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Satu Mering

Housing Environment and Enrichment for Laboratory Rats - Refinement and Reduction Outcomes

Doctoral dissertation

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ABSTRACT

Laboratory rats (*Rattus norvegicus*) are usually housed in polycarbonate, polypropylene or stainless steel solid bottom cages (SBCs) with bedding, although grid floor cages (GFCs) without contact bedding are also used. When housing rodents in laboratory conditions group housing and environmental enrichment are generally recommended. Due to the large number of rats used in biomedical research each year, it is important to offer them a proper housing environment thus satisfying their physiological and behavioural needs and ensuring their welfare.

In order to evaluate the effects of housing environment and environmental enrichment on the physiology and behaviour of Wistar rats, SBCs, GFCs and three different enrichment items were used. The extent of the use of items was assessed and the effects of group size, cage level in rack, litter and gender on the physiology and behaviour of rats were measured. The effects of housing environment and enrichment on the variability of research results and hence on the number of animals needed in an experiment were assessed with n-values obtained from SOLO Power Analysis and with N-ratios ($n_{larger} / n_{smaller}$). Additionally, the effects of housing modifications on two of the 3Rs – refinement and reduction – were evaluated in the experiments.

Smaller gnawing blocks were used effectively only in GFCs, suggesting a more enriching value in GFCs than in SBCs. Tubes and larger blocks enabled a wider range of behaviour patterns to be expressed and thus were more suitable enrichment items in SBCs than smaller gnawing blocks.

Enrichment items had only minor effects on physiology and behaviour of rats. Cage type had more profound influence on physiology and behaviour of rats than cage level or group size. The results, however, do not indisputably suggest the superiority of SBCs over GFCs, but rather that cage type in general may have physiological and behavioural consequences in rats.

Variation of research results may be influenced by housing modifications thus leading to more or less animals needed. Scientists should acknowledge these potential effects while designing animal experiments.

Welfare of Wistar rats was not threatened by any of the environmental modifications studied and environmental enrichment may act as refinement. In general, both concepts – refinement and reduction - can be applied simultaneously but in some cases one concept may interfere the application of the other.

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CAB Thesaurus: animal welfare; animal behaviour; enrichment; rats; rattus norvegicus, animal housing; cages; stress; variation; reduction

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Satu Mering

ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
AFOS	Alkaline phosphatase (other abbreviation ALP)
ALAT	Alanine aminotransferase (other abbreviation ALT)
ASAT	Aspartate aminotransferase (other abbreviation AST)
BAT	Brown adipose tissue
Ca	Calcium
CL	Cage level
CT	Cage type
CV	Coefficient of variation
EAT	Epididymal adipose tissue
FBW	Final body weight
GFC	Grid floor cage (without contact bedding)
GGT	Gamma-glutamyltransferase
GS	Group size
HPA	Hypothalamic -pituitary-adrenocortical
LDH	Lactate dehydrogenase
n	Number of animals needed
NLAC	National Laboratory Animal Center
N-ratio	$n_{larger} / n_{smaller}$
Pi	Phosphorus
SBC	Solid bottom cage (with contact bedding)
SD	Standard deviation
SPF	Specific pathogen free

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original papers, referred to in the text by Roman numerals **I - V**.

I Kaliste-Korhonen E, Eskola S, Rekilä T, Nevalainen T: Effects of gnawing material, group size and cage level in rack on Wistar rats. Scand J Lab Anim Sci 22: 291-299, 1995.

II Eskola S, Kaliste-Korhonen E: Effects of cage type and gnawing blocks on weight gain, organ weights and open-field behaviour in Wistar rats. Scand J Lab Anim Sci 25: 180-193, 1998.

III Eskola S, Lauhikari M, Voipio H-M, Nevalainen T: The use of aspen blocks and tubes to enrich the cage environment of laboratory rats. Scand J Lab Anim Sci 26: 1-10, 1999.

IV Eskola S, Lauhikari M, Voipio H-M, Laitinen M, Nevalainen T: Environmental enrichment may alter the number of rats needed to achieve statistical significance. Scand J Lab Anim Sci 26: 134-144, 1999.

V Mering S, Kaliste-Korhonen E, Nevalainen T: Estimates of appropriate number of rats: interaction with housing environment. In press. Lab Anim.

CONTENTS

1 INTRODUCTION

1.1 General

For the benefit of humans and other animals, research using animals has been and will continue to be necessary. This obliges the scientists to treat animals humanely. *The Principles of Humane Experimental Technique* was written by W.M.S. Russell and R.L. Burch as early as in 1959 in order to secure appropriate handling and use of animals. They introduced the concept of "the Three Rs", which is also nowadays (Declaration of Bologna 1999) established as a guide to improve the research using animals. The first "R" stands for Replacement meaning the "substitution for conscious living higher animals of insentient material". The second "R" stands for Refinement defined as "any decrease in the incidence or severity of inhumane procedures applied to those animals, which still have to be used". The third "R", Reduction, means "reduction in the number of animals used to obtain information of given amount and precision". (Russell and Burch 1959). However, it should be recognised that the application of "the Three Rs" should not jeopardise the validity of the research, but allow equally valid scientific results to be obtained as with or without "the Three Rs".

As long as research using animals is regarded as necessary, it is our duty to apply the concept of the Three Rs whenever applicable. This thesis focuses on two of the "Rs": Refinement and Reduction.

1.2 Characteristics of rats

The rat is a highly adaptable cosmopolitan creature generally considered as a social animal, but may also live a solitary existence (Weihe 1987). As a nocturnal animal, it is most active during the dark while rests during the light period. The rat has an exploring instinct, but is cautious, circumspect, and avoids danger (Weihe 1987). Although its eyesight is poor, its senses of hearing and smell are well developed (Weihe 1976, Sharp and La Regina 1998).

Male rats can reach maximum weights of 800 g and females of 400 g, but there are large strain differences. The average lifespan of the rat in a laboratory environment varies between 2.5 - 3.5 years depending on the strain and sex of animals (Weihe 1987, Sharp and La Regina 1998). Breeding in laboratory conditions has led to more tame animals compared to their counterparts in the wild, and laboratory rats habituate to repeated stimuli and can be trained to tolerate also unpleasant procedures, such as injections (Weihe 1987).

1.3 Rats as research animals

Rats have been used as research animals since the late 1800's. The earliest recorded laboratory breeding of albino and wild rats took place in Germany in 1877 (Weihe 1987). In nature,

the black rat (*Rattus rattus*) inhabited most of the Europe earlier, but nowadays the brown Norwegian rat (*Rattus norvegicus*) is more common and more widely spread than the black rat (Sharp and La Regina 1998). *Rattus norvegicus* is also the origin of various stocks and strains of today's laboratory rats.

The domestication of *Rattus norvegicus* is most likely a by-product of a popular early 19th century sporting event known as rat-baiting, in which a trained terrier dog tried to kill a group of wild rats (Porter 1993). In the USA, the period around 1890 is considered to be the time when rats were first used in research (Weihe 1987). In 1906, American physiologist, Henry H. Donaldson started a standardised breeding colony of albino laboratory rats in the Wistar Institute, Philadelphia and from that time on, the career of rats as research animals has flourished.

Rats and mice are the most commonly used laboratory animal species in biomedical research. In Finland, about 33.000 rats and 90.000 mice were used in experiments during 1999 (Ministry of Agriculture and Forestry 2000). The trend in the number of rats and mice used per year was declining until recently (Table 1). While keeping in mind the population of a country, these figures seem quite moderate compared to the numbers of animals used e.g. in Denmark, Belgium and especially in UK in 1996 (Fig. 1, Commission of the European Communities 1999).

Since the rat is the second most used species in biomedical research, it is important to know a proper housing environment that will meet physiological and ethological needs thus ensuring the welfare of the species.

Table 1. The number of animals used in procedures in Finland for selected species and the total number of research animals used per year (Ministry of Agriculture and Forestry 2000 and previous years).

	1999	**1998**	**1997**	**1996**	**1995**	**1994**	**1993**	**1992**	**1991**
Rat	33.131	30.660	32.110	36.316	42.391	40.023	58.168	52.300	53.844
Mouse	90.383	54.352	37.615	36.244	43.601	43.070	44.435	50.007	55.067
Rabbit	1.537	1.752	1.623	1.536	1.589	2.258	2.787	2.783	3.411
Dog	105	103	192	97	154	289	475	216	221
Cat	0	0	6	5	26	31	57	66	31
Fish	88.194	92.109	46.599	26.441	39.707	69.809	34.787	11.017	6.723
Total	230.326	195.261	131.896	110.659	139.980	180.057	159.116	147.133	148.779

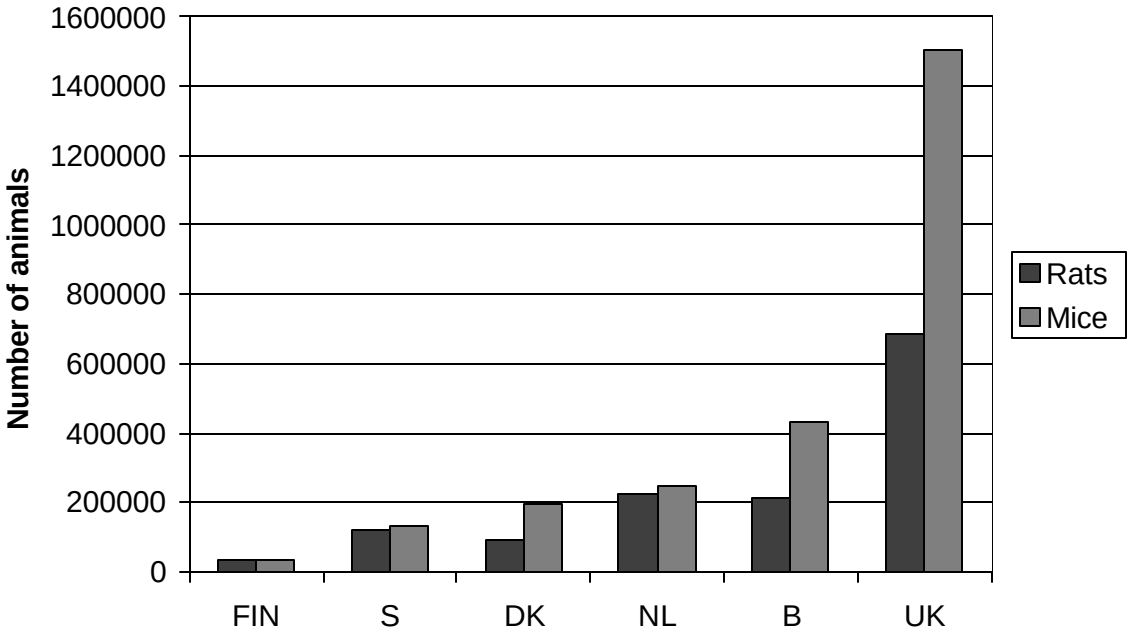

Figure 1. The number of rats and mice used per year in biomedical research in Finland, Sweden, Denmark, The Netherlands, Belgium and United Kingdom 1996 (Commission of the European Communities 1999).

1.4 Needs

In order to be able to fulfil an animal's needs, we have to know what they are. Already in the 1970's Abraham Maslow set up a hierarchical theory of human needs (Maslow 1970), in which the physiological needs (e.g. oxygen, food, warmth/coolness) were on the lowest level (foundation of the pyramid) and self-actualisation needs (i.e. a person's "calling") on the top. If a person is deprived of these basic physiological needs at the base of the pyramid, the person could or would die.

Application of Maslow's ranking of human needs to the needs of animals could result in a hierarchical organisation from highest to lowest priority: physical needs, safety needs, and psychological needs (Curtis 1985). They can also be categorised as life-sustaining, health-sustaining and comfort-sustaining needs (Hurnik 1988). The behavioural and physiological needs of an animal are not only species- and strain-specific, but may also relate to the individual's position and experience within a given social community (Stauffacher 1995).

The needs as such can be defined as requirements, that are fundamental to the biology of an animal, e.g. to obtain a particular resource or respond to a particular environmental or bodily stimulus (Broom and Johnson 1993). They can also be defined as essential for survival and reproduction, whereas the "wants" are the animal's cognitive representations of its needs (Duncan 1990). Presumably, basic needs of an animal must

be fulfilled to maintain a state of physical and psychological homeostasis (Clark et al. 1997a). The concept of a behavioural need is separate from that of a physical need, since it is the performance of the behaviour that is critical, not its consequences (Gonyou 1994).

Poole (1992) divided the behavioural needs into two categories; psychological needs, which appear to be unique to mammals, and ethological needs, which are experienced by all vertebrates. Mammals seem to be the only vertebrates that experience a need to behave in certain ways, that are not necessary for their immediate survival, such as leisure activities and exploration (Poole 1992). According to Poole (1992), mammals have a programme through which they are able to meet their behavioural needs. Four major requirements define this programme and an animal's demands for the environment: 1) the need for stability and security, 2) appropriate complexity, 3) an element of unpredictability and 4) opportunities to achieve goals. Since many mammalian species are solitary and some even prefer privacy, Poole did not include the need for social companions in the programme.

