Nutrition and Animal Welfare

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Chapter 5

NUTRITION AND ANIMAL WELFARE

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1. INTRODUCTION

One of the concepts of good animal welfare focuses on the fact that animals should function and feel well (Carter et al. 2001). There are two categories of basic values (see the text by Sørensen in this book). The first category is more or less objective, in that an animal should function well biologically and/or has the possibility to perform natural behaviours. This can be assessed by e.g. the absence of pathology and behavioural abnormalities such as stereotyped behaviour. The second category refers to the subjective approach, which relates to the inner mental state of the animals. An increased welfare is then related to an increased positive subjective state and reduced negative mental state (Fraser et al. 1997). In scientific research attempts are made to measure these subjective states indirectly by e.g. preference testing.

Each organism strives to maintain homeostasis, both physiologically and mentally. Homeostasis refers to a regulated state of internal stability or balance (Strubbe 2003). Such a state can never be a stable permanent situation, as there will always be fluctuations, such as activity versus resting, eating versus not eating, social interaction, etc., and it is essential that the homeostatic state is reached again. Eating a meal will automatically lead to a certain disturbance of the homeostasis (e.g. the thermogenic effect), but as long as this stays within certain limits, this offers no real threat. It is probably even stimulating animal welfare when fluctuations arise, as biological rhythms are at the basis of virtually all natural processes. Many functions show circadian rhythms, i.e. rhythms of approximately 24 hours.

E. Kaliste (ed.), The Welfare of Laboratory Animals, 51-80.

But these fluctuations should probably remain within an upper and lower boundary, otherwise the organism has more difficulties to return to the homeostatic state. In order to maintain welfare, it is possible that a certain amount of species-specific natural fluctuations in the feeding process have to be introduced in the laboratory setting in order to improve the welfare of laboratory animals.

Our goal in this chapter is to evaluate several aspects of nutrition in relation to the welfare of animals (focused on rodents) in experimental conditions. The aims are further to present essential knowledge and data that are known to prevent the occurrence of pathological and behavioural disorders. This knowledge can also contribute to a better standardisation of experiments. When doing animal experimentation, it is important to strive for standardisation within and between experiments, between institutes, nationally as well as internationally, in order to make data comparable (see also Haseman 1984, Roe 1994). In the more subjective approach, examples from results of different types of tests like e.g. preference testing will be provided, with a discussion of the possible welfare implications for the animal species involved. It is hypothesized that the more the environmental factors fit into the species-specific adaptive capacities (for being able to return to homeostasis) the better the welfare (Crok 2003).

By presenting the already published knowledge from animal experiments on nutrition and related animal behaviour, the insight into the laboratory animal in an experimental situation can be improved. By comparing the guiding principles of the concept of standardisation versus the natural rhythmicity, tools are given to researchers to make conscious and well-based decisions about the experimental design. Directly and indirectly this improved understanding can contribute to optimising the experimental conditions, leading to simultaneous improvement of animal welfare and experimental results.

2. GOOD BIOLOGICAL FUNCTIONING

Since this chapter focuses on nutrition and animal welfare, we first pose the question why eating is necessary. Food needs to be ingested, as many processes in the body depend on the (continuous) supply of energy and nutrients. Moreover, to maintain the body temperature at a certain level, which is critical for many vital functions in mammals, it is necessary to supply energy to the system. Nutrients like proteins and amino acids are necessary to build muscle tissue and keep it in good health, and minerals need to be a part of the diet e.g. for a proper bone mineralization as well as good muscle function. Certain trace elements and vitamins are essential factors in maintaining enzymatic functions in the body. By overconsumption during certain periods, energy deposits can be formed. This occurs for instance in hibernating animals. This is a basic mechanism applied by animals in nature, in order to prepare the organism for more difficult periods, in which no or hardly any food is available. On the other hand, the continuous supply of food to rodents in the laboratory, where the animals never have to make use of these reserves, may therefore not be the best way of feeding.

2.1 Nutritional requirements

From a general perspective, the nutritional requirements of farm animals and laboratory animals are similar. All require energy, protein, carbohydrate, lipid, macrominerals, vitamins and trace elements supplied in diets that should be palatable and free from chemical and biological contaminations (Ritskes-Hoitinga and Chwalibog 2003). Much information regarding farm animals may be applicable to laboratory animals, however, the aim of laboratory animal nutrition is not the efficient or maximum production, in contrast to the situation in farm animals. The documents published by the National Research Council provide currently the best documented basis for the nutrient needs of each animal species These guidelines are based on studies supporting maximum/optimum growth and production, which is not necessarily the same as goals in animal experimentation, where optimum performance and nutritionally unbiased results in biomedical experiments are the aims (Ritskes-Hoitinga and Chwalibog 2003). Fiber is not included in most nutrient requirements, but depending on the species, there can be a need to include fiber and/or add it to the diet for maintaining and/or improving good health. Rabbits and guinea pigs are examples of species where extra fiber will typically contribute to good biological functioning.

As regards energy requirements, animals will eat an amount of food determined by their energy need of that moment, mainly based upon the stage of life they are in (growth, maintenance, reproduction, lactation) (Beynen and Coates 2001). So, based on the energy density of the diet and the energy need, it can be calculated what the expected food intake will be under *ad libitum* conditions. It is beyond the scope of this chapter to give the exact calculations, and the reader is referred to Beynen and Coates (2001) and Ritskes-Hoitinga and Chwalibog (2003) for details. Upon changing the energy density of the diet, e.g. by adding fat, the animal will eat less food mass since fat contains more calories per gram. When changing the amount of fat in the diet, isocaloric exchange is advised in order to achieve optimal standardisation (see also Beynen and Coates 2001). Moreover, on a high fat diet animals (may) compensate for the higher caloric density and will therefore eat less of the other compounds. On the other hand, when diluting the diets, e.g. by adding fiber, one has also to make sure that the animals will still be able to ingest the necessary minimum amount of all essential nutrients. In other words, is the necessary ingested volume of diet too big in relation to the capacity of the stomach. Also the type of fiber needs to be chosen carefully: a certain short-type cellulose (Arbocel R B-00) is known to cause intestinal obstruction and death in rats (Ritskes-Hoitinga and Chwalibog 2003, Speijers 1987).

2.1.1 Fats

Essential nutrients need to be part of the diet in order to maintain good biological functioning. This will secure animal welfare and sound scientific results. De Wille et al. (1993) studied the influence of dietary linoleic acid levels on mammary tumour development. The control diet did not contain any linoleic acid (De Wille et al. 1993). This resulted in premature deaths of a substantial number of control animals as well as in biased results, as linoleic acid is essential for building cell membranes (National Research Council 1995). By leaving out dietary linoleic acid completely, it is possible that tumours cannot develop.

2.1.2 Proteins

Ewen and Pusztai (1999) showed that genetically modified potatoes could lead to crypt hyperplasia in the jejunum coinciding with an increased T lymphocyte infiltration. However, the diet which was fed to young growing rats contained only 6% protein. According to the guidelines of the National Research Council (1995) a young growing rat needs 15% high quality protein in the diet. Such a low protein diet as used by Ewen and Pusztai is therefore considered deficient for young growing rats, exerting a negative influence on the biological functioning, thereby compromising welfare as well as biasing results.

2.1.3 Vitamins

Dietary composition needs to fulfil species specific needs. For example, if a rat diet is fed to guinea pigs, disease and death will arise within a 14 days period due to vitamin C deficiency. Thus, vitamin C is an essential nutrient for the guinea pig, and not for rats. Another complicating factor arises from the shape of the pellet: the diameter of the pellets for rats is larger than for guinea pigs, as rats use their incisivi for gnawing. Guinea pigs will typically take the entire pellets into their mouth and grind it between their molars. So, besides knowledge on nutrient needs, species specific components of ingestive behaviour must be considered as well.

