & Yu 1982).

2.3.2. Endocrinology of energy metabolism

Energy metabolism is intricately regulated by both humoral factors and the nervous system and is governed by the hypothalamus. There are several regions, *i.e.* nuclei, in the hypothalamus controlling hunger and satiety. The brain stem and reward systems are also involved in central control of the energy metabolism. The hypothalamus is the target organ for several peripheral hormones and it also synthesizes neurotransmitters regulating the organism's energy balance. The central orexigenic (*i.e.* appetite increasing) substances in the hypothalamus include neuropeptides such as neuropeptide Y, melanin concentrating hormone, agouti-related peptide and galanin. These neuropeptides are upregulated during fasting and they stimulate appetite and increase food intake. The actions of the orexigenic substances are balanced by anorexigenic (*i.e.* appetite decreasing) neuropeptides in the hypothalamus. Melanocortins, glucagon-like peptide-1, a peptide called "cocaine and amphetamine related transcript" and also corticotropin releasing hormone (CRH) are some of the neuropeptides promoting satiety and decreasing food intake. (Rohleder, Kirschbaum 2007, Arora, Anubhuti 2006, Sainsbury, Cooney & Herzog 2002, Williams et al. 2004, Stanley et al. 2005)

The gastrointestinal tract monitors the nutrient intake and secretes several hormones in response to the quantity and quality of food eaten. The gastric mucosa secretes ghrelin, which is a peripheral orexigenic hormone. The secretion of ghrelin is associated with the timing of the last meal, *i.e.* its levels increase with the duration of time elapsing since the last meal. Ghrelin stimulates the production of neuropeptide Y and agouti-related protein, and thus increases appetite (Arora, Anubhuti 2006). Ghrelin is important in the short-term regulation of food intake, but its levels also reflect the long-term energy balance. Decreased caloric intake and low body weight correlate with elevated levels of ghrelin. (Gil-Campos et al. 2006, Hosoda, Kojima & Kangawa 2006, Klok, Jakobsdottir & Drent 2007, Popovic, Duntas 2005) In rodents, both plasma and gastric ghrelin levels have been observed to increase in DR regimes

ranging in severity from 30% to 75% of AL food intake (Barazzoni et al. 2003, Barazzoni et al. 2005, Gualillo et al. 2002). Ghrelin receptors are mainly located in the hypothalamus, but are also found in other tissues. In addition to increasing food intake, ghrelin also stimulates the secretion of growth hormone, corticotropin, corticosterone, mineralocorticoids, catecholamines, prolactin and possibly glucagon. Ghrelin has been reported to inhibit insulin secretion. These findings form the basis for the hypothesis of ghrelin acting as an anti-hypoglycemic hormone maintaining a euglycemic state. (Cummings, Foster-Schubert & Overduin 2005)

There is a greater number of anorexigenic hormones secreted from the gastrointestinal tract. These include peptide YY, cholecystokinin, enterostatin and bombesin. (Arora, Anubhuti 2006, Bray 2000)

Adipose tissue has only relatively recently been recognized as an important endocrine organ participating in the regulation of energy metabolism. Hormones secreted from the adipose tissue are called adipokines. The most thoroughly investigated adipokine is leptin. Its production is positively correlated with the amount of body fat. Leptin takes part in the long-term regulation of body weight and energy balance. Receptors for leptin are found in the hypothalamus, where leptin decreases the expression of orexigenic peptides and increases the expression of anorexigenic peptides, thus down-regulating appetite. Leptin secretion is upregulated by insulin and glucocorticoids and down-regulated by sympathetic nervous activity. (Klok, Jakobsdottir & Drent 2007, Bray 2000, Ahima 2006b, Ahima 2006a, Dallongeville, Fruchart & Auwerx 1998, Friedman, Halaas 1998) A decrease in the level of circulating leptin is a consistent finding in traditional methods of DR (Escriva et al. 2007, Barazzoni et al. 2005, Feuers et al. 1995, Feuers 1991, Gallardo et al. 2005, Maffei et al. 1995, Martin et al. 2007, Shimokawa, Higami 1999, Zhu et al. 2004). Resistin, tumour necrosis factor α, angiotensinogen, adipipsin and interleukin-6 are other adipokines and their secretions are also increased in obesity. These adipokines have similar effects on energy metabolism as leptin; they decrease food intake and body weight and increase energy expenditure. (Ahima 2006b)

Adiponectin is another hormone produced and secreted from adipose tissue involved in the long-term regulation of energy balance (Valassi, Scacchi & Cavagnini 2008). The circulating levels of adiponectin reflect the fat content of the body, but in an opposite manner to leptin. The levels of adiponectin increase when body fat mass decreases (Popovic, Duntas 2005, Shimokawa, Higami 1999, Sinha et al. 1996, Lafontan, Viguerie 2006, Mao, Hong & Dong 2006, Kahn et al. 1993).

Glucagon is secreted from the endocrine pancreas in response to hypoglycemia. The main function of glucagon is to elevate blood glucose levels. This is achieved by an increase in the breakdown of hepatic glycogen into glucose and gluconeogenesis in the liver and a decrease in glycolysis and in the synthesis of glycogen. (Bansal, Wang 2008) However, glucagon is not

able to increase appetite; on the contrary, it has been shown to decrease food intake. (Bray 2000)

Insulin is the counter-regulatory hormone for glucagon and is also secreted from the endocrine pancreas. Elevations in blood glucose and amino acid levels stimulate insulin secretion and a peak in insulin levels is observed after meals. (Gil-Campos et al. 2006, Klok, Jakobsdottir & Drent 2007) Insulin regulates glucose homeostasis at many levels, it increases glucose uptake from the bloodstream into muscle and adipose cells, it reduces the hepatic output of glucose and increases the synthesis of glycogen from glucose in the liver and in muscle cells (Gonzalez-Sanchez, Serrano-Rios 2007). Insulin not only exerts opposite effects compared to glucagon, but also directly suppresses glucagon secretion in pancreatic islets (Bansal, Wang 2008). It has been proposed that insulin also functions as a long-term regulator of energy balance. The peripheral actions of insulin are anabolic, whereas the central response to insulin secretion is decreased food intake (Dallman et al. 1995) When food intake is restricted, insulin levels decrease, as would be expected. DR also enhances the sensitivity of tissues to insulin, further decreasing the amount of insulin needed.

The hypothalamus-pituitary-adrenal (HPA) axis plays an important role in the regulation of energy metabolism. Corticosterone (CORT) is the active form of glucocorticoids in rodents and works as the final mediator of the HPA axis. It is secreted from the adrenal cortex in response to corticotrophin (ACTH) which in turn is secreted from the pituitary in response to hypothalamic secretion of CRH and vasopressin. CRH secretion can be increased by both orexigenic and anorexigenic peptides and indeed increased plasma CORT is observed both after eating and in DR or fasting. The secretion of corticosterone can also be regulated at the adrenocortical level. For example, leptin directly decreases the adrenocortical activity (Bornstein et al. 1997). CORT can be understood to function as a counterpart of insulin in regulating food intake and body weight. In the periphery, CORT induces a catabolic state promoting the mobilization of energy stores whereas centrally CORT acts as an orexigenic compound. (Rohleder, Kirschbaum 2007, Arora, Anubhuti 2006, Dallman et al. 1995, Dallman et al. 1993)

Adrenaline (ADR) and noradrenaline (NOR) are catecholamines secreted from the adrenal medulla, and NOR is also the neurotransmitter released from sympathetic nerve endings. These two catecholamines mediate the peripheral effects of the sympathetic nervous system (SNS). ADR and NOR are catabolic hormones that increase the availability of fuels in the bloodstream. ADR increases the level of free fatty acids (FFA) in the blood by stimulating lipolysis in adipose tissue. Gluconeogenesis in the liver is also increased and blood glucose levels are elevated. Somewhat surprisingly, hypoglycaemia suppresses the SNS and both glucose and insulin enhance the SNS activity. Leptin, on the other hand is known to stimulate the SNS. The sympathetic tone is decreased by DR and increased by overfeeding. This phenomenon is known as dietary thermogenesis, which is hypothesized to help maintain

a stable body weight by converting excess calories into heat energy and lowering the energy consumption when caloric intake is decreased. This theory is supported by noradrenaline's anorexigenic properties. (Bray 2000, Landsberg 2006) Table 2. shows a summary of the changes DR elicits in the metabolism and hormonal balance.

Table 2. Effects of DR on selected hormones and other variables. Please refer to text for references. Abbreviations: central anorexigenic peptides (CAP); central orexigenic peptides (COP); free fatty acids (FFA)

Effects of DR	
Adiponectin	↑
CAP	↓
Cholesterol	↓
COP	↑
Corticosteroids	↑
FFA	↓
Ghrelin	↑
Glucose	↓
Insulin	↓
Leptin	↓
Sympathetic tone	↓
Triglycerides	↓

2.3.3. Lipid metabolism

Lipids are a diverse group of compounds defined by their insolubility in water. Lipids serve various biological functions, not only as energy storage, but also as components of biological membranes and hormones. Lipids are obtained from the food and absorbed as fatty acids and monoglycerides and cholesterol. The body can also synthesize most, but not all, lipids from carbohydrates, the exception being the essential fatty acids. Excess energy is stored into the adipose tissue as triglycerides. In obese individuals, accumulation of triglycerides may be observed also in the liver. Each triglyceride molecule consists of three fatty acids and a glycerol moiety. When needed, the energy is released from the adipose cells into the bloodstream mainly in the form of fatty acids, *i.e.* after lipolysis. Cholesterols, triglycerides and phospholipids can also be found in the blood. Cholesterols are used as components of cellular plasma membranes and as precursors for steroid synthesis. (Lehninger, Nelson & Cox 1993)

The blood lipid profile reflects both the timing and composition of the last meal and the long-term energy balance of the organism. Increased levels of most lipids are present in the circulation after a meal. In the case of FFA, however, the opposite is true. FFA levels are reduced after feeding due to inhibition of lipolysis (Fuller, Diller 1970, Ip et al. 1977).

Lipid metabolism is under hormonal regulation. ADR and NOR are potent stimulators of lipolysis, increasing the availability of FFA for the tissues to

use as fuel. CORT is more crucial in blood glucose regulation, but it can also facilitate lipolysis. Insulin acts as an anabolic hormone decreasing the rate of lipolysis.

DR has profound effects on the lipid metabolism of rodents (Turturro, Duffy & Hart 1993). The overall adiposity correlates positively with the blood lipid levels. Decreased levels of serum cholesterol and triglycerides are consistent findings in rodents subjected to DR (Hubert et al. 2000, Keenan, Laroque & Dixit 1998, Turturro, Duffy & Hart 1993). Traditional methods of DR are also associated with lowered levels of serum FFA (Barazzoni et al. 2005, Curi, Hell 1986, Gonzalez et al. 2004a, Harris et al. 1994).

In humans, increased amounts of liver triglycerides can be associated with obesity (Kotronen, Yki-Jarvinen 2008). In rodents, however, liver triglycerides have been reported to be unaffected by DR (Barazzoni et al. 2005).

2.3.4. Stress reaction

The classic stress reaction was described already in the 1930's by Walter Cannon (1871–1945) and Hans Selye (1907–1982). Cannon invented the concept of the "flight or fight" reaction and described the role of adrenaline in the stress reaction. Selye discovered that glucocorticoids are also secreted in the stress reaction. He named the stress reaction the "general adaptation syndrome". (Sapolsky 2002) Both Cannon and Selye understood the stress reaction as a nonspecific response to different kinds of threats.

Today, the cascade of events known as the stress reaction or stress response has been characterized in great detail. Nonetheless, there remain controversies in the field of stress research. The main source of confusion seem to be the definitions of the terms "stress", "stressor" and "stress reaction". The stress reaction can be defined as an adaptive response to different circumstances outside or within the organism. With this definition, the stressors can also be positive events or emotions (eating, playing, exercise, sex) not causing any harm to the organism. On the other hand, the word "stress" can also be used to describe a failure of the organism to cope or adapt to the circumstances in which it is living. Stressors would then be only those circumstances that induce negative consequences for the individual. Another source of confusion is whether the term "stress reaction" is used to describe any reactions of the individual to a stressor or only to the activation of the HPA axis and other endocrine functions. Finally, there has been debate over the question of whether a universal stress reaction really exists or whether the reactions of an organism should be considered as stressor-specific.

For the purposes of this text, we will use a definition of stress based mainly on that proposed by G.P. Moberg. The stress reaction begins with the individual's perception of a potential threat (*i.e.* the stressor) to its homeostasis. The next step is the organism's response to the stressor. This

response can include changes in behaviour, endocrinology, immunology and nervous system activity. These changes constitute the actual stress reaction. The stress reaction is adaptive or defensive in its nature and functions to ensure the individual's survival. The stress reaction demands biological resources. If the biological cost is higher than the biological reserves, resources are shifted away from other functions. In this case, the consequences of the stress reaction are altered biological functions. If the biological cost of the stress reaction is sufficiently high, the organism may enter into a prepathological state, where it is predisposed to pathology. This state is called distress. In distress, the stress reaction has impaired the biological functions (reproduction, growth, immunocompetence) of the individual. The word "stress" can be understood as the general state of stressors that provoke a stress reaction. (Sapolsky 2002, Moberg 2000)

B.S. McEwen and S.M. Korte have introduced the concept of allostasis into welfare and stress discussion. This takes a more holistic view of the organism's struggle to adapt and cope with challenges. Homeostasis signifies the attempt to defend and maintain a constant state of equilibrium, whereas allostasis means to maintain homeostasis through change. Various systems in the organism are under constant change, reacting to different challenges with predictions of a new state of equilibrium. Maintaining allostasis has a price, *i.e.* the allostatic load. Quoting B.S. McEwen (1998)(McEwen 2004): "Allostatic load is the wear and tear on the body and brain resulting from chronic overactivity or inactivity of physiological systems that are normally involved in adaptation to environmental challenge".

One complication to the characterization of stress responses is the lack of a universal stress reaction. In contrast to the proposal of Cannon, it is now known that the stress reaction varies from one stressor to the next. There is also great inter-individual variation depending on former experiences, the timing and quality of previously encountered stressors, the coping style and the age, sex, strain and species of the individual. (Sapolsky 2002, Moberg 2000)

Despite this uncertainty of what a stress reaction really is, the next paragraphs will be dedicated to describing the stress reaction. The stress reaction will be described as one entity, but it should be emphasized that not all of these responses are seen in all individuals under all circumstances. The stress reaction described is merely an example of the possible responses an organism can elicit when encountering external or internal challenges. The endocrinology of the stress reaction will be characterized with greater detail than the rest of the responses.

