University of Groningen

Nutrition, Feeding and Animal Welfare

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Published in:
The UFAW Handbook on the Care and Management of Laboratory and Other Research Animals, Eighth Edition

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2010

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Savenije, B., Strubbe, J., & Ritskes-Hoitinga, M. (2010). Nutrition, Feeding and Animal Welfare. In R. Hubrecht, & J. Kirkwood (Eds.), *The UFAW Handbook on the Care and Management of Laboratory and Other Research Animals, Eighth Edition* (pp. 183-193).

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Download date: 21-06-2022

14 Nutrition, feeding and animal welfare

Bart Savenije, Jan Strubbe and Merel Ritskes-Hoitinga

Introduction

It is essential for the welfare of animals that adequate food, containing essential nutrients, is provided and ingested (National Research Council (NRC) 1995; Beynen & Coates 2001; Ritskes-Hoitinga 2004). Physical and mental processes are dependent on and influenced both by what is ingested, and when and how food is eaten (Ritskes-Hoitinga & Chwalibog 2003). Food ingestion is essential for homeostasis, a regulated state of internal balance (Strubbe 2003; Ritskes-Hoitinga & Strubbe 2004), although it always leads to some homeostatic disturbance, for example through the thermogenic effect. As many bodily functions naturally show biological rhythms, fluctuations due to food intake may result in good animal welfare, as long as the animal is able to return to the homeostatic state. Introducing species-specific fluctuations into the laboratory feeding process may, therefore, contribute positively to the welfare of laboratory animals.

This chapter provides some key concepts in nutrition, with the aim of helping animal care staff to prevent the occurrence of nutrition-based pathological and behavioural disorders in animals in their care. Using and implementing this basic knowledge will not only support good animal health and welfare, but will also contribute to standardisation and replicability of experiments. In the second part of the chapter, aspects related to feeding, rhythmicity and environmental factors and procedures are discussed. A range of examples is provided to illustrate how nutrition and feeding can influence animal welfare and experimental results. The authors suggest that better animal welfare can be achieved when nutrition is better adapted to the species-specific adaptive capabilities for returning to homeostasis (Ritskes-Hoitinga & Strubbe 2004). Thus, refining the feeding process by providing correct nutrition, together with appropriate foraging and feeding opportunities, should contribute to the animals' welfare as well as improve the quality of the science by reducing variation resulting from stress.

Proper study design

Minimum nutrient requirements

Animals' needs for macronutrients (lipid, carbohydrates, proteins) and the micronutrients (minerals, vitamins, trace elements) and water differ between species. For example, vitamin C is an essential nutrient for guinea pigs and primates, whereas rats and mice do not need to ingest it. For each animal species extensive scientific documentation on (essential) nutrient requirements has been assembled in the documents of the NRC (1977–2003). Estimated nutrient requirements for each species and stage of life are presented by the NRC (1977–2003). These recommendations are usually higher than the minimum requirements, as they are often based on the intention of obtaining maximum growth. Maximum growth is, however, probably not optimal for the health of laboratory animals. For example, *ad libitum* feeding can result in kidney degeneration in rodents, whereas food restriction prevents kidney degeneration completely (Hart *et al*. 1995). As these guidelines are currently the best available and are based on scientific evidence, it is advisable to use them until new scientific evidence comes available. However, it is important to be aware that there can be strain differences in nutritional requirements, and that this may also apply to genetically modified strains (Ritskes-Hoitinga 2004). For example, in phosphatidylethanolamine N-methyltransferase (PEMT) knockout mice, the *de novo* synthesis of choline is disrupted, so the NRC's minimum dietary choline recommendation for mice is likely to be insufficient for this strain (Zhu *et al.* 2003). In such cases it is necessary to establish the nutritional requirements of the strain, in order to provide a special diet fulfilling those particular needs.

It is also essential to provide for animals' differing needs during their various life stages (ie, growth, maintenance, reproduction, lactation or work). Failure to do this can lead to incorrect interpretation of experimental results as in the report that genetically modified potatoes could compromise the immune system of young rats (Ewen & Pusztai 1999). In this study, the diet only provided 6% protein; it was later concluded that the results were invalid, as young growing rats need 15% dietary protein (Horton 1999).

Manufacture of diet, chow versus purified diets, pellet hardness

Chow diets are the standard diets in most laboratory animal facilities, and are usually produced from natural ingredients. Manufacturers provide a whole range of diets adapted to particular animal species and their stage of life. Because

The UFAW Handbook on The Care and Management of Laboratory and Other Research Animals: Eighth Edition Edited by Robert Hubrecht and James Kirkwood
© 2010 the Universities Federation for Animal Welfare. ISBN: 978-1-405-17523-4

natural ingredients differ, the commercially available chow diets not only vary in composition between various companies, but can vary also between batches from the same company (Ritskes-Hoitinga & Chwalibog 2003; Ritskes-Hoitinga 2004).

Where natural-ingredient chows are used, it is advisable to obtain a batch analysis certificate with each batch used, to provide exact information on nutrient and contaminant levels. If a batch analysis certificate is not available, researchers will not be able to reject batches that may interfere with the results of their study (Nygaard Jensen & Ritskes-Hoitinga 2007). Moreover, unexpected outcomes where dietary composition may have been the cause would not be able to be explained. Some dietary chows are produced that are intended for use in several species, eg, rat, mouse and hamster. In these cases one has to be aware that individuals could ingest toxic levels of certain nutrients if they eat more than the intended amount of food where the concentration of the nutrient has been determined at the highest minimum concentration needed for one of the species, eg, iron in a diet for rat, mouse and hamster (Beynen & Coates 2001).