Several animal species perform coprophagy. This process is not prevented when animals are housed on grid floors, as they eat the faeces directly from the anus. Due to bacterial synthesis, animals supply themselves with adequate amounts of vitamin K and B12. As germfree animals lack this bacterial synthesis, they need to be fed with diets containing extra vitamins (Beynen and Coates 2001).

The goals of the experiment determine whether one wishes to leave out an essential nutrient. In case one examines the biological functioning of an essential nutrient, it may be worthwhile to leave it out entirely. However, in some cases it may be more worthwhile to use subdeficient levels instead; deficient levels will probably cause premature deaths, whereas the use of subdeficient concentrations are expected to lead to a more subtle pathology, providing more refined and detailed information on what the function of the nutrient is. This can be seen in line with the development of alternative tests in toxicological studies. Instead of using the LD50 (searching for a dose that kills 50% of the animals), the alternative fixed-dose-procedure provides more detailed and refined information on the working mechanism of a chemical that is tested.

2.2 Types of diets

The use of different diets between experiments and institutes can be a source of variation in experimental results. Therefore, major care should be given to the composition of diets, even when they do not affect health or lead to certain deficiencies. In laboratory animal units, generally two types of diets are used: 1) diets based on natural-ingredients (chow diets) and 2) purified diets.

2.2.1 Natural-ingredient diets

Natural ingredient diets, containing e.g. maize gluten, wheat, lard, corn oil and soya bean meal, are frequently used, as their usual composition makes it relatively easy to produce pellets, which are easy to handle. Also, these diets are generally much cheaper than purified diets. Many “standard” diets for growth/maintenance and reproduction are commercially available for each species. These diets are composed according to the species-specific needs in such a way that the concentration of all essential nutrients for each species and each condition (growth, reproduction) are met more than sufficiently. Depending on where the natural ingredients used in chow diets originate from (soil composition, use of pesticides, etc.) and the type of weather conditions they have been subjected to, the natural-ingredient diets can show large variation, which may be a cause of variation in experimental results (Table 5-1; Ritskes-Hoitinga et al. 1991). There is also variation between diets from different manufacturers (between-brand variation) and between batches from the same firm (between-batch variation) (Beynen and Coates 2001). Because a variable dietary composition can sometimes induce certain pathologies, variably compromised welfare in the form of reduced biological functioning can be the case. For instance, nephrocalcinosis is a condition which is induced in rats and rabbits as a result of higher dietary phosphorus levels (Ritskes-Hoitinga and Beynen 1992, Ritskes-Hoitinga et al. in print). Nephrocalcinosis has been found to be associated with reduced kidney function (Ritskes-Hoitinga et al. 1989, Al-Modhefer et al. 1986), indicating reduced biological functioning.

When producing natural-ingredient diets it is of importance that the minimum essential nutrient needs of each species are met, but at the same time it is essential to refrain from too high nutrient levels that can cause pathological conditions. A specific analysis certificate for each batch of diet is recommended, in order to make it possible for the buyer/researcher to judge the nutrient (and contaminant) levels before the start of each experiment.

Table 5-1. Batches of rodent maintenance diets from 10 different commercial firms fed to young female Wistar rats during a 4 week period (Ritskes-Hoitinga et al. 1991). The range of a different number of parameters is presented here.

Parameter	Group mean range
Growth (g/d)	2.5 – 3.5
Food intake (g/d)	12.1 – 15.6
Water intake (ml/d)	18.2 – 24.8
Incidence of nephrocalcinosis	0/6 – 6/6
Urinary pH	6.2 – 7.9
Caecal weight (g/100 g body weight)	1.8 – 3.9

2.2.2 Purified diets

Purified diets are much more expensive than natural-ingredient diets and are formulated from a combination of more purified macronutrients, pure chemicals, and ingredients of varying degrees of refinement. Purified diets are mainly used when the investigator wants to use a diet of quite a different composition than can be made from natural ingredients, e.g. when investigating (sub)deficient levels of nutrients (Zhou et al. 2003). By using purified diets there is more control of the ingredients and final dietary levels than in natural-ingredient diets, leading to more reproducible results within and between institutes (see also Ritskes-Hoitinga and Chwalibog 2003, Ritskes-Hoitinga et al. 1996). The nutrient levels are mostly close to the desired levels. By following the guidelines of the National Research Council (1995) and the American Institute of Nutrition (AIN93 diet, Reeves et al. 1993) guidelines, a standardised purified diet can be produced that fulfils the minimum required levels of essential nutrients of rodents. The nutrient levels are such that no pathology is induced due to uncontrolled variation or (unknown) superfluousness of certain nutrients.

One of the disadvantages of using purified diets is the fact that the composition is often such that it is unpelletable. Feeding the diet in a powder form to rodents may not meet their (essential?) need of gnawing. Gnawing makes sure that the continuously growing incisor teeth are shortened. If gnawing does not occur, incisor teeth can become too long which makes regular cutting necessary. Therefore, when feeding these soft diets, cage enrichment in the form of wood blocks/sticks may help to prevent these adverse effects, so that welfare becomes positively influenced. One of the disadvantages of using purified diets may be that they lack so far undiscovered nutrients, which can lead to deficiencies, thereby compromising biologically healthy functioning. One example for instance is that in the long-term, purified diets can cause degenerative problems and a decline in reproductive success (Ritskes-Hoitinga et al. 1993). Why this happens is unclear. Caution is warranted when using refined sources of protein (casein), as was demonstrated by Sanders et al. (1984). Five different types of casein were included in purified diets, and it appeared that not all casein sources could promote growth. When offering a choice situation, rats showed a reduced preference for the purified diet that they had experienced earlier which did not promote growth. What applies to different sources of protein, could also apply to other sources of nutrients. By allowing animals to select and compose their diets themselves, more insight in the necessary dietary elements can be made visible. Animals will learn and experience what is good for their well-being and can "tell" us their experiences in preference tests (see also 3.1 in this chapter).

2.2.3 Palatability

Palatability (defined as the relative acceptability of the feed, as determined by taste and texture) is another relevant factor that has to be taken into account, as the minimum level of essential nutrients must be ingested. When an unknown substance has to be mixed into the diet, palatability can be changed. Therefore it is wise to do a pilot study in a few animals, in order to judge the effect, before the real experiment is executed. The palatability of dietary fish oil for herbivorous rabbits was such, that a habituation period of about 6 months was necessary in order to make the rabbits ingest it in the proper amounts, i.e. as is required for a good health (Ritskes-Hoitinga et al. 1998).

2.2.4 Avoiding toxic levels

In order to make sure that animals do not become diseased as a result of toxic levels of nutrients and/or contaminants it is important to keep nutrient and contaminant concentrations similar to or below the "recommended" levels, unless the goal of the experiment is to examine this toxicity. The National Research Council documents provide background data on known toxic levels of each nutrient. In the case of contaminants, various guidelines provide maximum limits (British Association of Research Quality Assurance 1992, Environmental Protection Agency 1979, GV-Solas 2002, guidelines from production firms). These different guidelines do not necessarily provide the same maximum allowable concentrations. One of the most popular guidelines used by toxicologists worldwide is issued by the Environmental Protection Agency (1979). Remaining under these levels for all contaminants, will in general prevent animals and results from becoming negatively influenced. Which guidelines are used, will depend on the purpose of the experiment. If it is known that a certain contaminant will interfere with the particular purpose of an experiment, then the maximum tolerated level will be determined by the concentration known to cause interference. So in this case a specific maximum allowable level can be determined for the particular purpose of an experiment. For obvious reasons, by using purified ingredients, the level of toxic contaminants can be kept much lower than that found in natural-ingredient diets.