When a potential stressor is first perceived, the information is processed in the brain. The stress reaction is initiated only if the event is interpreted as a threat to the homeostasis of the organism. The limbic system and the reticular formation are involved in the brain's response to stress. The limbic system combines the perception of the stressor with emotions, thus enhancing the experience and increasing the likelihood of an appropriate

reaction the next time the same stressor is encountered. The reticular formation is involved in processing the sensory input and discriminating the essential from the trivial. The reticular formation makes it possible to focus on specific functions ignoring irrelevant information. (Joels et al. 2007, Palkovits 2002, Smith, Vale 2006)

The first line of response is often a behavioural one. Depending on the nature of the stressor and the proximity of the threat, this could be increased vigilance, freezing, crouching, fleeing, increased locomotor activity, increased communication with conspecifics *etc*. If the primary behavioural response is successful and the stressor is avoided, the rest of the stress reaction may be interrupted. (Rushen 2000)

The next response in the stress reaction is the activation of the SNS. The autonomic nervous system is controlled by the central autonomic network in the brain. This network consists of interconnected functional centres, such as the hypothalamus, basal forebrain and cerebral cortex. The functions of the central autonomic network are modulated by the main noradrenergic cell group of the brainstem, the *locus coeruleus*. The *locus coeruleus* synthesizes and stores NOR and releases it when the individual is aroused by stressors. The *locus coeruleus* can also be activated by CRH, the main orchestrator of the stress reaction. The SNS acts directly *via* neuronal pathways and also by stimulating the secretion of ADR and NOR from the adrenal medulla. SNS activation prepares the organism for vigorous physical exertion. Thus, oxygen and nutrients are transported to the muscles more efficiently by the circulation. Enhanced blood flow is achieved by increasing the heart and respiratory rate, elevating the blood pressure and by redirecting the blood flow from gastrointestinal organs to the locomotor system. Nutrients are released into the bloodstream by mobilizing glycogen from the liver and FFA from adipose tissue. (Sapolsky 2002, Gregory 2004, Matteri, Carroll & Dyer 2000, Kvetnansky et al. 1995, Kvetnansky, Kopin 1972, McCarty, Horwatt & Konarska 1988, Wortsman 2002)

Activation of the HPA axis begins simultaneously with the SNS arousal, but its effects are hormonally mediated and thus are slower than the immediate responses seen in systems innervated by SNS. Secretion of CRH and other hypothalamic hormones, such as vasopressin, begins the HPA response. ACTH secretion from the anterior lobe of the pituitary is increased and concomitantly glucocorticoids and mineralocorticoids from the adrenal cortex are secreted into the bloodstream. Mineralocorticoids elevate blood volume and pressure by increasing the reabsorption of sodium and water in the kidneys. Glucocorticoids are often thought to mediate the majority of the responses encountered in the stress reaction. However, it should be noted that CRH has direct effects independent of glucocorticoids. For example, CRH suppresses the secretion of growth hormone, decreases food intake and induces behavioural activation. (Sapolsky 2002, Matteri, Carroll & Dyer 2000, Johnson et al. 1992)

The elevation of plasma CORT concentration is a central feature of the stress reaction (Johnson et al. 1992, Sapolsky, Romero & Munck 2000, Stratakis, Chrousos 1995). CORT is normally secreted in hourly pulses with a marked circadian rhythm. The plasma concentration of CORT peaks at the beginning of the active phase, which is the beginning of the dark phase in rats. (Young, Abelson & Lightman 2004) In the stress reaction, the circadian rhythm becomes weakened and the levels of CORT are elevated throughout the day (Barriga et al. 2001). The actions of CORT in the stress response are diverse, ranging from physiology to behaviour. One important role of CORT is the control of energy metabolism. CORT has effects on appetite, fuel distribution and energy storage. Similar to the effects of the SNS, blood glucose levels are elevated by facilitation of the breakdown of glycogen stores and of gluconeogenesis in the liver. Proteolysis and lipolysis are enhanced. (Sapolsky 2002, Gregory 2004, Matteri, Carroll & Dyer 2000, Johnson et al. 1992, Sapolsky, Romero & Munck 2000) CORT also modulates the central nervous system's function in the stress reaction. There are three types of receptors in the brain with affinity for glucocorticoids: the nuclear mineralocorticoid receptors, the nongenomic mineralocorticoid receptors and the nuclear glucocorticoid receptors. The nuclear mineralocorticoid receptors have a high affinity for glucocorticoids and are saturated already at the baseline level of glucocorticoid secretion, presumably taking part in the maintenance functions of glucocorticoids. The nongenomic mineralocorticoid receptors mediate the rapid, excitatory effects that the glucocorticoids have on neuronal functions in the limbic areas. The nuclear glucocorticoid receptors have a lower affinity for glucocorticoids and mediate slower responses at a transcriptional level. The neuronal excitability is attenuated and inhibitory effects of serotonin are enhanced. These responses are proposed to take part in "shutting down" the stress reaction and normalizing the activity of the central nervous system some hours after the initial stress reaction. (Joels et al. 2008, de Kloet, Karst & Joels 2008)

The SNS and HPA axis are the main neuroendocrine systems involved in the stress reaction. However, various other functions of the organism are affected in important ways and are involved in the final outcome of a period of stress.

Immunological functions are a vital part of the organism's survival mechanisms. Glucocorticoids have long been recognized as potent immunosuppressants in pharmacology, but the effects of endogenous glucocorticoids on immunological functions are not straightforward. Other mediators of the immune response in the stress reaction include CRH and possibly the SNS. The acute phase of a stress reaction prepares the organism for immunological challenges. The immune response becomes intensified in the skin, lymph nodes and other tissues, which are the first line of defense when the organism encounters microbes or other harmful elements. The hepatic synthesis of acute-phase proteins is increased and both T- and B-cell responses can be enhanced (Johnson et al. 1992, Sapolsky, Romero & Munck 2000, Dhabhar 2002, Korte et al. 2005, McEwen 1998). It has been proposed

that the immunosuppressant effects of glucocorticoids are mediated *via* the nuclear glucocorticoid receptors and are intended to restore the normal level of immunological activity and to prevent the immune functions from over-reacting. The immunosuppressive actions of glucocorticoids include a decreased production of cytokines and cytokine receptors and suppression of leukocyte functions. Only when the stress reaction is prolonged to a pathologic extent, does one encounter an increased susceptibility to infectious and inflammatory diseases. The distorted functioning of the immune system in chronic stress can be manifested by weakened immune responses that expose the organism to infectious diseases; or inappropriate activity which increases the incidence of autoimmune diseases. One often repeated finding in animals suffering from a chronically activated stress reaction is atrophy of the thymus and other lymphoid tissues. (Sapolsky 2002, Johnson et al. 1992, Sapolsky, Romero & Munck 2000, McEwen 1998, Blecha 2000)

An inherent feature of the stress reaction is the negative energy balance that it creates in the organism. The responses elicited not only demand energy but also set the organism's metabolism towards a state of catabolism. The catabolic effects of the SNS, ADR and CORT have already been discussed. The release of nutrients into the circulation is further facilitated by the decreased insulin secretion and the elevated secretion of glucagon. Appetite is decreased by the actions of CRH. Glucocorticoids have been reported to increase plasma leptin levels (Newcomer et al. 1998). Leptin is known to decrease appetite by decreasing the secretion of neuropeptide Y and also by increasing the sensitivity of the cells to CRH (Makino et al. 1998). Supplementing the catabolic effect of the stress reaction, the levels of anabolic hormones such as growth hormone and gonadal hormones are decreased (Sapolsky 2002).

The responses described above serve to prepare the individual for the rigours of hunting or being hunted, vigorous physical activity, increased vigilance, fighting with conspecifics, cold exposure *etc*. In order to perform these functions efficiently enough to survive, other functions and activities are postponed. The down-regulated functions include reproduction, growth, digestion, repair and storage of nutrients. Maintenance behaviours such as sleeping, resting, eating and grooming are also suppressed.

If defining stress is controversial, so too is its quantitation. The animal's perception (*i.e.* the aversiveness) of the potential stressor can sometimes be assessed in behavioural tests. Some aspects of the actual stress reaction can be quantitated by measuring physiological variables such as plasma concentrations of CORT, ACTH and catecholamines, blood glucose, heart rate and blood pressure, immunological functions, behaviour and the size of adrenal glands and the organs of the immune system. More sophisticated methods include the dexamethasone suppression test and the ACTH challenge test which are claimed to more thoroughly characterize the function of the HPA axis. It is also possible to determine the expression of mineralocorticoid and glucocorticoid receptors in the brain. Nevertheless,

none of these measures is able to reveal the biological cost of the stress reaction or the success of the attempts to cope and adapt. The consequences of the stress reaction can also be assessed. Stress related pathology is a clear sign that the limits of adaptation have been exceeded. Commonly reported pathological findings are gastric ulcers, atrophy of the thymus, atrophy of the hippocampus, decreased growth or reproduction, abnormal behaviour (stereotypies, self-mutilation, apathy, increased aggression), increased morbidity and mortality *etc*. However, concentrating solely on the potential pathologic outcomes does not allow for the investigation of less extreme cases of stress.

It is easy understand how the stress reaction benefits the individual in the case of an acute stressor, such as the need to flee from a predator. However, the situation of chronic stress is much more obscure. It is not clear to what extent the prolonged stress reaction is adaptive or benefits the individual. Chronic stress has been proposed to be solely pathological, that the stress reaction has developed only to respond to acute stressors and that chronic stressors are not encountered in nature (Sapolsky 2002). The existence of truly chronic stress has been questioned. Chronic stress has been claimed to be a mere repetition of acute stressors without sufficient time to recover between bouts of stress (Moberg 2000, Ladewig 2000). On the other hand, it could be postulated that most species have developed appropriate responses to some genuine long-term stressors that they are likely to encounter, such as parasites, disease, cold and hunger.

Rodents are well adapted to periods of food shortage. Hunger induces a specific stress reaction in the organism. It has much in common with the classic stress reaction described above, but differs in some features. It has been suggested that the response to food shortage has evolved as an adaptive reaction allocating resources from reproduction to somatic maintenance (Masoro, Austad 1996). Table 3 provides a simplified comparison of the classic stress reaction and the stress-like reaction observed in DR.

An elevation of circulating glucocorticoids is a consistent finding in DR animals (Harris et al. 1994, Armario, Montero & Jolin 1987, Chacon et al. 2005, Han et al. 1995, Heiderstadt et al. 2000, Sabatino et al. 1991, Stewart et al. 1988, Patel, Finch 2002). The mechanisms inducing the rise in CORT are not clear. Many of the orexigenic and anorexigenic peptides are involved in activating the HPA axis (Rohleder, Kirschbaum 2007). The circadian rhythm of CORT secretion is modulated by DR and especially by the timing of feeding. The main peak in CORT is observed just before the main meal of the day. Although the levels of CORT are elevated in DR animals throughout the day, the peak values are especially high compared to AL animals. The free plasma CORT is elevated due to both increased secretion from the adrenal cortex and a decrease in the level of the CORT binding globulin in the circulation. (Patel, Finch 2002)

Table 3. A generalized comparison between a prolonged classic stress reaction and the response to DR. Please refer to text for references. Abbreviations: corticosterone (CORT); hypothalamic-pituitary-adrenal (HPA); sympathetic nervous system (SNS)

Chronic stress reaction	Response to DR
Induces a catabolic state. If prolonged, can lead to degeneration of tissues, e.g. muscle loss.	Energy reserves are used, but degeneration and other damage due to catabolism is avoided.
Immune functions are impaired and morbidity is increased.	Immune functions are altered, but morbidity is decreased.
Entire HPA axis is activated and circadian rhythms of CORT secretion can be disrupted.	Especially CORT secretion is increased. Circadian rhythms of CORT are affected by feeding time.
SNS is activated.	SNS activity is decreased.
Appetite is decreased.	Appetite is increased.
Hippocampal neuronal loss and a decline in cognitive functions can occur.	Aging-associated neurodegeneration and loss of cognitive functions is attenuated
Ability to cope with additional stressors is impaired.	Improved recovery from additional stressors.
Decreased activity, increased anxiety and increased depression in behavioural tests.	Increased activity, decreased anxiety and increased depression in behavioural tests.
Reproductive functions are impaired.	Reproductive functions are down-regulated, but the age-associated decline in reproductive functions is delayed.
Biological fitness and welfare are decreased.	Physical health is improved. Welfare?

It is not clear whether DR is perceived as a true stressor by the organism or whether the elevated levels of CORT are simply a means of energy regulation. The consequences of DR and states of chronic stress are blatantly different, yet similar endocrine responses are seen in both cases. DR and chronic stress are both associated with increased levels of CORT and decreased secretion of anabolic hormones (growth hormone, insulin, reproductive hormones *etc*.). Chronic stress is generally considered as being harmful to the organism and can lead to pathological changes such as impaired immune functions, muscle atrophy, hippocampal neuronal loss and decline in cognitive functions (Joels et al. 2007, Johnson et al. 1992, Sapolsky, Romero & Munck 2000, Patel, Finch 2002, Hibberd, Yau & Seckl 2000). DR, on the other hand, confers various health benefits despite the elevation of CORT. In addition, DR does not inflict such a state of catabolism as is observed in chronic stress. Even when the extent of caloric restriction is severe, the lean body mass is similar in AL and DR rodents (Yu et al. 1982, McCarter, Masoro & Yu 1982). DR is associated with increased gluconeogenesis both in the liver and in skeletal muscle, but in contrast to the classic stress reaction, glycolysis in inhibited by DR.

This contradiction has been named "the glucocorticoid paradox of caloric restriction" (Patel, Finch 2002). It is not clear how CORT can exert such opposing effects; or whether the diverse outcomes are in fact due to other factors differing between the two conditions.

The adrenocortical activation induced by DR is not identical with that observed in chronic stress. The classic stress reaction is characterized by the activation of the entire HPA axis (Johnson et al. 1992, Sapolsky, Romero & Munck 2000, Garcia et al. 2000), whereas DR is associated with decreased or unaltered circulating ACTH concentrations (Chacon et al. 2005, Han et al. 1995, Avraham et al. 2002). Another difference in the responses to DR and other stressors is the SNS. Activation of the SNS is an essential feature of the classic stress reaction. In DR, however, SNS activity is decreased (Landsberg 2006).

There are some findings indicative of negative stress-related effects of DR. The higher relative adrenal weights and lower relative thymus weights (Gursoy et al. 2001) and dysfunction of the brain serotonergic system (Jahng et al. 2007) observed in animals subjected to traditional methods of DR can be interpreted as pathological outcomes of chronic stress. However, it is difficult to assess whether and to what extent these effects are attributable to the stress of single housing, to infrequent meals or to DR *per se*.

DR also modifies immune functions. In general, immune functions are suppressed by DR (Giovambattista et al. 2000, Heresi, Chandra 1980, Jolly 2004). The dysfunction of the immune system caused by chronic stress predisposes the individual to morbidity. On the contrary, the immunosuppression in DR has been reported to confer benefits on the individual. DR has been observed to shorten the recovery time after surgery and to ameliorate autoimmune diseases (Masoro 1995, Jolly 2004, Klebanov et al. 1995, Shibolet et al. 2002). However, the laboratory environment is usually kept relatively free of pathogens thus making a functional immunologic defense less crucial. The consequences of reduced immune functions could be much more detrimental outside the laboratory.

Not only has the organism's response to DR been studied, but also the effects of DR on the organism's response to other stressors have been examined. There is much controversy about this topic, and the interplay between stress and nutrition is exceedingly complex. DR and fasting have been shown to blunt the HPA axis response to other stressors, such as restraint (Rohleder, Kirschbaum 2007, Dallman et al. 1995, Hanson et al. 1994). In DR and after fasting, blood glucose levels are low in spite of the elevated CORT levels. The low blood glucose has been shown to be one factor in mediating the decreased HPA response. When supplied with glucose or other carbohydrates before the stressor, the response is normalized (Gonzalez-Bono et al. 2002). The timing of the stressor in relation to the circadian rhythm of feeding and CORT secretion is also of importance. If the stressor is applied when the CORT levels peak, there is little additional response from the HPA axis. If the stressor is encountered when the baseline level of CORT is lower, then there is more extensive arousal of the HPA axis. (Dallman et al. 1993) It has been hypothesized that the elevated levels of CORT caused by DR activate the negative feedback mechanisms on the HPA axis and thus prevent the additional

stress reaction (Rohleder, Kirschbaum 2007). Several of the neuropeptides involved in regulating appetite are involved in modulating the HPA axis response to stressors. For example, some orexigenic peptides attenuate the stress-induced increases of CRH and ACTH. (Rohleder, Kirschbaum 2007) Nevertheless, DR has been shown to enhance the organism's ability to cope in variety of situations (Masoro 1995, Jolly 2004, Klebanov et al. 1995, Shibolet et al. 2002). It has been proposed that the stress reaction seen in DR can prepare the organism for other stressors (Masoro 1998). DR has indeed been reported to improve recovery from acute stressors, such as surgery and inflammation (Masoro 1995, Jolly 2004, Klebanov et al. 1995, Shibolet et al. 2002).

2.3.5. Mortality and morbidity

DR decreases morbidity and mortality (Allaben et al. 1996, Hubert et al. 2000, Keenan et al. 1999, Leakey, Seng & Alleben 2004, Duffy et al. 2004a, Duffy et al. 2004b, Masoro 2006c, Masoro 2005, Nelson 1988, Yu et al. 1982, Keenan et al. 1997).

DR retards age-associated deterioration (Masoro 2005, Fontana, Klein 2007, Kirkwood, Shanley 2005) and increases longevity in a variety of species ranging from insects to rodents and primates (Masoro 2006c, Masoro 2005). Longevity is increased in direct relation to the severity of the caloric restriction, peaking just before starvation. For example, the two year survival of Sprague-Dawley rats was 15% when the animals were fed AL. Moderate DR (75% – 80% AL) increased the survival to 40% – 65%. With a more severe DR regime (50% AL), the survival was further increased to 80%. (Keenan et al. 1999) The possibility of life extension has fascinated researchers and the mechanisms behind this phenomenon have been intensively studied.

Aging can be defined as "a process or group of processes occurring in living organisms that with the passage of time lead to a loss of adaptability, functional impairment and eventually death" (Masoro 2006a). There are several hypotheses (reviewed by E.J. Masoro 2005) about how caloric restriction exerts its antiaging effects. One theory is the oxidative damage attenuation hypothesis. Aging can be understood as an accumulation of oxidative damage in the organism and DR has been shown to attenuate the accumulation of oxidatively damaged molecules. This can be a consequence of a decrease in the production of reactive oxygen molecules, an increase in the efficiency of protective processes or an increased repair activity. A second theory is the attenuation of insulin-like signaling hypothesis. Hyperglycemia and hyperinsulinemia cause tissue damage similar to that seen in the aging process. Mouse strains with low levels of insulin-like growth factor-1 have a markedly increased longevity compared to other strains. DR is associated with decreased plasma levels of glucose, insulin

and insulin-like growth factor-1. These metabolic changes could be one factor accounting for the increased longevity observed in DR, although the causality has not been conclusively proven. A third theory is called the hormesis hypothesis. Hormesis has been defined by E.J. Masoro (2000) as "the beneficial actions(s) resulting from the response of an organism to a low intensity stressor". DR can be viewed as a low intensity stressor. The elevated level of CORT associated with DR could protect the organism from endogenous and exogenous stressors causing damage and aging-associated changes (Masoro 2006c, Masoro 2005, Han et al. 1995, Sabatino et al. 1991, Masoro 1998, Frame, Hart & Leakey 1998, Leakey et al. 1994, Masoro 2006b, Masoro 2000, Yu, Chung 2001).