Purified or semi-purified diets are formulated with a combination of natural ingredients, pure nutrients and ingredients of varying degrees of refinement, resulting in far more standardised compositions than natural-ingredient diets. For rodents the American Institute of Nutrition, AIN-93 diet (Reeves *et al*. 1993) is recommended, as the composition is such that (with slight modifications) it fulfils nutrient requirements (NRC 1995; Ritskes-Hoitinga 2004). Often, with these diets the composition is such that pelletting is not possible so that alternative types of presentation become necessary (Ritskes-Hoitinga & Chwalibog 2003). Because purified diets are constituted with a better control of dietary composition, more reliable and reproducible results are obtained (compare Ritskes-Hoitinga *et al.* 1989 and 1991). From the point of view of good science, purified diets ought to be used much more frequently than is currently the case. However, depending on composition, there can be problems with palatability and variation in intake: the choice of diets has to be made on a case-by-case basis. Pilot studies are advisable where the effect of the dietary composition is unknown and uncertain.

The hardness of pellets and blocks is measured as the amount of pressure that is required for crushing a pellet. If pellets are too hard (over 20 kp/cm^3), the growth of pre-weaned mice is reduced significantly (Koopman *et al*. 1989). This is partly because the young mice have to work hard to eat the pellets, and partly because the lactating females are not able to eat enough to satisfy the needs of both themselves and their young (Koopman *et al*. 1989).

About 30% of genetically modified mice have been reported to show signs of weakness and to have reproduction difficulties (FELASA Working Group *et al.* 2007), and this has led to the marketing of special high-fat diets for breeding transgenic mice. The eating problems may be the result of a 'general weakness' of genetically modified strains, but dental problems, muscular weakness, neurodegenerative disorders etc, could all contribute to a reduced food intake. The higher-fat diets marketed for these animals are not as hard, so mice do not need to work as much and can ingest food more easily. The disadvantage of a higher fat content is an increased risk that the mice will develop atherosclerosis. In addition to the various kinds of pelleted diets, manufacturers also produce so-called extruded diets, which have a softer composition and which provide greater availability of nutrients. The reader should consult the manufacturers for detailed information.

Contaminants and trace elements

Contaminants are defined as undesirable substances (usually foreign) which, when present at sufficiently high concentration in the food, can affect the animal and therefore the outcome of experiments (British Association of Research Quality Assurance (BARQA) 1992). Maximum permitted levels of dietary contaminants have been published by the United States' EPA (Environmental Protection Agency 1979); BARQA (1992); GV-Solas (2002). For certain contaminants like selenium, an essential trace element with a narrow safety margin, information on maximum safe levels is provided by the NRC (1995). A table comparing the maximum contaminant levels specified in the three guidelines listed above can be found in Ritskes-Hoitinga (2004). Which guidelines are followed will depend on the goal of the scientific procedure and on the necessity to protect welfare (Ritskes-Hoitinga 2004; Ritskes-Hoitinga & Strubbe 2004). The researcher should decide and choose maximum contaminant levels permissible in order to avoid compromising the results. When working under Good Laboratory Practice (GLP) guidelines the maximum levels must always be defined before the start of the study.

Avoiding toxic nutrient levels: quality assurance, diet and experimental outcomes

Quality control

Manufacturers design diets to contain (much) more than the required minimum nutrient densities. This helps to prevent nutrient deficiencies, even taking into account periods of storage during which some nutrients (eg, some vitamins) may be lost. Moreover, variation in nutrient concentrations in so-called standard chows can be quite considerable, due to the use of natural ingredients that vary in composition (Beynen & Coates 2001; Nygaard Jensen & Ritskes-Hoitinga 2007). This variation can affect results (Wainwright 2001; Ritskes-Hoitinga 2004) and sometimes nutrient concentrations may get close to toxic levels. It is therefore important to have an analysis certificate for each batch of diet used, so that experiments can be planned correctly and unwanted effects prevented. Diet can also be used in environmental enrichment strategies where the animals are provided with choice of diet or allowed to work for food. However, this approach complicates matters with respect to standardisation and quality control and can have adverse consequences. This topic is dealt with more extensively in a later section.

The NRC reports (NRC 1977–2003) provide information on the minimum nutrient requirements for each species, and on known toxic levels of these nutrients. Unless the goal of the experiment is to examine toxicity, nutrient levels that might cause toxic effects should obviously not be used, as this

would both compromise animal well-being and experimental results. Even when the goal is to examine nutrient toxicity, very high concentrations are not advisable as they may result in reduced growth and early death. From an animal welfare point of view, testing the dose–response relationship of several subtoxic concentrations is preferable to testing one or two high toxic concentrations (Ritskes-Hoitinga *et al*. 1998). Moreover, such a strategy will provide more detailed information on the metabolic effects of the nutrient, and this approach is therefore an important refinement.

Safety margins

Methionine is an example of a nutrient with a narrow safety margin. The minimum recommended level for mice during growth is 0.3% (NRC 1995). When studying the influence of higher dietary methionine levels on atherothrombosis in ApoE deficient mice, dietary methionine levels of 2.2% and 4.4% (W/W) proved to be toxic, resulting in reduced growth and premature death (Zhou *et al*. 2001; Ritskes-Hoitinga 2004). In a follow-up study, a dietary methionine level of 1.4% (W/W) did not give any obvious signs of toxicity, thereby resulting in better mouse health and welfare and a more reliable interpretation of study results (Zhou *et al*. 2003).

Toxic effects

The recommended nutrient levels for good health and those that cause toxicity vary between species. For sheep, the copper density of the diet is critical, as the difference between that for good health and that causing toxicity is very narrow. Errors in feed mixing can easily lead to mortality in this species (NRC 1995-Sheep 1985). Sheep are able to store sufficient reserves of copper to tide them over periods of up to 4–6 months when grazing copper-deficient forage (NRC 1985). When used in research, sheep are often fed pelleted chow as well as hay and grass on a daily basis, in order to avoid mineral and vitamin deficiencies. In these cases total copper intake must be kept below toxic levels. Moreover, because sheep are usually group-housed, a chow diet can lead to copper intoxication in some individuals, if the food intake is not distributed evenly among the individuals in the group. Characteristic symptoms copper intoxication are haemolysis, icterus (easily detected directly around the eyes) and haemoglobinuria (NRC 1985).