2.3 Choice of dietary composition in relation to optimal results and welfare

When designing the experimental dietary composition, important and relevant choices need to be made. These will partly depend on the goal of the study and the animal species. A thorough literature study should precede animal studies in order to avoid unnecessary duplication, suffering and biasing of experimental results. Pilot studies in a few animals under close observation are recommended in case the palatability and/or toxic effects of a certain diet/nutrient concentration are unknown. By using the information from pilot studies, experiments can be designed in a better way, so that an accurate number of animals can be used. Also post mortem examinations are important to reveal unwanted side-effects, as they may be relevant for the interpretation of the study as well as for the welfare. A few examples are outlined below.

Rabbits have often been used as a model in atherosclerosis research, since atherosclerotic plaques arise relatively easy (Jayo et al. 1994). When the effects of dietary fish oil on atherosclerosis in the rabbit were tested, it appeared that the amount of atherosclerosis in the aorta rose with increasing levels of fish oil in the diet (Ritskes-Hoitinga et al. 1998). This was quite unexpected, as dietary fish oil is associated with a reduced level of atherosclerosis in epidemiological studies in the human population (eskimo's) (Ritskes-Hoitinga et al. 1998). Detailed post mortem macro- and microscopic examinations revealed clear adverse effects of fish oil on the liver and a significant positive relationship was detected between the group levels of liver pathology scores and the quantity of atherosclerosis in the aorta. As the herbivorous rabbit usually does not eat fish, it is concluded that the rabbit is not a suitable animal model for testing these long-type unsaturated fatty acids. This is supported by anecdotical evidence about rabbits in the wild: in case of food scarcity, rabbits do eat dead fish found on the beach. As a result of eating the highly unsaturated long-chain fatty acids, the rabbits develop vitamin E deficiency symptoms (yellow fat disease). A similar picture of liver pathology like that resulting from fish oil feeding, arises when cholesterol is added to the rabbit diet in order to induce atherosclerosis. Cholesterol is not part of the normal rabbit diet, and relatively high concentrations of dietary cholesterol will induce liver pathology. It is therefore advisable to use dietary cholesterol levels that do not exceed 0.5 %, at least in cases where the diet contains added fat (Jayo et al. 1994), as this will induce atherosclerotic plaques, but no irreversible liver pathology. Testing fish oil in pigs does not lead to liver pathology: as the pig is omnivorous it can utilise these long type unsaturated fatty acids as well as dietary cholesterol.

Special diets used to induce atherosclerosis in mice are "western-type" diets containing about 21% fat and 0.15% cholesterol, and "atherogenic" diets, which contain 15% fat, 1.25% cholesterol and 0.5% cholic acid (Moazed 1998). This last diet is also referred to as "Paigen's diet". Historically, this diet was used to induce gallstones. This diet is known to be hepatotoxic and to induce a proinflammatory state (Moazed 1998). When using the Paigens diet, atherosclerotic plaques can be induced, however, hepatotoxicity and gallstones are induced simultaneously. Hepatotoxicity may interfere with the development of atherosclerotic lesions, as was seen in rabbits (Ritskes-Hoitinga et al. 1998). Therefore it is considered necessary to (at least) evaluate the condition of the liver. As gallstones are very painful in humans, it may be the case in mice as well.

The use of transgenic mouse models instead of feeding "extreme" diets to wild type mice models may be a good alternative solution, as it then becomes possible to leave out e.g. the cholate from the diet. On a normal chow diet, apoE-deficient mice develop plasma cholesterol levels that are at least 10 times as high as in wild type mice and most of the cholesterol is in the form of the highly atherogenic VLDL (Very Low Density Lipoproteins) form (Moazed 1998). Apo-E deficient mice develop atherosclerotic lesions on a normal chow and on a Western type diet, however, lesions develop more rapidly and at an earlier age on the Western type diet. The atherosclerotic lesions in apoE-deficient mice have strikingly similar pathological characteristics and anatomical distributions as compared to humans (Moazed 1998). The LDL-receptor-deficient mouse (LDL = Low Density Lipoproteins) does not develop atherosclerosis on a normal chow diet, but this can be induced by feeding a high fat diet. Lesion characteristics are the same as in the apoE-deficient mouse, but lesion formation is better controllable by dietary changes (Moazed 1998). Plasma cholesterol levels are lower than in the apoE-deficient mice and thereby more human-like. Although the use of transgenic animal models is preferable in certain studies, there are also negative welfare aspects surrounding their use. In a survey of the information on transgenic mouse strains submitted by researchers to the Danish Inspectorate, 30% of the strains were reported to have considerable welfare problems (Thon et al. 2002). A reduced breeding performance is one of the reported problems associated with breeding transgenic animals. This has led to the special production of high fat diets (9% fat) on the market, in order to attain higher reproduction levels in transgenic strains (Tobin 2003). An increased fat content also decreases the hardness of the food pellets, likely to have a very significant effect on food intake of weaker animals (Tobin 2003). From the point of view that welfare encompasses good biological functioning, the development of transgenic strains exerting poor breeding performance can be considered a negative development. By modifying dietary composition, this welfare disturbance is tackled succesfully. Whether the development of these strains is acceptable is an ethical question, which falls outside the scope of this chapter.

2.4 Methods of food administration

2.4.1 Feeding schedules

Certain feeding schedules can be a possible tool in the experimental design to achieve increased standardisation. Depending on the species and the scientific question, *ad libitum* or restricted feeding schedules are applied. In food restriction, one restricts the energy supply, while still ensuring nutritional adequacy, i.e. that all essential nutrients are supplied in the required minimum amounts (Hart et al. 1995). For dogs, pigs, cats, monkeys, etc. it is considered bad veterinary practice to feed *ad libitum*, as the animals will become obese (Hart et al. 1995). Therefore these species are fed restricted rations. Rabbits are sometimes fed *ad libitum*, at other times restricted, dependent on the laboratory policy. Rodents are fed *ad libitum* in the majority of all experimental studies. *Ad libitum* means that food is available at all times.

Feeding rodents *ad libitum* may be attractive from a practical point of view, however, on the basis of results from long-term toxicological studies, it can be questioned whether this is a sound scientific or welfare approach. *Ad libitum* feeding leads to clearly negative long-term health effects as compared to restricted feeding (75% of *ad libitum* intake): more obesity, shorter survival time, more degenerative kidney and heart disease, more cancer at an earlier age (Hart et al. 1995). *Ad libitum* feeding schedules in Wistar rats can induce severe kidney degenerative disease at an age of 6-9 months. Food restriction prevents this kidney degeneration. Also in the short-term, food restriction can have favourable effects. The relative body weight reduction 48 hours after surgery (jugular cannulation) is smaller in food restricted animals as compared to *ad libitum* fed rats (both at 3-4 and 17-18 months of age) (Hart et al. 1995). It is possible however, that this effect is mainly caused by the reduced size of the body fat compartments in the food restricted animals. It is claimed that animals become more "robust" when fed restrictedly, i.e. they can cope better with experimental stressors/procedures (Keenan 1999). Diluting the diet, e.g. by including a higher fiber content under *ad libitum* conditions, does not give the same positive health effects as compared to dietary restriction. It may be that a certain fasting period each day has a more positive health effect than feeding an energy diluted diet *ad libitum*. Thus, it is mainly the energy intake which is associated with body weight and the degree of obesity that is causal in these health affecting processes.