In addition to living longer than AL fed animals, DR animals are also physically healthier. The incidence of neoplastic diseases is significantly lower in DR animals (Hubert et al. 2000, Duffy et al. 2004a, Keenan, Soper 1995). DR also reduces the incidence of degenerative kidney diseases (Hubert et al. 2000, Duffy et al. 2004b), endocrine disturbances (Keenan et al. 1996, Keenan, Soper 1995) and other common causes of morbidity in laboratory rodents (Hubert et al. 2000, Duffy et al. 2004b). The decreased morbidity has been attributed specifically to decreased caloric intake. Modifications of the diet composition have failed to produce such universal health benefits (Keenan et al. 1999).

Reproductive functions may be an exception to the otherwise consistent phenomenon of improved health and biological functioning. It has been claimed that increased longevity and reduced reproductive performance are inherently linked outcomes of DR (Koochmeshgi 2004b, Koochmeshgi 2004a). The evolutionary basis for this would be to increase the likelihood of the animals living long enough to survive the hard times and use energy and resources to reproduce when sufficient energy intake is again guaranteed (Shanley, Kirkwood 2000). In support of this theory, moderate DR has indeed been shown to impair reproductive functions at an early phase, but to prolong reproductive life by delaying the age-associated decline in reproductive functions (Chen et al. 2005, Nelson et al. 1995, Selesniemi, Lee & Tilly 2008, Sharov et al. 2008). Severe DR leads inevitably to down-regulation of reproductive functions, especially in females (Martin et al. 2007, Rehm et al. 2008). Recently, it has been proposed that the mechanisms governing aging and longevity may be independent of the regulation of fecundity (Partridge, Gems & Withers 2005). It appears to be possible to combine life extension with unhampered reproductive success when the degree of DR is correct; not too mild to be inefficient in prolonging longevity and not too severe to impair fecundity (Johnston et al. 2006, Rocha et al. 2007).

2.3.6. Circadian rhythms

Circadian rhythms are seen in many behaviours, motor activity, body temperature, endocrine functions and most other physiological variables. Circadian oscillations are produced endogenously, but the timing and phase of the rhythms is set by environmental cues, *i.e*. zeitgebers. The suprachiasmatic nucleus of the hypothalamus is the central pacemaker in rats and other mammals which is predominantly entrained by the light-dark rhythm. There are also peripheral oscillators which are readily entrained by the timing of feeding. (Strubbe, Woods 2004, Mendoza 2007, Wu et al. 2008) The timing of feeding in relation to the light-dark rhythm has profound effects on the circadian rhythms of the organism (Damiola et al. 2000, Madrid et al. 1998, Nelson, Halberg 1986, Nelson, Scheving & Halberg 1975, Philippens, von Mayersbach & Scheving 1977). Each feeding method has its effects on the circadian rhythms of the animals.

When fed AL, laboratory rats maintain a similar eating rhythm as that of wild rats. It has been estimated that 70% – 85% of the daily food intake occurs during the dark phase, the majority of which is consumed at the beginning of the dark hours (Barnett 1963, Calhoun 1962, Spiteri 1982, Strubbe, Woods 2004, Zucker 1971). Rats will readily adopt a new feeding schedule and eat whenever food is offered, but there is evidence that complete metabolic adaptation does not occur. If given the opportunity, the animals will return to their original feeding pattern almost immediately (Spiteri 1982, Ritskes-Hoitinga, Chwaliborg 2003).

In DR, if the daily ration of food is offered at the beginning of the dark phase, the circadian rhythms are generally altered in such a way that the zenith of the curve is at the same time as in AL feeding but its amplitude is heightened, thus making more prominent the diurnal variation. The most common way of implementing DR is to give one meal per day during the light phase. In this case, the circadian rhythms do not shift completely to accommodate the "unnatural" feeding time, but show a disrupted, bimodal pattern. Feeding entrains the animals' circadian rhythms only partly when there is a dissonance between the feeding schedule and the light-dark rhythm. (Damiola et al. 2000, Nelson 1988, Wu et al. 2008, Nelson, Scheving & Halberg 1975) No matter how the DR regime is timed, the DR animals' circadian rhythms differ from those of their AL fed controls. It may be difficult or even impossible to differentiate the effects of the actual caloric restriction from those of the disturbed circadian rhythms. It has been reported that the response to pharmacological compounds can be altered if rodents are fed during the light phase (Toates, Rowland 1987, Claassen 1994). This methodological shortcoming may be easily overlooked when the results of AL and DR groups are compared. Furthermore, it would be difficult to arrange identical eating schedules for DR and AL animals with the conventional methods of feeding.

2.3.7. Behaviour

The physiological consequences of different DR methods are the main focus of this study. However, a brief overview of the impacts of DR on behaviour is necessary in order to be able to discuss the overall influence of different DR methods on laboratory rodents.

AL feeding makes it easier to group-house rats in socially harmonious groups. In the wild, aggressive encounters are rarely seen between the members of the same group (Barnett 1951). The aggression observed is mostly territorial and not linked to food resources. Breeding females can defend their nesting sites and established groups often fight strange males entering their territory (Lore, Flannelly 1977, Barnett 1951, Hurst et al. 1996, Hurst, Barnard & West 1996). Rats sometimes try to secure their own portion by hoarding food and eating it somewhere else, but there is no fighting involved in this behaviour (Barnett 1951).

In the laboratory environment, it is difficult for rats to use the competitive strategies seen in the wild, *i.e.* to hoard food, to escape from conflict situations *etc.* (Hurst et al. 1997). When laboratory rats are presented with food competition paradigms, usually one of the animals gains dominance over the food resources (Lore, Flannelly 1977, Hoshaw et al. 2006, Millard, Gentsch 2006). Once this hierarchy is established, very little direct aggression or fighting is observed (Lore, Flannelly 1977, Hoshaw et al. 2006, Millard, Gentsch 2006). However, the subordinate animals have been reported to develop serious stress-related pathology and to suffer from higher mortality than the dominant individuals (Hoshaw et al. 2006, Blanchard et al. 2001, Blanchard, McKittrick & Blanchard 2001, Blanchard et al. 1995, Martinez, Calvo-Torrent & Pico-Alfonso 1998). Nonetheless, the subordinate animals still choose social contact rather than social isolation if given the opportunity (Hurst et al. 1997).

For some reason, the situation is different in the case of traditional DR regimes. There are reports of severe aggression and even cannibalism occurring in DR regimes, where the rats are fed only once a day (Pugh, Klopp & Weindruch 1999, Adams et al. 1994, Pahlavani, Vargas 2001). It should be emphasized, however, that even AL feeding does not completely resolve the issue of social stress in laboratory rats. The detrimental effects of hierarchy can be found also in AL fed groups (Hurst et al. 1999).

The effects of DR have also been evaluated in different behavioural tests but the results are to some extent inconsistent.

DR increases general activity both in behavioural tests such as the open field (Heiderstadt et al. 2000, Geng et al. 2007, Smith et al. 2005, Smith, Metz 2005) and also in the home cage (Means, Higgins & Fernandez 1993). DR has been observed to exert anxiolytic effects in the open field and in the elevated plus-maze (Heiderstadt et al. 2000, Genn et al. 2003a, Genn et al. 2003b, Inoue et al. 2004, Levay et al. 2007). DR also increases exploratory behaviour in rats measured with a hole-board (File, Day 1972).

DR has been shown to cause depression-like changes in Porsolt's forced swimming test (Porsolt et al. 2001). DR animals become immobile quicker and spend more time immobile than AL animals in the Porsolt's forced swimming test (Jahng et al. 2007, Chandler-Laney et al. 2007). The immobility is interpreted as behavioural despair. The longer periods the animal is immobile, the more depressed it is considered to be. DR animals have been reported to suffer from anhedonia (*i.e.* decreased responsivity to reward), another behavioural change associated with depression (Bekris et al. 2005, Grippo et al. 2005) and for example, DR rats drink less sucrose water than their AL counterparts (Chandler-Laney et al. 2007). In addition to these behavioural findings DR has also been reported to cause some neurochemical changes typical of depression (Jahng et al. 2007, Chandler-Laney et al. 2007, Krieger et al. 1980, Stanley et al. 1989).

The effects of DR on cognition are also not unambiguous. In most studies, DR has been found to protect animals from the age-related decline in cognitive functions (Geng et al. 2007, Adams et al. 2008, Fitting et al. 2008, Goodrick 1984, Stewart, Mitchell & Kalant 1989, Pitsikas et al. 1990) or even improve their spatial learning abilities (Stewart, Mitchell & Kalant 1989, Kant et al. 1988). However, DR has also been associated with impaired learning (Yanai, Okaichi & Okaichi 2004) and in one study DR rats were reported to be too restless and hyperactive to concentrate on the learning task (Smith, Metz 2005).

2.3.8. Welfare

There is a paucity of studies directly addressing the welfare impacts of different feeding methods in laboratory rodents. However, the vast databank on physiological and behavioural effects of DR characterized in other fields of research (*e.g.* the mechanisms underpinning longevity, obesity, metabolic syndrome, appetite control and eating disorders) has been reviewed to approach the subject (Ritskes-Hoitinga, Strubbe 2004, Rowland 2007, Toth, Gardiner 2000). More information is available with respect to production animals, especially fowl and swine (D'eath et al. 2009).

The term "welfare" has been defined in several different ways. It is the definition of welfare used which determines how welfare is assessed and how the effects of any given feeding method will be interpreted.

D.M. Broom (1986) has defined welfare in the following way: "The welfare of an individual is its state as regards to cope with its environment". This refers both to the "cost" of attempting to cope and to the success of these attempts. If the cost of coping is high or the attempts to cope unsuccessful, the animal's welfare will be negatively affected. This can be measured by using biological variables. D.M. Broom lists the following indicators of poor welfare: increased mortality, increased morbidity, reduced growth, decreased fecundity, immunosuppression, adrenal activity and abnormal

behaviour. In this definition, measures of biological fitness are emphasized in the assessment of welfare. However, D.M. Broom acknowledges that the environmental adversities threatening the welfare of an individual include *e.g.* frustration of behavioural needs, fear, pain and lack of control over the environment. These are all threats that primarily affect the animal's feelings and then cause secondary, observable damage or abnormalities in the biological functions of the animal. Some of the indicators of poor welfare are also measures or consequences of a chronic stress reaction. D.M. Broom defines stress as "an environmental effect on an individual which overtaxes its control systems and reduces its fitness or seems likely to do so". (Broom 1998, Broom 1996, Broom 1991b, Broom 1991a, Broom 1990, Broom 1988, Broom 1986)

The previously mentioned definition of stress by G.P. Moberg (in section 2.3.4. Stress reaction) has much in common with D.M. Broom's concept of stress. However, the term "stress" used by D.M. Broom would translate into "distress" by G.P. Moberg, who also recognizes forms of stress where welfare or biological functions are not yet threatened. G.P. Moberg states that "The key to determining when stress affects animal's welfare is the biological cost of the stress. When the biological cost of coping with the stressor diverts resources away from other biological functions, such as maintaining immune competence, reproduction or growth, the animal experiences distress. During distress, this impairment of function places the animal in a prepathological state that renders it vulnerable to a number of pathologies." (Moberg 2000)

Welfare can also be defined in other ways, which emphasize feelings (Duncan 1996, Simonsen 1996) or preferences (Dawkins 2003, Sandoe 1996) of the animal or the naturalness of the way the animal is kept (Rollin 2007). A more thorough review of the welfare impacts of DR with regard to the different ways of defining welfare is presented in article IV.

In this text, the definitions used for welfare and for the relationship between stress and welfare are based on D.M. Broom and G.P. Moberg, if not otherwise stated. Next, an attempt will be made to interpret the welfare impacts of DR using these definitions.

DR has been shown to decrease morbidity and mortality (Allaben et al. 1996, Hubert et al. 2000, Keenan et al. 1999, Leakey, Seng & Alleben 2004, Duffy et al. 2004a, Duffy et al. 2004b, Masoro 2006c, Masoro 2005, Nelson 1988, Yu et al. 1982, Keenan et al. 1997) and to cause other benefits such as improved coping with environmental challenges (Masoro 1995, Jolly 2004, Klebanov et al. 1995, Shibolet et al. 2002). These findings would suggest that the welfare of DR rodents is better than that of AL rodents.

Many of the responses elicited by DR could also indicate decreased welfare compared to AL animals. DR quite obviously retards growth and can also decrease reproductive functions (Martin et al. 2007, Rehm et al. 2008). Immunosuppression has also been observed in DR rodents (Giovambattista et al. 2000, Heresi, Chandra 1980, Jolly 2004).

The results of the behavioural tests are difficult to interpret using only the definition of D.M. Broom. As mentioned above, DR has been shown to decrease anxiety and increase activity in behavioural tests, both of which can be interpreted as signs of increased welfare. Rodents subjected to stressful events generally become less active (Ottenweller et al. 1992, Ottenweller et al. 1989) and more anxious (*i.e.* less explorative and more fearful) (Ottenweller et al. 1992, Ottenweller et al. 1989, Archer 1973, Hogg 1996, Lister 1990, Pellow et al. 1985, Rodgers, Dalvi 1997, Rodgers, Cole 1993). However, DR might affect behaviour directly by increasing locomotor activity and food searching behaviour (Heiderstadt et al. 2000, Geng et al. 2007, Means, Higgins & Fernandez 1993). This could disturb the interpretation of behavioural tests and observations.

On the other hand, DR has been shown to produce depression-like neurochemical and behavioural changes (Jahng et al. 2007, Chandler-Laney et al. 2007) indicating decreased welfare (Cryan, Mombereau & Vassout 2005, Cryan, Valentino & Lucki 2005). Also increased aggression has been reported in DR animals (Pugh, Klopp & Weindruch 1999, Pahlavani, Vargas 2001).

The heightened adrenocortical activity observed in animals subjected to DR is difficult to interpret from the welfare point of view. Assessing welfare by measuring the stress reaction is never straightforward. A stress reaction does not equate to a welfare problem. A stress reaction can certainly be aroused without any suffering being involved. The overall well-being of an individual cannot be assessed solely by the stress reaction. (Rushen 1991) Moreover, it is argued that measures of stress do not correlate with well-being (Moberg 1987).

Nevertheless, chronic stress is considered a serious threat to welfare (McEwen 2004, Johnson et al. 1992, Korte et al. 2005, Korte, Olivier & Koolhaas 2007, Korte 2001, Wiepkema, Koolhaas 1993). The impacts of stress on welfare depend on what the "price" of attempting to cope with the stressor is. The negative outcomes of stress emerge only when the organism can no longer cope with the stressor (Johnson et al. 1992, Korte et al. 2005, Broom 1996, Goldstein, McEwen 2002, McEwen, Seeman 1999).

In the case of DR, the stress reaction's interpretation in terms of welfare is even more challenging. The stress reaction typical of DR can be understood as the organism's adaptation to reduced food intake (Frame, Hart & Leakey 1998). Frame (1998) states that "The overall profile of these changes is one of improved defense against environmental stress." It has even been suggested that the beneficial effects of DR on health and longevity may be mediated by the increased activity of the adrenal cortex associated with DR regimes (the hormesis hypothesis) (Han et al. 1995, Sabatino et al. 1991, Masoro 1998, Frame, Hart & Leakey 1998, Leakey et al. 1994, Yu, Chung 2001).

The stress reaction observed in DR is generally not associated with the negative outcomes of other states of chronic stress (Patel, Finch 2002). However, there are some observations of stress-related pathology, such as

gastric lesions, indicating decreased welfare in DR rodents (Heiderstadt et al. 2000, Rehm, Sommer & Deerberg 1987, Nakamura et al. 1990).

Even if the stress reaction observed in DR animals is considered a benign metabolic adaptation, the possibility of DR posing a serious threat to the welfare of the animals cannot be excluded. Next, some of the threats and challenges associated with the traditional methods of DR are presented.

To begin with, it is very difficult to estimate the amount of food that should be given to laboratory rats to ensure their well-being. AL feeding cannot be used as a reliable measure of the optimal food intake; it is quite clearly more than the animals need, more than is healthy for them and more than they would eat in the wild. Moderate DR (80 % – 85 % AL) provides in a nutritional sense enough for the animals. Moderate DR enhances physical health and increases longevity and can also improve fitness and coping capabilities. However, suffering can occur despite impeccable physical health (Dawkins 1990).