The European Convention (1986) sets recommendations for the environment of laboratory animals on the basis of needs: any restriction on the extent to which an animal can satisfy its physiological and ethological needs shall be limited as far as practicable. The European Commission's international workshop recommends that rodent cage environment should satisfy the physiological and ethological needs of resting, grooming, exploring, hiding, searching for food and gnawing (Brain et al. 1993). According to the Swiss Ordinance on Animal Protection (1981), experimental animals should be kept "in such a way as not to interfere with their bodily functions or their behaviour, or overtax their capacity to adapt" (reviewed by Stauffacher 1995). The consequence of unsatisfied needs in either the short term or the long term will be poor welfare (Broom and Johnson 1993).

1.5 What is welfare?

Even though the concept of welfare (or well-being in the United States, Madden and Felten 1995) is widely used, its definition is not yet clear (Newberry 1995, Clark et al. 1997a, Rowan 1997). It has been stated that animal welfare is a vague concept that can neither be viewed in a purely objective manner nor simply described, defined or assessed (Clark et al. 1997a). It has also been argued that welfare is entirely a question of the animal's mental, psychological, and cognitive needs (Duncan and Petherick 1991). However, according to Broom (1986) the welfare of an individual is its state as regards its attempts to cope with its environment and it can be assessed precisely. Rowan (1997) also defines welfare as an animal's ability to cope with or adapt to internal and external stressors.

Welfare also includes the absence of such adverse phenomena as pain, distress, hunger, disease and suffering (Rowan 1997). The absence of these adverse phenomena is also included to the concept of "Five Freedoms", which are the factors likely to influence the welfare of animals (FAWC 1993, Harri-

son 1988, Clark et al. 1997a). These are: 1) freedom from thirst, hunger and malnutrition, 2) freedom from discomfort, 3) freedom from pain, injury and disease, 4) freedom to display most normal patterns of behaviour and 5) freedom from fear and distress. However most of these adverse effects are subjective phenomena, which makes it difficult to apply them to a group of animals instead of an individual (Morton and Griffiths 1985).

Welfare is a complex dynamic internal state that varies on a continuum from very good to very poor and also in its manifestations (Broom 1988, Clark et al. 1997a). The biggest problem in welfare research is that there is no agreement about what good welfare involves at the most general level (Hurnik 1988). Even though the coping systems do succeed, the welfare can be poor, if the coping is possible only with difficulty or takes much time and energy (Broom 1988). It has been stated that animals exhibiting normal behaviour are more likely to have better welfare than those that cannot - however, we also need to know what normal behaviour is for each species (Gonyou 1994). According to Stauffacher (1995), all behaviour that leads to successful growth, avoiding harm, successful maintenance of bodily functions, and (potentially) to successful reproduction are said to be ”normal”. However, research should not just try to simulate an animal’s natural habitat and ecology (National Research Council 1992, reviewed by Clark et al. 1997b). Simply providing natural materials and settings will not ensure natural behaviour or welfare (Markowitz and Line 1990).

Decisions about animal welfare involve complex judgements based on many sources of information (Rushen and de Passillé 1992). Reaching a universally acceptable definition of welfare is probably impossible because of the way people define the quality of non-human animal life, a phenomenon that depends on an individuals personal experiences, views and values (Moberg 1985).

1.6 How to measure welfare?

As stated above, animal welfare is a complex phenomenon, and one component of welfare does not necessarily tell the whole truth. Different indices of welfare measure different components rather than welfare per se (Rushen and de Passillé 1992). In the case of welfare indicators, the absence of evidence is not necessarily evidence of absence (Broom 1988, Patterson-Kane et al. 1999). Assessment of animal welfare can include a combination of the animal’s appearance, performance, behaviour, productivity, disability, injury, disease, longevity, mortality and also include the condition of an animal’s environment (Clark et al. 1997b). According to Brain et al. (1995), combining behavioural, physiological (such as hormonal assays and heart rate) and immunological measurements as well as injury, growth and reproductive performance, may provide a more complete indication of welfare.

In general, factors that determine welfare are poorly understood, and means of assessing welfare are still to be validated (Clark et al. 1997a). Physiological measures of welfare can include e.g. growth, heart and respira-

tory rate, hypothalamic-pituitary-adrenocortical (HPA) activity, immune system function, injuries, diseases, reproduction, productivity and survival. Behavioural indicators of poor welfare may include inability to carry out normal behaviour, misdirected behaviour and attacks on conspecifics (Broom 1988). Behavioural changes can be measured with preference tests, home cage behaviour monitoring and open-field tests. However, the interpretation of some of these measures in terms of welfare may be difficult. Some indicators are good for evaluating short-term while others are more appropriate for evaluating long term effects. Moreover, large differences between species and individuals exist in the responses and same responses may be present both in adverse and pleasant situations.

1.6.1 Physiological measures

1.6.1.1 Growth

Remarkable weight loss in adults or lack of weight gain in juveniles is usually a clinical sign of pain or distress and may in animals indicate severe environmental conditions (Morton and Griffiths 1985, Broom and Johnson 1993). Weight loss may also be an indicator of a disease process (Sharp and La Regina 1998). On the other hand, decreased weight gain may be due to a more stimulating environment compared to conventional housing (Augustsson 1999), or simply due to reduced eating: e.g. rats that have alternative outlet for gnawing "need" do not gnaw/eat because of boredom (Fiala et al. 1977).

1.6.1.2 HPA activity

The hypothalamus excretes ACTH-releasing factor (adrenocorticotropic hormone), which causes the release of ACTH from the pituitary gland. ACTH travels via the bloodstream to the adrenal cortex and stimulates the release of corticosteroids, including glucocorticoids. Glucocorticoids such as hydrocortisone and corticosterone are directly related to stress and emergency situations, since they facilitate the conversion of stored fat and proteins to usable forms of energy (Green 1994).

According to Rushen (1991), claims about animal welfare based on data regarding the HPA activity should be viewed with scepticism because of the lack of consistency between the results of different studies. A simple determination of the plasma glucocorticoids is not a definitive measurement of welfare (Moberg 1987). Indeed, a single measurement provides little information about the welfare of an animal over a period of more than a few hours, since there is a diurnal rhythm in glucocorticoid activity and adaptation of the adrenal cortex response to environmental challenges (Broom and Johnson 1993). Furthermore, glucocorticoid levels, like also heart rate, can be raised for a variety of reasons, which may also be associated with pleasant situations (Broom 1988, Morton 1997). Many forms of emotional arousal, such as mating, anticipation of food, or minor procedures like handling, stimulate the release of glucocorticoids and other hormones (Clark et al. 1997b).

Long-term effect of ACTH is suggested to cause an increase in the adrenal weight with increased number of adrenocortical cells. This should enable adrenal cortex to maintain a high rate of corticosteroid hormone output for longer periods (reviewed by Nussdorfer 1986). Accordingly, long-term stress may cause hypertrophy of adrenals and increased serum corticosterone levels. However, Gómez et al. (1996) demonstrated a negative correlation between adrenal weight and increased plasma corticosterone levels in rats.

1.6.1.3 Other measures

Other physiological measures of welfare may include heart rate, immunological measures, injuries, diseases, reproduction, productivity and lifespan. Heart rate can increase in normal and pleasant situations, such as exercise, other physical exertion or mating. It may also change when animals are preparing for emergency actions (Broom 1991a). In a situation of possible threat, autonomic nervous system increases heart rate and blood supply to the muscles, thus helping the body to be ready for action or movement (Green 1994). Broom and Johnson (1993) suggest that heart rate is a useful measure of welfare in the short term, but of little value when comparing long-term conditions, such as the quality of housing. On the other hand, long-term conditions can affect changes in heart rate, which occur in test situations (Broom and Johnson 1993).

Glucocorticoids excreted from adrenal cortex also suppress immune system in addition to the effects on stored fat and protein (Green 1994, Stratakis and Chrousos 1995). A decreased thymus weight may also result from the increased secretion of glucocorticoids (Gray 1991, Manser 1992). The function of the immune system can be measured e.g. with antibody production, T-lymphocyte function and macrophage activity (Broom and Johnson 1993).

Injuries and diseases are quite simple indicators of poor welfare: broken bones and fever certainly have effects on welfare. However, the effects on welfare depend upon the extent of damage, what the animal must do to combat a disease or how much the animal suffers because of the disease or injury (Broom and Johnson 1993).

For many different reasons (stress, injury, inadequate environment), an animal's welfare may be threatened. This may also be seen as reduced reproduction. In the case of productivity (e.g. meat, milk etc.), measurements should be based on the performance of individual animals rather than the facility as a whole (Duncan and Dawkins 1983). The total productivity of a facility may be high even though some individual animals have poor welfare with reduced production.

When considering an animal's lifespan, the welfare of an individual is suggested to be better, if its life expectancy is longer in one environment than in the other (Hurnik and Lehman 1988, Broom 1991b). However, the effects of diseases, "quality of life" and metabolic rate are important factors to take into consideration together with survival while evaluating animal welfare.

1.6.2 Behavioural measures

1.6.2.1 Stereotypies

Stereotypies are repetitive, invariant sequences of movements or actions that are fixed in form and orientation with no obvious goal or function (Fox 1965, Broom 1983, Dantzer 1986, Mason 1991). Stereotyped behaviour may arise from maladaptation to an environment or from malfunction of the sensory, integrative, decision-making or motor systems (Broom and Johnson 1993). Boredom or lack of environmental enrichment have been considered possible causes of stereotypies (Gonyou 1994). Broom (1983) suggests that the welfare of an animal is compromised if 5-10 % of animal's active time is spent on stereotypic behaviour. According to Wiepkema et al. (1983) the welfare of animals is threatened if 5 % of all animals exhibit stereotypic behaviour. However, it has also been argued that in some cases, animals performing stereotypic behaviour may be under less stress than those not performing, since stereotypies help to reduce physiological responses to stress (reviewed by Rushen and de Passillé 1992, Mench 1998). Clark et al. (1997b) also state that atypical behaviours are not necessarily associated with reduced welfare, since an animal may simply express atypical behaviours while coping with a new situation.

1.6.2.2 Preference tests

A technique to measure what is good for animals is to observe their preferences and to measure how hard (strength of preference) animals will work for the preferred event or object (Broom 1988). This technique assumes that animals know what is good for them and that the choice automatically ensures better welfare. However, animals (like humans) may not always choose the alternative, which is best for them on the long run (Duncan 1978, Mench 1998). Preference tests are said to be most suitable when answers are wanted to relatively specific questions. However, choices of animals ultimately only indicate relative preferences, they do not necessarily indicate that a less preferred alternative will lead to suffering (Rushen and de Passillé 1992). Furthermore, depending on the motivational level (breeding season, hunger), animals can voluntarily tolerate considerable discomfort (Broom and Johnson 1993), thus distorting the results of the test.

1.6.2.3 Open-field tests

The open-field test is commonly used test in behavioural studies (Walsh and Cummins 1976, Royce 1977, Ossenkopp and Mazmanian 1985, Overmier et al. 1997, Patterson-Kane et al. 1999). Animals, which show high levels of activity (locomotion) and have low defecation scores in the open-field test, are considered less emotional than animals showing the opposite (Archer 1973, Walsh and Cummins 1976). In contrast, increased activity can also be an indication of "active escape" or explorative behaviour (Archer 1973, Igarashi and Takeshita 1995). Results from open-field tests vary between different laboratories, mainly because of the lack of agreement on equipment and procedures. The length of test might be one of the most important variable (Patterson-Kane et al. 1999). It would be more

desirable to record the temporal dynamics of motor activity than just the total time, which may in part account for the disagreement in results (Walsh and Cummins 1976, Markel and Galaktionov 1989, Patterson-Kane et al. 1999).

1.7 Housing environment

1.7.1 Cage type

Usually rats are housed in polycarbonate, polypropylene or stainless steel cages with bedding. Grid floor or wire mesh floor cages (GFCs) without contact bedding are also used. However, GFCs are not generally recommended and it has been suggested that GFCs should contain at least a solid area for the animals to rest on (Weihe 1987, Brain et al. 1993, Multilateral Consultation 1997). According to preference tests, rats prefer to rest in solid bottom cages with bedding (SBCs) and use GFCs mainly during active periods (Manser et al. 1995, Manser et al. 1996, Blom et al. 1996, van de Weerd et al. 1996). Housing in GFCs has been criticised because it may cause feet problems in animals and it does not allow animals to fulfil nest-making and digging behaviours (Brain et al. 1993). On the other hand, Nagel and Stauffacher (1994) did not find differences in resting and exploration behaviours or adrenal weight and corticosterone concentration for rats housed in GFCs compared to animals housed in SBCs. Moreover, Manser et al. (1995) did not find differences in body weight gain, food consumption or water consumption between rats housed in SBCs or in GFCs.

1.7.2 Conspecifics

Group size and housing density are important factors to consider when housing rodents. In general it is preferable to keep laboratory rodents in groups rather than as individuals, but care must be taken to ensure that the groups are harmonious and stable (Brain et al. 1993). In particular, group housing for male mice may not be possible due to high levels of aggression (Festing 1979). Isolation on the other hand may lead to an altered emotional or fear responses to novel stimulations (Gentsch et al. 1981, Holson et al. 1991). It may also provoke variations in plasma glucose, triglycerides and total cholesterol levels (Pérez et al. 1997) or improve sexual performance in male rats (Swanson and van de Poll 1983). The possibility to see, smell or hear conspecifics may reduce the aggressiveness of singly housed male rats (Hurst et al. 1997), thus social housing can be considered as one form of enrichment (Sharp and La Regina 1998).