Keenan (1999) has demonstrated that *ad libitum* feeding is our least controlled experimental factor in the laboratory. Considerable variation in experimental results from rodents on *ad libitum* feeding schedules arose during the 1980's to 1990's. One of the possible explanations could be the continuous selection of faster growing individuals in outbred strains (Keenan 1999). Feeding rodents at around 75% of the *ad libitum* food intake is recommendable for long-term toxicity studies in order to make a sufficient number of animals survive the requested 2 year period (Hart et al. 1995). Under *ad libitum* feeding conditions, the body fat content can be more than 25% (Toates and Rowland 1987). By restricting food intake to 85% of *ad libitum* intake, the body fat content will be less than 10%, which is similar to that found in wild-captured animals (Toates and Rowland 1987). In order to make sure that all individual animals eat the same amount of food, it is advised to individually house the animals (Keenan 1999). However, the consequence may be a reduced welfare due to the level of food restriction and isolation, as rodents are social animals. In order to improve welfare, one of the labour intensive possibilities is to feed animals individually, whereafter they come back into the group. In line with agricultural practice, we are currently examining the use of microchip and computer technologies to develop automated feeding devices for feeding individual rats restrictedly under permanent group housing conditions. This is expected to improve health (cause less pathologies) and at the same time fulfil the need for social housing.

2.4.2 Pair-feeding

If voluntary food intake of the test group differs from the controls, it may be necessary to equalize the intake between the groups. This can occur when the palatability of the test diet is negatively influenced, or in cases where the substance influences health negatively, causing a reduced food intake in an indirect manner. From the point of view of standardisation, the test and control animals must ingest a similar amount of food at a similar time of day, otherwise it will become impossible to solely judge the effect of the test substance. Whether a reduced food intake has a negative influence on biological functioning, depends on to what level the food intake becomes lowered. As the NRC requirements are based on obtaining maximum growth, a level of 75% of the NRC recommendations is still considered sufficient to fulfil all needs. Four possible methods for achieving pair-feeding are: (1) weighing; (2) coupling of food dispensers; (3) gavage/permanent stomach cannula; (4) feeding machine. A feature of method (1) is that the amount of food eaten by the test group is weighed, and the control group is fed the same amount of food the next day. A disadvantage of this method can be that the total amount of food is provided at once to the control animals, perhaps at an unnatural time point of the day (the light resting phase in rodents). This may induce quite a different eating pattern in the controls as compared to the test animals, preventing proper comparison between test and control animals. In method (2), by using food dispensers, rats can be trained to obtain a pellet by pressing a lever. By coupling food dispensers of one test and one control animal, the dispenser of the control rat will provide a pellet at exactly the same time as the test animal "asks for" and eats a pellet. This system creates an optimal standardisation of amounts eaten and ingesting patterns. In method (3) permanent stomach cannula's and gavage make it possible to provide all animals with exactly the same amount of food at the same time points (Balkan et al. 1991). In method (4), feeding machines with regulated opening of valves make it possible to open and close food hoppers at the same time points of the day in test and control animals.

Meal training can be another possible solution as a pair-feeding schedule. Rats can easily be trained to ingest the required amount of food in a limited amount of time. Within a week they can learn to ingest the necessary food intake in e.g. 2 meals of 0.5 hr during the light phase (Ritskes-Hoitinga et al. 1995). Also one meal of 2 hours per day can be sufficient for a rat to obtain the necessary energy and nutrients. This will depend on the physiological stage of the animal. The exact time of day when the food is offered is critical in relation to the welfare, and is discussed under the section on natural behaviours. There are clear species differences in the rhythmic patterns and training/learning abilities. Although rats can be trained within one week to eat one or two meals per day during the light phase, hamsters could not cope with a schedule of one meal of 2 hours per day. A very rapid drop in body weight (20% in one week) occurred in these hamsters, making it necessary to stop this experiment (Ritskes-Hoitinga, unpublished observations). A restricted feeding schedule will usually lead to a more efficient handling of the diet, which leads to e.g. a slower gastrointestinal passage time and a hypertrophy of the gastrointestinal wall. This ensures that the animal can cope with the situation, despite the fact that the feeding schedule can be unphysiological and uncomfortable.

2.4.3 Gavage / stomach-tubing

When compounds such as pharmaca or nutrients are to be tested, it is important to avoid the possible negative influence of a bad taste on food intake. Therefore, gavage is used in order to apply meals/substances directly into the stomach. Upon comparison of the application of a meal directly in the stomach with the voluntary eating of a similar meal, a difference in experimental results was obtained (Vachon 1988). After the voluntary consumption, the results were more comparable to results from human studies. Disadvantages of stomach tubing are that it omits the oropharyngeal process, thereby omitting the physical effects of chewing and the addition of salivary enzymes that initiate the digestive process. Gavage can also cause stress to the animals, thereby suppressing gastrointestinal activity. By training the animals to become accustomed to the procedure and giving them positive rewards, the use of the stomach tubes becomes gradually less stressful. Another possibility is to insert permanent cannulas in the stomach (or small intestine), which makes it possible to apply substances without stress directly into the stomach or small intestine (Strubbe et al. 1986a).

These permanent cannulas are e.g. used to measure the (satiety) effects of filling the gastrointestinal system with purified nutrients such as glucose at different anatomical locations. These cannulas can also be used to make sure that chemicals and radioactive substances are applied directly into the gastrointestinal system without risk of contamination of the environment and the person executing it.

3. POSSIBILITY TO PERFORM NATURAL BEHAVIOURS

3.1 Behaviour in the laboratory

Although restricted feeding schemes and solitary housing have advantages for standardisation and execution of experiments, there are also opinions that restricted feeding schedules and solitary housing may affect welfare adversely, because it is unnatural. However, it is questionable whether natural conditions always induce better welfare, since nature provides the free-living animals with predation and various kinds of infectious threats. Although predation and health risks like infections are usually excluded, there are still many disadvantages, even when natural condition-like situations in the laboratory are introduced. The introduction of various natural behavioural repertoires for the rat is discussed. Under *ad libitum* feeding conditions, rats will eat most of their meals during the dark phase and only a few meals during the light phase (Spiteri 1982). This is considered a remainder from natural behaviour where the light period is more dangerous due to a heavy predation risk and is probably genetically determined. There are approximately 2-3 peaks in food intake during the dark phase. The first peak arises in the first few hours after the start of the dark phase and is probably needed to compensate for the energy deficit which has arisen during the light (resting) phase. In the middle of the night a relatively small peak is seen. During dawn another peak occurs, even though the stomach is still filled. The dawn peak is considered necessary to build reserves, in order to successfully overcome energetically the dangerous light phase where predation pressure is the highest. This dawn peak will occur typically in the 2 hours before it gets light. Upon changing the dark-light schedule, the dawn peak will always shift to the 2 hours before the start of the light phase. When food is removed in the 2 hours before the start of the light phase, the rat will not learn to consume extra food at an earlier time point, but compensates during the day. By eating in advance of the period of sleep which is during the light phase, the rat does not need to come into the open when the predation pressure is high. Thus, this feeding behaviour is probably the result of natural selection and genetically determined in the body clocks. The circadian clock directs/controls the daily feeding activity, and is located in the suprachiasmatic nucleus (SCN), which is also called light entrainable oscillator (LEO). If the SCN is lesioned, the typical day-night rhythm in food intake behaviour immediately disappears (Figure 5-1, Strubbe et al. 1987).