Dietary phosphorus (P) levels are critical in mice, rats and rabbits, as excessive concentrations lead to soft tissue calcification (NRC 1995; van der Broek 1998; Ritskes-Hoitinga *et al.* 2004a). This typically affects the kidneys in rabbits and (female) rats, whereas in mice more widespread calcification involving kidney, tongue and heart is seen. Kidney calcification can negatively affect kidney function (Ritskes-Hoitinga *et al.* 2004a, 2004b). Not all dietary P is available to mice and rats as they cannot access that bound to phytates, whereas rabbits can access all dietary P because of the role of their intestinal microflora (NRC 1995-Rabbits 1977). The recommended dietary P level (at a calcium level of 0.5%) for growing mice and rats is 0.3 % (NRC 1995), and for rabbits 0.2% (NRC 1977). This minimum recommended dietary (available) P level for rabbits is also the maximum recommended level (Ritskes-Hoitinga *et al.* 2004a).

Deficiencies

Usually manufacturers ensure that the nutrient densities of diets exceed the minimum requirements, which helps to prevent nutrient deficiencies following storage. Providing animals with the opportunity to forage and/or select what they want to eat in addition to a complete chow diet, means that intake needs to be carefully monitored. For example, it is well known that providing sunflower seeds to parrots *ad libitum*, in addition to a complete diet, can lead to vitamin A deficiency, as the parrots prefer to eat sunflower seeds. Similarly, scattering corn and wheat in the substrate provides rodents with foraging opportunities, and is thought to be enrichment. However, the amount that is provided needs to be calculated and monitored. Rodents eat according to energy need, so it is important to ensure that most of the energy intake is not from the corn and wheat, as this will suppress intake of the complete diet, potentially leading to mineral and vitamin deficiencies. In summary, it is important to calculate the amount of treats that can be provided without disturbing the balance of nutrients required for health.

Foraging is an important activity of primates in the wild, and can be profoundly affected by captivity (Wolfensohn & Honess 2005). Collecting and eating food is necessary to satisfy physiological, behavioural and social needs, and it is therefore important to consider both forage and eating time when feeding primates. Small portions should be provided unpredictably rather than providing large portions in a predictable schedule (Wolfensohn & Honess 2005). Tables listing the nutrient densities of foods commonly used for primates are presented in the *Nutrient Requirements of Non-Human Primates* (NRC 1995-Nonhuman primates 2003). The size of the food pieces should be such that the primates can hold them, as primates like to manipulate their food. It is important to keep track of the nutrient balance of the total ration presented, as commercial diets are formulated to have a balanced composition according to the animals' needs and this balance may be disturbed if inappropriately large amounts of additional items that do not have a balanced composition are consumed. It is often recommended that a varied diet should be provided (eg, fruit supplements with the chow diet), and care is needed to ensure that the balanced composition of the total diet is maintained (Wolfensohn & Honess 2005). As dietary supplements are also given for environmental enrichment or as part of positive reinforcement training, the selection of these treats and the amounts given are crucial. Treats that are nutritionally complete or high in moisture and low in calories, such as fresh fruit and vegetables, are better choices than nuts and raisins, as these are energy dense and nutritionally incomplete. Data on the nutrient composition of commonly used food supplements are provided by Wolfensohn and Honess (2005) and the Foods Standards Agency and Institute of Food Research (2002). Age-related disorders in primates are often related to nutrition. Dietary restriction that does not lead to essential nutrient deficiencies may tend to increase longevity and to decrease the incidence and age of onset of age-related degenerative conditions. Dominant animals may become obese (Kemnitz 1984) as they may dominate access to food. This can be modified by changing spatial distribution and mix of food types.

Isocaloric exchange

When a standard complete food is offered *ad libitum*, animals ingest according to their energy need. Energy need depends on the life stage, and is generally defined in MJ metabolisable energy per metabolic weight ($kg^{0.75}$): for growth the need is typically recommended as 1.2 MJ/$kg^{0.75}$/day (but this depends upon rate of growth in relation to adult body mass and species that grow very rapidly, eg altricial birds, have greater daily requirements per metabolic weight than more slowly growing ones such as primates), for maintenance the need is typically about 0.45 MJ/$kg^{0.75}$/day, for gestation, about 0.60 MJ/$kg^{0.75}$/day (depending on species and litter size) and for lactation, about 1.3 MJ/$kg^{0.75}$/day (depending on species and other factors). Metabolic weight is used to make proper comparisons between species, correcting for size-related variation in metabolic rates. Because fat in the diet has an energy density 2.25 times higher than that of carbohydrate and protein, changing the fat concentration will result in a change in energy concentration of the diet and thus on amount of food ingested. If the fat content of the diet is changed, compensation is necessary by changing carbohydrates (or proteins) on the basis of calories (isocaloric exchange) not weight, This is the only way to ensure that the intake of all nutrients, except for fat and carbohydrate (or protein), remains similar in test and control group. Only isocaloric exchange isolates the effect of a change in fat (and carbohydrate) content in the diet and makes it possible to reliably interpret study results. Examples of how this is done can be found in Beynen and Coates (2001), Ritskes-Hoitinga and Chwalibog (2003) and Ritskes-Hoitinga (2004).