The animals can suffer from hunger, especially if the DR regime includes long periods of food deprivation. It has been suggested that rats adapt well to 24 hour rhythms in feeding (*i.e.* feeding once a day) but if they are fed for example every 48 hours, the metabolic stress increases considerably. The aversiveness of hunger in the traditional methods of DR is difficult to assess. Motivation to eat can be investigated in setups where the rats have to work for their food. The amount of food the rats will work for decreases when the work load increases. These rats will maintain a lower body weight than AL rats, suggesting that a part of the AL food intake is "extra" from the rat's motivational point of view. It is also likely that the experience of hunger is relieved when the food portion is offered, even if it is less than the normal AL food intake. (Rowland 2007, Toth, Gardiner 2000)

The possible disruption of circadian rhythms is another concern. As mentioned earlier, the timing of feeding in relation to the light-dark cycle can have a profound impact on the scientific integrity of the data obtained from the experimental animals. Circadian rhythms are also important to consider from the welfare point of view. It is not known how much an animal's welfare is affected when its endocrine circadian rhythms are disrupted, as is the case when rodents are fed during the light phase in DR regimes. (Ritskes-Hoitinga, Strubbe 2004) Also appetite follows a circadian rhythm; thus the animals might suffer from an unpleasant period of intense, unsatisfied appetite during the beginning of the dark phase if they have been fed during the daytime. (Bellinger, Mendel 1975) The animals will adapt better to a DR regime if it mimics the typical feeding schedules of the species (Toth, Gardiner 2000).

The opportunity to express species-specific behaviours related to eating, *i.e.* foraging in rats, is often overlooked in laboratory animal housing systems. This is true for both AL and the traditional DR feeding regimes. However, the problem might be further emphasized in DR, where rodents have less behavioural possibilities relating to food and presumably to a

higher motivation to eat. Foraging has been proposed to be a behavioural need in rats (Dawkins 1990, Balcombe 2006, Dawkins 1988). A behaviour can be called a need when the "performance of behaviour itself does have motivationally significant consequences which are not necessarily related to functional requirements" (Hughes, Duncan 1988). In the case of rats and foraging, this does seem to be the case, the animals will engage in foraging behaviours (Johnson, Patterson-Kane & Niel 2004) and will choose to work or explore for their food (Inglis et al. 2001, Inglis, Forkman & Lazarus 1997, Mitchell, Becnel & Blue 1981) even when food is offered AL.

Boredom is generally understood as a feeling experienced when there is a lack of novel stimuli (or an internal disinterestedness towards the environment). The term can also be used describe the pathological state of an individual created by prolonged exposure to an impoverished environment. The animal is impaired in its ability to pay attention or focus on the environment. (Wemelsfelder 1994) In any case, chronic boredom is a form of suffering and has been suggested to cause not only behavioural malfunction, but also neurological damage (Broom 1998). Korte (2007) raises the important prospect that environmental hypostimulation may be as serious a threat to the wellbeing of an individual as environmental hyperstimulation. It has been proposed that optimal welfare is associated with short-lasting responses to stressors protecting the animal from boredom (Wiepkema, Koolhaas 1993). The standard laboratory environment may be considered impoverished in any case, but traditional methods of DR often additionally deprive the animals of the company of conspecifics and of the opportunity to eat and manipulate food throughout the day, leaving them sometimes with nothing but the bedding material.

The welfare threats of different feeding regimes can also be evaluated from the point of view of frustration. Unsatisfied appetite, unfulfilled need for foraging, boredom and inability to perform eating behaviours at the appropriate time can all lead to frustration. Stress has been defined as a "failure to achieve proper feedback when it attempts to act according to its own motivational state" (Jensen, Toates 1997). This definition of stress is very much like that of frustration. The word "frustration" is derived from the verb "to frustrate" *i.e.* "to prevent (a plan or action) from progressing or succeeding" or "to prevent (someone) from doing or achieving something" or "to cause (someone) to feel dissatisfied or unfulfilled" (Oxford Dictionaries, *www.askoxford.com*). Frustration can be understood as an emotional response to circumstances where one is obstructed from arriving at a personal goal. The standard laboratory housing systems have been claimed to impair the behavioural and neurological development of rats due to a frustration of behavioural needs, such as foraging, gnawing and exploring (Balcombe 2006). Frustration has been proposed to be an important source of suffering in animals (Broom 1998, Dawkins 2003, Dawkins 1988).

A major welfare concern in the traditional methods of DR is social isolation. The animals are usually housed individually to avoid aggression-related problems and to ensure equal food intake of each individual. Social isolation is recognized as a potent stressor for rats and can lead to diminished welfare (Krohn et al. 2006, Brenes, Rodríguez & Fornaguera 2008, Perello et al. 2006, Serra et al. 2005). Isolation has even been reported to decreases longevity (Menich, Baron 1984, Perez et al. 1997). Rats are highly motivated to work for the company of conspecifics, suggesting either that social isolation is aversive or that company is pleasurable or otherwise valued (Patterson-Kane, Hunt & Harper 2002). The ability to cope with different stressors is decreased in individually housed rats (Karim, Arslan 2000, Balcombe 2006). Current European legislation recommends that rats be housed individually only if there is justification based on veterinary, welfare or experimental grounds (2007/526/EC).

In conclusion, the traditional methods of DR pose several threats on the welfare of rodents. The actual caloric restriction might not be the main welfare concern. The adversities imposed on the animal by the traditional methods of DR are more likely related to social isolation, the periods of fasting and the inability to perform species-specific behaviours at appropriate times.

2.4. 3R alternatives in dietary restriction

The 3R principle in laboratory animal science was first launched in 1959 by W.M.S. Russell and R.L. Burch. The 3Rs stand for replacement, reduction and refinement. The principle states that when possible, the use of animals should be replaced by non-sentient alternatives, such as in vitro methods. When using experimental animals, the number of animals used should be reduced to a minimum by good experimental design and statistics and the method chosen should be as refined as possible from the welfare point of view without compromising the scientific value. (Russell, Burch 1959)

DR methods may have implications for two of the Rs, reduction and refinement. There has been much debate about whether an AL fed rodent is the optimal experimental model. The AL rodent is argued to be obese and to suffer from malnutrition; DR feeding would "normalize" the animal's metabolism and make it a better research model with less confounding morbidity (Keenan et al. 1999, Keenan, Laroque & Dixit 1998, Turturro, Duffy & Hart 1993). Thus, from the scientific point of view, the traditional methods of DR could be seen as improvements compared to AL feeding. On the other hand, the traditional methods of DR can be argued to act against the refinement goal by disrupting the circadian rhythms of the animals and subjecting them to social isolation. Moreover, disrupted circadian rhythms can make more difficult the comparison between study and control groups and may therefore complicate the interpretation of research results. Social

isolation can produce animals with disturbed and less flexible behavioural patterns and a higher susceptibility to stressors. This can also be understood as anti-refinement.

Reduction can be achieved in DR fed rodents by two mechanisms, increased longevity and decreased inter-individual variation. The longevity of laboratory rats has decreased significantly over the past decades. For example, the two-year survival rate of male Fischer 344 rats in the 1970's was about 80 %, whereas in the 1990's the survival of the same strain was reported to have decreased to 36 % (Keenan, Laroque & Dixit 1998). At the same time, the rats are growing more quickly and achieving higher and higher body weights (Keenan et al. 1996). An increasing number of animals per study are needed for safety evaluations to compensate for the increased mortality. This is especially true for long-term safety evaluations, where a fixed number of the control animals must survive the two-year study (OECD). In order to ensure this, today the experimental groups have to be larger than before. It is not known whether there is causality between more rapid growth and increased mortality. Nevertheless, increasing longevity with DR could translate into millions fewer animals being used annually (Hubert et al. 2000).

DR can also lead to reduction also by decreasing inter-individual variability. AL feeding is claimed to be the least controlled variable in animal experiments. AL fed animals have extensive variations in body weight, survival and tumour incidence (Keenan et al. 1996). DR has been proposed to represent a solution to these problems (Allaben et al. 1996). Indeed, traditional methods of DR have been shown to reduce the inter-individual variation in several variables, *e.g*. body weight and liver to body weight ratio (Duffy et al. 2001, Leakey, Seng & Allaben 2003, Carney et al. 2004, Leakey et al. 2003). Moderate DR (75-80 % AL) has been called a "powerful reduction tool" (Ritskes-Hoitinga, Savenije 2007). The decreased variation seen in animals subjected to traditional methods of DR are probably caused by a more uniform food intake instead of reduced food intake as such. The animals are housed alone and given a precalculated portion of food each day and the majority of the animals will consume their whole portion immediately. This means that the food intake and the timing of feeding are highly standardized. Another hypothesis of the mechanics of the decreased variation observed has to do with "normal" and "abnormal". It is proposed that normal phenomena are normally distributed within a population and tend to have a small variation. Abnormal phenomena, on the other hand, are supposedly often skewed in their distribution and exhibit a larger variation. If the body weight maintained in DR is more "normal" for the rat as a species than the body weight in AL, then this theory might also be considered to explain the differences seen in variation. (Macri et al. 2007, Garner 2005)

2.5. Finding a good feeding method

Choosing a feeding method for laboratory rodents is a real dilemma. Different aspects of welfare can be affected in opposite directions. Even though DR may significantly improve the physical health of laboratory rodents, its implementation often exposes the animals to adverse housing conditions such as social isolation and disturbed diurnal eating rhythms. From a scientific point of view, balancing between the effects that a chosen feeding method can have on variation, the validity of the research model and on the research results is also complicated. Both welfare and scientific integrity can be compromised simply to achieve practicality, as one might argue has happened with the current practice of AL feeding. The ideal feeding method would provide optimal nutrition and the health benefits of restricted caloric intake without threatening the welfare of the animals or the scientific value of the experiments.

One way to solve the problem is to continue to feed the rodents AL, but to modify the diet composition. One example of this is the nonpurified diet called the NTP-2000, which was formulated for the National Toxicology Program (USA). It was developed to decrease the morbidity and mortality associated with unlimited access to the commonly used laboratory animal diets. NTP-2000 has an increased content of fibre and fat and a decreased content of protein, but a similar energy content compared to other diets. Rats fed AL on this diet grow more slowly, have an increased survival and a decreased incidence and severity of nephropathy and cardiomyopathy compared to AL fed animals on other diets. (Rao, Morris & Seely 2001, Rao 1997)

High fibre diets are believed to confer various health benefits for humans and results from rodents have been used to back up this claim. High fibre diets can result in decreased weight gain and seem to be rather well tolerated by the animals. The high fiber diets have been shown to improve blood glucose and lipid levels (Aleixandre, Miguel 2008), to normalize the metabolic disturbances in rats with induced diabetes (Yamashita et al. 1980) and to retard aging-associated changes in the nervous system (Joseph et al. 1995). However, the utility of high fibre diets in resolving the problems associated with AL feeding has been questioned. Rats readily increase their food intake to compensate for the dilution of energy. In addition, longevity and other physiological variables have been reported to be unaltered by consumption of a diet containing 16 % crude fiber (standard rodents diets contain less than 5 % of crude fiber). (Keenan et al. 1999)

Another solution is the feeding station (Laboratory Feeding Systems, Denmark). It is a small feeding pen situated within the cage. Individual animals are identified with microchips, which are recognized when the animal enters the pen. A detailed, individual feeding regime can be programmed into the system. This feeding station provides the opportunity to combine group-housing with DR. There is a possibility to control and

record the feeding at an individual level. However, this invention might be too expensive and high-tech to be implemented into laboratory animal facilities on a large scale.

In one study, rats were offered four different choices related to feeding: AL feeding in a standard cage, AL feeding with access to only a small portion of the food hopper, AL feeding with additional wooden gnawing sticks and food provided in a "foraging device", a metal plate where the food was mixed into gravel. There was no difference in the weight gain between the different AL feeding methods. The rats compensated for the limited access to the food hopper by increasing the time spent eating. The foraging device was favoured by the animals in a preference test and the rats spent large amounts of time looking for food in the gravel container. However, the weight gain was significantly increased in the foraging group. (Johnson, Patterson-Kane & Niel 2004) Thus the foraging device does not solve the dilemma of feeding; its value is limited to enrichment.

Food consumption and weight gain can also be regulated by housing conditions. As a general rule, animals housed with environmental enrichment gain less weight than animals in barren environments (Fiala et al. 1977, Spangenberg et al. 2005, Eskola, Kaliste-Korhonen 1998). The same principle applies for the social environment, group-housed animals gain less weight than animals housed in social isolation (Menich, Baron 1984, Perez et al. 1997).

The subject of this dissertation is a novel feeding method for laboratory rats. The diet board is an attempt to solve the dilemma of feeding. The diet board offers the possibility of combining moderate DR with group-housing and unaltered circadian rhythms. The diet board also provides enrichment, thus further adding its value as a refinement alternative to traditional methods of DR. The purpose of this study was to begin the validation process of the diet board by assessing its effectiveness in controlling body weight and characterizing its impacts on physiology, welfare and result variation.

3. Materials and methods

All of the results presented here and published in articles I – IV are obtained from one single experiment. All variables have been measured from each animal and all analyses are performed with data obtained from all of the 60 animals utilized in that study.

The study was done in the National Laboratory Animal Center (NLAC), University of Kuopio, Finland. The laboratory animal facilities are approved and meet the demands of national and European legislation (license number MMM 1107/712–86). The study protocol was reviewed and approved by the Finnish National Ethics Committee (license number ISLH-06-66). The experimental phase took place from January to April in 2007.

3.1. Animals and housing

A total of 60 barrier bred male Wistar (HsdBrlHan:WIST) rats (bred at NLAC, Kuopio, Finland) were used. The breeding unit and the experimental unit were free of the pathogens listed in the FELASA recommendations for health monitoring (Nicklas et al. 2002). The animals entered the experiment at seven weeks of age. The animals were kept in groups of three in solid bottom stainless steel cages with open wire-mesh hoppers (Franke Finland Ltd, Naarajärvi, Finland). All cages (48.5 x 28.5 x 20.0 cm) were kept in a cubicle. Ambient temperature was 21 ± 1 °C and relative humidity 55 ± 15 %. Room illumination followed a 12/12 h cycle, switching lights on at 0700 h and off at 1900 h. Tap water in polycarbonate bottles was available at all times. The diet used was Lactamin R36 (Lantmännen, Kimstad, Sweden) available either from the diet board or AL from the food hopper (availability is described in more detail in the study design section). Aspen chips (Tapvei Ltd, Kaavi, Finland) were used as bedding; and aspen wool (Tapvei Ltd, Kaavi, Finland) as nesting material; both were renewed at the cage changes which were conducted twice a week.

3.1.1. The diet board

The diet board (See Figure 2. for a photograph of the diet board) consisted of two aspen boards (35.0 x 12.2 x 2.7 cm) placed into the cage in the form of a cross made by intersecting the boards. Two corners (6.0 x 6.0 cm) of each board were removed to facilitate the rats' movement within the cage. Twenty vertical holes (Ø 12.5 mm) with a 2 to 3 mm slot open to the side of the board were drilled into each board. These holes were filled with food pellets (Lactamin R36 (Lantmännen, Kimstad, Sweden)) that were fixed in place by autoclaving the board (121 °C, 20 min, 220 kPa, Finn-Aqua 121821

D, Steris Finn-Aqua, Tuusula, Finland). The control animals were provided with autoclaved aspen boards of the same configuration, but without the drill holes or food.

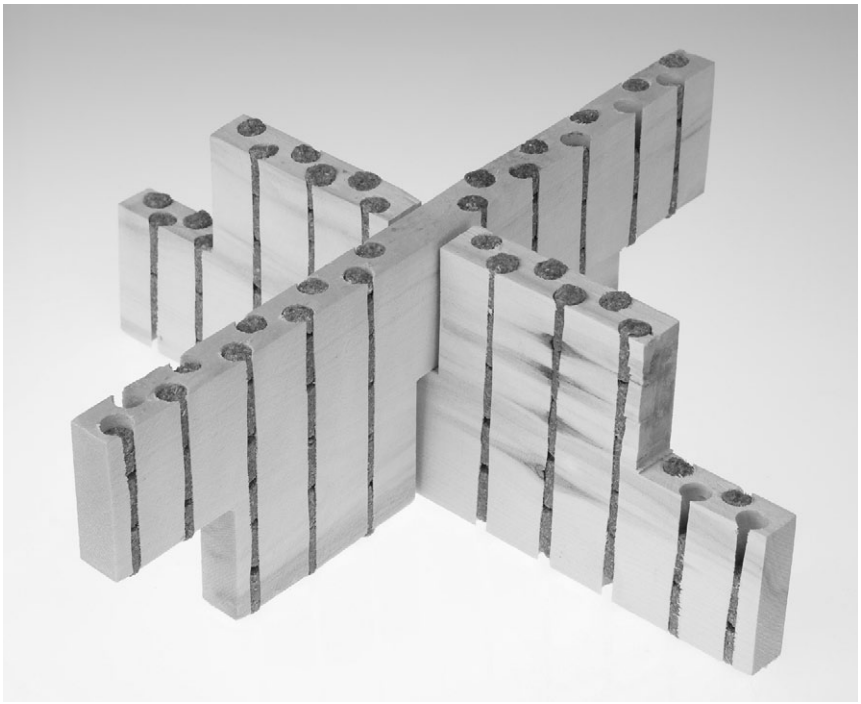

Figure 2. The diet board

3.2. Study design

The rats were divided into two groups (n=30 in each group). The diet board group was fed exclusively from the diet board throughout the experiment. The control group was fed AL, *i.e.* food was always available in unrestricted quantities in the food hopper.