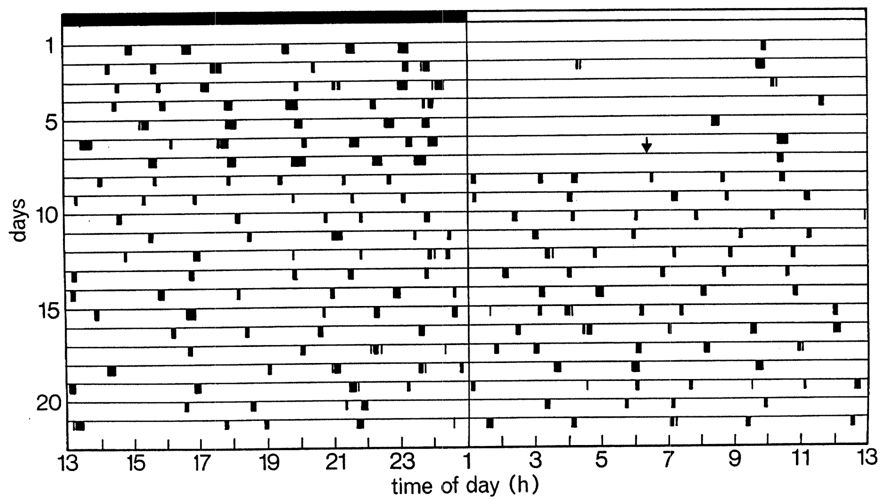

Figure 5-1. Daily pattern of food intake of a rat on consecutive days. The black squares represent bouts of feeding activity. On day 7, the arrow marks the time of electrolytic lesion of the suprachiasmatic nucleus. The horizontal black bar indicates the dark phase. (From: Strubbe et al. 1987)

Rats can be trained to eat one or two meals per day, but the internal rhythm of the circadian clock is maintained. Therefore, in these scheduled feeding designs, there is a strong interference with the circadian feeding behaviour and concomitant gastrointestinal physiology. As soon as the *ad libitum* food intake is reinstalled, rats will immediately revert to their original pattern of food intake (Spiteri 1982). Well-known effects are gastrointestinal problems associated with jetlag and shiftwork in humans, when subjects are “forced” to eat at a different time in the daily cycle to that dictated by the internal clock.

Although food intake on a regular chow diet can be used for numerous experiments, nature provides a greater variety of ingredients. The question can be posed whether a greater variety and the opportunity to choose and select is better for welfare? When rats were given the choice between various diets providing energy from different sources, the rats chose carbohydrate rich diets in the evening hours, and fat rich diets just before the start of the light period (see Figure 5-2, from Strubbe 1994a). The function may be that carbohydrate rich diets will quickly provide the animal with an easy accessible energy source, necessary to stop the deficits that have arisen during the light phase. On the other hand, eating fat rich diets (with their higher energy content per gram) creates energy reserves for covering the next light phase, with the initial fasting/resting phase (Strubbe 1994a). Another example is that monkeys which had access to a herb garden in the Zoo Apenheul, made a selection of specific herbs which was in accordance to their health state.

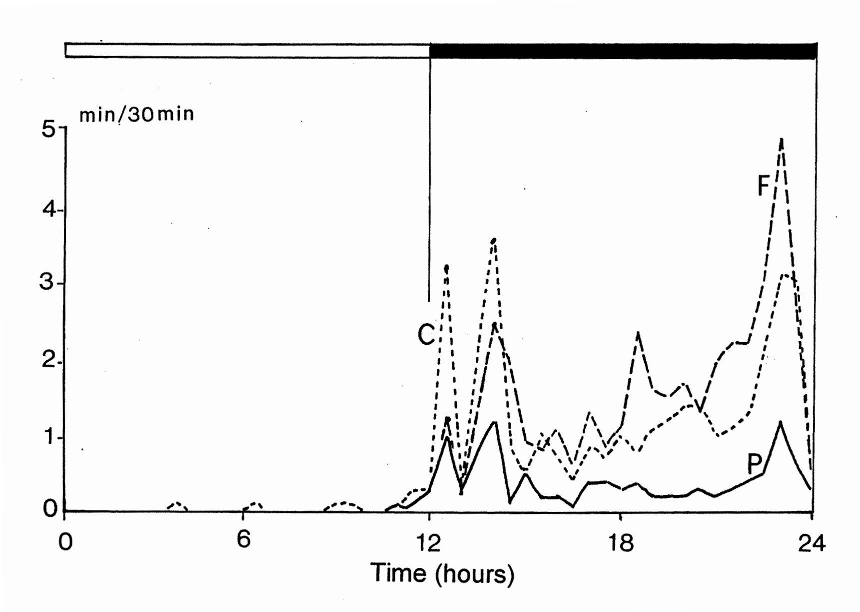

Figure 5-2. Daily rhythm of time spent consuming macronutrients in the rat. C = Carbohydrate, P = Protein, F = Fat. (From Strubbe 1994a)

Food intake is adjusted in accordance with energy expenditure. At lower ambient temperatures, the extra heat loss will be compensated by increased food intake. During increased levels of energy expenditure, the rat will in first instance compensate by increasing meal size. Thereafter, the meal frequency will change as well, when an increased meal size is not sufficient. During lactation, especially the third week postpartum, females will even eat meals during the initial stages of the light phase as well, in order to meet the high energy demands (Strubbe and Gorissen 1980). In diabetic animals the loss of glucose via the urine is immediately compensated by increasing meal size, whereas frequency and timing of meals are unaffected (Strubbe 1994b).

Usually, ingestive behaviour is associated with food-anticipatory behaviour, which in the rat is expressed as increased locomotor behaviour. This is particulary clear when rats are kept on a restricted feeding schedule, e.g. a one hour meal per day. This food-anticipatory behaviour gradually disappears when *ad libitum* conditions are reinstated (Stephan 1984, Brinkhof et al. 1998). If the SCN is lesioned, the rats will still display the food anticipatory activity (Stephan 1984). This suggests that another rhythmic clock determines meal-associated rhythms (the Food Entrainable Oscillator = FEO). Food anticipatory activity may be a functional reaction, in that e.g. the body temperature rises, probably by increasing the efficacy of enzymatic processes involved in nutrient handling (Strubbe and van Dijk 2002).

3.2 Experimental design and circadian rhythms

Experimental animals can be diurnal or nocturnal, i.e. being active during the light or dark phase, respectively. Some species do not have a clear circadian rhythm, e.g. the guinea pig is active during the entire 24 hours per day and has short periods of rest in between activity periods. Many experiments in the laboratory are performed during daytime, when nocturnal animals like rats are normally asleep (Toates and Rowland 1987). If food is presented during the light phase only, rats quickly learn that food has to be ingested at that time as well. However, the circadian pacemaker rhythm remains and does not adapt: as soon as ad lib conditions are reinstated, the rats will immediately return to the original natural ingestive pattern of food intake (Spiteri 1982, Strubbe et al. 1986b, Strubbe 1994b, Brinkhof et al. 1998). By feeding only during the light phase instead of the dark phase, substantial differences in physiology, neurochemistry and behaviour can result. Also the response to pharmacological agents can be different (Toates and Rowland 1987, Claassen 1994). The circadian pacemaker rhythm does not change, but the feeding schedule dictated by the experimental design demands adaptative behaviour. Feeding rats during the light phase can be compared to the effects of shift work in humans. Shift work is associated with disturbed physiology, pathological stomach bleeding, disturbed sleeping behaviour and disturbed food processing in humans. This is because the physiological and behavioural responses are not in accordance with the internal biological clocks. This can result in reduced health and welfare and negative interference with experimental results, unless of course shift work is the focus of study. The following experiment will illustrate how this "shiftwork" induces a less optimal digestive processing of food. The bile flow in rats provided with a permanently implanted bile fistula, shows a clear circadian rhythm under *ad libitum* feeding conditions, with the highest levels during the dark phase, where most food intake takes place (Vonk et al. 1978). Feeding six meals of 0.5 hr each throughout the dark phase gives a virtually identical bile flow response as that under "real" *ad libitum* feeding conditions (Figure 5-3, Strubbe unpublished observations). When rats are offered six meals during the light period only, the bile flow shows a distinctly different pattern: there is a reduced bile flow at night time, and there is a peak in the bile flow, each time a meal is eaten. As the absolute level of the bile flow (ml/hr) is less when being fed during the light phase as compared to the feeding of six meals during the dark phase, the digestive process is expected to be less efficient. Therefore, if it is the goal to study rats that eat e.g. for a few hours per day, feeding the rats during the dark phase instead of the light phase is in better accordance with their gastrointestinal physiological activity patterns. In order to make this practically possible, the dark phase can be shifted to a time point of the day which is more convenient for the experimenter/personnel.