Aspects of feeding

Rhythms of feeding in nature versus the laboratory situation

Living organisms in nature are continuously influenced by external stimuli, many of them having regular or rhythmic patterns. These include lunar/tidal, solar/daily and seasonal/yearly patterns of light, temperature, food availability, and so on. Because these environmental rhythms are usually quite predictable, animals can usually adapt their physiology to cope with them. The ability to anticipate critical environmental events has clear advantages and survival value in nature, and may be related to the fact that predictability and controllability often have a stress-reducing effect on animals. Species have evolved adaptive anticipatory strategies through the process of natural selection with the result that many species have innate behaviours that are environmentally and temporally appropriate, such as hibernation, migration and seasonal reproduction. Other behaviours that maximise reproductive success include eating patterns that minimise exposure to predators or harsh environments while maximising food consumption. Learning can modify the timing of innate feeding behaviours based upon whether past behaviours were successful in providing adequate nutrients or not. Hence, animals are able to adjust their patterns of ingestive behaviour to adapt to a wide spectrum of environmental conditions, as long as these conditions are predictable (Strubbe 1994b).

These evolved characteristics and strategies are still present in animals in the laboratory today, although domestication has led to changes (Strubbe 1999); chickens housed in battery cages spend less time voluntarily foraging for food than wild jungle fowl (Ritskes-Hoitinga & Strubbe 2004). It is necessary to know what these genetically determined characteristics are and whether and how one must to adapt to them in the laboratory, because they can impact on the animals' well-being and the experimental results. For instance, for practical reasons most scientific tests on animals in the laboratory are performed during the light phase. However, for nocturnal animals this seriously interferes with sleeping behaviour. Regular and predictable changes found in nature are usually absent in the laboratory. The very important light–dark cycle is normally replicated artificially in the laboratory and kept constant throughout the year, in order to synchronise daily rhythms. However, it is not necessary to mimic all natural conditions to produce better welfare for the laboratory animals. Reducing or eradicating infectious diseases in the laboratory clearly improves animal welfare and experimental results.

One of the best-understood rhythmic patterns is the 24 h circadian cycle that underlies many physiological processes and behaviour. The rat is the species that has been most extensively investigated with regard to rhythms and their links to feeding, because this animal exhibits most of the general characteristics of mammalian timing systems. Rats are nocturnal and this remains the case when they are maintained under experimentally controlled light–dark rhythms in the laboratory. Under undisturbed *ad libitum* feeding conditions in the laboratory, rats maintained on a 12 h dark and 12 h light schedule eat most of their total daily food during the dark hours, with peaks at the beginning (dusk) and end (dawn) of the dark period (Kersten *et al.* 1980).

Whereas the dusk peak is needed to compensate for the lower energy stores in the body following the resting phase, the dawn peak has a more anticipatory function to maintain adequate energy over the forthcoming resting phase (Strubbe *et al.* 1986a). Even before the dawn peak the stomach is hard packed (Armstrong *et al.* 1978) and rats do not reduce feeding when liquid food is infused into the stomach (Strubbe *et al.* 1986a). This indicates that satiety signals are neglected during this period in order to allow preparatory 'over-consumption'.

While each rat may have its own characteristic feeding pattern, such patterns usually do not deviate much from the common pattern in free-feeding animals in the laboratory and from animals in nature. This pattern represents a conserved, probably genetically determined, natural behaviour that, in the wild, would reduce the risk of predation.

Light aversiveness

Nocturnal feeding in rats could, at least in part, be a product of light avoidance. Light is known to be aversive to some nocturnal species and in particular to albino species. When rats are provided with smaller and darker nest boxes inside their cages, they spend most of their time during the light

phase inside these nest boxes. This remains true when the food hopper and water bottle are placed at a location remote from the nest box (Strubbe *et al*. 1986b; Brinkhof *et al.* 1998). During the dark phase, rats eat in close proximity to the food hopper, whereas when they eat during the light phase, they make rapid excursions from the nest box to the food hopper, take a morsel of food and quickly return to the nest box to consume it. Consumption of food in the nest box increases with light intensity and can therefore be used as a measure of the light's aversiveness (Brinkhof *et al.* 1998; Strubbe & Woods 2004). Aversion to light should, therefore, be taken into account when assessing feeding activity in rats during the light phase. In commonly used rat and mouse cages, the layer of food pellets in the food tray offers some protection from the light. Extremely bright light is, however, never advisable since it may induce retinal damage (Schlingmann *et al*. 1993, 1995). The message here is that the presence of a burrow will certainly increase animal welfare for nocturnal animals, and for rats large burrows are recommended (Jegstrup *et al*. 2005).

Circadian rhythms

When an animal lives in an environment in which it is exposed to predictable light cycles every 24 h, its behavioural patterns entrain on (or synchronise with) the light cycle of the environment. Important patterns can be revealed when the normal light–dark cycle is absent. Thus, when light is kept constant (either with continuous light or continuous dark), animals are no longer able to synchronise with the environment. The result is that many behavioural patterns, including feeding patterns, are governed by a, now unentrained, endogenous oscillator. This operates at a 'free running' rhythm with a daily period that is near, but not identical, to that of one rotation of the earth. From this the term, circadian has been derived (circa = approximately, dies = day). The free running period (tau) is dependent on the individual and is genetically determined, so that in the absence of a light–dark change, or other effective cue, animals' rhythms will be at different phases, which could increase experimental variance. For this reason, a constant light–dark cycle is usually maintained in the laboratory. It is therefore important always to control and check the lighting conditions in the experiment rooms and when experiments are performed in the laboratory, it is extremely important to do this under entrained conditions, unless specific chronobiological experiments require a 'free run' design.

The anatomical site of the light-entrainable oscillator or clock that controls circadian rhythms has been the subject of considerable investigation. The hypothalamic suprachiasmatic nucleus (SCN), which lies just dorsal to the optic chiasm, has been identified as the site of the clock that generates circadian rhythms in mammals. When the SCN is lesioned in mammals, there is immediate and permanent disruption of the circadian rhythm of food intake and the animal's feeding pattern becomes arrhythmic (Strubbe *et al.* 1987).