1.8 Enrichment

Environmental enrichment is not a new concept – already in the 1860s Alfred Russell Wallace developed a surrogate primate mother made of buffalo skin in an attempt to reduce a captive infant orangutan's self-clasping behaviour (Wallace 1869). Even though the basic idea of environmental enrichment is generally clear, scientists have not been able to reach a full consensus for its explanation.

According to Chamove (1989), the goal of enrichment is to alter behaviour so that it is within the range of the animals' normal behaviour. If normal behaviour is the goal, then we need to know the normal behaviour of each species (Gonyou 1994). Is it the one, which is expressed in the wild or the one animals are performing in their life-lasting environment? Purves (1997) suggests that the goal of enrichment is to make the animals' environment as natural as possible. This suggestion can be criticised with the adaptation ability of animals and with the fact that natural conditions have also negative aspects such as predation, starvation and diseases (Markowitz and Line 1990, Poole 1992, Clark et al. 1997b, Patterson-Kane et al. 1999). Furthermore, it has been stated, that domestic species as well as some animals commonly maintained in captivity may cope with and adapt to life in captivity so that all aspects of life in a natural setting are not necessary (Clark et al. 1997b).

According to Newberry (1995), environmental enrichment may be defined as modifications of the environment resulting in an improvement in the biological functioning of captive animals. However, the concept of environmental enrichment has also been used in studies in which the impacts have not been beneficial (Haemisch et al. 1994, Haemisch and Gärtner 1997). To clarify the use of the word "enrichment": to enrich; increase or enhance the wealth, quality or value (The New International Webster's Comprehensive Dictionary of The English Language 1996), it may be more advisable to use the phrase "environmental modifications" until the beneficial effects are proven. The confusions in definitions also indicate that evaluation of enrichment provided is still required.

Environmental enrichment may also be said to be a measure, which allows animals to show a rich repertoire of species-typical behaviour patterns, while reducing or eliminating abnormal behaviour (such as apathy, stereotypies, self mutilation and excess aggression). This may be achieved by providing a more stimulating environment (Sales 1997). Complex artificial situations can often satisfy behavioural needs through environmental enrichment (Markowitz 1982). Symptoms of distress in animals can be reduced or eliminated by providing an animal with opportunities to work or play (Poole 1992). The species-typical behaviour does not always mean the behaviour expressed in the wild, since many species are sufficiently flexible to accept and enjoy substitutes (Poole 1992). For example chimpanzees in captivity spend hours working with computer games (Matsuwa 1989).

1.8.1 What has been used?

Enrichment can be social (conspecifics or human contacts), nutritional (foraging for food or additional food), physical (toys, objects or shelter), sensory (auditory or olfactory stimuli) and psychological (tasks or learning) or combinations of these (Baumans 1997). During the last few decades, a wide variety of enrichment items have been used to modify the cage environment of captive animals. Nesting material has most commonly been offered for mice (van de Weerd et al. 1997a, Eskola and Kaliste-Korhonen 1999a) while rats and

rabbits have often been given items for gnawing and shelters for hiding (Brooks et al. 1993, Orok-Edem and Key 1994, Chmiel and Noonan 1996). The variety of items and modifications used increases with larger animals; ropes, trees, branches, straws, coconuts, bowling balls, infant teething toys, mobiles, hanging objects, scratching posts, hollow tubes etc. (reviewed by Beaver 1989). Basically, the items should be safe for both animal and care taker, and economical for use with large numbers of animals. The items should also be easy to clean or replace and suitable so that animals use them (Orok-Edem and Key 1994, Chmiel and Noonan 1996, Baumans 1997). Enrichment items should also allow animals' control over their environment, since this may have an impact on their welfare (Townsend 1997, Manser et al. 1998).

1.8.2 Effects on physiology and behaviour

Environmental enrichment studies have been focused on the detection of differences in the group means attributable to enrichment and on the evaluation of importance of these differences. These have been used to find the appropriate enrichment for each species.

Enrichment may influence the physiology and behaviour of animals, which may have impact on the suitability of these animals for other experiments. Animals in an enriched environment may have a heavier and thicker visual cortex, more extensive dendritic branching of neurones, and more synapses per neurone in the brain than animals in a less enriched environment (Black et al. 1989, Greenough and Black 1992). Rats reared in complex environments have lower body weight than isolation-reared rats (reviewed by Fiala et al. 1977), whereas mice from enriched conditions were found to weigh more than mice housed under standard conditions (van de Weerd et al. 1997b). Enriched environments may also retard vaginal opening in female rats, while isolation may improve sexual performance in male rats (Swanson and van de Poll 1983). In mice, the cage design may influence emotionality (Chamove 1989) and aggression (McGregor and Ayling 1990). In rats, environmental enrichment may decrease the defensive behaviour during behavioural testing (Klein et al. 1994) or ameliorate behavioural deficits and improve cognitive performance (Patterson-Kane et al. 1999, Young et al. 1999). Additional consideration with enrichment items is their chemical composition, which may have effects on drug metabolism and enzyme induction in cases where items can be eaten or they cause emissions (Ferguson 1966, Vesell 1967, Potgieter et al. 1995).

Since enrichment is intended for all experimental groups, the possible (positive) effects should distribute equally to all experimental groups without causing biased results. However, depending on the nature of a study (toxicological, behavioural, neurological etc.) the possible effects of enrichment on responses of animals should be taken into consideration.

1.9 Variation

1.9.1 Causes and control of variation

Experimental variance can be considered under four main categories:

biological, pre-analytical, analytical and pharmacological (Davies 1998). These categories are not mutually exclusive; one cause of variance may appear under several categories. Biological variance includes features such as the genetic background and microbiological status of an animal. This variability can often be reduced by using more genetically uniform animals such as inbred strains or F1 hybrids (Beynen et al. 1993, Festing 1996). The use of specific pathogen free (SPF) animals is also a good choice, since sub-clinical infections can drastically increase the variability of results obtained from animals (Beynen et al. 1993).

Pre-analytical variance includes the adaptation of animals to a new environment and defined sampling times and techniques. It has been recommended to habituate animals to a new environment for 2-3 weeks subsequent to any change, since moving animals from one environment to another may increase stress and hence increase variability (Beynen et al. 1993, Morton and Griffiths 1985). The effects of circadian rhythm on hormones and other bodily functions are well known (Davies 1998), thus an accurate sampling time (especially in blood samples) is important. Moreover, the sampling technique may influence various blood and clinical chemistry variables (Leard et al. 1990, Sonntag 1986, van Herck et al. 1999).

A common analytical cause of variation is the differences in skill of the staff performing the analysis. Limiting activity, one technique or the handling of certain samples/organs, to one individual, can reduce intra-individual variation. The validation and accuracy of analytical method is also important, especially when the sample sizes and biological differences are small. Special consideration is required to ensure that the analytical method employed is appropriate for the species being evaluated and that compounds under evaluation do not interfere with the assay (Davies 1998).

Pharmacological variation is partly composed of biological variation, since no two individuals respond in an identical manner to an administered drug (Davies 1998). These differences are not known beforehand and therefore cannot be controlled.

A key point in designing good experiments is to control the variability of the experimental material (Festing 1994). Additional ways to reduce variability are to increase the accuracy of the measurement (new measurement tool), use littermates or matched pairs of animals, or use an animal as its own control (Mann et al. 1991). By randomisation the inter-individual variation is divided approximately equally between control and test groups, when the information about individual responses is not available (Beynen et al. 1993). If the measured values depend of certain characteristic (e.g. time, litter), randomised block design can reduce the variability (Mann et al. 1991, Festing 1992, Beynen et al. 1993). Even though the housing conditions and treatments can be standardised quite effectively, some amount of variation (e.g. random variation in measurements and in individuals, Mann et al. 1991) must be accepted.

1.9.2 Enrichment and variation

Within-group variation relates to the number of animals needed in an experiment. If the expected difference in group means (i.e. effect size), statistical power and significance level are not changed but the within-group variation of animals increases, more animals are needed to detect the biologically important treatment effect (e.g. Cohen 1988, Erb 1990, Festing 1992).

It has been claimed that environmental enrichment decreases the variability in the research results, thus reducing both the need to duplicate experiments and the numbers of animals used (Purves 1997). According to Baumans (1997), animals from an enriched environment may better cope with environmental variations and hence would be less reactive to stressful experimental situations. In general, stress is suggested to cause increased inter-individual variation (Beynen 1992). Improvement in housing environment leading to reduced pain and distress results in "better experiments", perhaps requiring fewer animals (Brain et al. 1995). A few studies seem to support this suggestion: petting rats for 10 minutes a day for one week reduced variability in learning task (West and Michael 1987). In mice the provision of a tube in addition to nesting material decreased the variability in physiological parameters (Eskola and Kaliste-Korhonen 1999b). However, there are also examples of an opposite effect: nesting material, nest boxes and wood bars for climbing increased variation in open-field test, urine protein and organ weights of inbred mouse strains, depending on the strain and test (Tsai and Hackbarth 1999). Furthermore, blood corticosterone levels in male mice together with traits linked to lipid metabolism were particularly susceptible to disturbances by environmental enrichment and the number of animals needed increased (Gärtner 1998).

1.10 Scope of the thesis

The aims of the experiments in this thesis were to evaluate with Wistar rats:

1) the extent of use of enrichment items,
2) the effects of aspen blocks and tubes on physiological and behavioural parameters,
3) the effects of cage type, cage level, group size, litter and gender on the physiological and behavioural parameters of rats, and
4) the effects of environmental modifications on variation of results and on the number of animals needed in a study.

The overall goal of this thesis was to use the information obtained in evaluation of the welfare of rats and to assess the relation of two of the 3R's - refinement and reduction – in different housing designs.

2 MATERIALS AND METHODS

The materials and methods from the original papers of this thesis (**I-V**) are summarised below. More detailed descriptions can be found in the respective sections of the original papers. The papers are based on four different experiments:

Paper **I** - Experiment 1 (pilot study)
Paper **II** - Experiments 2 and 3
Paper **III** - Experiment 4
Paper **IV** - Experiment 4
Paper **V** - Experiments 1, 2 and 3.

A summary of the study designs is presented in Table 2.

2.1 Animals and housing conditions

Animals used were barrier bred outbred male (**I-V**) and female (**I, III, IV, V**) Wistar rats (WH, Hannover origin) housed conventionally (NLAC, Kuopio, Finland). All studies were carried out in the NLAC, University of Kuopio. The animals in Experiment 4 were chosen from eight litters, three females and three males from each and allocated into three groups of four animals at weaning; control, tube and block group. Each group consisted of animals from eight different litters (**III, IV**). Litter details were not identified in Experiments 1-3.

Animals were housed at an ambient temperature of 20 ± 2 °C with relative humidity between 47 - 72 %. The light/dark cycle of the animal room was 12:12 hours with lights on at 07.00 a.m. Rats were housed either in stainless steel solid bottom cages (48x28x20 cm with a wire lid) containing bedding (SBC, **I-V**) or in grid floor cages (45x38x19.5 cm, wire diameter 1.6 mm and mesh size 10x10 mm) without contact to bedding (GFC, **I, II, V**). The direct or indirect bedding used was aspen (*Populus tremula)* chips (4HP, Tapvei Oy, Kaavi, Finland).

The housing types used were 1) housing in SBC throughout the experiment (SBC), 2) housing first in SBC and transfer into GFC (Transfer) and 3) housing in GFC after weaning until the end of experiment (GFC). The number of animals per cage ranged from one to four (Table 2).

2.2 Enrichment

Three kinds of aspen (*Populus tremula*) items were used for enrichment (Fig. 2): smaller gnawing blocks (1x1x5 cm, 2-5 g, **I, II** and **V**), larger blocks (6x6x6 cm with penetrating drilled holes, diameter of 1.9 cm on each side, **III** and **IV**) and rectangular tubes (20x12x12 cm with 1.5 cm wall thickness, **III** and **IV**) (Tapvei Oy, Kaavi, Finland). The smaller gnawing blocks were used because they were expected to fulfil the species-specific need, i.e. gnawing need of rats. The size of the larger block was based on earlier studies and chosen so that it would last for at least one week. The shape of it was modified from the study by Chmiel and Noonan (1996). The shape of the tube was chosen to fulfil the natural tendency of rats for hiding and also from the manufacturing point of view (easy to produce). The tube was large enough to allow the entry of also larger rats (about 250 g), but still there would be enough space to move inside the cage. Aspen was chosen as a material, because it was the same material as bedding used.

Table 2. The summary of experimental designs. SBC = solid bottom cage with bedding, GFC = grid floor cage without contact bedding, Transfer = housed in SBCs prior transfer into GFCs, FBW = final body weight, EAT = epididymal adipose tissue, BAT = brown adipose tissue, x = measured, - = not measured.