The animals were divided into study groups and cages in the following manner. Ten litters with a minimum of six males were ordered for the study, and six males from each litter were chosen for the experiment. The six males were chosen randomly but excluding aberrantly large or small male pups. These six siblings were housed together in one cage from weaning until the beginning of the study. On a Friday, when the animals were six weeks old, each of the sextets was divided at random into two cages; one cage designated for diet board feeding and the other cage for AL feeding. Thus both the diet board group and the AL group consisted of ten cages with three male siblings from the same litter in each cage. The cages' location in the cage rack was randomized to yield an equal number of

each group at each horizontal level. The animals were weighed and ear-tattooed before they were placed in the new cages. All animals continued to be fed AL during the following weekend to allow them to recover from the re-grouping and handling.

The experiment began on the following Monday, when the rats were seven weeks old. The diet boards and plain boards were placed in the cages and the food hoppers of the diet board cages were emptied. The animals were taken into the study in three cohorts, all following the same week-day routine. The cages were changed twice a week, on Monday afternoons (1200–1400 h) and Friday mornings (0900–1100 h). New boards were provided at each Monday cage change. The study lasted ten weeks. (Figure 3. shows a summary of the study design)

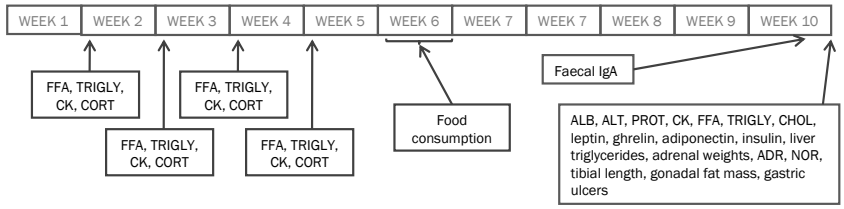

Figure 3. The schedule of the experiment. Abbreviations: adrenaline (ADR); albumin (ALB); alanine aminotransferase (ALT); cholesterol (CHOL); creatine kinase (CK); corticosterone (CORT); free fatty acids (FFA); immunoglobulin A (IgA); noradrenaline (NOR); protein (PROT); triglycerides (TRIGLY)

3.3. Humane endpoints

Three different age-specific criteria for humane endpoints were established. From seven to eight weeks of age, the criterion for euthanasia was a 15 % loss in body weight. From eight to 13 weeks of age, the criteria for euthanasia were: failure to gain weight during a two-week period or a 5 % loss of body weight during one week. Thereafter, the criterion was a 10 % loss in body weight during one week. In all age groups, the humane endpoints included dehydration, unexpected disease, trauma, dental problems or any other clinical signs of suffering.

3.4. Data collection and sampling during the study

3.4.1. Weighing

The animals were weighed (Sartorius 1B31 (1–32000 g) Sartorius-Werke GMBH, Göttingen, Germany) twice a week when the cages were changed on Mondays (1200–1400 h) and Fridays (0900–1100 h).

3.4.2. Blood samples

Blood samples were collected four times during the study, on the Mondays of weeks 2, 3, 4 and 5 of the experiment. All of the samples were taken at 0900–1100 h. Samples were taken from *Vena saphena* by piercing a hole in the vein with a 25 G needle (Hem, Smith & Solberg 1998). The blood ran freely from the vein to the collecting tube. The sample volume was at the maximum 0.5 ml. The blood was left to coagulate in the plastic tube for 10–15 minutes. The samples were centrifuged at 12 000 rpm for 15 minutes at room temperature (Eppendorf centrifuge 5412, Eppendorf, Germany). The serum was removed and placed into clean plastic tubes. The serum was frozen immediately at -20 °C.

3.4.3. Food consumption

Food consumption of the animals was measured from five study cages and five control cages during the sixth experimental week. The control animals' food consumption was measured simply by weighing the food in the food hopper in the beginning and at the end of the week. The diet board animals' food consumption was measured by measuring the length of eaten food pellets in the holes in centimeters. From this length, the weight was calculated. These measures of course represent the total food usage, consumption and wastage together.

3.4.4. Faecal samples

Cage-specific faecal samples were collected from every cage on the Friday of week ten. Six hours after the routine Friday cage change, all faecal pellets found in the cages were collected, weighed and frozen at -20 °C.

3.5. Terminal data collection and sampling

3.5.1. Euthanasia

The animals were euthanized at the end of the ten-week experiment, on the Monday of week 11. The animals were first weighed and then anesthetized with a mixture of oxygen and carbon dioxide (1:1). A final blood sample was collected from the anesthetized animals by cardiac puncture, after which the animals were immediately euthanized with 100 % carbon dioxide.

3.5.2. Final blood sample

The final blood sample was collected by drawing blood straight from the heart with an 18G needle into a clean plastic syringe. The sample volume was 5–10 ml. The syringe was immediately emptied into a plastic tube. The blood was left to coagulate in the tubes at room temperature for 10–15 minutes. The blood was centrifuged at 3600 rpm for 15 minutes at 4 °C (Megafuge 1.0R, Heraeus instruments, Germany). The serum was distributed into several plastic tubes. The tubes were cooled in solid carbon dioxide. The serum was frozen at -80 °C.

3.5.3. Measurements and visual inspection

A post-mortem examination was performed without delay after euthanasia. Both adrenal glands were carefully dissected and weighed. The gonadal fat surrounding the testicles was dissected and weighed (Sartorius 2842 (0.0001–160 g) Sartorius-Werke GMBH, Göttingen, Germany) The right tibia was dissected and the shaft length was measured from the cranial intercondylar area to the medial malleolus with a vernier calliper (Central Tools Inc., Cranston, RI, USA). The ventricle was cut open and observed visually for ulcers. Other abdominal and thoracic organs were assessed visually for macroscopic changes.

3.5.4. Tissue samples

Both adrenals were carefully dissected, weighed and immediately frozen in liquid nitrogen. The liver was removed, washed in 0.9 % NaCl solution, divided into three plastic tubes and frozen in liquid nitrogen. All tissue samples were transferred into a -80 °C freezer for storage.

3.6. Analyses

3.6.1. Serum biochemistry

Alanine aminotransferase (ALT), albumin (ALB), total protein (PROT), creatine kinase (CK), triglycerides (TRIGLY), FFA and total cholesterol (CHOL) concentrations in serum were determined from the terminal blood samples. The serum concentrations of CK, TRIGLY and FFA were also determined from the blood samples taken on weeks two, three, four and five of the experiment.

Kinetic methods according to International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) (37°C) were used for ALT (Bergmeyer, Horder & Rej 1986) and CK (Horder et al. 1990) analyses. The colorimetric bromcresol purple method was used for ALB (Pinnell, Northam 1978) and the Biuret method for PROT (Burtis, Ashwood 1996) analyses. CHOL (Roschlau, Bernt & Gruber 1974) and TRIGLY (Röschlau, Bernt & Gruber 1974) levels were measured by enzymatic colorimetric methods. FFA was analyzed using an enzymatic colorimetric method (Wako NEFA C ACS-ACOD Method, code 994-75409E, Wako Chemicals GmbH, Germany). A Konelab 60i Clinical Chemistry Analyzer (Thermo Electron Co, Vantaa, Finland) was used for all of these analyses with reagents from Thermo Electron Co (Vantaa, Finland).

3.6.2. Serum corticosterone

Total serum corticosterone was analyzed from the blood samples taken on weeks 2, 3, 4 and 5 with ImmuChemTM Double Antibody Corticosterone 125I RIA Kit (MP Biomedicals LLC, New York, NY, USA). The serum was diluted with phosphosaline gelatin buffer (pH 7.0 +/- 0.1) 1:200. 100 µl of this dilution was used for the assay. 200µl of corticosterone-^{125}I label was added followed by 200 µl of corticosterone-3-carboxymethyloxime. The samples were incubated at room temperature for 2 hours and then 500 µl of precipitant solution was added. All of the above mentioned reagents were provided in the kit. The samples were centrifuged at 2300 rpm for 15 minutes at room temperature (Eppendorf Centrifuge 5810 R, Eppendorf, Germany). The supernatant was removed from the tubes. The radioactivity of the precipitates was measured with a 1260 Multigamma II -gamma counter (LKB Wallac, Sollentuna, Sweden).

3.6.3. Faecal immunoglobulin A

Immunoglobulin A (IgA) was extracted from the faecal samples (Pihl, Hau 2003). The concentration of IgA in the samples was determined with a Rat

IgA Quantitation Kit (Bethyl, Montgomery, TX, USA) using a Multiskan Ex microtiter reader (Thermo Electron Corp., Waltham, MA, USA).

3.6.4. Serum ghrelin, leptin, adiponectin and insulin

Serum ghrelin, leptin, insulin and adiponectin levels were analyzed from the terminal blood samples. Commercial kits were used to determine the serum concentrations of insulin (Rat Ultrasensitive ELISA kit, Mercodia AB, Uppsala, Sweden), ghrelin (Total Ghrelin RIA kit; Linco Research, St. Charles, MO, USA), leptin (Rat Leptin RIA kit; Linco Research) and adiponectin (Rat Adiponectin ELISA kit, Linco Research).

3.6.5. Adrenal content of adrenaline and noradrenaline

High performance liquid chromatography with coulometric electrochemical detection (Scheinin et al. 1991) was used to determine the concentrations of ADR and NOR in adrenal glands. The tissue samples were homogenized in 0.1 M perchloric acid, proteins were precipitated by centrifugation, and the supernatants were extracted with activated alumina (ANTON, SAYRE 1962). Authentic reference standards and the internal standard, 3,4-dihydroxybenzylamine, were purchased from Sigma-Aldrich (St. Louis, MO, USA). ADR and NOR concentrations were measured separately for the right and left adrenals.

3.6.6. Liver triglycerides

Liver triglycerides were isolated using the Folch method for extraction and measured using Free Glycerol Reagent F6428 and Triglyceride Reagent T2449 (Sigma-Aldrich, St. Louis, MO, USA) for quantitation (Ruohonen et al. 2008).

3.7. Statistical analyses

The appropriate sample size in the experiment was estimated with the Resource equation method (Festing 2002); the degree of freedom for error was 17. The statistical software packages used to process and analyze the data were SPSS 14.0 for Windows (SPSS Inc., Chicago, IL, USA) and SAS version 9 for Windows (SAS Institute Inc. Cary, NC, USA). The graphs were drawn with SigmaPlot 10.0 software (Systat Software Inc., San Jose, CA, USA).

3.7.1. Comparisons between groups

The differences between the diet board and AL groups were analyzed with linear mixed models using all collected data. A random litter effect was included in the models in order to accommodate for the possibility of correlated outcomes among siblings. The variables that were not normally distributed were log-transformed before the statistical analysis.

The results of normally distributed variables are presented as arithmetic means and standard deviations (SD). Model-based estimates for differences of the means and their 95 % confidence intervals (CI) are also reported. For variables that were not normally distributed, the results are presented as model-based estimates of the geometric means, their ratios and their 95 % CI.

3.7.2. Comparisons between groups in repeated measurements

Linear mixed models for repeated measurements were used for variables measured at multiple times from the same subjects. Time, treatment and their interaction were included into the models as fixed effects. In addition, siblinghood was included as a random effect in the models in order to account for possible dependencies due to the hierarchical nature of the data. The variables that were not normally distributed were log-transformed before the statistical analysis.

Growth rates were estimated by multilevel growth models involving litter- and rat-specific intercepts, linear and quadratic terms. The period of the introduction of the feeding intervention appeared to influence the growth profiles and was therefore omitted from the estimation of the growth curves.

The results are presented as the averages of the model-based estimates of the geometric means, their ratios and their 95 % CI.

3.7.3. Correlations

Correlations were analyzed using Spearman's correlation test on the original data. The correlation coefficients are analyzed separately for the diet board and AL groups.

3.7.4. Comparisons of inter-individual variation between groups

The equality of variances was investigated from the residual variance terms (obtained from the linear mixed model analyses) with Levene's test.

4. Results and discussion

The diet board was developed to offer an alternative method of feeding laboratory rats. The ultimate goal was to devise a solution that would offer the possibility of combining DR with group-housing and unaltered circadian rhythms. Such a DR method would not only provide a more welfare friendly way of restricting the caloric intake of laboratory rats but was also expected to promote better science by eliminating the confounding factor of disrupted circadian rhythms.

The diet board is a simple feeding device, where the food pellets are placed into vertical drill holes in the wooden boards. The board is present in the cage all of the time. The restriction on feeding and weight gain is created by the difficulty of extracting the food pellets from the board *i.e.* the rats have to gnaw the wood in order to reach the pellets. Since there are four arms in the diet board, there are always enough feeding spots for each of the three individuals in the cage. This was expected to make it possible to group-house the animals without encountering aggression-related problems.

The purpose of this study was to start the validation process of the diet board as a DR method. In this study, some of the diet board's basic features were characterized to determine the general usability of the invention and also to define the focus of the research group's future experiments.

4.1. Efficacy of the diet board

4.1.1. Weight gain

The diet board was successful in reducing the weight gain of the rats. The study began when the animals were seven weeks of age. At that time, the rats of the DR group weighed 118 $\pm$ 20 g ($\pm$SD) and those of the AL group 119 $\pm$ 21 g. The growth curves of the animals can be seen in Figure 4. The difference between the two groups was established during the first week of the feeding intervention. The DR animals did not gain weight and the AL animals continued to grow. By the end of the first week, the difference in the mean weights of the groups was 34 g (95 % CI: [28,40]), which was statistically significant ($p<0.0001$).

The first week of the diet board feeding was undoubtedly a trial for the rats, although none of the rats reached the pre-defined humane endpoints. A visual, unquantified observation was that the diet board rats seemed fearful and restless during the first week. In this study, the food source was abruptly changed from AL feeding to the diet board without any overlap. In pilot studies a period of adaptation was provided for the animals.

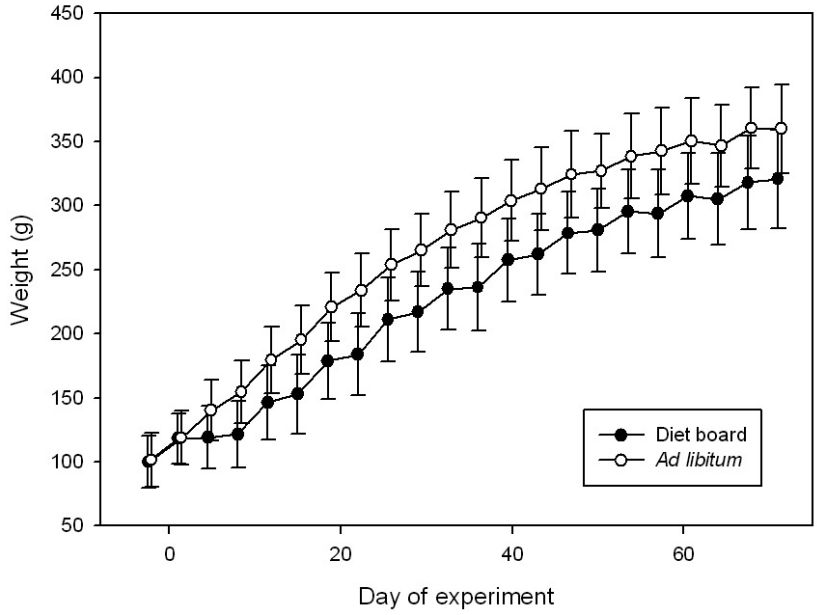

Figure 4. The weight gain of diet board and ad libitum fed rats during the ten week experiment. The rats were seven weeks old at the beginning of the experiment. The error bars represent the standard deviations.

The animals were allowed one week of AL feeding from the hopper with the diet board also present in the cage. However, the food pellets in the diet boards were left untouched and the first week of exclusive diet board feeding resulted in a similar cessation of growth. This observation is in contradiction with the known phenomenon of contrafreeloading, where rats have been observed to work for food even when it is available also "for free" (Inglis, Forkman & Lazarus 1997). However, it has been argued that the contrafreeloading phenomenon is mostly exploratory in its nature (Inglis et al. 2001, Forkman 1996). Perhaps the diet board rats did explore (sniff, lick, climb *etc*.) the diet board enthusiastically, but still chose to obtain their food in the easiest possible way. Rats are neophobic with new foods and will decrease their food intake for a number of days when presented with any novel food (Berdoy, Macdonald 1991). This does not, however, seem to be a likely explanation for the delay in eating from the diet board. The food pellets in the diet board were the same food that the rats had been used to eating for their whole lives. The diet board itself was aspen; this was not a novel material for the rats, since their bedding and nesting material were also made of aspen. One simple explanation could be that the animals were not used to gnawing wood and it required a couple of days before they mastered the new skill.

After the first week, both DR and AL rats grew steadily. The growth curves exhibited the same slope with the rats growing at similar rates. The

DR group did not catch up with the AL group during this ten-week study and once established, the difference in body weights was significant throughout the study ($p<0.0001$). At the end of the study, the model-based estimate for the difference of the means was 38 g (95 % CI: [25,62], $p<0.0001$). The rats in the DR group reached an average body weight of 321 $\pm$ 38 g and those in the AL group 360 $\pm$ 35 g.

