



Netherlands National Committee  
for the protection of animals  
used for scientific purposes

# Motivation by restriction?

*Starting points for controlled fluid  
and food intake in neurocognitive  
research from a 3Rs perspective*

Opinion of the Netherlands National Committee for the  
protection of animals used for scientific purposes (NCad)  
commissioned by the former minister of Agriculture



## The NCad and its methods

The Netherlands National Committee for the Protection of Animals Used for Scientific Purposes (NCad: *Nationaal Comité advies dierproevenbeleid*) was founded to protect animals used for scientific and educational purposes. By providing advice, knowledge exchange and developing national and international networks, the NCad seeks to significantly contribute to minimising the use of laboratory animals both domestically and internationally. Assessment of the ethics of animal procedures and the Replacement, Reduction, Refinement principles (The Three Rs: 3Rs) are central to this aim.

## NCad members

Herman Koëter (chair), Henriëtte Bout, Reineke Hameleers, Coenraad Hendriksen, Wim de Leeuw, Jan-Bas Prins and Pieter Roelfsema.

Under Article 9: Exemption, paragraphs 3 and 4, of the Administrative Regulations, one member did not take part in the final discussion and decision-making process regarding the Codes of Practice (CoPs) for non-human primates (NHPs). The NCad was subsequently unable to achieve a consensus on the preferred method of approach for determining minimum fluid intake for young primates.

With four votes in favour and two votes against, 30 ml/kg of metabolic weight was set as the minimum volume.

# Summary

For neurocognitive research, experiments are performed, whereby animals must perform activities to obtain an insight into the activity in the functioning brain. To motivate animals to perform these activities, their fluid or food intake must be restricted to such an extent that they become hungry or thirsty.

In this opinion report, the Netherlands National Committee for the protection of animals used for scientific purposes (NCad: *Nationale Comité advies dierproevenbeleid*) provides starting points for controlled fluid and food intake during neurocognitive research for rodents and non-human primates (NHPs) from the perspective of the 3Rs (Replacement, Reduction and Refinement).

The NCad is of the opinion that a Synthesis of Evidence (SoE) conducted prior to the research must be used to ascertain whether the research can be performed without the use of animals. Should this not be possible, it must be established whether the behavioural tasks can be taught without fluid or food restrictions. The motivation method least harmful to the animal must always be chosen. The NCad has prepared a decision tree (page 25), which helps the researchers to choose the most refined research design. Should restriction be necessary, the general framework and the Codes of Practice for rodents and non-human primates apply, which are enclosed with the opinion as a guideline.

The NCad recommends the following:

1. Use the “no, unless” principle to begin with and do not apply any restrictions unless there are well-substantiated scientific arguments and/or compelling public interests.
2. Promote the development and application of replacement methods for fluid or food restrictions.

Do this by:

- promoting the acquisition of more knowledge and understanding of possible replacements in the field of neurocognitive research;
  - making this subject part of the target situations that are being developed within fundamental and applied scientific research;
  - investing in technological developments and innovations that involve no laboratory animals in this field.
3. Use this framework and Code of Practice (CoP) as reference documents pursuant to Article 10 of the Experiments on Animals Act (Wod: *Wet op de dierproeven*). The following specific activities or situations should be especially prevented:
    - simultaneously applying fluid and food restrictions
    - “push days”
    - withdrawal of food for more than 24 hours
    - withdrawal of fluid for more than 24 hours
    - restriction of pregnant animals
    - restriction of protocols that could cause severe discomfort.

Do this by:

- calling on established licensees to implement the framework and the CoPs and to monitor compliance;
- having the Netherlands Food and Consumer Product Safety Authority (NVWA: *Nederlandse Voedsel- en Warenautoriteit*) use these CoPs as a reference when supervising the design and completion of this type of research.

4. Safeguard and – where possible – improve the welfare of animals that are used for teaching behavioural tasks for the purpose of neurocognitive research.

Do this by:

- requesting that the Animal Welfare Body (*IvD Platform*) promotes the knowledge of positive reinforcement with and without restriction by means of targeted training and knowledge sharing;
- initiating a partnership between users and breeders in which the possibilities for raising animals in an enriched environment are explored;
- calling on establishment licensees to train animals in their familiar environment (home cage) as much as possible;
- periodically evaluating the CoPs and modifying them in the event of new insights.