Importantly, a SCN lesion does not induce blindness, and during the dark phase rats will still eat in the vicinity of the food hopper and in the light they will still take each food pellet back to the nest box. SCN lesions also disrupt many other behavioural and physiological circadian rhythms. For example, in restriction experiments where rats are forced to eat and drink during the light phase, there are strong interacting influences between food intake and sleep (Spiteri *et al*. 1982; Strubbe *et al.* 1986b; Brinkhof *et al*. 1998; Ritskes-Hoitinga & Strubbe 2004). As soon as the *ad libitum* food intake is reinstated, rats revert to their original pattern of food intake (Spiteri 1982). Rats forced to eat during the light phase suffer gastrointestinal problems, comparable to the problems associated with jetlag and shift work in humans (Ritskes-Hoitinga & Strubbe 2004). Interacting effects between circadian clock influences and behaviour and physiology are abolished in rats with SCN lesions (Strubbe *et al*. 1987; Strubbe & van Dijk 2002). This suggests that circadian pacemaker activity in the SCN normally dominates the temporal patterning of food intake, water intake and sleeping behaviour and is not shifted permanently by long-term shifts in food or water availability. As humans work mostly during the light phase, researchers must carefully consider in the implications of their choices as to how and when they feed and treat nocturnal animals in the laboratory.

Memory for feeding time and feeding schedules

In the laboratory, feeding schedules can be part of the experimental design. Rats are quick learners and will readily adapt to these feeding schedules, however, in other rodent species, such as the mouse and hamster, this can be much more difficult, if not impossible. Hamsters on a schedule of one meal of 2 h/day experienced a very rapid drop in body weight (20% in 1 week), making it necessary to stop this experiment (Ritskes-Hoitinga, unpublished observations). It is essential to know and adapt to the characteristics of the species in feeding studies, and where there is uncertainty, pilot studies are recommended. If food restriction is a scientific necessity, feeding meals at certain times can be used to achieve the restriction. Sometimes exact coupling of food intake between test and control groups is necessary in order to promote standardisation, so-called 'pair feeding' (see below).

Meal feeding

Although feeding schedules are used to increase standardisation of experiments, they are, mostly, quite unnatural and artificial. Such schedules interfere with the natural rhythms of drinking, eating and sleeping as dictated by the circadian oscillators. Interference will also occur with the rhythms of many (food-related) physiological processes. When food is consumed, especially large meals, the food itself perturbs many ongoing physiological processes that are closely regulated by the body. As obvious examples, blood glucose concentration and metabolic rate both increase during and after meals, and the effects are greater when larger meals are eaten. In order to minimise the impact of these challenges, the well prepared individual can make meal-anticipatory responses that lessen the magnitude of the meal-induced

perturbations. This has obvious advantages for an individual who, due to environmental constraints, is forced to consume all of its daily food in one or two very large meals. Rats can easily be trained to ingest the required amount of food in a limited amount of time. Within a week's time, rats can be trained to ingest their daily food intake in two meals of 0.5 h each during the light phase (Ritskes-Hoitinga *et al*. 1995). One meal of 2 h/day can be sufficient for an (adult) rat to obtain the necessary energy and nutrients but this does depend on the physiological state of the animal. Although rats can be trained to adapt to eating one or two meals per day, 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. The time of day when the food is offered is critical to physiology and welfare. Meal-feeding during the dark phase as compared to during the light phase, resulted in bile flow and physical activity being comparable to *ad libitum* feeding (Ritskes-Hoitinga & Strubbe 2004) and resulted in more normal physiological responses.

Animals can eventually adapt to feeding schedules in which fewer, larger meals are eaten, and this is usually associated with more efficient handling of the diet, including a slower gastrointestinal passage time and hypertrophy of the gastrointestinal wall. These changes help the animal to cope with the situation, despite the fact that the feeding schedule can be unsuited to its physiology and uncomfortable.

Virtually every digestive or metabolic variable that has been investigated changes in anticipation of meals. These meal-related changes are often called cephalic responses, because many of them are initiated by signals from the brain. When six equal meals are spread over the daily cycle with equal inter-meal intervals, these anticipatory responses are not seen (Strubbe 1992; Kalsbeek & Strubbe 1998). However, where rodents are used and the study requires a schedule of one to two meals per day, these anticipatory responses do occur. Generally it is preferable to feed more often as rats normally eat eight to ten meals per day. Welfare is certainly affected when forced artificial schedules are used.

Restricted feeding

Severe obesity and diabetes mellitus are becoming increasingly prevalent in Western societies, and reducing body weight by adequate control of food intake is one of the best therapies. However, it is also one of the most difficult treatments in humans, since it usually induces a feeling of a permanent state of hunger and as a consequence stress and mood problems, such as depression, occur. This is also the case in several husbandry systems, for instance in broiler breeders, where severe long-term food restriction is applied during rearing to prevent health and reproduction problems at a later age (De Jong *et al.* 2003).

In food restriction studies, instead of food being provided *ad libitum*, the energy supply is restricted, while still ensuring nutritional adequacy, ie, that all essential nutrients are supplied in the required amounts (Hart *et al*. 1995). For dogs, pigs, cats, monkeys and many other animals it is considered bad veterinary practice to feed *ad libitum*, as the animals will become obese (Hart *et al*. 1995).

Long-term food restriction

Rodents are fed *ad libitum* in the majority of experimental studies. Feeding rodents *ad libitum* may be attractive from a practical point of view because no special feeding system or special care is required. However, on the basis of results from long-term toxicological studies, it is questionable whether this is a sound scientific or welfare approach.