The weekly undulation in the growth curves of the diet board rats (growth from Monday to Friday, maintenance of weight from Friday to Monday) can be explained by the accessibility of the food in the diet board. New diet boards were provided every Monday and although the food never ran out, it was easier to access the food at the beginning of the week. The rats preferred to eat from the top of the diet board downwards following the open slots. The bottom part was possibly more difficult to gnaw. To solve this problem in our future studies, a small pilot study was done where the diet board was flipped upside down on Thursdays. This procedure abolished the undulation in the growth curves. However, in our recent studies (Inhilä, K., unpublished) the rats have changed their eating habits and now gnaw the top and bottom equally. The rats are older and the slots in the diet board are very slightly wider, but otherwise the setup has been identical. It seems that laboratory rats are flexible in their eating habits and may adopt different ways of consuming food from the diet board depending on the situation.

The diet board rats gained 15 % less weight than the AL group, this being equivalent to mild to moderate levels of DR.

4.1.2. Adiposity and skeletal growth

The adiposity of the rats was estimated by weighing the gonadal fat at the end of the study. The gonadal fat mass has been demonstrated to correlate directly with the body fat content in mice (Rogers, Webb 1980, Webb, Rogers 1979), but to our knowledge no such correlation has been decisively established in rats. The weight of the gonadal fat was 4.47 $\pm$ 0.90 g in DR rats and 5.66 $\pm$ 1.06 g in AL rats (model-based difference -1.12 g (95 % CI: [-1.62,-0.77])), *i.e.* the AL rats had 30 % more gonadal fat than the DR rats ($p<0.0001$).

The rats in this study were from seven to seventeen weeks of age, hence they were still growing. In order to determine whether the diet board feeding affected the skeletal growth of the animals, the length of the right tibia was measured at the end of the study. The mean tibial length was 3.97 $\pm$ 0.11 cm in the DR rats and 4.07 $\pm$ 0.09 cm in AL rats (model-based difference -0.11 cm (95 % CI: [-0.14,-0.07])). Although this difference was statistically significant ($p<0.0001$), it only amounted to a 3 % difference between the groups.

The diet board seems to hinder skeletal growth only minimally. The 15% difference in body weights could be mostly attributed to decreased adiposity

in the DR rats. This is in accordance with other studies, where the lean body mass was very little affected by DR (Yu et al. 1982). In another study, mild DR (72–79 % AL), corresponding to the level of restriction now achieved with the diet board, did not cause changes in the musculoskeletal system, whereas moderate (68–72 % AL) and marked (47–48 % AL) DR decreased the size of the bones and muscles of the rats (Keenan et al. 2005).

4.1.3. Food consumption

The one-week food consumption of the animals was measured during the sixth week of the experiment. (Figure 5. shows a photograph of a gnawed diet board) The mean weekly food consumption per cage was 384 $\pm$ 40 g in the DR cages and 449 $\pm$ 31 g in the AL cages. Thus, each DR rat consumed 128g (18 g per day), while each AL rat consumed 150 g (21 g per day) of food during that week. The difference in absolute food consumption is *ca*. 15 %, but when proportioned to the body weight, DR and AL animals consumed an equal amount of food per gram of body weight. This phenomenon is well known in DR research; the animals' body weight is adjusted to the energy intake and the energy intake/body weight ratio of animals on different levels of DR stays the same (Masoro 2005). The caloric intake of the animals in this study was roughly estimated as 0.2 kcal/g body weight/day.

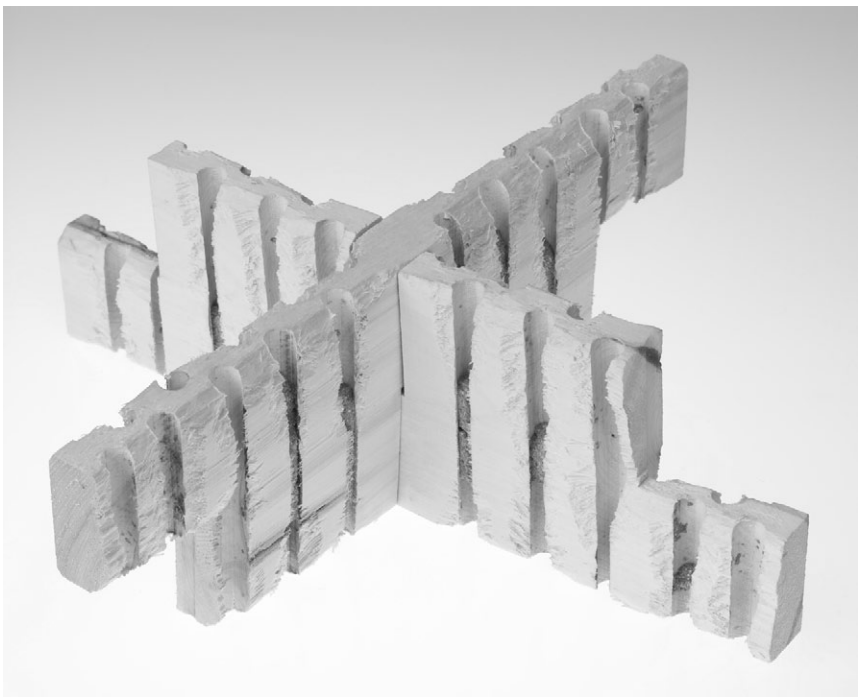

Figure 5. A consumed diet board

This is somewhat more than in for example Keenan's (2005) study, where the caloric intake per gram of body weight per day was 0.15 kcal. The level of DR in Keenan's study was 72–79 % of AL food intake, whereas in this study the level of DR on week six was 85 % of the AL food intake. From these figures, one can calculate that the level of caloric intake in the AL group in Keenan's study was lower than in our study. This could be explained by the fact that the animals were older in Keenan's experiment and past the age of fast growth. (Keenan et al. 2005)

4.2. Applicability as an alternative to AL

The diet board can be seen as an alternative to the practice of AL feeding. The diet board might offer valuable improvements to the routine housing of laboratory rats by promoting a healthy body weight without disturbing research results. We hypothesized that diet board feeding would not cause changes in variables other than those directly linked to energy metabolism. To test this hypothesis, several more or less arbitrarily chosen clinical chemistry variables were analyzed at different time points of the study.

Blood samples were taken from the animals on study weeks two, three, four and five and at the end of the ten-week experiment. Serum CK was analyzed from each of these samples, whereas serum ALB, total proteins PROT and ALT were analyzed only from the final blood samples. The terminal blood samples collected from anaesthetized animals were considerably larger those taken during the study, therefore more variables could be measured at the end of the study. The numeric values and distributions can be seen in Table 4.

Table 4. Values and distributions of clinical chemistry variables. Abbreviations: alanine aminotransferase (ALT)

		Diet board				*Ad libitum*			
Variable	**time**	**mean**	**quartile 1**	**median**	**quartile 3**	**mean**	**quartile 1**	**median**	**quartile 3**
ALT (U/l)	week 10	43	36	42	47	44	37	44	48
Albumin (g/l)	week 10	14	13	14	15	14	13	14	14
Total protein (g/l)	week 10	61	58	61	64	61	58	62	63
Creatine kinase (U/l)	week 2	3120	1623	2640	4227	2606	1327	1937	3458
Creatine kinase (U/l)	week 3	3957	1458	2535	4660	2274	1173	1663	3335
Creatine kinase (U/l)	week 4	2318	1023	1862	2785	1454	741	1440	2113
Creatine kinase (U/l)	week 5	1708	867	1624	2394	1797	728	1364	2205
Creatine kinase (U/l)	week 10	245	116	149	203	387	126	144	347

The levels of CK were significantly higher in DR rats during the experiment ($p<0.03$). The average ratio of the geometric means was 1.28 (95 % CI: [1.03,1.61]). In the final blood samples, no differences in CK levels were found between DR and AL rats ($p>0.05$).

CK is an intracellular enzyme found predominantly in muscle tissues, such as skeletal muscles and the heart and also in the brain. CK catalyzes the reaction from creatine to phosphocreatine and also from phosphocreatine to creatine. These reactions are used in the mitochondrial energy metabolism of the muscle cells. At times of exertion and catabolism, glycogen is used as an energy source (glycolysis) and the muscle cells synthesize ATP (and creatine) from ADP and phosphocreatine. During rest, the phosphocreatine reserves are built up again using ATP and creatine. (Lehninger, Nelson & Cox 1993, Wallimann et al. 1998) The serum levels of this enzyme rise when the permeability of the muscle cell membranes is increased. This can occur for example if there is muscle damage or even after strenuous exertion. In kidney failure, the serum levels of CK have also been observed to increase due to its decreased renal clearance. (Schlattner, Tokarska-Schlattner & Wallimann 2006)

In rats, DR has been reported to attenuate the aging-associated decline in intracellular levels of CK in the brain (Aksenova et al. 1998) and to accelerate the replenishment of phosphocreatine after hypoxic injury to the heart muscle, possibly suggesting a higher intracellular level of CK in the DR animals (Shinmura, Tamaki & Bolli 2005). On the other hand, DR has been shown to reduce mitochondrial energy metabolism in skeletal muscle (Ardawi et al. 1989, Johnson et al. 2006, Madapallimattam, Law & Jeejeebhoy 2002). Elevated levels of circulating CK have been observed after total food deprivation in voles (Mustonen, Saarela & Nieminen 2008) and in turkeys (Szabo et al. 2005). Different stressors have been associated with increased serum CK levels (Mitchell et al. 1999, Sandercock et al. 2001) and it has been suggested that the increased CORT secretion during the stress reaction could decrease muscle membrane stability and thus cause an efflux of the intracellular enzymes (Malheiros et al. 2003).

The reason for the elevation of CK in the diet board rats is unclear. It is unlikely that the exertion required to gain access to the food would be enough to cause actual muscle cell damage. It is equally improbable that the rats would have voluntarily spent enough time fasting to result in a severe state of catabolism. The diet board rats did have higher levels of CORT compared to the AL group. Whether this had an impact on the integrity of the muscle cell membranes, is also questionable. However, the difference in the serum CK observed in this study was minor compared to the changes previously reported in animals subjected to stressors and fasting.

In ALT, ALB and PROT, no statistically significant differences ($p>0.05$) were found between the DR and AL rats in this study. ALB and PROT have elsewhere been reported to decrease following 45 % AL (Hubert et al.

2000), 70 % AL (Snyder, Towne 1989) and 80 % AL (Roe et al. 1995) levels of food restriction. In this study, the level of restriction was less severe.

The applicability as an alternative to AL feeding cannot be conclusively determined by these results. It is likely that the majority of variables not directly related to energy metabolism would be little, if at all, affected by diet board feeding in young, healthy animals. However, it could be that the diet board's effects become more prominent when the animals grow older. The level of DR achieved by the diet board (85 % AL) could be enough to delay changes and morbidity associated with aging, thus complicating the comparison of results with previously published results obtained from AL fed rats. On the other hand, one could argue that if nothing is changed, then nothing can be improved. In order to solve some of the problems associated with AL feeding, the diet board should have at least some of the beneficial effects associated with DR.

4.3. Applicability as an alternative to traditional methods of DR

The diet board functions as a method of restricting food intake, weight gain and adiposity in laboratory rats. Nonetheless, it is possible that the diet board could have only face validity as a method of DR. Face validity means that a method or measurement produces similar results as the reference method but might differ in the mechanisms by which the apparent similarity is produced. A method with only face validity cannot be used as a reliable model, since the results will be unpredictable and not truly representative of the phenomenon studied. True construct validity means that the method or model is truly representative of the construct it is meant to represent. In other words, the findings obtained with this method or model can be generalized to the construct it models. (Carmines, Zeller 1979, Trochim, Donnelly 2006, Guion 1980) We wanted to evaluate the level of construct validity of diet board feeding in respect to traditional methods of DR. If there was a high in construct validity, then the mechanisms of action would be comparable to those seen in traditional methods of DR. An attempt was made to characterize the effects of diet board feeding on hormonal regulation and other variables associated with energy metabolism. High construct validity would make the diet board feasible for use in research projects requiring a mild to moderate level of DR. Of course, in order to represent a refined method of DR, the diet board is expected to differ in some qualities from the traditional methods of DR. However, it is important to evaluate whether the physiological phenomena induced by diet board feeding are comparable to those observed in the traditional methods of DR.

4.3.1. Lipids

A reduction of overall adiposity, fat deposits and circulating lipids is a well documented consequence of DR (Hubert et al. 2000, Keenan, Laroque & Dixit 1998, Turturro, Duffy & Hart 1993, Snyder, Towne 1989).

In this study, serum TRIGLY and FFA levels were analyzed from blood samples taken at experimental weeks two, three, four and five and at the end of the experiment. Serum CHOL was analyzed only from the final blood sample. In addition, the liver triglyceride content was quantified at the end of the experiment from the *post mortem* tissue samples.

Diet board feeding resulted in significantly ($p<0.0001$) lower values of TRIGLY throughout the study from week two to five. The average ratio of the geometric means was 0.77 (95 % CI: [0.68,0.87]. However, no differences were detected in TRIGLY at the end of the experiment on week ten ($p>0.05$).

CHOL was also lower in the diet board group. The mean concentration of CHOL was 1.55 $\pm$ 0.29 mmol/l in DR rats and 1.73 $\pm$ 0.35 mmol/l in AL rats (model-based difference -0.17 mmol/l (95 % CI: [-0.32,-0.03], $p=0.02$)).

No differences were found in FFA between DR and AL animals during the study from weeks two to five ($p>0.05$). At the end of the experiment, the DR group showed significantly ($p=0.0005$) lower values of FFA. In DR rats, the mean FFA concentration was 0.29 $\pm$ 0.15 mmol/l and in AL rats 0.44 $\pm$ 0.18 mmol/l (model-based difference -0.15 mmol/l (95 % CI: [-0.23,-0.07]). See Table 5. for the numeric values.

These findings are in agreement with the existing literature. TRIGLY and CHOL correlate well with the general adiposity of the individual, but the levels of FFA are subject to different interpretations. The concentration of FFA in the circulation is a balance between the release of FFA from adipocytes by lipolysis and the uptake of FFA into the liver and oxidation by muscle and other tissues. (Gonzalez-Yanes, Sanchez-Margalet 2006) On one hand, the level of FFA reflects the general adiposity. Decreases in serum FFA are seen in rodents subjected to DR (Barazzoni et al. 2005, Curi, Hell 1986, Gonzalez et al. 2004b) and increased levels of FFA are observed in the majority of obese individuals (Boden 1998). On the other hand, lipolysis is also under intricate short-term regulation. FFA are released into the bloodstream when there is a demand for energy in the tissues. Lipolysis is accelerated by the SNS and to a lesser degree by CORT (Jaworski et al. 2007, Lafontan et al. 1997, Nonogaki 2000, Saleh, Sniderman & Cianflone 1999). Increased levels of FFA are observed in catabolic states such as fasting (Davis, Rho & Sullivan 2008, Finn, Dice 2006) and also when the stress reaction is activated (Sampaio-Barros et al. 2003, BARRETT 1964, Curzon, Knott 1975, Dimsdale, Herd 1982, Haller, Kiem & Makara 1996, Khan, Forney & Hughes 1964).

The lower levels of FFA in DR animals seen in this and other studies might be mediated by the decreased sympathetic activity associated with

Table 5. Values and distributions of hormones and lipids

		Diet board				Ad libitum			
Variable	**time**	**mean**	**quartile 1**	**median**	**quartile 3**	**mean**	**quartile 1**	**median**	**quartile 3**
Cholesterol mmol/l)	week 10	1.55	1.39	1.6	1.8	1.73	1.46	1.69	2.1
Liver triglycerides (mg/g)	week 10	1.1	0.3	1.1	1.6	1.1	0.4	1.0	1.6
Free fatty acids (mmol/l)	week 2	0.50	0.33	0.50	0.60	0.54	0.44	0.52	0.63
Free fatty acids (mmol/l)	week 3	0.48	0.32	0.46	0.55	0.48	0.40	0.48	0.55
Free fatty acids (mmol/l)	week 4	0.47	0.35	0.47	0.59	0.44	0.34	0.40	0.57
Free fatty acids (mmol/l)	week 5	0.50	0.39	0.47	0.56	0.51	0.4	0.51	0.60
Free fatty acids (mmol/l)	week 10	0.29	0.17	0.28	0.38	0.44	0.25	0.45	0.58
Triglycerides (mmol/l)	week 2	1.17	0.80	1.14	1.41	1.42	1.12	1.33	1.60
Triglycerides (mmol/l)	week 3	1.26	0.96	1.22	1.63	1.59	1.10	1.50	2.00
Triglycerides (mmol/l)	week 4	1.36	1.06	1.38	1.57	1.68	1.28	1.66	1.97
Triglycerides (mmol/l)	week 5	1.44	1.16	1.38	1.71	1.83	1.37	1.80	2.10
Triglycerides (mmol/l)	week 10	1.39	1.19	1.40	1.53	1.63	1.11	1.60	1.85
Corticosterone (ng/ml)	week 2	84.9	22.9	81.7	144.9	86.4	15.9	44.9	109.4
Corticosterone (ng/ml)	week 3	64.2	11.6	43.0	104.6	32.5	0.0	2.4	55.0
Corticosterone (ng/ml)	week 4	217.7	0.2	31.5	220.7	39.8	0.0	10.4	51.5
Corticosterone (ng/ml)	week 5	72.2	8.8	53.0	121.6	35.8	0.0	9.6	61.2
Ghrelin (pg/ml)	week 10	1764	1405	1741	2147	2705	1624	2175	3680
Leptin (ng/ml)	week 10	3.5	2.4	3.2	4.7	4.6	1.4	4.6	6.6
Insulin (µg/l)	week 10	1.2	0.8	1.2	1.5	1.6	0.7	1.2	2.7
Adiponectin (µg/ml)	week 10	13.9	11.9	13.3	16.8	14.9	12.6	13.7	16.1

DR, which in turn could be explained by the lower plasma leptin level seen in DR animals. Leptin is known to increase the sympathetic tone (Nonogaki 2000). This finding of decreased FFA in the diet board rats suggests the animals are not in a state of catabolism or stress.