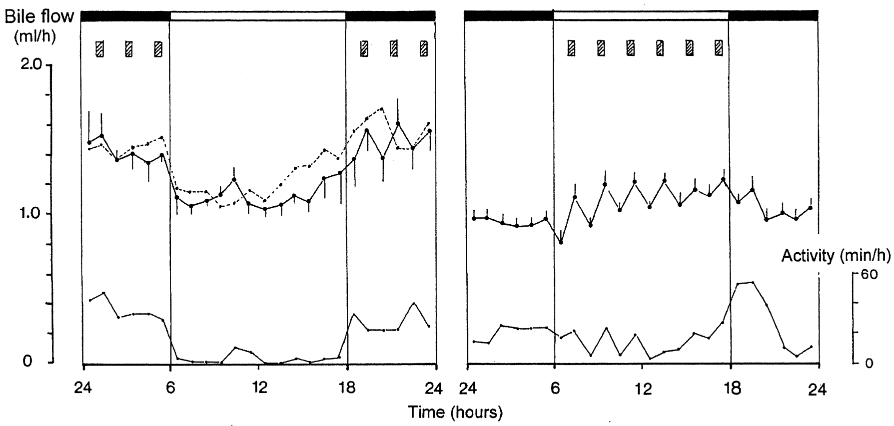

Figure 5-3. Bile flow and physical activity in rats during the daily cycle. Bile flow was measured with permanently implanted bile fistulas. In the lower graphs a measure for general locomotor activity is depicted. The left side of the figure shows the bile flow in a normal *ad libitum* condition (dotted line) and when a meal schedule is offered in 6 meals (dashed bars) spread over the dark phase (black bar on top). The right part of the figure shows the bile flow and concomitant activity when meals are spread over the light phase. (Strubbe unpublished observations)

Rabbits fed a restricted amount of food just before the dark phase, when in nature most of the food is eaten (Hornicke et al. 1984), have a low frequency of stereotyped behaviour (Krohn et al. 1999), compared to rabbits fed a restricted amount of food in the morning or rabbits fed *ad libitum*. As stereotyped behaviour is thought to reduce well-being, these results indicate that by feeding animals a restricted amount of food at a natural time point of the day, welfare can be increased. Not only the timing of providing the food is important, but also the schedule. For instance, when providing rabbits with a restricted amount of food (60% of *ad libitum*) at the beginning of the light phase (7.30 am), a significantly increased food conversion and water intake occurred, compared to rabbits that had food freely available from 7.30 am to 2.30 pm, even though the food intake in grams per day was similar (Table 5-2; Ritskes-Hoitinga and Schledermann 1999). These examples illustrate that it matters how a restricted feeding schedule for a certain species is executed.

Table 5-2. The influence of various dietary restriction schedules in rabbits during a 6 week period. (Ritskes-Hoitinga and Schlederman 1999)

Parameter	AL	FR	NFR	DFR	Probability (P)
Growth (g/d)	30.1±1.6 [a]	13.5±0.3 [b]	18.2±1.5 [b]	30.1±3.0 [a]	<0.05
Food intake (g/d)	206±18 [a]	125±0 [b]	126±3 [b]	213±7 [a]	< 0.05
Food conversion (g food/g growth)	6.8±0.3 [a]	9.3±0.2 [b]	6.9±0.5 [a]	7.1±0.7 [a]	< 0.05
Water intake (g/d)	255± 34 [a]	381±46 [b]	244±44 [a]	284±6 [a]	< 0.05

AL = *Ad libitum* feeding, fresh food supplied at 7.30 am each day
FR = 60% of the AL amounts eaten was provided at 7.30 am each day
NFR = Food was freely available from 7.30 am to 2.30 pm.
DFR = Food was freely available from 2.30 pm to 7.30 am.
Means and SD are given for 3 rabbits per group.
Results in the same row not bearing the same superscript are significantly different.

Rats in the wild show a neophobic reaction towards novel objects in their environments and will often avoid them for days. Novel food evokes a similar careful behavioural pattern. After carefully sampling a small amount of unknown food, the rat learns to distinguish safe from poisonous foods, and nutritionally poor foods from those of a higher value (Nott and Sibly 1993). When food has proven to be "safe", larger amounts will be eaten by the rats. Knowledge of these basic behaviours is relevant, in order to design experiments effectively. Upon changing dietary composition in meal-trained rats (cross-over design), a typical neophobic response results in an immediately reduced food intake (Ritskes-Hoitinga et al. 1995). If one wants to perform a cross-over design, animals must have been accustomed to this typical dietary composition in an earlier phase. So, when they are exposed to this diet a second time, they will not reduce their food intake. Diets varying in macronutrient composition can influence behavioural and physiological stress responses, as was found by Buwalda et al. (2001). A high dietary fat level appeared to have an ameliorating effect on behavioural and physiological parameters as compared to a diet with a high carbohydrate level, after rats were exposed to psychosocial or physiological stressors. So it has to be established whether rats wish to eat "functional foods" in order to cope better with stressful situations.

3.3 Group housing

Rats in the wild live in colonies and have social interactions. The question can be posed whether group housing is always favourable for welfare or not? The way we house the animals in the laboratory and the husbandry conditions can influence both the amount of food they ingest as well as their meal patterns. Whether an animal is group or individually housed has implications for the food intake and the results. Individually-housed mice of both sexes had a higher food intake than group-housed mice (2, 4 or 8 mice per cage) (Beynen 2001, Chevdoff et al. 1980) The mice housed individually or at 2 per cage had a higher body weight and body weight variability than the other groups. In case the mice were housed 8 per cage, an increased gastritis frequency occurred compared with individually housed mice (Chevdoff et al. 1980). These results indicate that 4 mice per cage was the optimum group size in this particular study. Peters and Festing (1990) showed that the maximum body weight gain at different cage densities depended on the cage size and the mouse strain chosen: the inbred Balb/c thrived best in a high density housing, whereas the outbred MF1 had a higher body weight gain during low density housing. If mice are housed in a group, they usually lie together, thereby reducing the total surface area as compared to individual housing. A reduced heat loss per animal in the group is the result, and due to this "behavioral thermoregulation" the food intake becomes reduced. It has been hypothesized that individuals from an inbred strain may produce identical pheromones, which may make them more tolerant to high density (Peters and Festing 1990).