Ad libitum feeding leads to long-term negative health effects compared with restricted feeding (75% of *ad libitum* intake). These include shorter survival time, increased rates of obesity, degenerative kidney and heart disease, and cancer at an earlier age (Hart *et al.* 1995). Food restriction can have other positive effects on health. For instance, the relative body weight reduction 48 h 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 claimed that animals become more 'robust' when food intake is restricted, that is, that they can cope better with experimental stressors and related procedures (Keenan *et al.* 1999). Diluting the diet, for example, by including a higher fibre content under *ad libitum* conditions, does not give the same positive health effects as compared to food restriction (Hart *et al*. 1995).

In most instances, health problems due to being overweight are not considered a problem, because laboratory animals do not live to an age at which problems occur. However, for research into aging and kidney physiology, there are clear reasons to use food restriction instead of *ad libitum* feeding. Long-term food restriction is also very useful when long-term reductions of body weight are required, such as in obesity and type 2 diabetes research.

Keenan *et al*. (1999) have demonstrated that *ad libitum* feeding is the least controlled experimental factor in the laboratory. Considerable variation in experimental results from rodents on *ad libitum* feeding schedules was seen during the 1980s to 1990s in toxicology (Keenan *et al*. 1999). One possible explanation could be the continuous selection of faster growing individuals in outbred strains (Keenan *et al*. 1999). As rapid growth has often been considered an indicator of good health, robustness and leading to better reproduction, heavier individuals may have been selected for the breeding process. However, faster growth tends also to be associated with a shorter lifespan. By selecting within outbred colonies on the basis of fast growth, subpopulations may arise, leading to more variation within outbred populations. Feeding rodents at approximately 75% of the *ad libitum* food intake is recommended for long-term toxicity studies in order to make certain that a sufficient number of animals survive the required 2-year period (Hart *et al.* 1995). Under *ad libitum* feeding conditions, body fat content can be more than 25% (Toates & Rowland 1987). By restricting food intake to 85% of *ad libitum* intake, body fat content will be less than 10%, which is similar to that found in wild-caught animals (Toates & Rowland 1987). In order to make sure that all individuals eat the same amount of

food, it is advised to house them individually (Keenan *et al*. 1999). However, this may have a negative welfare impact because of social isolation, as rodents are social animals. In order to solve this, one possibility is to feed group-housed animals individually. In line with current agricultural practice, microchips, computer technology and automated feeding devices have been used for feeding individual rats restrictedly under permanent group housing conditions. This strategy needs to be further developed.

An aspect of food restriction that deserves much more attention is the issue of standardisation and reduction. Under *ad libitum* feeding conditions, animals can freely choose the amount of food eaten. By definition, animals are fed a standardised amount of food under restricted feeding conditions, which automatically improves standardisation of experiments. Upon testing chloral hydrate in rats under *ad libitum* versus food-restricted conditions, it was found that a significant difference in the liver enzyme activity of LA (lauric acid) between control and test group occurred at a dose of 100 mg/kg (Leaky *et al*. 2003; Ritskes-Hoitinga 2006; Ritskes-Hoitinga *et al.* 2006). Under *ad libitum* conditions 86 animals per group were necessary, whereas only 7 animals were needed under food-restricted conditions to prove that this difference was statistically significant (Figure 14.1). This indicates that a reduction of 92% in the number of animals used was possible under these conditions because the magnitude of the effect was much greater under restricted feeding conditions than under *ad libitum* feeding, which is a well known effect of food restriction (Hart *et al.* 1995).

However, it is not only from the point of view of the numbers of animals used, that welfare issues associated to food restriction need to be addressed. Long-term food restriction may (temporarily) cause feelings of hunger. This can be a strong stressor leading to abnormal behaviour such as stereotypies and will affect animal's welfare by causing mood problems (Dixon *et al.* 2003). One study with rabbits showed that a lower frequency of stereotypical behaviour occurred when rabbits were offered a restricted amount of food at their natural time of the day compared with restricted feeding at an unnatural time of day and as compared to *ad libitum* feeding (Krohn *et al.* 1999). This indicates that feeding a restricted amount of food at the 'proper' time is beneficial for welfare, even when compared to *ad libitum* feeding. When a restricted feeding schedule is used, it should be remembered that as soon as food becomes available again, the animal will ingest a large meal in order to compensate for energy deficits from the fasting period.

Short-term food deprivation

Short-term food deprivation, such as overnight fasting or other food deprivation schedules, is often used in research. For example, fasting may be necessary when blood samples need to be lipid-free for certain analyses. These short-term food restriction periods (ie, hours) are needed for certain experiments and can also improve standardisation and reproducibility of experiments. However, food deprivation for a period of 24 h results in a different metabolic state than under *ad libitum* feeding conditions (Strubbe & Alingh Prins 1986). After rats have been deprived of food for a period of 24 h or more, all glycogen deposits have been used. Food deprivation in rats for a period of 12 h leads to a shift from carbohydrate to fat metabolism (Strubbe & Alingh Prins 1986). An excellent overview of how food restriction influ-