5. Promote and guarantee the knowledge of different motivation methods among the parties involved. Have the Central Authority for Scientific Procedures on Animals (CCD: *Centrale Commissie Dierproeven*) assess whether this knowledge is sufficiently guaranteed in the project application and have the NVWA ensure that this knowledge is guaranteed in the workplace, e.g. by using training records.

Do this by:

- requiring established licensees to have a Synthesis of Evidence conducted prior to the project to analyse the options for having an animal perform behavioural tasks without a restriction protocol and, if this is not possible, to choose a protocol that is least harmful to the welfare and health of the animals;
- requiring established licensees to provide a satisfactory explanation and substantiation of the choice for a motivation method in the project application. In addition to the aspects that are already mandatory, the following points should be explicitly addressed and substantiated here:
  - the method used and the period within which the tasks are taught
  - the conditions for learning and performing behavioural tasks
  - preconditions for accommodating and caring for the animals
  - criteria for the level and duration of the restriction, should this appear to be necessary.

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# Introduction

Approximately 270.000 animal procedures (269.267 in 2015, NVWA) were carried out in the Netherlands within the context of fundamental scientific research and in applied and conversion-oriented research. A portion thereof (40.130 in 2015, NVWA) was carried out for the benefit of research into the brain and the nervous system. Within this research domain, experiments are carried out that involve animals being required to carry out a behavioural task (primarily rodents and non-human primates (NHPS)) (*see Appendix 9 for some examples*). The objective of this research is to gain insight into activity in the working brain, for example to gain a better understanding of processes and disorders in the brain and to develop new treatment techniques. In these types of experiments, the animals may be required to perform a specific activity that deviates from their natural behaviour and where it may be necessary that the animal repeat the activity within a specific time interval.

The animal will not carry out the activity willingly. Therefore it must be motivated in a certain way to learn the activity and to have it repeat the activity. The facilitation of such motivation will usually involve the use of a reward in the form of fluid or food following the correct execution of a behavioural task. An animal may receive a reward in the form of an “extra treat”, such as fruit juice or sweets, but also in the form of water or everyday dry food. The more frequently the animal is required to carry out the task, or the more complex/aversive the task is to the animal, the more the animal will have to be motivated. In order to motivate an animal, it must be restricted in fluid or food intake. If the animal cannot fulfil its need for fluid or

food, and consequently is thirsty or hungry, the value to the animal of such a reward will increase. The animal is subsequently more prepared to carry out the requested task repeatedly in order to obtain fluid or food (*please also see Appendix 9 for some examples*).

The degree to which and duration that fluid or food is restricted will vary according to the type of experiment. This also means that the extent to which the welfare of the relevant animals is affected, either for the short or long term, will vary. In the scientific community, the inconvenience that animals experience is generally ranked as mild or moderate, in accordance with the classification for discomfort as included in the Experiments on Animals Act (WOD), Article 10b, paragraph 1<sup>1</sup>.

There are societal and political concerns regarding this method of research, referring to the intrinsic value of the animal, which is also the key principle on which legislation governing animal procedures is based. An important issue in this matter is whether the objective of the research outweighs the damage inflicted on the intrinsic value of the animal and whether or not methods are available that do not involve animal procedures or do not require restriction to achieve the intended objective.

If there is no alternative available that does not involve animal procedures, and in addition that restriction is necessary, then the welfare of the relevant laboratory animals must be guaranteed by identifying in advance where and how the effects of restriction on the animals' welfare can be limited.<sup>2</sup> The opinion will use the definition used by Utrecht University: “An animal can be said to be in a good

state of welfare if it is capable of adapting to prevailing environmental conditions and in so doing to achieve a state that it experiences as positive.” (Utrecht University<sup>3</sup>).

In 2015, Parliament questioned the Minister of Agriculture during a General Consultation (AO) of the permanent committee of Economic Affairs on these research methods in NHPs. In response, the Minister pledged to request that NCad issue an opinion on the matter.