Another aspect of group housing concerns the possible interference of the social status of the individuals. Behavioural and ingestive patterns can be influenced by the social structure in the group. A dominant animal may prevent others from eating at certain times. On the left side of Figure 5-4 food intake patterns of a socially compatible group of S3 rats have been given: no perceivable dominant rat is present in this colony. The normal feeding pattern with a clear dawn peak can be seen for all individuals. On the right side, the dominant animal (D) prevented all the other rats lower in hierarchy from having a dawn peak at the natural time point. Some had to shift to an earlier time point during the dark phase, and some even shifted to the beginning of the light phase (Strubbe and Koolhaas, unpublished observations). It is so far unclear whether the phase of circadian rhythm had shifted permanently or not.

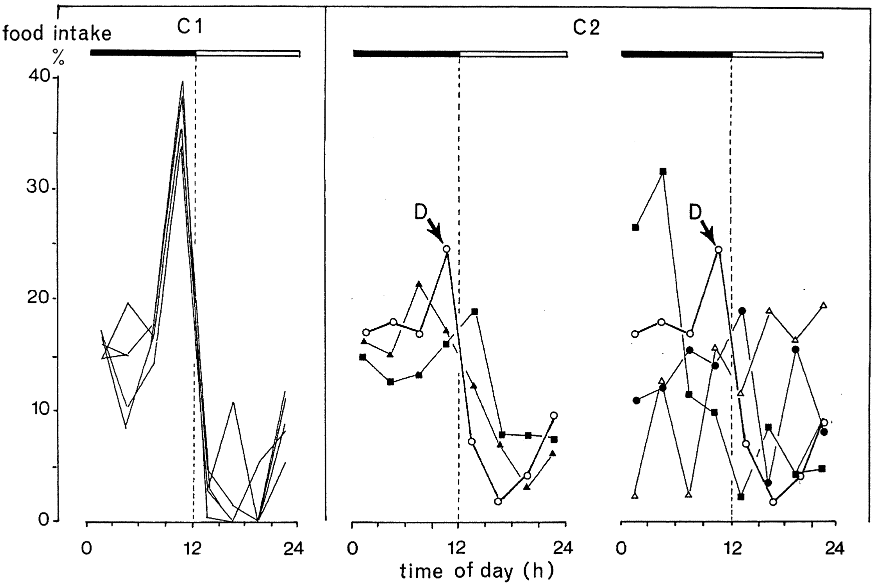

Figure 5-4. Feeding activity of members of two rat colonies. The left part of the figure shows a relatively calm colony (C1) without a dominant rat. Rats are feeding together at dawn at the feeding place. In the right part, the feeding rhythm of a colony (C2) is depicted where a dominant rat (indicated by D) is present. On the left side of C2, the D and subdominant rats are depicted. Notice that D eats at the same time (dawn peak) as the members of colony C1 and that the subdominants eat in the proximity of the dawn peak. In the right part of C2, the feeding activity of the other more submissive members of the colony are depicted in comparison with D. The "dawn peak" of the submissive animals shifts more than those of the subdominant rats. The dark phase is indicated by the black bars on the top of the figures. (Strubbe and Koolhaas, unpublished observations)

Thus, group housing can also differentially influence the experimental outcome, either by influencing food intake/meal patterns and/or due to other unknown factors. For instance, by feeding a high phosphorus diet (0.5% P) to females of the RP and LE strains, nephrocalcinosis was induced in the RP strain only. The severity of nephrocalcinosis in the group-housed RP rats was significantly higher than in individually-housed animals (Ritskes-

Hoitinga et al. 1992). This illustrates the interaction that can occur between genetic and environmental (dietary composition and housing) factors. Thus although group housing is more natural than individual housing, no clear-cut general guidelines on the optimum group size can be given. It depends on the purpose of the experiment, the species, the strain of animals used, the sex, the age, the experimental design, etc., which optimum group size needs to be chosen. Social interactions in group-housed animals can also lead to (chronic) stress responses, thereby compromising the welfare of some individuals and reducing the standardisation of results. We observed in a colony of female group-housed Beagle dogs, that even though the animals had been separated for the duration of the meal (half an hour), they could vomit up to 4-6 hours later (Ritskes-Hoitinga et al. unpublished observations). It is clear that this caused a large variation in results, as the stomach contents were eaten by one other individual. In the case of dogs it is very important to socialize and train the animals accurately in order to establish social compatible groups. A good socialisation and training program will make dogs feel secure and will create a stable hierarchy in the group. In the case of male animals it may be unwise and impossible to group-house them under laboratory conditions, due to aggressive attacks without possibilities for escaping or hiding. Therefore it is likely that the formation of compatible social groups will add to achieving standardisation of results as well as optimal welfare.

3.4 The nutritional state of fasting

Fasting (food deprivation) is commonly used in pharmacological research. It can be used to standardise experiments: an overnight fast assures that the last meal has been taken at least 12 hours before. This guarantees that there cannot be any interference with this last ingested meal. However, as a result of fasting, the animals are in a quite different metabolic state (Strubbe and Prins 1986). Sometimes it is necessary to have an empty stomach to judge the absorption and bioavailability of medicine after oral application, without interference of dietary components. In other experiments animals are made hungry, so that they will immediately eat a meal as soon as it is offered, or use food as a reward to quickly train animals. The time which is used for fasting varies considerably: rats are sometimes fasted up to 72 hours (Claassen 1994). When fasted blood samples need to be collected, usually rats are fasted overnight. Vermeulen et al. (1997) examined how quickly the rat stomach is emptied. They found that the stomach is empty after just 6 hours of food deprivation. If one wishes the intestines to be empty as well, a 22 hour period of food deprivation is necessary (SGV newsletter 2001). When the food deprivation period in rats exceeded a 6 hour period during the dark phase, a clearly increased locomotor and grooming behaviour and increased hair contents in the stomach was detected (Vermeulen et al 1997). No stereotyped behaviour was seen in the rat, in contrast to food-deprived mice (Schlingman et al. 1993). Because of their small body size and high metabolic rate, mice are extremely sensitive to fasting and may die when it lasts too long. The increased locomotor behaviour in rats fasted for longer than 6 hours was thought to be the result from an increased food searching behaviour. For nutritional studies, fasting is not recommended, as the effects of the diet/nutrients are the goal and not the effects of fasting. In some cases fasting cannot be avoided, as high levels of blood lipids can increase turbidity in the blood, which makes the analysis of certain nutrients impossible. From the point of view of the rat and sound results, a fasting period exceeding 6 hours during the dark phase is not necessary when the goal is to obtain an empty stomach. Thus, dependent on the scientific question, one can apply the fasting condition. However, one has to be realize that a different metabolic rate is present at the time of testing due to the fasting condition. Thus, although, long fasting periods can reduce welfare, short-term fasting can be used to standardise experiments.

3.5 Essential needs in the environment

The execution of certain natural behaviours in the laboratory setting are an important prerequisite for good animal welfare, as they represent basic essential needs. It is a well-known fact that zoo-animals develop stereotyped behaviour, because some essential needs, especially in the field of feeding and locomotion are not met. Also caged laboratory mice develop stereotyped behaviour. In the agricultural setting pigs can develop e.g. sham-chewing and tail-biting as a stereotyped behaviour, as their need for rooting for food is not satisfied. Chickens normally use a large part of their awake hours searching, scratching and pecking for food. When they are housed in batteries, many develop feather pecking behaviour, which may be the result from not fulfilling their food searching needs. Which essential needs have to be fulfilled, depends on the species and circumstances. Each species has its own specific needs, which need to be taken into account. An attempt has been made to prioritise categories of needs at the Dahlem workshop nr. 87 in Berlin (Broom 2001). Providing rats and mice with food, water, social contact and the possibility to sleep are presuppositions for basal welfare. Deficiencies of these factors will lead to poor welfare. By providing the possibility of mating, climbing, nest building, rearing and additional space (curiosity satisfaction), conditions are created for good welfare (Gartner 2002).