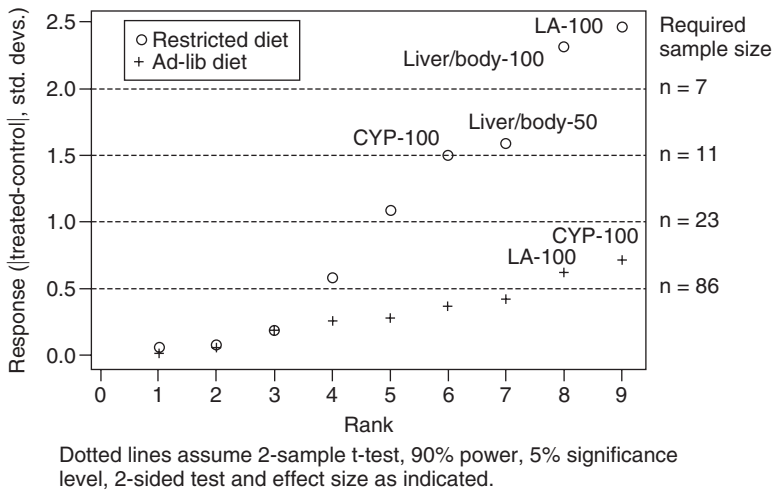

Figure 14.1 Chloral hydrate caused a significant increase in the liver enzyme activity LA-100 (LA is lauric acid, 100 refers to a dose of 100 mg/kg). Under *ad libitum* conditions, 86 animals were needed in order to show a statistically significant difference, whereas under food restriction conditions, only seven animals were necessary to find a significant difference between the treatment and control group (ie, a 92% reduction). Food restriction also leads to an increase in effect size as compared to *ad libitum* conditions (Leaky *et al*. 2003; Ritskes-Hoitinga 2006). 'Effect size' is the magnitude of the difference between the treated and control group, calculated by taking the absolute differences between the treated and control means and dividing this difference by the standard deviation for each group; 'rank' indicates the numerical position of a certain parameter in the magnitude of the effect size; crosses refer to *ad libitum* fed groups, circles refer to restrictedly fed groups; CYP-100 refers to liver enzyme activity measurement of the enzyme CYP at a dose of 100 mg/kg; liver/body is the liver/body weight ratio at the dose of 50 and 100 mg/kg. (Dr. Michael Festing is greatly acknowledged for these calculations.)

ences the physiology and well-being of various species has been published by Rowland (2007).

Pair feeding

If a test group's voluntary food intake differs from that of the controls, it is advisable to take steps to equalise the intake between the groups. Such differences can occur when the palatability of the test diet is negatively influenced, or where the substance has a negative effect on appetite or health, resulting in reduced food intake. For standardisation, test and control animals must ingest a similar amount of food at a similar time of day, otherwise it will be impossible to judge the effects of the test substance and/or procedure independently of its effects on food intake. Because NRC requirements are based on obtaining maximum growth rate, a level of 75% of the NRC recommendations is still considered sufficient to fulfil all needs.

There are four possible methods for achieving pair feeding and these are: (1) weighing; (2) coupling of food dispensers; (3) gavage/permanent stomach cannula; and (4) feeding machine. In the first method 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 daily food supply is provided at once to the control animals, perhaps at an unnatural time point of the day (eg, perhaps during the resting phase in rodents). This may induce quite a different eating pattern and overloading of gastrointestinal processes in the controls as compared to the test animals, thereby preventing proper comparison between test and control animals. In the second method, using food dispensers, rats can be trained to obtain a pellet by pressing a lever. By coupling food dispensers for test and control animals, the dispenser of the control rat provides a pellet at exactly the same time as the test animal 'asks for' and eats a pellet. This system standardises amounts eaten and ingesting patterns. In the third method, permanent stomach cannulation 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). However, inserting a permanent stomach cannula requires a surgical procedure and both the stomach cannula and gavage method omit the oral digestive process. In the fourth method, feeding machines with regulated opening of valves make it possible to open and close food hoppers at the same times during the day for test and control animals. Meal training is another possible solution for a pair-feeding schedule.

Gavage feeding

In research on pharmaceutical agents or nutrients, it can be important to avoid any negative influence of taste on food intake. In such cases, gavage is used to introduce substances directly into the stomach. Gavage may also be required for radioactive isotopes or immunosuppressants, where exact dosing is required and health-damaging substances must not be spilled into the environment. However, the application of a meal directly in the stomach as compared to voluntary eating of a similar meal, can affect experimental results (Vachon *et al*. 1988). In this study by Vachon *et al*. (1988), voluntary consumption yielded results more comparable to those from from human studies. A disadvantage of stomach tubing is that it bypasses all the oropharyngeal processes, including the physical effects of chewing and the addition of salivary enzymes that initiate the digestive process. Gavage can also cause stress to the animals, resulting in suppression of 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 directly into the stomach or small intestine without stress (Strubbe *et al*. 1986a) and without risk of contamination of the environment and the person executing it. These permanent cannulas are used, for example, to measure the satiety effects of filling the gastrointestinal system with purified nutrients at different anatomical locations. After the initial surgical procedure, rats can walk freely in their home cage. Due to the presence of the cannula attachment to the skull, individual housing is often used, however, this will impact on the animals' welfare. Social housing is possible if the cannula attachments are protected from bites by a metal cap.

Working for food

When food is always available, but not 'free', animals readily change their feeding patterns based upon other kinds of constraints. When an energy cost is placed on gaining access to food, rats change their strategy to minimise total daily work while maintaining a constant body weight (Collier *et al*. 2007). Specifically, as the cost of gaining access to food increases (eg, the number of responses an animal must make to gain access to food; or some other aspect of physical effort), two changes occur: the rats eat larger meals, and they eat fewer meals (Figure 14.2).

It is difficult to determine how much time/energy ought to be spent on working for food. The choice the animals make does not necessarily optimise long-term 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 in ways that impact on their needs for optimal welfare. Comparing the behaviour of the white leghorn chicken and its ancestor, the wild jungle fowl, a remarkable difference has occurred in food searching behaviour. When given the choice between freely available food from a plate and food that was hidden in the bedding in a semi-natural environment, the white leghorn chose to search for food in the bedding and consumed 30% of their feed from this source, whereas wild jungle fowl consumed 70% of the total diet from the hidden food (Ritskes-Hoitinga & Strubbe 2004). This indicates that there may be a behavioural adaptation as a result of domestication and selecting strains for increased production.