#### Fluid and food restriction in the Netherlands

Fluid and food restriction for neurocognitive research is carried out by various research institutes in the Netherlands, primarily on rats and mice. It is unclear how many of these institutes apply this method and how many rodents are concerned, however, subsequent inquiries made with the NVWA have shown that this type of research is carried out in many academic centres. In 2014, two institutes in the Netherlands used 15 non-human primates (NHPs) for neurocognitive research.<sup>4</sup> As of 2016, there is one more institute in the Netherlands where NHPs are used for this type of research.

#### Legal frameworks and guidelines

Both European and national legislation set requirements for the nutrition and care of laboratory animals, prior to, during, and following animal experiments.<sup>5</sup> Several other countries (United States, United Kingdom, Canada, Australia) have specific legislation in place on fluid and food restriction as a method to motivate animals to carry out a behavioural task. In Australia, deprivation<sup>6</sup> is illegal and restriction may only be applied if the animals in question will not suffer any lasting adverse effects.<sup>7</sup> In the UK, any form of restriction

must be reduced to a minimum to allow the animal to fulfil its physiological and ethological needs. In the US, deprivation, meaning full restriction from fluid or food, is prohibited as a training method; however, partial restriction of fluid and food intake is widely used. In addition, primarily in the United States, various research institutes have set up internal guidelines. In Canada, experiments in which deprivation is applied for a period equal to one when the absence of water or food would occur naturally in the wild are classified as causing minor to no discomfort.

#### Terminology

A number of terms are used both in the literature and in the research, as well as in discussions on this subject, which are interpreted in various ways. In order to avoid any lack of clarity for the readers of this opinion, the framework (*Appendix 1*) indicates which terms have been used in this opinion and provides a definition for this terminology.

The terms restriction and deprivation are both used in the literature and in the field. Occasionally these concepts are used interchangeably, with the same definition, while on other occasions they are defined differently. Restriction is almost always interpreted as a reduced amount of fluid/food that is offered to the animal or a restriction of the duration during which the fluid/food is offered. Deprivation is sometimes used as a synonym for restriction, but is also defined as a total withdrawal of fluid/food (associated with a specific length of time, e.g. 24 hours) or the omission of a specific nutrient, for example. Due to the various interpretations of the concept of deprivation, this opinion will use the term restriction.

### Types of restriction

It is primarily the restriction of food that can also be applied for other reasons, for example, for pre-operative fasting or for research into nutrition. In addition, other types of restriction can be applied, such as restriction of sleep or social interaction. Restriction of fluid or food can also be used as a motivator within other research than neurocognitive research that requires a behavioural task to be performed, such as in the study of regeneration in paraplegia. In accordance with the request for an opinion issued by the Minister, this opinion will only focus on the restriction of fluid or food in laboratory animals within the context of neurocognitive research. The opinion makes no judgement on the content of the behavioural tasks and/or the objective of the research, but only focuses on the procedures with regard to motivation of animals to perform a behavioural activity.

### Realisation and structure of the advisory report

The NCad has developed two Codes of Practice – one for NHPs and one for rodents – on the basis of information obtained by two working groups (an NHP working group and a Rodents working group), from the literature and from a consultation of community group. An overarching document has been drafted that acts as a coordinating framework, containing general frameworks of research methods in which laboratory animals are motivated to carry out certain cognitive behavioural tasks. Both working groups also contributed to this framework using their specific expertise. The working groups consisted of experts in the field of neurocognitive research, animal welfare, ethics, animal behaviour and veterinary care.

## Request for opinion

On 22 March 2016, the NCad received the following request for an opinion from the Minister for Agriculture:

*At the request of the House of Representatives, I provided information together with the State Secretary of Education, Culture and Science regarding the method of water deprivation in non-human primates that is used in brain research.*

*Although the method of controlled fluid intake is common international practice, I wish to investigate whether there is room for improvement of the animal welfare involved with this method or regarding the use of alternative methods.*

*As such, I have requested that the NCad look into possible improvements to research methods in which water and/or food intake is limited with the aim of increasing laboratory animals' motivation to perform tasks and regarding possible alternatives to these methods. I would also like to request that the research into other animal species be included in this investigation.*

*I request that the NCad draw up a set of "best practices" for these research methods by the end of the year and that these "best practices" be published and promoted at the European level.*