	Experiment 1 Papers I and V	**Experiment 2 Papers II and V**	**Experiment 3 Papers II and V**	**Experiment 4 Papers III and IV**
Total number of rats	78	54	36	48
Housing type	Transfer	SBC, GFC, Transfer	SBC, Transfer	SBC
Group size per cage	1, 2, 3 or 4	3	3	4
Sex	Females and males	Males	Males	Females and males
Enrichment	Gnawing block	Gnawing block	Gnawing block	Tube or larger block
n per test group	6 to 8	9	9	8
Age at weaning (weeks)	3	4	4	3
Age at transfer (weeks)	14	8	8	-
Age at euthanasia (weeks)	14 and 18	8 and 12	11	8
Period in SBC (weeks)	11	4 and 0	7 and 4	5
Period in GFC (weeks)	4	0 and 4	0 and 3	-
Behavioural measurements	Use of enrichment (g) Time spent with items Open-field test	Use of enrichment (g) Open-field test	Use of enrichment (g)	Use of enrichment (ml) Time spent with items
Physiological measurements				
Food consumption	x	-	-	-
FBW	x	x	x	x
Growth	x	x	x	x
Thymus	x	x	x	-
Adrenal glands	x	x	x	x
Spleen	x	x	x	-
EAT	-	x	x	-
BAT	-	x	x	x
Serum corticosterone	-	x	-	x
Clinical chemistry	-	-	-	x

2.3 Use of enrichment items

The items were changed to new ones once a week. The use of items was recorded by measuring the weight loss of blocks (**I** and **II**) or by measuring the volume gnawed (**III**). The volume gnawed (ml) was transformed into the weight loss (g) in this thesis to ease the comparisons. Furthermore, the enrichment item-related activity was analysed with 24 h video recordings in Experiment 1 (**I**) and with 9.5 h video recordings in Experiment 4 (**III**).

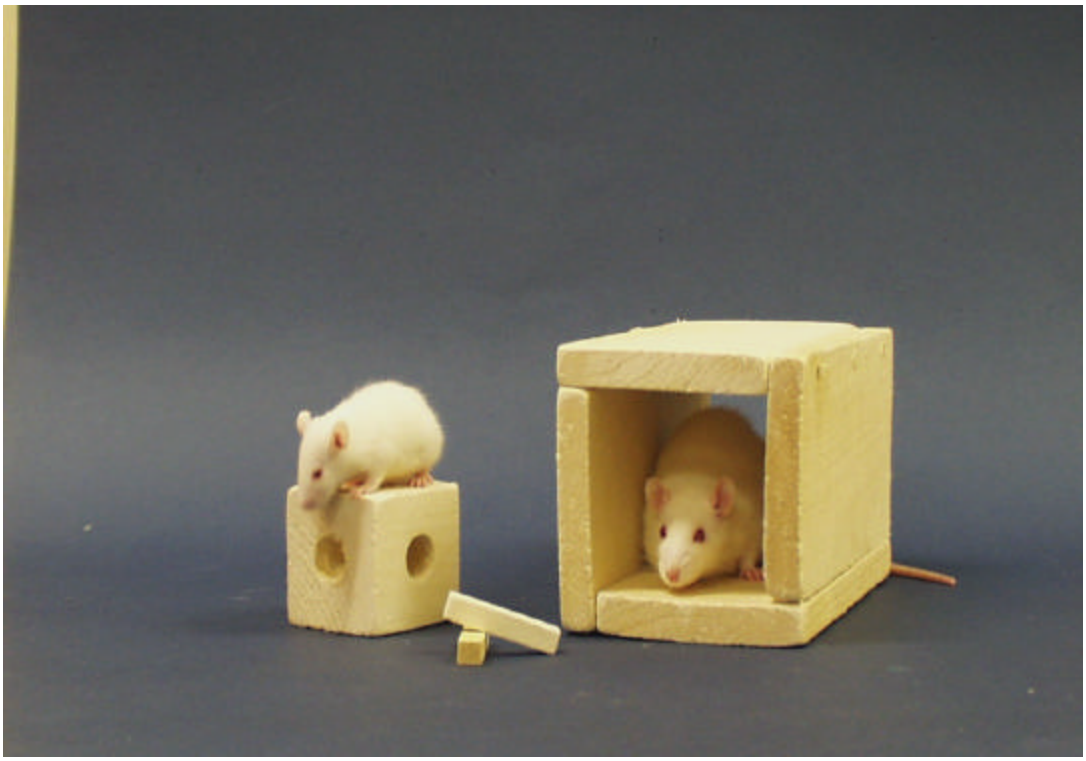

Figure 2. The smaller gnawing blocks, larger block and rectangular tube used as enrichment items in the experiments.

2.4 Physiological measurements

The food consumption per cage was assessed only in Experiment 1 (**I**) for three days during each of the first four weeks (animals at the age of 3-6 weeks, housed in SBCs). Growth of the animals was measured by weekly weighing, except in Experiment 4, in which animals were weighed at the age of 3, 7 and 8 weeks (**III**). At the end of the experiments final body weights (FBW) and weights of adrenal glands, thymus (excluding Experiment 4), spleen (excluding Experiment 4), epididymal adipose tissue (EAT) (excluding Experiments 1 and 4) and brown adipose tissue (BAT) (excluding Experiments 1 and 4) were analysed. Furthermore, serum corticosterone levels were determined in Experiments 2 and 4 (**II, IV**), and other clinical chemistry parameters (AFOS, ALAT, ASAT, LDH, GGT, Pi, Ca, cholesterol, triglycerides,

creatinine, total bilirubin and protein) in Experiment 4 (**IV**).

2.5 Open-field test

The behaviour of animals was tested with 5 minute open-field tests in Experiments 1 and 2 (**I, II**). In Experiment 1, the open-field test was conducted once when the animals were at the age of 8 weeks (**I**). In Experiment 2, the animals in SBCs and in GFCs were tested once at age of 8 weeks, while the animals transferred into GFCs were tested twice, first at age of 8 weeks and again at age of 12 weeks when they had been housed in GFCs for 4 weeks (**II**). The open-field arena was white and circular, with a diameter of one metre, surrounded by a 50 cm high grey wall. Animal behaviours defined as walking, standing alert (= active but not walking), rearing, grooming and defecation were monitored by video recordings. The total frequency and duration, as well as the latency to the first onset of any behaviour were determined from the video recordings.

2.6 Number of animals needed and N-ratio

Based on mean ± standard deviation (SD) (Table 2 in paper **V** and Table 6), SOLO Power Analysis; one-sample mean (1991) was used to estimate the smallest number of animals needed (n) to detect an arbitrarily chosen 20 % difference in the group means (i.e. effect size) of physiological parameters, when the within group variation (SD) varied, significance was set at p=0.05 and statistical power at 0.90. The smallest accepted value for n was two. Since n-value is based on relation of mean and SD, n-value increases when SD becomes wider or when the expected change in group means becomes smaller and contrary. The main effects to be evaluated were enrichment (**IV-V**), litter (**IV**), cage type (**V**), and group size (**V**).

N-ratios (n_{larger} / $n_{smaller}$) for the effects of enrichment, cage type and group size were calculated to compare the number of animals needed in "treatment" group to that in control group. The effects of enrichment were studied by comparing the n-values of block/tube-groups with the n-values of control-groups. SBCs were considered as "controls" and GFCs as "treatments" while evaluating the effects of cage type and singly housed animals were "controls" and two, three or four animals per cage were "treatments" in the case of group size. The impact of litter could not be evaluated with N-ratios, since this group does not have control group. If the n-value in "treatment" group was smaller than that in control group, negative sign was added to the N-ratio to indicate the reduced number of animals needed. Positive N-ratio directly indicates, how many times more animals are needed in "treatment" group in comparison to control group. If the N-ratio is 1, the value for n in the "treatment" group is equal to the n-value in the compared group. N-ratio cannot be between –1 and 1.

2.7 Statistical analyses

Data was processed with different versions of SPSS/PC+ and SPSS for Windows statistical packages (V3.1 in paper **I**, V5.1 in paper **II**, Release 6.1.4 in papers **III** and **IV**, Release 9.0.1 in paper **V**: SPSS Inc., Chicago, IL, USA). The distribution of the data separately in

each experimental group (e.g. SBC with blocks, SBC without blocks etc.) was tested with Kolmogorov-Smirnov test and regarded as normally distributed if the statistical significance of the test was $p>0.05$. In most cases the experimental unit, i.e. the unit for statistical analysis, was an individual animal. Even though the experimental unit (the entity which can be assigned independently of other units to a treatment) can also be considered to be a cage, an individual animal was chosen in order not to loose valuable information.

In Experiment 1, the weight losses of blocks were measured per cage and further divided by the number of animals to obtain the weight losses per animal. This data was used to evaluate associations of individual physiological parameters with use of enrichment items. In Experiments 2, 3 and 4, the experimental unit regarding the weight losses of blocks and use of items was a cage. The relative times (%) animals spent with items in Experiment 4 were means from four repeated recordings and analysed with Multivariate analysis of variance (**III**).

The statistical methods used were chosen as follows. Normally distributed data was analysed with One-way analysis of variance (one dependent parameter with one independent variable with more than two groups), Multiway analysis of variance (one dependent parameter with several independent variables) and Multivariate analysis of variance (several dependent parameters with several independent variables). Paired t-test was used with two related variables and more than two repeated measures were analysed with Multivariate analysis of variance with repeated measures. Non-parametric data and normally distributed data with heterogeneous variances were analysed with Mann-Whitney U-test (two independent variables) and with Kruskal-Wallis One-way analysis of variance (one dependent parameter with one independent variable with more than two groups). Two or more not normally distributed and related variables were tested with Friedman test.

Post-hoc analysis for normally distributed data was Scheffe's test and for non-parametric data Multiple comparison between groups according to Siegel (1988). The open-field observations were subjected to a factor analysis with orthogonal VARIMAX rotation and Kaiser normalization in order to reduce the number of behavioural variables. The correlation of food consumption with weight loss of blocks was estimated with Pearson's correlation coefficient (**I**). Organ weights were adjusted for body weight by analysis of covariance using body weight as covariate (**I, II**) or by using relative weights (organ weight / FBW) (**IV**).

The N-ratios in paper **IV** were not statistically analysed, but One-Sample t test was later adopted (**V**). The absolute N-ratios were transformed with natural logarithm in order to get them normally distributed. One-Sample t test was used to evaluate, if these transformed N-ratios in different housing environments would differ from zero in general (log-transformed N-ratio is zero if n-values are equal in "treatment" and control groups).

3 RESULTS

The main results of the original papers (**I-V**) are summarised below.

3.1 Weight losses of enrichment items

In general, rats housed in SBCs with bedding gnawed enrichment items only 1-2 g / animal / week regardless of the shape of the item, group size or sex (Table 3). In Experiments 1-3, the gnawing behaviour was significantly increased in animals housed in or transferred into GFCs without contact bedding (range 2-8 g / animal / week). The weight loss of gnawing blocks occurred mainly during the dark period and it was largest on the third shelf of the rack (**I**).

Table 3. Rounded means of weight losses of blocks (g/animal/week) in four different experiments, when the rats were of age 5 to 18 weeks. The grey area indicates the period when animals were in GFCs. B = block, T = tube, n = number of animals per experimental group.

Experiment / Age (weeks)	5-6	7	8	9	10	11	13-14	15-16	17-18
Experiment 1									
Males 1/cage (n=6)	1	1					2	8	6
Males 2/cage (n=6)	2	2					1	5	6
Males 3/cage (n=6)	2	4					1	6	7
Males 4/cage (n=8)	2	4					1	4	6
Females 1/cage (n=6)	2	3					2		
Females 2/cage (n=6)	1	2					2		
Females 3/cage (n=6)	1	2					1		
Females 4/cage (n=8)	2	2					1		
Experiment 2									
Males 3/cage (n=9)	1	1							
Males 3/cage (n=9)	3	4							
Males 3/cage (n=9)	1	1		3	2	2			
Experiment 3									
Males 3/cage (n=9)	1	1	1	1	1				
Males 3/cage (n=9)	1	1	5	4	4				
Experiment 4									
Males 4/cage B (n=8)	< 1	< 1	1						
Females 4/cage B (n=8)	< 1	< 1	1						
Males 4/cage T (n=8)	< 1	< 1	< 1						
Females 4/cage T (n=8)	< 1	1	1						

3.2 Time spent with enrichment items

During Experiment 1 (time-lapse recording system, 6 pictures / s), single housed rats in SBCs spent on average 93 s in contact with the smaller gnawing blocks during the 24 h monitoring. Pair housed animals and three animals per cage spent on average 84 s and 103 s per animal, respectively (**I**). These times represent less than 1 % of the total 24 h period. However, in Experiment 4 (time-lapse recording system, 1 s per min, instantaneous sampling at 1 min interval, 4 animals per cage) rats with larger blocks spent on average 7 % of their time in contact with the item during 9.5 h monitoring (the average from summarised dark and light periods). Furthermore, the animals with tubes spent 78 % of their time with the item (**III**). Most of the contacts occurred during the dark period (**I, III**). However, rats with tubes were in contact with the item more during the light than dark period. These animals spent over 80 % of their time inside the tube during the light period (**III**).

The possible sex differences in contact times with enrichment items were analysed in Experiment 4 (**III**). During the dark period, female rats spent more time on top of the tube than males. Correspondingly, male rats with tubes spent more time elsewhere in the cage than females. During the light period gender differences were not detected. Furthermore, in animals with blocks the gender differences were not detected in either light or dark periods or in any of the behaviours.