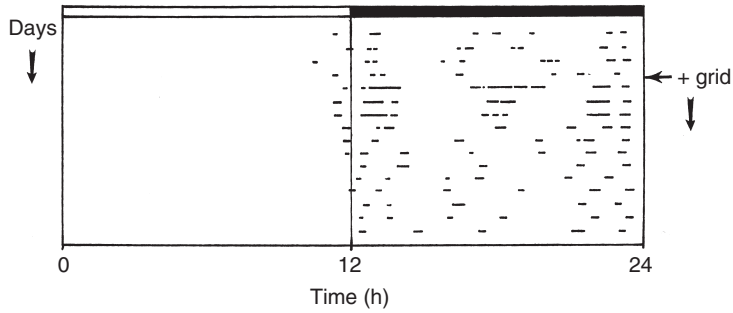

Figure 14.2 Daily pattern of food intake of a rat on consecutive days. The black lines represent bouts of feeding activity. On day 5, the distance between the food hopper bars was reduced, requiring increased effort to obtain food through the bars. Notice the large meals of extremely long duration in the beginning. After a few days, the animals could work their way through the narrowed food hopper bars just as fast as before. Reproduced from Ritskes-Hoitinga, M. and Strubbe, J.H. (2004) Nutrition and animal welfare. In: *The Welfare of Laboratory Animals*. Ed. Kaliste, E., pp. 51–80. With kind permission of Springer Science and Business Media.

Diets

A change is likely in the animal's feeding strategy if diet and palatability are changed. However, after a few days the free-feeding pattern will return to the original state with the same meal frequency (Strubbe & van Dijk 2002; Ruffin *et al*. 2004).

A regular chow diet can be used for animal maintenance and during numerous types of scientific procedures but, in nature, a greater variety of ingredients is often available and consumed. So, is greater variety and the opportunity to choose and select better for the welfare of the animals? 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 resting period (Strubbe 1994a, 1994b). The same time-dependent pattern of macronutrient selection was also seen in humans (van het Hof *et al.* 1997). The evolutionary explanation may be that carbohydrate-rich diets will quickly provide the animal with a source of readily available energy, necessary to supply the energy deficits that have arisen during the resting phase. The selection of macronutrients is governed by several neurotransmitters in the central nervous system such as galanin, neuropeptide Y (NPY) and serotonin (Strubbe 1994a; Kyrkouli *et al*. 2006). At present, in the authors' laboratory, the animals are often only offered one standard diet throughout the day but varying the diets offered can improve their welfare. It has been reported that monkeys that had access to a herb garden in the Zoo 'Apenheul' at Apeldoorn in the Netherlands, made a selection of specific herbs according to their health state[1]. Food selection is individually determined, and it may be better for welfare to permit individual selection of foods. At first glance, this may appear to conflict with attempts to standardise experiments, however, if individual needs are properly met, standardisation of experiments can be improved by adopting this approach. It will be a challenge to define how this should be achieved in practice.

Normal feeding patterns in rats also vary with their physiological and metabolic state. When energy demands are increased, such as during lactation or during forced daily exercise, rats first increase the size of meals up to their normal maximal meal size and later increase the number of such meals as energy demands increase further (Strubbe & Gorissen 1980).

Group housing

Although restricted feeding schemes and solitary housing have advantages for the standardisation and execution of experiments, restricted feeding schedules and solitary housing may have welfare consequences. Animals of social species ought to be housed socially, unless there are clear reasons for doing otherwise. When harmonious social housing is not possible, then all possible efforts should be made to improve animal welfare in other ways.

Housing and husbandry conditions can influence both the amount of food animals ingest, and the animals' feeding patterns. Whether an animal is group or individually housed has implications for food intake and results of scientific procedures. Individually housed mice of both sexes were found to have higher food intake than mice housed in groups of two, four or eight per cage (Chvedoff *et al*. 1980; Beynen & Coates 2001). Mice housed individually or at two per cage had a higher body weight and body weight variability than the other groups. When the mice were housed at eight per cage, gastritis occurred more frequently compared with individually housed mice (Chvedoff *et al*. 1980). These results indicated that four mice per cage was the optimum group size in this particular study. Peters and Festing (1990) showed that 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 in low-density housing. If mice are housed in a group, they usually lie together, reducing their total surface area. Reduced heat loss per animal in the group is the result and, due to this 'behavioural thermoregulation', food intake is reduced (De Vries *et al.* 1993; Woods & Strubbe 1994). If test compounds are incorporated in the food, the investigator should be aware of the phenomenon that group size influences intake and, therefore, the intake of the compound.

Behavioural and ingestion patterns can also be influenced by the social structure in the group. A dominant animal may prevent others from eating at certain times. Food intake patterns of socially compatible groups of S3 rats showed the normal feeding pattern with a clear dawn peak for all individuals. In contrast in a group that was socially incompatible, the dominant animal prevented the rats lower in

[1]http://www.kennislink.nl/publicaties/wat-kunnen-wolapen-ons-leren-over-gezondheidsbevorderend-gedrag

hierarchy from having a dawn peak at the natural time. Some had to shift to an earlier time during the dark phase, and some even shifted to the beginning of the light phase (Ritskes-Hoitinga & Strubbe 2004). It is unclear whether the phase of circadian rhythm had shifted permanently.

Thus, group housing can influence experimental outcome, either by influencing food intake and feeding patterns or by other factors. In one case, incompatibility between individuals in a group of dogs led to stress and vomiting of food until 8 h after the meal. Ingestion of this vomited food caused a disturbance of the experimental results. Upon changing the treatment and handling of the group of dogs in many ways, a social compatible group of individuals led to more reliable and reproducible results (Ritskes-Hoitinga *et al*. 2006).

Concluding remarks

When carrying out research using animals, it is a challenge to feed them in such a way that their health and welfare is maintained and results are reliable and reproducible. To do this requires knowledge of nutritional and behavioural requirements. This chapter provides an overview of factors that must be taken into account when feeding animals in the laboratory. Much more research is needed into how different feeding methods can be used as enrichment in order to improve laboratory animal welfare.

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