*I wish the NCad every success in carrying out this request.*

*Yours faithfully,*

*Martijn van Dam  
Minister for Agriculture*



# Recommendations

An exploratory survey has shown that research methods that involve the restriction of the fluid or food supply, aimed at increasing motivation for laboratory animals to carry out activities, is frequently applied to rodents (mice and rats) in addition to NHPs. As such, this opinion will focus on the aforementioned animal species. It has also been shown that within neurocognitive research carried out in the Netherlands, fluid *and* food restriction never take place simultaneously.<sup>8</sup>

The NCad is aware that currently the motivation of animals to carry out activities takes place using fluid or food restriction, both within the Netherlands and abroad. This method is documented, common and relatively easy to carry out. However, it should be noted that restriction constitutes an impairment of the welfare, and consequently of the intrinsic value, of the animals concerned.

A Synthesis of Evidence (SoE),<sup>9</sup> should be conducted to ascertain whether the research can be performed without the use of animals. Should this not be possible, it must be established whether the behavioural tasks can be taught without fluid or food restrictions. The motivation method least harmful to the animal must always be chosen. The decision tree on page 25 is designed to help researchers select the most refined research design. If restriction is necessary, the framework and CoPs attached to this opinion should be used as guidelines. The framework and the CoPs have been drafted in consultation with experts from the field and contain clear guidelines and preconditions that established licensees must meet.

Considering the foregoing, the NCad makes the following recommendation to the Minister of Agriculture, Nature and Food Quality (LNV):

1. Use the “no, unless” principle as a starting point and do not apply any restrictions unless there are well-substantiated scientific arguments and/or compelling public interests.
2. Promote the development and application of replacement methods for fluid or food restrictions.

Do this by:

- Promoting the acquisition of more knowledge of and insight into possible replacements in the field of neurocognitive research;
  - Making this subject part of the target situations<sup>10</sup> that are being developed within fundamental and applied scientific research;
  - Investing in technological developments and innovations without laboratory animals in this field.
3. Use this framework and Code of Practice (CoP) as reference documents pursuant to Article 10 of the Experiments on Animals Act (Wod: *Wet op de dierproeven*). The following specific activities or situations should be especially avoided here:
    - Simultaneously applying fluid and food restrictions
    - “Push days”
    - Withdrawal of food for more than 24 hours
    - Denying fluids for more than 24 hours
    - Applying restrictions on pregnant animals
    - Restriction protocols that could cause severe discomfort.

Do this by:

- calling on established licensees to implement the framework and the CoPs and to monitor compliance;
  - having the NVWA use these CoPs as a reference when supervising the design and implementation of this type of research.
4. Safeguard and – where possible – improve the welfare of animals that are used for teaching behavioural tasks for the purpose of neurocognitive research.

In addition to the provisions of the framework and the CoPs, do this by:

- requesting that the Animal Welfare Body (IvD Platform) promote the knowledge of positive reinforcement<sup>11</sup> with and without restriction by means of targeted training and knowledge sharing;
- having a partnership initiated, for example by the NCad (or IvD Platform), between users and breeders in which the potential for animals to grow up in an enriched environment are explored and given shape, to allow animals to be taught to deal with complex/aversive situations at an early stage. The IvD Platform can subsequently be requested to take charge of further development;
- calling on establishment licensees to train animals in their familiar environment (home cage) as much as possible;
- periodically evaluating the CoPs and modifying them in the event of new insights.

5. Promote and guarantee the knowledge of different motivation methods among the parties involved. Have the Central Authority for Scientific Procedures on Animals (CCD: *Centrale Commissie Dierproeven*) assess whether this knowledge is sufficiently guaranteed in the project application and have the NVWA ensure that this knowledge is guaranteed in the workplace.

Do this by:

- requiring established licensees to have a Synthesis of Evidence conducted prior to the project to analyse the options for having an animal perform behavioural tasks without a restriction protocol and, if this is not possible, to choose a protocol that is least harmful to the welfare and health of the animals;
- requiring established licensees to provide a satisfactory explanation and substantiation of the choice for a motivation method in the project application. In addition to the aspects that are already mandatory, the following points should be explicitly addressed and substantiated here:
  - the method used and the period within which the tasks are taught
  - the conditions for learning and performing behavioural tasks
  - preconditions for accommodating and caring for the animals
  - criteria for the level and duration of the restriction, should this appear to be necessary.