3.3 Food consumption (I)

Food consumption was measured in animals housed in SBCs before transfer into GFCs. The presence of blocks, cage level in rack and group size had no effect on food consumption in animals of either sex (Table 4). However, the consumption of the blocks increased with increasing food intake in males (Pearson's coefficient 0.94, $p<0.01$), but not in females.

3.4 Growth and FBW

In general, the presence of enrichment items, cage type, cage level in rack, group size or litter did not influence the group means of growth and FBW (Table 4). However, in Experiment 2 (**II**), the animals with gnawing blocks had lower total weight gain and FBW in both cage types than animals without gnawing blocks (Table 4). Furthermore, the animals housed in GFCs had greater FBW and total weight gain than animals housed in SBCs (Table 4). As expected, male rats achieved greater FBW and gained weight faster than female rats (**III**, Table 4).

Table 4. Summary of the effects of enrichment, cage type (CT), cage level (CL), group size (Group), sex (M=males) and litter on physiological parameters of Wistar rats measured during four experiments. FBW = final body weight, EAT = epididymal adipose tissue, BAT = brown adipose tissue, Cortico. = serum corticosterone, SBC = solid bottom cage, GFC = grid floor cage, ⇑ = increase, ⇓ = decrease, ne = no effect, - = not analysed.

	Experiment 1			Experiment 2		Experiment 3		Experiment 4			
	Block	**CL**	**Group**	**Block**	**CT**	**Block**	**CT**	**Block**	**Tube**	**Sex**	**Litter**
Food consumption	ne	ne	ne	-	-	-	-	-	-	-	-
FBW	ne	ne	ne	**ß**	**Ý (GFC)**	ne	ne	ne	ne	**Ý M**	ne
Growth	ne	ne	ne	**ß**	**Ý (GFC)**	ne	ne	ne	ne	**Ý M**	ne
Thymus	ne	ne	ne	ne	ne	ne	ne	-	-	-	-
Adrenals	**ß**	ne	ne	ne	**Ý (GFC)**	ne	**Ý (GFC)**	ne	ne	**ß M**	ne
Spleen	ne	ne	ne	ne	ne	ne	ne	-	-	-	-
EAT	-	-	-	ne	ne	ne	ne	-	-	-	-
BAT	-	-	-	ne	ne	ne	**Ý (GFC)**	-	-	-	**Ý ß**
Cortico.	-	-	-	ne	**ß (GFC)**	-	-	ne	ne	**ß M**	**Ý ß**
Clinical chemistry	-	-	-	-		-	-	**ß tot.bilirubin**	ne	**Ý ß**	**Ý ß**

3.5 Other physiological parameters

In Experiment 1 (**I**), adrenal weight decreased in animals housed with gnawing blocks in GFCs compared to animals housed without gnawing blocks (Table 4). The adrenals were also enlarged in animals housed in GFCs when compared to animals housed in SBCs (**II**), although the presence of gnawing blocks did not have an effect in these cases (Table 4). Furthermore, male rats had smaller adrenal weights than females (**IV**, Table 4). Serum corticosterone levels were lower in animals housed in GFCs compared to animals housed in SBCs (**II**), but the presence of enrichment items had no effect (**II, IV**) (Table 4). Corticosterone levels did vary between litters, and males had lower levels than females (**IV**).

In Experiment 3, the BAT weight was on average greater in rats housed in GFCs than rats housed in SBCs (**II**). The presence of blocks had different effects depending on the cage type; in SBCs the blocks had no effect on the weight of BAT but in GFCs it was decreased (see Table 1 in paper **II**). In Experiment 2 the BAT weight was not dependent on the presence of gnawing blocks or cage type (Table 4). Furthermore, the BAT weights differed between litters (**IV**). Thymus and spleen weights were not influenced by the presence of gnawing blocks, cage type, and cage level in rack or group size (Table 4). Neither was the EAT weight affected by the presence of gnawing blocks or cage type (**II**).

Clinical chemistry parameters (AFOS, ALAT, ASAT, LDH, GGT, Pi, Ca, cholesterol, triglycerides, creatinine, total bilirubin and protein), showed that only total serum bilirubin levels were influenced by the presence of enrichment items (Table 4); animals with blocks seemed to have lower levels than animals with tubes or control animals (**IV**). Furthermore, male rats had higher serum AFOS and ALAT activities, whereas females had higher creatinine, protein and LDH levels (**IV**). The serum AFOS, Pi and cholesterol levels varied slightly between litters (**IV**).

3.6 Open-field behaviour

In Experiment 1, the presence of smaller blocks had no effect on the open-field behaviour of male rats (**I**) (Table 5). However, in Experiment 2 rats with gnawing blocks in GFCs were more active in the central area of the arena during the last 2.5 min of the test than animals without blocks (**II**). After transfer into GFCs, rats with gnawing blocks were more active in the peripheral area during the first half of the test and showed less standing alert behaviour than animals without blocks (**II**). Furthermore, animals housed in GFCs showed less grooming behaviour than animals housed in SBCs and after transfer into GFCs, rats were less active in the central area (**II**). Defecation behaviour was not affected by the presence of blocks, cage type, cage level or group size (**I, II**) (Table 5).

In Experiment 1, single housed rats were less active and moved slower from the periphery to the central area of the arena in comparison to group-housed animals (**I**). Furthermore, the groups of four animals reared sooner and groups of three or four animals reared more than groups of two or single housed animals. Animals living on the highest shelf of the rack showed a longer latency time in rearing and shorter latency times in grooming (Table 5).

Table 5. Summary of the effects of enrichment (Block), cage type (CT), cage level (GL), group size (Group) on the behaviour of Wistar rats in open-field test. ne = no effect, ⇑ = increase, ⇓ = decrease. The other parameters were not influenced by grouping variables.

	Experiment 1			Experiment 2	
	Block	**CL**	**Group**	**Block**	**CT**
Open-field					
Activity in peripheral area	ne	ne	⇓ single housed	⇑ after transfer	ne
Activity in central area	ne	ne	⇓ single housed	⇑ in GFC	⇓ after transfer
Standing alert	ne	ne	ne	⇓ after transfer	ne
Time spent grooming	ne	ne	ne	ne	⇓ GFC
Latency to groom	ne	⇓ in highest shelf	ne	ne	ne
Latency to rear	ne	⇑ in highest shelf	⇓ 4 animals	ne	ne
Defecation	ne	ne	ne	ne	ne

The behavioural parameters of the open-field test in Experiment 1 were also analysed with factor analysis (**I**). The six factors extracted, accounted for 75 % of the variance. Factor 1 was clearly loaded with explorative and general activity behaviours, such as walking, rearing and activity in the peripheral area. It accounted for 39 % of the variance. The grooming behaviour was loaded on Factor 2, rearing behaviour on Factor 3, first minute activity on Factor 4 and general central activity on Factor 5. Defecation variables were separately loaded on Factor 6 (**I**). The group size, cage level and presence of blocks had no effect on the separate factor scores of animals according to ANOVA analysis (**I**).

3.7 Number of animals needed

The means ± SDs and their respective n-values for Experiments 1-3 are presented in Table 2 in paper **V**. The same values for Experiment 4 are presented in Table 6. According to SOLO Power Analysis, the smallest number of animals needed in experimental groups and in different experiments ranged from 2 to 296.

Table 6. Means±SDs and the smallest number of animals needed (n) to detect arbitrarily chosen 20 % effect size (calculated with SOLO Power Analysis) in physiological parameters of male and female Wistar rats in Experiment 4.

		Experiment 4	
		Males	**Females**
FBW (g)	*Control*	215±24 (3)	163±13 (2)
	Block	217±20 (2)	161±17 (3)
	Tube	212±16 (2)	165±11 (2)
Growth (g)	*Control*	174±22 (3)	124±10 (2)
	Block	176±17 (3)	122±15 (4)
	Tube	172±14 (2)	125±11 (2)
Adrenals (mg)	*Control*	47±10 (9)	53±12 (11)
	Block	45±10 (10)	57±6 (3)
	Tube	43±7 (7)	57±9 (6)
BAT (mg)	*Control*	334±76 (11)	311±55 (7)
	Block	363±65 (7)	270±60 (11)
	Tube	312±47 (5)	247±34 (5)
Ca (mmol/l)	*Control*	3±0,1 (2)	3±0,1 (2)
	Block	3±0,1 (2)	3±0,1 (2)
	Tube	3±0,1 (2)	3±0,1 (2)
Creatinine (µmol/l)	*Control*	44±2 (2)	46±5 (2)
	Block	44±4 (2)	48±4 (2)
	Tube	45±1 (2)	48±4 (2)
Protein (g/l)	*Control*	62±3 (2)	66±2 (2)
	Block	60±3 (2)	69±4 (2)
	Tube	62±2 (2)	68±3 (2)
Pi (mmol/l)	*Control*	3±0,2 (2)	3±0,4 (3)
	Block	3±0,3 (3)	3±0,3 (3)
	Tube	3±0,4 (3)	3±0,3 (3)
Cholesterol (mmol/l)	*Control*	3±0,3 (3)	3±0,4 (6)
	Block	3±0,3 (3)	3±0,5 (8)
	Tube	3±0,4 (5)	2±0,4 (4)
ALAT (U/l)	*Control*	57±7 (4)	49± 7 (4)
	Block	58±8 (4)	45±7 (6)
	Tube	60±16 (15)	47±9 (9)
AFOS (U/l)	*Control*	523±116 (11)	296±36 (4)
	Block	522±123 (12)	255±65 (14)
	Tube	575±111 (9)	300±37 (4)
ASAT (U/l)	*Control*	95±21 (11)	137±46 (25)
	Block	84±14 (6)	93±49 (59)
	Tube	91±16 (7)	87±10 (3)
Triglycerides (mmol/l)	*Control*	2±0,4 (9)	2±0,6 (28)
	Block	3±1,0 (32)	2±0,3 (7)
	Tube	2±0,3 (7)	2±0,7 (26)
GGT (U/l)	*Control*	5±0,5 (3)	4±1,4 (22)
	Block	3±1,5 (42)	3±1,2 (27)
	Tube	5±0,8 (7)	3±2,0 (78)

		Males	Females
Tot. bilirubin (mmol/l)	*Control*	2±1,1 (64)	2±1,0 (67)
	Block	1±0,7 (78)	2±0,5 (23)
	Tube	2±0,6 (25)	2±0,9 (38)
LDH (U/l)	*Control*	469±160 (25)	1009±363 (29)
	Block	615±294 (49)	718±679 (192)
	Tube	479±144 (20)	680±333 (52)
Corticosterone (ng/ml)	*Control*	441±295 (96)	452±299 (94)
	Block	232±130 (55)	621±566 (178)
	Tube	173±72 (38)	505±450 (170)

In all four experiments and in different experimental groups, the estimated n-values for FBW and growth remained similar and were mainly around two or three (Table 2 in paper **V** and Table 6). The n-values for the weights of adrenals, BAT and EAT were mostly around 10. However, in Experiment 1 the pair housed animals with gnawing blocks had the n-value of 32 in adrenal parameter and in Experiments 2 and 3 animals housed in SBCs with gnawing blocks had the n-value of 19 and 34 in EAT parameter (Table 2 in paper **V**). The n-values for spleen weight ranged from 2 to 10 in Experiments 1-3 and those of thymus weight ranged from 2 to 27 (Table 2 in paper **V**).

The n-values were highest and varied the most for the corticosterone parameter (ranged from 32 to 296, Table 2 in paper **V** and Table 6). The n-values for LDH parameter also varied greatly (ranged from 20 to 192, Table 6). From the other serum parameters, the n-values of Ca, creatinine, protein, Pi, cholesterol, ALAT and AFOS varied the least (ranged from 2 to 15), whereas n-values of ASAT, triglycerides, GGT and total bilirubin were more variable (ranged from 3 to 78) in Experiment 4 (Table 6).

3.7.1 Number of animals needed by litter

According to n-values, the serum ASAT, GGT, triglycerides, LDH, corticosterone and total bilirubin were the most sensitive parameters in both genders for the effects of litter, i.e. to genetic factors (n-values ranged from 2 to 273, **IV**). The growth, FBW, BAT and adrenal weights in addition to serum Ca, cholesterol, creatinine, Pi, protein, ALAT and AFOS were the least sensitive parameters for genetic factors.

3.8 N-ratio

Based on the above results, the N-ratios were calculated ($n_{larger} / n_{smaller}$) to indicate the number of animals needed in treatment groups in comparison to control groups (summarised from each 4 experiments in Fig. 3 and 4). The effects of enrichment, cage type and group size were evaluated. The summary of N-ratios is presented in Table 7.