3.6 Working for food

Rats in nature need to find their food in the neighbourhood of their nest. Therefore, they have to perform action. In the laboratory situation the food is always close or delivered at fixed times, i.e. there is no need for seeking for food. Rats with a permanent stomach cannula, had learned to supply food directly into the stomach by pressing a lever (Strubbe et al. unpublished observations). The rats were able to maintain their normal body weight, however, "sham chewing" near the food hopper was observed. This may indicate that the satisfaction of oropharyngeal factors are an essential part/need of the ingestive process.

Rats have been made to put more effort for obtaining food by shortening the distance between the food hopper bars (Figure 5-5, Strubbe, unpublished observations). The first time they had to work their way through, they spent a considerable amount of extra time gnawing their way through the decreased spaces to gnaw off the food. But, after a certain period they worked through the narrowed distance between the bars much more efficiently. The final result was that they used about the same amount of time on the ingestive process as compared to the situation before. This indicates that working for food may quickly loose its value as a possible enrichment factor.

Another example of working for food is when rats choose between working for food or eating freely available components (Kaufman and Collier 1981). Sunflower seeds with and without hulls were offered. After about 5 days, rats developed a strong stable preference for seeds without hulls. The range of preferences depended on the individual rat: 68.6 – 94.9% of total sun flower seed intake consisted of seeds without hulls. This indicates that rats preferred the food item with the lowest handling cost after a habituation period of about 3-7 days, but that the preference was not absolute (Kaufman and Collier 1981). The fact that the rats did not develop an absolute preference could be a failure of the rat to discriminate, or could be an adaptive strategy that makes it possible for the foraging animals to monitor a changing environment, or may indicate a partial preference, i.e. part of an optimal diet needs to be without hulls. Rats are expected to monitor the consequences of consumption indirectly, as the consequences of ingestion are usually not immediate. They will judge the caloric and nutrient content, but are also sensitive to the cost it takes in time and energy in order to obtain a certain food (Kaufman and Collier 1981).

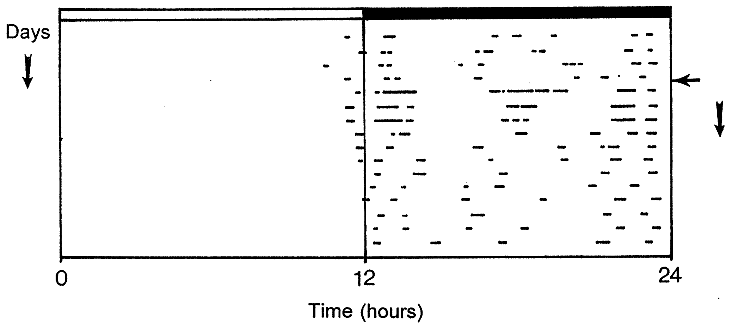

Figure 5-5. Daily pattern of food intake of a rat on consecutive days. The black lines represent bouts of feeding activity. On day 5, the distance between the foodhopper bars was shortened (arrows), requiring increased effort to obtain food through the bars. Notice the large meals of extremely long duration in the beginning. After a few days, the animals could work their way through the narrowed food hopper bars just as fast as before (Strubbe unpublished observations).

It is difficult to determine how much time/energy ought to be spent on working for food. The choice the animals make is not necessarily leading to an improved welfare. Sometimes the animals are inclined to eat a large amount of easily available food fast, in order to make energy deposits. This in turn, may have a negative impact on the animals health and welfare in the long-term. As a result of domestication and selection, animals may change. This may also change their needs for optimal welfare. Upon comparison of the behaviour of the white leghorn and its ancestor the wild jungle fowl, a remarkable difference occurred in the food searching behaviour. The chickens got the choice between freely available food and food that was hidden in a semi-natural environment. The white leghorn chose to eat 70% of the total amount of food from the freely available food. In contrast, the wild jungle fowl ingested 70% of the total diet from the hidden food (Jensen 2003). This illustrates that there is a behavioural adaptation to an increased production level.

4. CONCLUDING REMARKS

In this chapter many experimental conditions in relation to feeding have been discussed and advantages/disadvantages for animal welfare have been evaluated. What implications do they have for research on animals with regard to the animals welfare and the standardisation of experimental results?

Providing animals with food that fulfils their species-specific nutrient needs is a necessary item for securing basal welfare and reliable experimental results. However, in what way we need to provide the food in order to maintain or increase animal welfare is an important question. Is it enough to fulfil essential nutrient needs, or do we need to do more? Is it important for the animal to have the possibility to select from various food items, i.e. have more influence on composing the diets themselves? When rats were offered various diets differing in macronutrient composition, their preference varied with the time of the night. This is an indication that the needs may vary during the circadian cycle. More investigations are needed to solve whether self-selection of food is important for animal health and welfare. Whether it is important for the animals' well-being to work for obtaining food, remains a subject of debate. When given the choice, domesticated chickens clearly show a lower preference for obtaining food by working for it as compared to their wild ancestors. However, the percentage of food obtained by working is still 30%. Results from preference tests with rats give the same result. This indicates that animals in captivity still like to work for food, at least to a certain extent. The method that can be used to make animals work for food will depend on the species-specific behaviour and needs. In case a method is chosen, it is not certain that it will remain satisfactory as it can quickly loose its novelty value as was shown in the example where rats initially had to increase their efforts for obtaining food through narrowed distance between food hopper bars.

As *ad libitum* feeding in long-term toxicity studies have been clearly associated with negative health effects, restricted feeding is advised. How and when to feed is important for the animals' welfare, as e.g. the frequency of stereotyped behaviour in rabbits is increased when they are fed *ad libitum* or restrictedly at an "unnatural" time point of day as compared to restricted feeding at a "natural" time point (just before the dark phase). Stereotyped behaviour will induce increased variation in results, as the degree of behaviour will vary for each individual and thereby the effects on energy utilisation. We believe that an adaptation of feeding schedules to the normal circadian patterns will contribute positively to welfare as well as standardisation of results. Feeding at "unnatural" times will cause wider fluctuation and perturbed physiology and will bring animals out of homeostasis, thereby decreasing welfare. Although group housing of social species is preferred over individual housing, it may be unpractical (e.g. catheterized animals, aggressive males) and a source of unwanted extra variance. Dependent on the species, sex, experimental goals, etc. groups should be composed and monitored carefully, as group composition and behaviour can compromise standardisation and welfare.

Whether variation in food items provided is an essential factor to increase welfare is a matter of debate. Providing this dietary variation may be in conflict with the striving for standardisation of results, as the choice of individuals may be different. Also the choice of individuals during the circadian cycle may vary, contributing to a higher variation in experimental results. An indirect indication of how animals subjectively value the provision of food variation is obtained in preference testing, also involving the level of effort animals are willing to invest for obtaining this goal. By evaluating the level of investment an animal is willing to give, an indication of the motivation to reach a certain goal can be obtained.

Trying to improve welfare and fulfilling the animals' needs for a good welfare may give a potential conflict with trying to standardise experiments and reducing variation in results. On the other hand, an improved welfare may also lead to an animal that is more in balance physiologically and psychologically, thereby resulting in reduced variation as it can cope better with environmental challenges. This will depend on the circumstances and parameters measured. By carefully monitoring the animals' species-specific physiology and behaviour in experimental studies exploring the relation between nutritional factors, welfare and variation in results, more insight into these factors can be obtained.

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