In addition to the serum lipid profile, the liver triglyceride content was also measured but no difference was detected between the diet board and AL groups ($p>0.05$). In humans, obesity is sometimes associated with increased liver triglycerides, but this can be considered a pathological change linked with metabolic disturbances (Kotronen, Yki-Jarvinen 2008). In rodents, liver triglycerides have been unaffected by DR (Barazzoni et al. 2005). This can be interpreted as a sign either that AL fed rodents are not as pathologically obese as some humans or that lipid metabolism and triglyceride distribution differs between species.

These findings indicate that the effects of diet board feeding on lipids are comparable to those in other obtained with methods of DR.

4.3.2. Endocrinology of energy metabolism

To further characterize the rats' metabolic responses to diet board feeding, the serum levels of ghrelin, leptin, adiponectin and insulin were analyzed from the final blood samples. No differences were detected in the leptin, adiponectin and insulin concentrations between diet board fed and AL rats ($p>0.05$). Table 5. shows the numeric values and distributions.

The lack of differences in these hormone levels is not consistent with the existing literature. Decreases in leptin and insulin are consistent findings in rodents subjected to DR (Escriva et al. 2007, Barazzoni et al. 2005, Gallardo et al. 2005, Maffei et al. 1995, Martin et al. 2007, Shimokawa, Higami 1999, Keenan et al. 2005, Gonzalez et al. 2004b, Chiba et al. 2002, Johansson et al. 2008, Marinkovic et al. 2007, Martin et al. 2008). Less is known about the relationship between adiponectin and DR, but adiponectin levels have been observed to increase in DR (Escriva et al. 2007, Martin et al. 2007, Zhu et al. 2004).

An important factor in these hormones is their circadian rhythmicity governed by both the light-dark rhythm and the timing of feeding. In AL animals, the peak in the plasma leptin concentration occurs late in the dark phase, just before the beginning of the light phase. The nadir occurs during the light phase. (Kalsbeek et al. 2001) However, this rhythm can be shifted by altering the feeding time. A peak in serum leptin occurs some hours after food intake. (Ahima, Prabakaran & Flier 1998, Saladin et al. 1995, Xu et al. 1999) Thus, if the DR animals are fed during the light phase and the AL animals eat during the dark phase, no single time of sampling can represent the optimal hormonal phase for the two groups. Depending on the timing of the meals and sampling, the differences between DR and AL animals could be exaggerated or underestimated. This could be one explanation for the failure to detect differences in this study. The final blood samples were taken early in the light phase, when the insulin and leptin levels would be expected to be at their nadir, if the animals follow a normal eating rhythm feeding mainly during the beginning of the dark phase. According to preliminary results, the diet board animals seem to have a similar circadian rhythm as their AL counterparts (Kemppinen et al. 2008). It could be that possible differences in insulin and leptin secretion were not detectable during the nadir of the secretion. In support of this explanation, in one study, the major difference in insulin levels between DR and AL animals was the amplitude of the peak (higher in AL animals) occurring after feeding, not the basal levels (Harris et al. 1994). In another study, the basal insulin levels were lowered only in the DR group that was fed during the light phase. The DR group given food during the dark phase did not differ from the AL group in their basal insulin levels (Haouari-Oukerro et al. 1994).

The lack of significant differences in the serum levels of leptin, insulin and adiponectin could also be explained by factors other than the circadian

rhythms. The diet board offers a relatively mild level of DR, possibly not provoking as prominent endocrine alterations as more severe regimes of DR. Furthermore, diet board feeding does not include periods of fasting for the animals. Most, if not all commonly used methods of DR mean that the animals are fasting for 12 to 23 hours per day. Many of the findings claimed to represent the impact of caloric restriction *per se* could actually be reflections of altered circadian rhythms or food deprivation instead of restriction. It cannot be concluded whether the diet board simply does not elicit similar endocrine changes as traditional methods of DR; or whether the changes are too subtle to have been detected in this study. Our research group has conducted an experiment characterizing the circadian rhythms of several hormones of diet board fed rats, but unfortunately these results have not been analyzed at present.

Rather surprisingly, in this study the ghrelin levels were significantly ($p<0.001$) lower in the diet board group. The model-based estimates of the geometric means for the groups were 1700 pg/ml (95 % CI [1380;2100]) in DR rats and 2400 pg/ml (95 % CI [1940;2970]) in AL rats. The ratio of the geometric means was 0.71 (95 % CI [0.607;0.83]). This finding is in contradiction to reports in the literature. Ghrelin is an orexigenic hormone increasing food intake. Ghrelin levels rise in response to both short-term and long-term decreases in food intake (Gil-Campos et al. 2006, Hosoda, Kojima & Kangawa 2006, Klok, Jakobsdottir & Drent 2007, Popovic, Duntas 2005, Barazzoni et al. 2003, Barazzoni et al. 2005, Gualillo et al. 2002, Yang et al. 2007). However, there are some examples where DR protocols have failed to increase serum ghrelin (Johansson et al. 2008) or even resulted in decreased ghrelin levels (Martin et al. 2007).

The explanation behind the higher ghrelin levels in AL rats in this study might be the timing of the meals. Although diet board rats show similar rhythms of circadian activity compared to the AL rats, it could be that the diet board rats distribute their food intake in a more scattered manner throughout the night. The AL animals could have more easily obtained a larger meal at the beginning of the dark phase, providing more time for the ghrelin levels to rise before the final blood samples taken at the beginning of the light phase. In rats allowed to feed only during the light phase, the plasma ghrelin levels were lower in the DR animals during the light phase, although the 24 h average was still higher in the DR group (Bodosi et al. 2004). The higher levels of FFA observed in the AL rats in this study support this explanation, since recent feeding suppresses lipolysis (Fuller, Diller 1970, Ip et al. 1977). Our research group has done further experiments where the feeding behaviour of diet board fed rats was analyzed with 24 h video recording in the home cage. According to the preliminary results, the diet board rats spend more time eating during the night than the AL controls (Inhilä, K. unpublished).

We explored the data also by analyzing the correlations between different variables measured at the end of the experiment. Spearman's

correlation test revealed several statistically significant associations between the variables. The significant correlations included a negative correlation between ghrelin and TRIGLY and also between ghrelin and leptin in both AL and DR rats. Leptin correlated positively with serum triglycerides and gonadal fat mass in all rats. These associations have been confirmed in other studies (Gil-Campos et al. 2006, Hosoda, Kojima & Kangawa 2006, Popovic, Duntas 2005, Barazzoni et al. 2003, Gallardo et al. 2005, Frederich et al. 1995). In the DR group, the gonadal fat mass displayed more significant correlations with other variables than in the AL group. This might be explained by the fact that only adipose tissue can increase almost limitlessly. The other variables are much more tightly regulated in the organism. Thus, the rest of the variables were no longer in proportion with the adiposity of the AL animals. Some of the correlations are graphically illustrated in Figure 6.

In addition to the above-mentioned hormone analyses, concentrations of serum CORT were measured at study weeks two, three, four and five. The average levels of serum CORT over time differed significantly ($p<0.0001$) between the diet board and AL rats, with the diet board group displaying higher values. The model-based estimates of average geometric means were 30.41 ng/ml (95 % CI [16.37,56.64]) for the diet board rats and 11.22 ng/ml (95 % CI [6.03,20.89]) for the AL rats. The ratio of the geometric means was 2.72 (95 % CI [1.67,4.41]). The elevation of circulating CORT is an integral part of the organism's metabolic response to decreased energy intake and has been repeatedly observed in rodents subjected to DR (Harris et al. 1994, Armario, Montero & Jolin 1987, Chacon et al. 2005, Han et al. 1995, Heiderstadt et al. 2000, Sabatino et al. 1991, Stewart et al. 1988, Patel, Finch 2002). It could be suggested that CORT is one of the primary responses to decreased energy intake, thus being a more sensitive responder to mild levels of DR than the other hormones measured. In support of this explanation, a rather severe DR regime (45 % AL) was associated with increased CORT but no differences in adiponectin or ghrelin levels (Johansson et al. 2008). The CORT levels in this study were measured during the first half of the ten week experiment and the rest of the hormones at the end of the experiment. It could also be that by the tenth week, the diet board animals had already adapted so well to the feeding regime that fewer differences were found.

These results indicate that the diet board elicits only minor effects on the hormonal balance regulating energy metabolism. Apart from ghrelin results, these findings are not in contradiction with the known effects of DR.

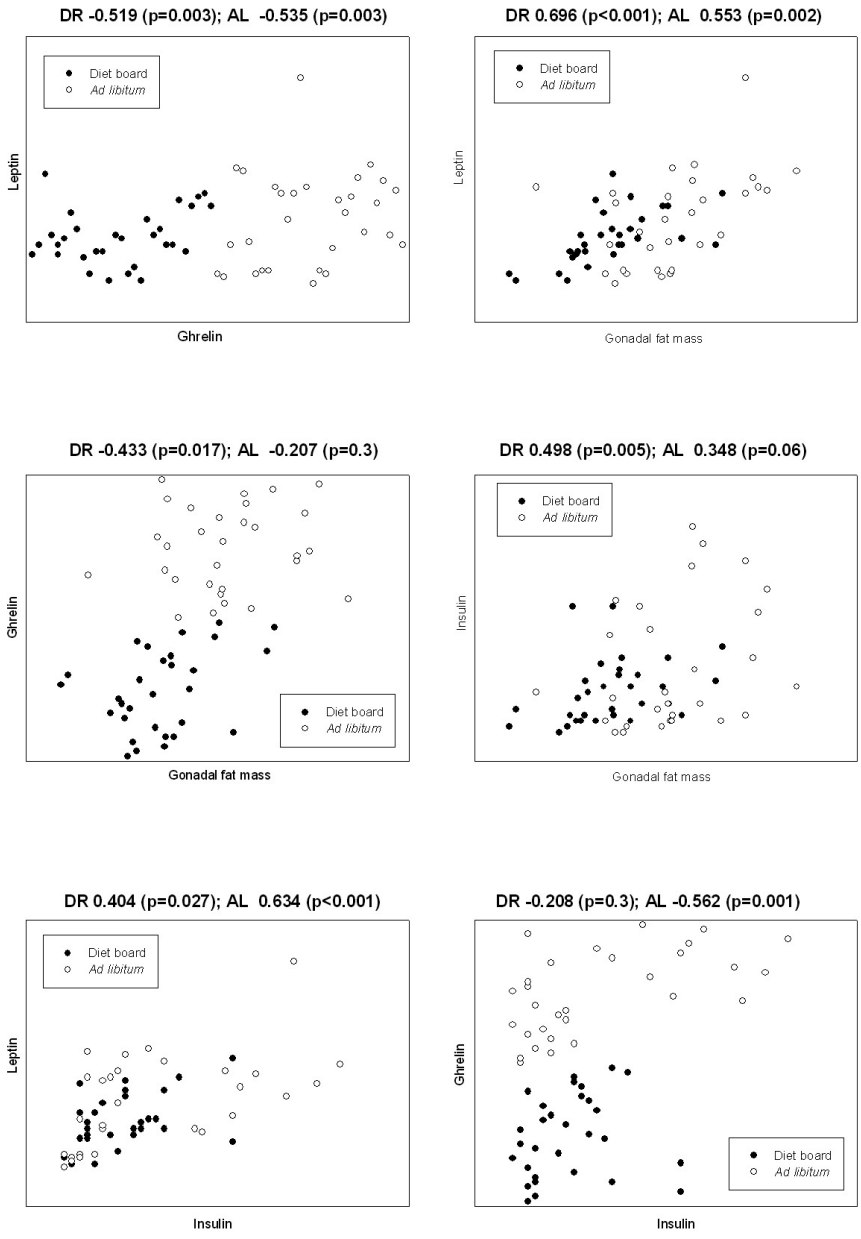

Figure 6. Correlations of selected variables. The values above the figures are correlation coefficients and the respective p-values from Spearman's correlation test.

4.4. Refinement potential of the diet board

The value of the diet board as a refinement alternative can be evaluated in comparison to AL feeding or against the traditional methods of DR. The diet board could be considered a refinement alternative to AL if it solved some of the problems attributed to AL feeding, *i.e.* decreased mortality and morbidity. These variables were not within the scope of this study, so in this respect, the refinement value remains unresolved. Our research group has begun a two-year experiment recording the mortality and morbidity of diet board fed rats but unfortunately, no results are available yet.

As a refinement alternative to the traditional methods of DR, the diet board does seem promising. The refinement features include the possibility of group-housing, unaltered circadian rhythms and the enrichment value of the diet board. When mild to moderate dietary restriction is needed, the diet board could result in both better science and better animal welfare.

4.4.1. Welfare

The elevated serum CORT values observed in the diet board rats should primarily be interpreted as a metabolic adaptation to DR and not as a sign of threatened welfare. Although higher than in the AL animals, the serum CORT levels in the DR rats do not indicate a welfare problem. In the case of a strong, possibly maladaptive stress reaction, a deterioration of the circadian rhythm of serum CORT could be expected. The levels of serum CORT would no longer show a clear zenith and a nadir, but instead would be elevated throughout the day. (Barriga et al. 2001, Perello et al. 2006, Apter, Eriksson 2006) The difference between the CORT levels of unstressed and stressed animals is most prominent during the nadir, which occurs at the beginning of the light phase in rats following a normal feeding rhythm (Barriga et al. 2001, Armario, Montero & Jolin 1987, Dallman 1993, Solberg et al. 2001). The circadian rhythms of CORT were not measured in this study, but the serum samples were taken during the anticipated nadir of CORT secretion. Although the diet board group had significantly higher values of CORT, the absolute concentrations of serum CORT were relatively low in both groups. Basal serum CORT levels in rats subjected to different types of chronic stress range from 60ng/ml up to as much as 700ng/ml (Ottenweller et al. 1992, Ottenweller et al. 1989, Hardy et al. 2002). Thus the results of this study indicate that the diet board group did not lack a nadir in its CORT secretion and was not in a state of chronic stress.

The other variables used to assess the welfare of the rats were chosen to reflect the possible prepathological or pathological outcomes of a prolonged stress reaction. The prepathological or pathological changes indicate that the biological cost of trying to cope with the stressor has been too high and that the organisms' biological functions are endangered. If this happens, the

organism enters a state of distress, which is considered a sign of a serious threat to the welfare of the individual. (Moberg 2000)

To assess the possible effects of diet board feeding on immunological functions, the secretion of faecal IgA was measured on week ten. Impaired immune function has been named as an important indicator of decreased welfare (Moberg 2000, Broom 1996, Broom 1991b, Korte, Olivier & Koolhaas 2007).

IgA is secreted into saliva, tears and the gastrointestinal tract. It protects the body from pathogens by eliminating them at the mucosal membranes (Valdimarsdottir, Stone 1997). In humans, stressful situations have been found to both increase and decrease the amount of IgA secreted into saliva (Deinzer, Schuller 1998, Hucklebridge, Clow & Evans 1998). In rodents, quantification of faecal IgA secretion is more commonly used to assess stress reactions. It can be done with minimum disturbance to the animals, *i.e.* the results are not affected by the sampling itself. Faecal secretion of IgA is negatively correlated to faecal corticosterone secretion in rats (Royo et al. 2004). In rodents, chronic stress (*e.g*. social stress, social isolation) is associated with decreased IgA secretion into the faeces (Eriksson et al. 2004) and also into the saliva (Guhad, Hau 1996). The IgA secretion in response acute stressors does not change to such a significant extent (Royo et al. 2004).

In this experiment, the diet board group had a significantly (p=0.002) lower rate of faecal IgA secretion than the AL group. The variable used in the statistical analyses was the absolute secreted amount of IgA per hour per cage (ng/h). The model-based estimate of geometric means for the groups was 78900 ng/h (95 % CI [45600,136100]) in the DR cages and 167100 ng/h (95 % CI [96600,289000]) in the AL cages. Thus the average individual secretion rates were 26300 ng/h/diet board rat and 55700 ng/h/AL rat. The ratio of the geometric means was 0.47 (95 % CI [0.32,0.70]). Table 6. lists the numeric values and distributions.