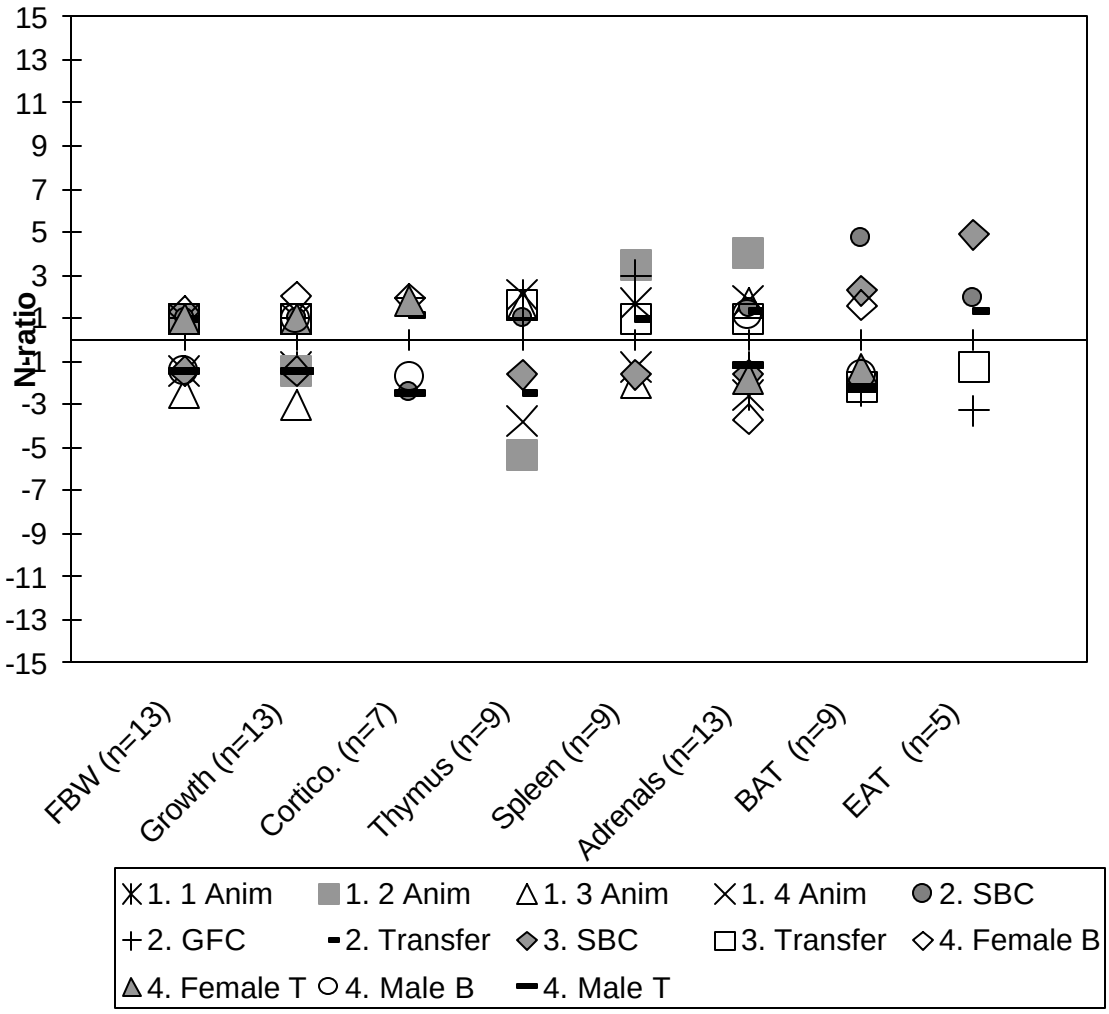

Figure 3. The effect of enrichment items on the required animal number regarding physiological parameters of Wistar rats. SOLO Power Analysis was used to calculate the smallest number of animals needed (n) to detect arbitrarily chosen 20 % difference in group means in four experiments, when significance was set at p=0.05 and statistical power at 0.90. N-ratios (n_{larger} / $n_{smaller}$) were calculated to compare the n in enrichment group to the n in control group in Experiment 1 (1.; group sizes 1 to 4), Experiment 2 (2.; SBC, GFC and Transfer), Experiment 3 (3.; SBC and Transfer) and in Experiment 4 (4.; females and males with tubes (T) and blocks (B)). Positive N-ratio indicates how many times more animals are needed in enrichment group in comparison to control group. Negative N-ratio indicates how many times more animals are needed in control group in comparison to enrichment group. If N-ratio is 1, n-values in enrichment group equals n-values in control group. N-ratio can not be between –1 and 1. SBC = solid bottom cage, GFC = grid floor cage, Transfer = animals first housed in SBCs and then transferred into GFCs, BAT = brown adipose tissue, EAT = epididymal adipose tissue, Cortico. = serum corticosterone, n = number of comparisons (N-ratios).

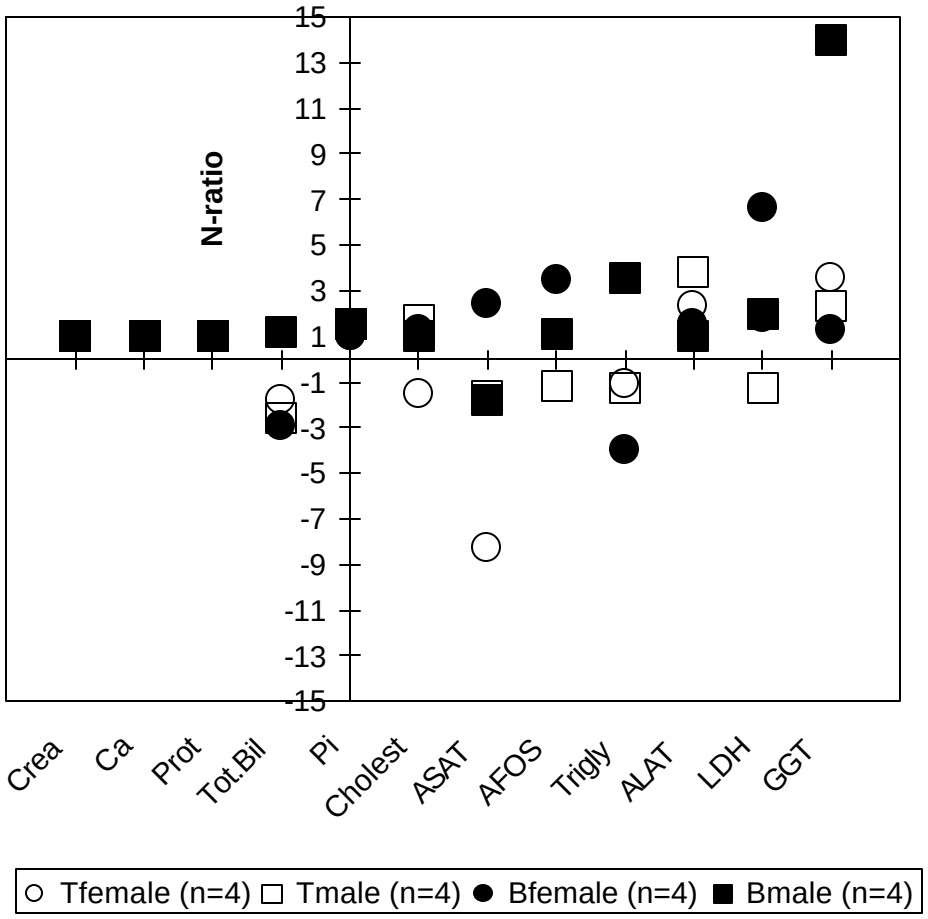

Figure 4. The effects of enrichment items on the required animal number regarding clinical chemistry parameters of Wistar rats. N-ratios (n_{larger} / $n_{smaller}$) were calculated to compare the n in enrichment group (T = tube, B = block) to the n in control group in Experiment 4, separately for females and males. The parameters on the right side of Y-axis have at least one N-ratio greater than 1.5. n = number of comparisons (N-ratios).

3.8.1 Effect of enrichment

According to N-ratios, the FBW and growth were again the least sensitive parameters for the presence of enrichment items in any of the experimental designs (Fig. 3). In general the presence of enrichment items decreased the number of animals needed regarding FBW and growth parameters, except in females with larger blocks (N-ratios = 1.3 for FBW and 2 for growth, Fig. 3).

From the physiological parameters the n-values of BAT and EAT varied the most according to the presence of enrichment: the presence of gnawing blocks in SBCs and larger blocks in cages of females increased the number of animals needed (N-ratios ranged from 1.6 to 4.9, Fig. 3). However, the presence of enrichment items in other experimental groups mainly decreased the number of animals needed (N-ratios = -3.3 to 1.3, Fig. 3). The adrenals and spleen parameters were most sensitive for the presence of gnawing blocks when rats were pair housed (N-ratios = 4 and 3.5, respectively, Fig. 3). The n-values of thymus and corticosterone parameters were also altered by the presence of enrichment items (N-ratios = -5.4 to 2.1 and -2.5 to 1.9, respectively).

From the clinical chemistry parameters the n-values of GGT, LDH and ALAT levels varied the most according to the presence of larger blocks and tubes (N-ratios varied from -1.3 to 14, Fig. 4). Cholesterol, ASAT, triglycerides and AFOS parameters were also influenced by the presence of tubes and blocks (N-ratios varied from -8.3 to 3.6), but not as strongly as ALAT, LDH and GGT (Fig. 4). The presence of tubes and larger blocks altered also the n-values of total bilirubin (N-ratios = -2.9 to 1.2) and Pi (N-ratios = 1 to 1.5) levels and had no effect at all on the n-values of creatinine, Ca and protein parameters (N-ratios = 1, Fig. 4).

Table 7. The ranges of N-ratios (n_{larger} / $n_{smaller}$) of physiological and clinical chemistry parameters in four Experiments when the effects of enrichment (Block, Tube), group size (Group) and cage type (GT) on Wistar rats were evaluated. Negative sign indicates smaller number of animals needed in "treatment" group in comparison to control group. When the N-ratio is 1, the n in "treatment" group equals n in control group. FBW = final body weight, BAT = brown adipose tissue, EAT = epididymal adipose tissue, Cortico. = serum corticosterone, Triglyc. = triglycerides, Tot.bilirub. = total bilirubin, - = not analysed.

	Experiment 1		**Experiment 2**		**Experiment 3**		**Experiment 4**	
	Block	**Group**	**Block**	**CT**	**Block**	**CT**	**Block**	**Tube**
FBW	-2.5-1	-1.5-1	1	1	-1.5-1	-1.5-1	-1.5-1.3	-1.5-1
Growth	-3-1	-2-1.5	-1.5-1	1	-1.5-1	-1.5-1	1-2	-1.5-1
Adrenals	-2.6-4	-2.2-6.4	-1.3-1.5	1-2	-1.6-1	-2.7- -1.7	-3.7-1.1	-1.8- -1.2
BAT	-	-	-2.4-4.7	-2-5.7	-2.2-2.3	-2.8-1.8	-1.6-1.6	-2.2- -1.4
EAT	-	-	-3.3-1.9	-6.3-1	-1.3-4.9	-2.6-2.4	-	-
Spleen	-2-3.5	-1.7-3.3	-1.7-3	-2.5-2	-1.6-1	-1.3-1.2	-	-
Thymus	-5.4-2.1	-3-3.9	-2.5-1	1.3	-1.6-1.6	-1.6-1.6	-	-
Cortico.	-	-	-2.4-1.9	2-9.3	-	-	-1.7-1.9	-2.5-1.8
Ca	-	-	-	-	-	-	1	1
Creatinine	-	-	-	-	-	-	1	1
Protein	-	-	-	-	-	-	1	1
Pi	-	-	-	-	-	-	1-1.5	1-1.5
Cholesterol	-	-	-	-	-	-	1-1.3	-1.5-1.7
ALAT	-	-	-	-	-	-	1-1.5	2.3-3.8
AFOS	-	-	-	-	-	-	1.9-3.5	-1.2-1
ASAT	-	-	-	-	-	-	-1.8-2.4	-1.6- -8.3
Triglyc.	-	-	-	-	-	-	-4-3.6	-1.3- -1.1
GGT	-	-	-	-	-	-	1.3-14	2.3-3.6
Tot.bilirub.	-	-	-	-	-	-	-2.9-1.2	-2.6- -1.8
LDH	-	-	-	-	-	-	2-6.6	-1.3-1.8

According to the One-Sample t test, all the transformed N-ratios of physiological parameters (FBW, growth, adrenals, BAT, EAT, spleen and thymus) differed significantly from zero, when comparing control and enrichment groups (Table 8). The N-ratios of Ca, creatinine and protein could not be tested since the SDs were zero (N-ratios were 1, Table 8).

From the clinical chemistry parameters, only the transformed N-ratios of corticosterone and total bilirubin levels differed from zero (Table 8).