Table 6. Values and distributions of welfare-related variables. Abbreviations: adrenal (adr.)

		Diet board				*Ad libitum*			
Variable	**time**	**mean**	**quartile 1**	**median**	**quartile 3**	**mean**	**quartile 1**	**median**	**quartile 3**
Right adrenal (g)	week 10	0.0305	0.0281	0.0295	0.0339	0.0319	0.0288	0.0316	0.0365
Left adrenal (g)	week 10	0.0323	0.0294	0.0321	0.0351	0.0346	0.0322	0.0333	0.0384
Adrenaline in right adr. (µmol)	week 10	50.61	43.74	49.51	56.42	52.69	44.32	51.14	58.96
Adrenaline left adr. (µmol)	week 10	49.38	43.91	48.93	54.57	55.07	48.39	55.10	63.69
Noradrenaline in right adr. (µmol)	week 10	12.56	9.37	12.96	14.01	13.81	10.46	13.60	17.09
Noradrenaline in left adr. (µmol)	week 10	14.08	11.36	14.10	15.90	17.07	13.33	16.98	20.12
IgA secretion (ng/h/cage)	week 10	96100	50700	75300	146700	223500	94600	185800	372900

Diet board feeding thus appeared to affect the faecal IgA secretion. It is not known what rate of secretion is required to ensure adequate immune defence in the gastrointestinal tract. No morbidity was observed in any of the rats during this study, but the immunological challenges were presumably scarce in the laboratory environment. The absolute secretion of IgA was analyzed instead of the secretion rate in relation to body weight or the weight of the faeces. This was done in order to avoid a false negative result due to the obvious fact that the DR rats weigh and defecate less. The majority of the difference in body weight is adipose tissue. It was presumed that the secretion rate of IgA would not be in proportion to adiposity but rather to the size of the gastrointestinal tract of the animal. It is not known if the IgA secretion is in some way directly affected by the amount of food eaten or if the diet board rats had significantly smaller gastrointestinal tracts due to decreased quantity of ingesta. Compared to the results of Eriksson (2004) and Royo (2004), the IgA secretion rates of both diet board and AL rats are low. Eriksson (2004) reported secretion rates of approximately 30 mg IgA/24 h/kg bodyweight. Converted to the same unit, the secretion rates of this study are only 2–4 mg/24 h/kg bodyweight. This is most likely due to the fact that the secretion rates in this study were extrapolated from the samples taken during the light phase, when the animals defecate much less than during the dark hours (Eriksson et al. 2004). It is not clear why the secretion rates measured during the light phase in Royo's (2004) study are also many times higher compared to those observed in this study.

The size and catecholamine content of the adrenals were also analyzed. Enlarged adrenals can be a sign of chronic activation of the HPA axis and their catecholamine content reflects the activity of the SNS.

The right and left adrenals were dissected and weighed directly after euthanasia. The absolute weights of the right adrenal glands were similar in both groups, but the AL group had slightly but significantly ($p=0.024$) larger left adrenal glands. The mean weight of the left adrenal gland was 0.0323 $\pm$ 0.004 g in the diet board rats and 0.0346 $\pm$ 0.0042 g in the AL rats. The model-based difference of the means was -0.002 g (95 % CI [0.004,0.000]). Hypertrophy of the adrenal glands associated with chronic stress consists mainly of the thickening of the adrenal cortex (Bassett, Cairncross 1975); in this study the structure of the adrenals was not analyzed. This result is not consistent with the elevated serum CORT levels observed in the diet board rats. There is a concept of fluctuating asymmetry, which is defined as "small, randomly directed deviations from perfect symmetry that would be expected in bilateral structures" (Knierim et al. 2007). Fluctuating asymmetry is a measure of developmental stability, which can be affected by many factors *e.g.* different environmental stressors. This measure has been introduced as a method of welfare assessment and does indeed correlate with other measures of welfare (Knierim et al. 2007). The only bilateral organ measured in this study was the adrenal gland. The adrenals are not the best organs with which to undertake interpretations about fluctuating asymmetry, since

the left and right adrenal are inherently asymmetrical and differentially innervated by the SNS (Gerendai, Halasz 1997). In this study the left adrenal glands were larger than the right adrenal glands in both groups, but the difference was more pronounced in the AL group. The difference between the weights of the left and right adrenal was 50 % greater in AL rats. This could be interpreted as a sign of fluctuating asymmetry indicating that the AL group has been subjected to stressors perturbating the development of the adrenals. More likely, it is a consequence of the higher SNS activity associated with AL feeding compared to DR (Bray 2000, Landsberg 2006).

In support of this latter speculation we found significantly higher catecholamine levels in the AL rats. The ADR and NOR contents were analyzed separately for the right and left adrenal. In the right adrenal, no differences ($p>0.05$) were detected in ADR or NOR content. In the left adrenal, the AL group showed significantly higher values in both ADR and NOR. The ADR content of the left adrenal was 49.37 $\pm$ 2.33 µmol/adrenal in the diet board rats and 55.12 $\pm$ 2.34 µmol/adrenal in the AL rats. The model-based difference was -5.74 µmol/adrenal (95 % CI [-9.54,-1.94], $p=0.004$). The NOR concentration was 14.08 $\pm$ 1.20 µmol/adrenal in the diet board rats and 17.16 $\pm$ 1.20 µmol/adrenal in the AL rats. The model-based difference was -3.07 µmol/adrenal (95 % CI [-4.63,-1.52], $p<0.0001$). These results are in accordance with the existing literature. The decreased adrenomedullar activity in DR animals has been explained as representing an effort to save energy at times of negative energy balance (Bray 2000, Landsberg 2006). One mechanism behind this phenomenon is leptin, the levels of which are higher in AL animals. Leptin has been shown to increase sympathetic outflow (Nonogaki 2000) and on the other hand to inhibit the HPA axis (Bornstein et al. 1997, Heiman et al. 1997). The heightened SNS activity in the AL animals in this study can also be discussed from the hormesis (Masoro 1998) point of view. Animals subjected to DR are proposed to cope better with different challenges (Masoro 1995, Klebanov et al. 1995) *i.e.* the diet board animals might show smaller responses to the minor stressors of everyday life. AL feeding provides little challenges for the animal, and thus fails to prepare the animal to encounter stressors. AL animals might repeatedly "overreact", causing the observed difference in the SNS. This is, however, just speculation which cannot be verified by our results. In line with the heightened adrenomedullary activity of the AL animals is the previously mentioned finding of higher FFA levels in the AL animals. ADR stimulates lipolysis thus increasing plasma FFA levels (Kvetnansky et al. 1971).

At the end of the experiment, a *post mortem* examination was performed on each animal. The animals were examined for any gross, visual abnormalities in the integumentum, dentition or internal organs. The stomach of each animal was checked for gastric ulcers since it stress is known to predispose rodents to ulceration of the gastric mucosa, *i.e.* stress ulcers (Martinez-Augustin, Sanchez de Medina & Sanchez de Medina 2000). Furthermore, gastric lesions have been found in mice subjected to

traditional methods of DR (Rehm, Sommer & Deerberg 1987, Nakamura et al. 1990). In this study, no gastric ulcers or any other lesions were found in any of the rats from either group.

It is not unambiguous to measure the state of welfare in animals on any DR regime using stress-related variables. The metabolic response to DR is similar to that observed in the stress reaction, and yet the negative consequences of chronic stress are often not encountered in animals subjected to DR. In this study, there is evidence of a heightened adrenocortical activity in the diet board rats. This does not, however, seem to be a sign of chronic stress or distress, since the animals showed no stress-related pathology. The AL animals, on the other hand, had a heightened SNS activity. It is hard to judge whether the AL rats differed from the diet board group simply because of the lowered SNS activity associated with DR or whether there was a true increase compared to a normal, baseline function of the SNS.

Our research group has made an attempt to assess the welfare of diet board rats using several different variables, *e.g.* home-cage behaviour, behavioural tests and telemetric recordings of heart rate, blood pressure, heart rate variability and activity. These results are not yet analyzed, but will be published in the near future by K. Inhilä *et al*.

4.4.2. Group-housing

The diet board offers the possibility of group-housing rats, whilst maintaining them on a DR regime. This is one of the most promising refinement features of this feeding method. In this study, the AL control animals were also housed in groups to be able to compare the effects of the diet board *per se* and not that of social isolation *vs*. group-housing. The rat is a gregarious animal that prefers the company of other rats over solitude (Hurst et al. 1999). The disadvantages of individual housing are well documented. Social isolation can result in decreased welfare (Krohn et al. 2006, Serra et al. 2005). Social isolation can also be a confounding factor in the interpretation of research results. Individually housed animals have been reported to show altered behavioural responses, impaired cognitive abnormalities, hypersensitivity to stressors, increased variation of results and disrupted circadian rhythms (Hall 1998, Karim, Arslan 2000, Perello et al. 2006, Perez et al. 1997, Gentsch, Lichtsteiner & Feer 1981, Holson et al. 1991, Sharp et al. 2003). Social housing can have its drawbacks; rats kept in colonies establish strong hierarchies which can cause distress for the subordinates and also accentuate the differences between individuals (Blanchard, McKittrick & Blanchard 2001). However, these problems do not usually arise is normal laboratory conditions where animals are housed in small same-sex groups (Hurst et al. 1999). Furthermore, according to current European legislation, rats should be

housed individually only with justification on experimental, veterinary or welfare grounds (2007/526/EC).

In this study, no problems attributable to group-housing were observed. The diet board provides the opportunity for the animals to eat simultaneously, without having to compete for food. There was no evidence of fighting or heightened aggression. No differences were detected in the variances of body weights and CORT levels between the diet board and AL animals. This suggests that the rats in the diet board cages were growing as evenly as the AL animals and thus had even access to food within the group. It could also indicate that the diet board rats did not establish more pronounced hierarchies within their cages than the AL rats. In strong hierarchies, the CORT levels of the dominant and subordinate animals differ considerably with the subordinate animals usually showing higher levels of CORT (Martinez, Calvo-Torrent & Pico-Alfonso 1998, Hardy et al. 2002, Albeck et al. 1997).

4.4.3. Enrichment

Another refinement aspect of the diet board is its putative enrichment value. The diet board can be understood to provide several different enrichment functions. The diet board is an object dividing the cage into compartments. This is in accordance with the European legislation recommending that the complexity of the cage environment should be increased by enrichment (2007/526/EC). The diet board might also provide the rats with the possibility to express some aspect of the species-specific foraging behaviour. In addition, the diet board can be explored, climbed over and gnawed. (See Figure 7. for the diet board in the cage environment.)

Figure 7. The diet board in the cage environment

The enrichment value could make the diet board a potential refinement alternative to AL feeding. Environmental enrichment has been shown to have positive effects on the welfare of rats (Brenes, Rodríguez & Fornaguera 2008, Belz et al. 2003, Fernandez-Teruel et al. 2002, Newberry 1995, Olsson, Dahlborn 2002, Soffie et al. 1999, Van Der Harst et al. 2003). However, the effects of enrichment on scientific research using rats are not clear. Enrichment can affect research results and has been reported to both increase and decrease inter-individual variation (Kaliste, Mering 2004).

Compared to the traditional methods of DR, the diet board has a clear refinement value. Apart from the general positive effects of enrichment, the diet board could help the animals to cope better with the possible adversity of DR. The traditional methods of DR not only leave the animals hungry, but also restrict their feeding-related behavioural repertoire both temporally and qualitatively. The diet board allows the animals to act on the hunger impulses and thus offers them some control over their environment. The possibility of taking relevant behavioural action in response to the motivational state will protect the animal from stress (Jensen, Toates 1997). Psychological variables are important in determining the individual's perception of a potential stressor. Increasing the animal's control over its environment and the predictability of the stressors can decrease the negative consequences of stressors (Sapolsky 2002, Broom 1991b, Broom 1991a, Balcombe 2006). It has been argued that having to work for food can increase welfare by promoting the animal's experience of control over the environment and by providing stimulus and feedback from the environment (Balcombe 2006). For example, in swine, the possibility of trying to find food has been shown to decrease the adverse effects associated with DR (Appleby, Lawrence 1987).

4.5. Reduction potential of the diet board

DR has been advocated as a method to obtain more uniform research results (Keenan et al. 1999, Keenan, Laroque & Dixit 1998, Keenan et al. 1996, Turturro et al. 1997, Turturro et al. 1996) and has indeed been associated with decreased result variation (Duffy et al. 2001, Leakey, Seng & Allaben 2003, Carney et al. 2004, Leakey et al. 2003). Diet board feeding, however, does not seem to possess this kind of reduction potential.

The inter-individual variation was analyzed from the majority of variables, leaving out only some of the repeated measurements to avoid false positive results and IgA, which was analyzed at the cage level, thus leaving a very small number of observations for the statistical analysis. The variables investigated were: FFA, TRIGLY, CK and CORT on week five; FFA, TRIGLY, CK, ALT, ALB, PROT, CHOL, ghrelin, leptin, insulin, adiponectin, liver triglycerides, weights of the adrenals, ADR and NOR contents of the adrenals, tibial length and gonadal fat at the end of the experiment and

body weights on weeks two and ten. No differences ($p>0.05$) were found between the diet board and AL groups in the variances of any of the variables. Figure 8. illustrates the variation in selected variables.

A simple explanation is that diet board feeding does not offer any more control over an individual's food intake than AL feeding. The animal's food intake will be affected by its motivation to eat and other characteristics varying from one individual to other. It has been shown that when the work load required to obtain food increases, the rats will eventually maintain a lower body weight and will not work very hard to obtain more food (Collier, Hirsch & Hamlin 1972). We had hypothesized that the diet board animals would also be willing to work only for the amount of that food they needed, reducing the effect of excess eating and thus decreasing variation. This phenomenon might be created by increasing the difficulty of obtaining food from the diet board by altering its dimensions.

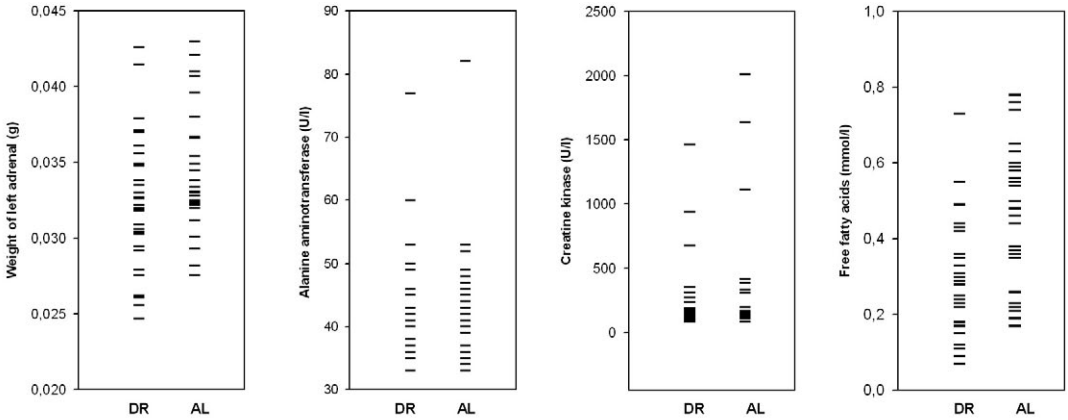

Figure 8. Variation in selected variables (week 10). Abbreviations: diet board group (DR); *ad libitum* group (AL)

4.6. Limitations

There are some important limitations in the applicability and practicality of the diet board. The diet board does not offer the possibility of controlling food intake. The diet board also offers a very limited possibility of recording food intake. If the animals are group-housed, their individual food intake cannot be recorded.

The diet board is not suitable for rats younger than seven weeks of age. We did some pilot studies to determine the earliest possible starting age. Rats younger than seven weeks lost more than 15 % of their body weight during the first week of diet board feeding. The period of adaptation should be at least one week, during which the animals are not additionally challenged.

This means that animals younger than eight weeks cannot be used for studies where they would be fed with the diet board.

Furthermore, the diet board is not suitable for animals in poor condition, or those with malocclusion or any other impairment affecting their ability to gnaw.

Finally, a practical commercial version of the diet board does not yet exist.

Conclusions

I. The diet board is suitable for dietary restriction in group-housed rats. The diet board provided mild to moderate dietary restriction (85 % of AL feeding) and resulted in a 15 % difference in weight gain. The diet board reduced adiposity very significantly (30 %) and hindered skeletal growth only minimally (3 %).

II. Diet board feeding was not associated with any major changes in variables unrelated to energy metabolism. The diet board could be used an alternative to *ad libitum* feeding without unduly confounding research results.

III. The diet board elicited milder endocrinological responses than traditional methods of dietary restriction. However, these results were not in contradiction with the known effects of caloric restriction. The effects on lipid metabolism were comparable to those reported for traditional methods of dietary restriction.

IV. The diet board can be considered a refinement alternative to traditional methods of dietary restriction. The diet board provides the possibility of restricting the food intake of group-housed rats without disrupting their circadian rhythms.

V. The result variation did not differ between the diet board and ad libitum fed rats in any of the analyzed variables. Thus the diet board does not seem to possess reduction potential.

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