Table 8. N-ratios (n_{larger} / $n_{smaller}$) of physiological and clinical chemistry parameters for enrichment when n-values of enrichment groups were compared with the n-values of control groups. Positive N-ratio indicates how many times more animals are needed in enrichment group in comparison to control group. Negative sign was added to indicate how many times more animals are needed in control group in comparison to enrichment group. The absolute N-ratios were transformed with natural logarithm and analysed with One-Sample t test (t and p-values presented) with test value = 0 to evaluate if N-ratios in general would differ from one (= equal n-values in both groups). FBW = final body weight, BAT = brown adipose tissue, EAT = epididymal adipose tissue, Cortico. = serum corticosterone, Nt = analysis could not be performed due to the lack of SDs. Statistically significant effects are in **bold.**

	Number of comparisons	Range	t	p-value
FBW	n=13	-2.5 - 1.3	2.84	**0.015**
Growth	n=13	-3 - 2	3.03	**0.010**
Adrenals	n=13	-3.7 - 4	4.60	**0.001**
BAT	n=9	-2.4 - 4.7	6.45	**0.000**
EAT	n=5	-3.3 - 4.9	3.07	**0.037**
Spleen	n=9	-1.6 - 3.5	3.69	**0.006**
Thymus	n=9	-5.4 - 2.1	3.55	**0.008**
Cortico.	n=7	-2.5 - 1.9	5.77	**0.001**
Ca	n=4	1	Nt	
Creatinine	n=4	1	Nt	
Protein	n=4	1	Nt	
Cholesterol	n=4	-1.5 - 1.7	2.72	0.072
ALAT	n=4	1 - 3.8	2.25	0.110
AFOS	n=4	-1.2 - 3.5	1.32	0.280
ASAT	n=4	-8.3 - 2.4	2.66	0.077
Triglycerides	n=4	-4 - 3.6	2.18	0.117
GGT	n=4	1.3 - 14	2.41	0.095
Tot. bilirubin	n=4	-2.9 - 1.2	3.53	**0.039**
LDH	n=4	-1.3 - 6.6	2.32	0.103

3.8.2 Effect of cage type

The effects of cage type (housing in GFCs in comparison to housing in SBCs) on n-values were evaluated in Experiments 2 and 3 (**V**). In addition to serum corticosterone levels (N-ratios = 2 to 9.3), the n-values of BAT (N-ratios = -2.8 to 5.7) and EAT (N-ratios = -6.3 to 2.4) varied the most when housed in GFCs in comparison to SBCs (Table 7). The n-values of adrenals (N-ratios = -2.7 to 2), spleen (N-ratios = -2.5 to 2) and thymus (N-ratios = -1.6 to 1.6) parameters varied also in different cage types. Regarding FBW and growth parameters, housing in GFCs either did not alter (N-ratios = 1) or it decreased (N-ratios = -1.5) the number of animals needed in the experimental groups in comparison to the housing in SBCs (Table 7).

According to One-Sample t test, the n-values of BAT and thymus parameters differed significantly between these two cage types (transformed N-ratios differed from zero, Table 3 in paper **V**), while the other parameters did not.

3.8.3 Effect of group size

The effects of group size (group housing in comparison to single housing) on the n-values were evaluated in Experiment 1 (**V**). For the adrenal parameter, group housing with gnawing blocks increased the number of animals needed (N-ratios = 2.2 to 6.4) compared to single housing, whereas group housing without gnawing blocks decreased it (N-ratios = -2.2 to -1.4, **V**). In contrast, group housing animals with gnawing blocks decreased the number of animals needed as regards the thymus weight (N-ratios = -3 to -1.4), whereas group housing without gnawing blocks either had no effect (N-ratio = 1) or increased it (N-ratios = 2.7 to 3.9, **V**). The N-ratios as regards the spleen parameter varied from -1.7 to 3.3, but a clear pattern was not seen.

According to One-Sample t test, the n-values of FBW were similar in singly and group housed animals (transformed N-ratios did not differ from zero, Table 3 in paper **V**). The n-values of adrenals, spleen, thymus and growth parameters differed in singly and group housed animals.

4 DISCUSSION

At first sight, environmental enrichment seems quite a simple and ideal method to improve animals' welfare. We provide animals what they need or want in order to enhance and ensure their physiological and psychological well-being. This may work with animals held in zoos or with endangered species to be released into a natural habitat, but not necessarily with laboratory animals (Galef 1999). While manipulating the environment of laboratory animals, one should not forget possible consequences on the research results.

In order to evaluate the efficiency of enrichment programmes, we need objective measures for the physiological and psychological changes attributable to welfare. Furthermore, we need to recognise that optimal environments are likely to vary from one species to another, from one sex to the other, and in different age classes (Galef 1999). This calls for further research in the area of environmental enrichment and especially critical evaluation of enrichment provided is required.

One or two indicators, which are or are not "normal", do not mean that the welfare of an animal is good or vice versa (Broom and Johnson 1993). Plain physiological measurements do not allow comprehensive evaluation of the effects of housing environment on welfare, therefore behavioural measurements are also recommended (Rushen and de Passillé 1992, Patterson-Kane et al. 1999).

4.1 Use of enrichment items

Gnawing and burrowing are normal behaviour patterns in rats (Sharp and La Regina 1998). Their incisors grow continuously so the animals must gnaw to wear off new tooth growth (Sharp and La Regina 1998). Gnawing of wooden blocks has been suggested to reduce the incidence of overgrown teeth (Orok-Edem and Key 1994). In laboratory conditions, burrowing tendency can be seen in the animals' preference for cages containing a shelter (Townsend 1997). The shelter may also enable an escape from cage mates or bright light (Manser et al. 1998) and satisfy wall-hugging tendencies or enable rearing and climbing (Townsend 1997).

The smaller gnawing blocks (**I, II**) were effectively gnawed in GFCs (Table 3). The three to four fold weight loss of blocks (4-8 g/animal/week) in GFCs compared to that observed in SBCs also remained at a constant level throughout the studies. These findings indicate that the items were actually used in GFCs and animals did not lose interest in them over time. In conclusion, smaller gnawing blocks seemed to have more enriching value for rats housed in GFCs than in SBCs. Accordingly, the negligible use of blocks in SBCs indicates, that this kind of item did not give any additional enrichment for rats beyond bedding.

The extent of gnawing of tubes and larger blocks in SBCs (**III**) was equal to the minimal gnawing of smaller blocks in SBCs (**I, II**). However, animals spent on average 7 % (blocks) to 78 % (tubes) of their time in contact with these items. The results indicate that tubes and larger blocks were not extensively used for gnawing, but were used for other purposes by the animals:

the block was large enough to stand on and it was easily moved within the cage, while the tube provided shelter from light. The avoidance of cage mates is unlikely, since all four animals squeezed themselves inside the tube at the same time. During the dark period, other behaviour patterns were more prominent (e.g. standing on top of, or beside tubes), but rats still spent over 20 % of their time inside the tube.

Controllability of an environment is psychologically and biologically important to animals (Sambrook and Buchanan-Smith 1997). It has also been suggested to have an impact on animals' welfare (Townsend 1997, Manser et al. 1998). Rats in the present studies had control over light intensity by choosing the inside of the tube or the other parts of the cage. In control cages or in cages with blocks, animals appeared to escape light by positioning themselves under the food hopper, where the light intensity was about half of that present elsewhere. The effects of humidity or temperature inside the tube were not measured.

Only minor gender differences were observed in the contact times with the tube. Females were probably more curious about the surrounding environment than males, since they spent more time on top of the tube than males. Overall, a greater variety of behaviours were possible in cages with tubes and rats spent more time with the tube than with the block. Therefore, it can be suggested that the tube was a more suitable and attractive enrichment item for rats in SBCs than blocks.

In comparison to the blocks, it could be argued that due to the larger size of the tube it was difficult to avoid contacts with it. Since rats with tubes spent also a considerable proportion of their time elsewhere in the cage, this was presumably not the case in these studies.

With the use of smaller gnawing blocks, the effects of group size, cage level, cage type and gender were evaluated. Group size or gender had no influence on the use of items. Cage level in rack had some effects. Gnawing of the blocks was largest on the third shelf of the rack, but clear explanation for this could not be found (**II**). Cage type was the most important factor; gnawing of blocks occurred mainly in GFCs and was minimal in SBCs.

It is well established that the principle characteristics of good environmental enrichment items are safety (both for animals and care takers), practicality, low cost, easy sanitation or replacement and suitability for the enrichment purpose so that animals use them and receive some benefit of them (Line 1987, Cubbitt 1992, Watson 1993, Orok-Edem and Key 1994, Chmiel and Noonan 1996, Baumans 1997). The enrichment items studied in the present thesis undoubtedly easily fulfilled most of the characteristics.

All items were made of the same material (aspen) as bedding, hence new materials were not introduced into the cages. As hardwood it is a more suitable material for enrichment than softwood, since softwood may have enzyme-inducing effects (Vesell 1967, Weichbrod et al. 1988) probably due to the presence of volatile organic compounds (Bang and Ourisson 1975). The best way to reduce the volatile organic compounds if softwood is used is to autoclave the material (Nevalainen and

Vartiainen 1996). The items in the present experiments kept their structure even after ten autoclaving cycles thus enabling their use for several weeks without compromising hygiene.

4.2 Effects on Physiology

4.2.1 Enrichment items

Presence of smaller gnawing blocks, cage level in rack, group size or gender did not change the food intake of rats in SBCs (**I**). This is in agreement with Watson (1993) who was also unable to find consistent effects of inanimate object material (nylon ball, nylon bone) on the food intake or body weight of single housed rats.

Presence of either blocks or tube did not have major effects on the group means of FBW and growth or the weights of thymus, adrenals, spleen, EAT and BAT (Table 4). The decreased FBW and growth of rats with gnawing blocks in Experiment 2 can be regarded either as a positive effect or as an adverse effect. In general, well growing animals are considered to be healthy and have good welfare whereas stressed or sick animals have decreased weight gain. However, results from numerous studies indicate that decreased weight gain from restricted calorific intake tends to increase lifespan, decrease the incidence of degenerative diseases and delay the onset of neoplasia in the rat (Sharp and La Regina 1998).

Since the food intake or home cage behaviour were not monitored in Experiment 2, it is not known whether the decreased FBW and growth were due to reduced food intake or increased energy consumption related to gnawing blocks. Perhaps the presence of smaller gnawing blocks fulfilled the gnawing need of animals thus reducing the gnawing of food pellets (Fiala et al. 1977). The rats in this experiment were not malnourished or in poor condition and group means of spleen, thymus, EAT and BAT weights in addition to clinical chemistry parameters were not influenced by the presence of enrichment items. Hence, reduced weight gain may be considered as a positive consequence, leading to reduced fatness in laboratory rats who have a tendency to obesity. The value of this finding is, however, weakened by the fact that it was observed only in one out of four experiments.

The decreased adrenal weights were detected in rats with smaller gnawing blocks in Experiment 1. Enlarged adrenals are often suggested to be a consequence of adverse stimuli (reviewed by Brain and Benton 1979 and Nussdorfer 1986, Manser 1992, Clark et al. 1997b). This is not necessarily always the case as discussed later in connection with cage type (4.2.2). However, if adrenal hypertrophy is considered to be an indicator of stress, rats housed with smaller gnawing blocks would be less stressed than control animals. This finding was seen only in one experiment, which prevents drawing of strong conclusions.

4.2.2 Cage type

Cage type had more profound influence on the physiology of rats than enrichment items. The decreased FBW and growth in SBCs may also be considered as positive effects, as discussed above. They may be due to a more stimulating environment in cages with bedding and hence increased energy consumption. Moreover, housing in

GFCs may lead to a greater food intake due to boredom. Fiala et al. (1977) showed that isolation-reared rats weigh more than rats reared in a complex environment because they eat more, which was thought to be due to boredom. However, single housing of rats in this study did not reveal any effects on food consumption.

Adrenals were heavier in GFCs than in SBCs, which may indicate increased stress in animals housed in GFCs (**II**). However, serum corticosterone levels were lower in GFCs, which, on the other hand, may indicate reduced stress. Negative correlation between adrenal weights and serum corticosterone levels was also demonstrated by Gómez et al. (1996). These contradictory results show the importance of multiple measurements when evaluating housing effects on the welfare of animals. They also show the interpretation difficulty when stress indicators point to opposite directions. Although high glucocorticoid levels in chronic stress conditions have been reported, the relevance of corticosterone measures as long-term indicators of stress has been criticised (Rushen 1991, Broom and Johnson 1993). Moreover, changes both in corticosterone levels and in adrenal weights may be artefacts. Serum corticosterone levels of animals change rapidly due handling (Gärtner et al. 1980, Manser 1992) while adrenal weights can be affected by the dissection technique. These results indicate that absolute or relative adrenal weight and serum corticosterone levels may not be the most reliable parameters for evaluating the long term effects of housing environment on the welfare of rats.

The group means of spleen, thymus and EAT weights were not influenced by cage type. The weights of BAT were greater in GFCs than in SBCs in Experiment 3. This may indicate thermoregulatory response for colder environment, which is not, however, in accordance with temperature measurements (0.5 ^{o}C higher in GFCs than in SBCs, **II**). The significant statistical interaction of cage type and blocks regarding BAT weight seemed to result from the fact that rats in GFCs with smaller gnawing blocks had lower BAT weights than rats without them, whereas rats in SBCs with or without blocks had similar BAT weights. This finding was not present in Experiment 2, suggesting that this may be a random phenomenon.

Male rats were heavier and grew faster than female rats (**IV**). As expected, gender had an impact on organ weights. The lower adrenal weight in males is in accordance with the literature (Tucker 1997). The serum corticosterone levels were also lower in males than in females. The sex, age and strain differences in circulating corticosterone levels are well known (reviewed by Evans 1996).

The results in this thesis indicate the minor influence of cage level and group size on the physiological indices of welfare in rats. However, group housing of rodents is preferred to single housing as long as the groups are stable and aggressive behaviour is not expressed (Brain et al. 1993). In general, the presence of conspecifics enriches animals' environment and gives them more stimuli (Broom and Johnson 1993).

The genetic background (as determined by litter) influenced some of

the physiological and serum parameters measured (BAT, AFOS, Pi and cholesterol). This is not surprising due to the wider genetic variability within outbred stocks (Festing 1992, Festing 1996). Random allocation of littermates into all groups takes into account genetic factors and provides better experimental design.

4.3 Effects on behaviour

Repetitive and stereotyped behaviours are important indicators of long-term welfare problems (Broom and Johnson 1993). Among these studies, the home cage behaviour was recorded in Experiments 1 and 4. The focus of these recordings was on the monitoring of the use of items. Stereotypic behaviour was not observed.

The open-field test was conducted in Experiments 1 and 2. The presence of smaller gnawing blocks had no influence on the open-field behaviour of rats in Experiment 1 (**I**) and had only minor effects on the behaviour in Experiment 2 (**II**). The animals with gnawing blocks in GFCs or after transfer into GFCs seemed to be slightly more active than animals without blocks. However, the differences between groups were small and the other variables (grooming, rearing, defecation) were not affected. It appears that the presence of smaller gnawing blocks had no impact on the emotion-based behaviour of rats.

Rats transferred into GFCs in Experiment 2 expressed less locomotion activity in the central area of the arena than before transfer (**II**). The changes were small, but may be indicative of a more fearful behaviour (Walsh and Cummins 1976). However, the age of animals or repetition of the test may have influenced the behaviour. Furthermore, rats housed in GFCs or transferred into them groomed less than animals housed in SBCs, indicating a reduced ability to habituate to the test situation (File et al. 1988). Housing in SBCs may have provided more stimulation for rats, leading to more active and less reactive behaviour in a novel situation.

Singly housed animals were less active and reared less in the open-field than group-housed animals (**I**). They also moved from the periphery to the central area of the arena more slowly. The group housed animals had the shortest latency to rear. These results may indicate that singly housed rats were more timid while group housed animals were more explorative (Archer 1973, Walsh and Cummins 1976, Ossenkopp et al. 1994, Patterson-Kane et al. 1999). Rats on the highest shelf of the cage rack reared later and groomed earlier than animals housed on other parts of the rack. These animals were probably more habituated to high light intensities (200 lux inside a cage during light period) than other animals (light intensities 105 lux and less), and thus more familiar with the high light intensity of the open-field (380 lux in the centre). Other behaviours were not sensitive to the cage level, probably indicating the minor importance of cage level on timid and explorative behaviours.

The factor analysis was used to evaluate the open-field behaviour in Experiment 1 (**I**). From this analysis, a general activity factor could be derived, accounting for 39 % of the variance. Other clear factors were not obtained, even though individual behaviour vari-

ables were loaded on separate factors, e.g. grooming on Factor 2 (9 % of variance), rearing on Factor 3 (8 % of variance) and defecation on Factor 6 (5 % of variance). The lack of significant effects of group size, cage level and presence of smaller gnawing blocks on the factorial scores of animals indicates that in general, behaviour of rats was not dependent on the environmental factors tested.

4.4 Number of animals needed

The adequate number of animals used in a research ensures the detection of biologically significant treatment effect. However, above a certain point, the increase in animal numbers does not add precision of an experiment, but is simply a waste of animals (e.g. Erb 1990, Mann 1991, Festing 1992, Festing 1997). If the inter-individual variation of animals increases, but treatment effect, statistical power and significance level are not changed, more animals are needed to detect the important treatment effect (e.g. Cohen 1988, Erb 1990, Festing 1992). Accordingly, if the variability of animals and test factors can be limited, the number of animals needed can be smaller without the loss of precision (Festing 1993).

Pre-evaluation of sample size when possible is recommended by some scientific journals (e.g. Laboratory Animals 2000). However, the n-values assessed by power analyses are mathematical estimates and these analyses require pilot studies or some other ways to assess information about the effect size and variation of the material. In the present experiments retrospective power analysis was used to evaluate the impact of changes in variability on the number of animals needed in the experiments.

In the present study, the smallest number of animals needed to detect the arbitrarily chosen 20 % difference in group means with statistical power at 0.9 and significance at $p=0.05$ varied from 2 to 296 regarding the physiological parameters (Table 6 and Table 2 in paper **V**). The n-values needed for FBW and growth parameters were the smallest (around two or three) in all experiments and in different experimental groups. The most variable and highest n-values were observed for corticosterone parameter (varied from 32 to 296). These results indicate that when FBW and growth measures are used, the probability of detecting group differences of 20 % is high even with small sample size. On the other hand, the detection of treatment effect requires a much larger number of animals, when serum corticosterone levels are studied. Apparently, to show a group difference of 20 % in corticosterone levels is difficult. However, a significant group difference of 70 % in corticosterone levels was found with 10 animals per group in Experiment 2 (**II**).

The n-values of other physiological parameters (weights of adrenals, BAT, EAT, spleen and thymus) were generally less than 10 with only some exceptions. This indicates that when experimental conditions are equal in all groups, the common practise of animal experiments to use 5-10 animals per group should be large enough to detect a treatment effect.

The n-values of clinical chemistry parameters (Ca, creatinine, protein, cholesterol, triglycerides, total bilirubin, Pi, ALAT, AFOS, ASAT, GGT and LDH)

varied from 2 to 196. The results suggest that environmental, genetic and/or analytical factors have the least effect on measures of creatinine, protein, cholesterol, Ca, Pi, ALAT and AFOS. Number of animals needed for the detection of treatment effects in these parameters can again be the commonly used 10 animals per group. However, triglycerides, total bilirubin, GGT, ASAT and LDH parameters were more variable, which calls for caution, when the animal numbers needed are predicted.

An alternative method to evaluate differences in within-group variation may include comparisons of variances. Gärtner (1990 and 1998) used coefficient of variation (CV = SD / 0). It is also a good method, which takes into account both group means and standard deviations. Third method to evaluate differences in within-group variation is to analyse group variances e.g. with F-test (two groups) or with Bartlett-Box test (more than two groups). These tests, like all tests of homogeneity of variances, require normally distributed data. In this thesis n-values and N-ratios were used to illustrate the practical consequences of small changes in variability on the appropriate number of animals needed in an experiment.

4.5 N-ratio

N-ratios were calculated to enable comparisons between different housing environments and enrichment items and their effects on the number of animals needed. Furthermore, transformed N-ratios (normal logarithm) were tested with One-Sample t test to evaluate if N-ratios in general would differ from zero, which would indicate statistically unequal n-values in "treatment" group in comparison to control group.

According to N-ratios, the n-values of FBW and growth were the least sensitive for the effects of housing environment and for the presence of enrichment items (Fig. 3, Table 7). The housing in GFCs, presence of enrichment items or group housing reduced or had no effect at all on the number of animals needed with these parameters. Even though the N-ratio for growth in females with larger blocks was two, thus indicating the two fold number of animals needed in this group in comparison to the control group, the actual n-values were small: two and four (Table 6). Conversely, N-ratios for the corticosterone levels were also in general about two or less, but the actual n-values were substantially higher varying from 32 to 296 (Table 6 and Table 2 in paper **V**). The results indicate that even though N-ratio seems quite a useful indicator of effects on variation, the actual n-values should not be overlooked.

The results also show how important it is to understand the difference between statistically significant difference and practically important effect. According to One-Sample t test, the N-ratios of FBW and growth significantly differed from one (transformed N-ratios from zero), when enrichment group was compared to control group (Table 8). However, when the actual n-values are examined, they are in general two or three (only once five and six). The statistically significant difference probably emerges from the fact that these two exceptions altered otherwise so homogeneous variance and that the number of comparisons was 13.

From other physiological parameters, BAT and EAT were the most sensitive ones for the effects of cage type and for the presence of enrichment items (N-ratios -6.3 to 5.7, Table 7). The weights of thymus, spleen and adrenals were somewhat medium responsive (except the weight of adrenals in pair housed rats with smaller blocks 6.4), indicating minor sensitivity for environmental modifications in comparison to BAT and EAT parameters. The statistical analysis of N-ratios also revealed significant unequality in n-values (Table 3 in paper **V** and Table 8). These differences were significant even with the number of comparisons of nine and five.

From the clinical chemistry parameters GGT, LDH and ALAT levels seemed to be the most susceptible to the effects of enrichment items according to N-ratios, whereas creatinine, Ca, protein and Pi were the least sensitive ones. However, according to One-Sample t test, only the N-ratios of total bilirubin differed significantly from zero. The lack of statistical significances is probably due to small sample size (four comparisons). In general, the n-values of serum determinations seemed to be more sensitive to environmental factors than organ weights. This higher variability may be due to (pre)analytical procedures. By developing procedures and methodologies this variability is easier to control contrary to biological (inter-individual) variation. The susceptibility in the present experiments means that some parameters seemed to be more responsive to the environmental factors than others. This does not necessarily mean that more animals are needed. In several cases the number of animals needed was smaller in the treatment group than in the control group.

In general, the N-ratio seemed a suitable tool to evaluate effects of housing modifications on the variation of research results and to illustrate the change in n-values of two groups. However, n-values calculated with SOLO Power Analysis are based on group means and SDs, hence it is more applicable to normally distributed parameters. Furthermore, totally different magnitudes of n-values may produce equal N-ratios thus not showing the real variability of a parameter. Moreover, the arbitrarily chosen 20 % difference in group means may not be biologically significant effect size for all parameters. In overall, n-values and N-ratios are illustrative parameters, which can also be statistically analysed.

4.6 General conclusions and evaluation of welfare

Two concepts of the Three Rs – Refinement and Reduction - were dealt with in this thesis. Furthermore, the applicability of these concepts and the influence of environmental modifications on them were evaluated.

In general, the enrichment items were used by rats in terms of contact time and amount gnawed. This indicates that they were species-appropriate and rats gained some value of using them. However, the suitability of these items depended on the housing environment. In SBC, rats had no interest on smaller gnawing blocks, but in GFCs they seemed to have more enriching value. Larger blocks and especially tubes seemed to be suitable enrichment tools for rats in SBCs. However, their value as enrichments in other housing environments should be tested before general recommendations can be made. In conclusion, depending on the circumstances, individual enrichment may or may not give benefit to animals. The enrichment items used in this thesis fulfil the modified concept of refinement: method, which alleviates or minimises potential pain, suffering and

distress, and which enhances animal well-being (Declaration of Bologna 1999).

Enrichment items, cage type, cage level in rack and group size did not have any clear adverse effects on the physiology and behaviour of rats as measured with differences in group means. In most cases there was no effect at all. When effects were seen, they were inconsistent and in some cases contradictory. The results indicate, that recommendations or prohibitions based on results from one or even two experiments should not be made. In conclusion, housing in GFCs compared to housing in SBCs may have some adverse effects. However, according to these studies, the effects were small and inconsistent thus probably not threatening the welfare of rats. Even though the results from these studies do not clearly support the superiority of SBCs over GFCs, SBCs may be recommended for rodent housing according to preference tests (Manser et al. 1995, Manser et al. 1996, Blom et al. 1996, van de Weerd et al. 1996). If it is necessary to house rodents in GFCs, appropriate enrichment may alleviate the possible adverse effects.

Reduction concept includes methods for obtaining comparable levels of information from the use of fewer animals, or for obtaining more information from the same number of animals (Declaration of Bologna 1999). In this thesis, housing environment and environmental enrichment altered the variability between animals and hence influenced the number of animals needed. The number of animals needed was both reduced or increased depending on the modification and in several cases it remained unchanged. It appears that the effects of environmental modifications on the variability of animals should be evaluated case by case. When the refinement attempts increase the number of animals needed, one has to evaluate the importance of both concepts; whether to apply refinement and possibly improve animal welfare or to decide against refinement. It is obvious that enrichment is a research variable as such and can affect the number of animals needed, as any environmental condition can. Scientist should acknowledge this while designing experiments. Worldwide generalisations and recommendations are very difficult to make, but while applying the concepts of the Three Rs, the reliability of results should not be jeopardised.

In conclusion, the effects on physiological and behavioural parameters indicate that any of the environments studied were not clearly superior or inferior to the others, and the general welfare of rats was not threatened in any of them. One can argue however, that the welfare indices used in the experiments were not sensitive enough to detect impacts of these housing modifications. Enrichment items were used by animals thus possessing some value for them and probably improving animals' welfare. The effects of environmental modifications on the number of animals needed may or may not be beneficial, but in most cases one can provide environmental enrichment for rats without endangering the experimental results.

5 CONCLUSIONS

1. Enrichment items were used effectively, thus they possess enriching value for rats. This however, depends on the housing environment. In general, these kind of environmental enrichment items can be recommended, but their usability in all environments requires further investigation.
2. Enrichment items had only minor effects on the physiology and behaviour of rats. Thus the application of these enrichment items does not threaten research results but possibly improves animals' welfare.
3. Cage type had some influence on the physiology and behaviour of rats in comparison to cage level or group size. The effects were, however, small and they neither improved nor endangered the welfare of animals. The results do not indisputably suggest the inferiority of GFCs and with additional enrichment these possibly adverse effects may be alleviated.
4. Variability of rats may be influenced by environmental enrichment and other housing modifications thus leading to more or less animals needed in experiments.
5. Refinement can be applied in the form of environmental enrichment. Reduction, however, may be influenced by refinement attempts. In general, both concepts can be applied simultaneously but in some cases one concept may interfere the application of the other.

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