# Substantiation of the opinion

*Sub 1: Use the “no, unless” principle as a starting point and do not apply any restriction unless there are well-substantiated scientific arguments and/or compelling public interests.*

The NCad does not consider the means but rather the objective of the research crucial to choosing a research method, with the 3Rs being the guiding principles. Research that requires animals to be motivated to carry out a specific behavioural task must, in principle, make use of a method without restriction.

*Sub 2: Promote the development and application of replacement methods for fluid or food restrictions.*

The NCad has, through expert consultations, found that there are ongoing innovative technological developments that could, in time, result in a decrease of the use of laboratory animals. The NCad recommends that the Minister have the target situations in relation to neurocognitive research included in the Agenda for Innovation Without Laboratory Animals (*Agenda Proefdiervrije Innovatie*) in order to implement and accelerate (future) developments that are ongoing within this research domain.

Such developments may include the application of techniques that measure the activity of a variety of brain cells simultaneously, for example using cellular imaging techniques and electrode arrays that measure the activity of multiple nerve cells (instead of the activity of a

single nerve cell at a time). The NCad recommends that the Minister not only invest in such technological developments, but also in animal-free innovations in order to replace animal procedures.

*Sub 3: Use this framework and Codes of Practice (CoP) as reference documents pursuant to Article 10 of the Experiments on Animals Act (WOD) (see appendices 1, 2 and 3)*

In consultation with experts, the NCad has drawn up preconditions and frameworks, which have been incorporated into a Framework and Codes of Practice. As such, these documents contain the prevailing views of the experts in the field and can be regarded as a point of reference for the interpretation of Article 10 of the Experiments on Animals Act.

It is vital that use of the framework and the CoPs be promoted by actors in the field (Central Authority for Scientific Procedures on Animals (CCD), researchers, Animal Welfare Body (IvD), Animals Ethics Committee (DEC), NVWA) in order to guarantee and improve animal welfare as best and as much as possible. The Codes of Practice provide clear guidelines for the selection of restriction protocol that negatively affects animal welfare as little as possible, in the absence of other options. The framework drafted by the NCad outlines the aspects that must be considered by a researcher in order to arrive at a suitable choice for a motivation method and, as such, provides guidelines for Animal Welfare Bodies (IvD), Animal Ethics Committees (DEC) and the Competent Authority (CCD) to assess whether this is a suitable choice made on the correct grounds.

The Codes of Practice include lower limits for the minimal amounts of fluid/food that an animal must consume during a restriction protocol. These amounts are determined on the basis of input provided by experts, scientific literature and with the application of the precautionary principle. These lower limits serve to protect animals from health and/or behavioural problems in the short and long term.

No uniform information could be obtained from the literature or consulted experts regarding young NHPs and the definition of “animals in a phase of growth” and the minimum volume of fluid to be provided. There is also a lack of scientific substantiation to provide a uniform opinion on the fluid needs of young animals for the fluid restriction protocols that are currently being applied.

The NCad, in its final decision, discussed two approaches to this issue, in which either the precautionary principle or practical expertise prevails:

- In the application of a precautionary principle, a minimal amount is determined that fulfils the physiological needs of the animal. Based on the various lower limits for fluid volumes for young animals to consume that are currently used in practice, the value is set at a minimum of 30 ml/kg metabolic body weight per day.
- Due to the absence of conclusive scientific substantiation, the everyday monitoring of the health and welfare of the individual animal must be guaranteed by including a number of specific relevant observations in the restriction protocol, which must be

reported and recorded daily. The restriction protocol must be adapted as soon as the physiological, clinical and/or behavioural parameters begin to deviate.

The NCad by a majority decision decided to recommend the precautionary principle (minimum 30 ml/kg metabolic body weight).

In the explanatory note to the vote, both dissenting members stated that given the absence of scientific substantiation, the determination of a generic value for the minimum volume of fluid intake required by a young animal is neither possible nor desirable. An approach in which the minimum amount is determined as a derivative of the individual free intake of fluid is preferred.

*Sub 4: Safeguard and – where possible – improve the welfare of animals that are used for teaching behavioural tasks for the purpose of neurocognitive research.*

The NCad deems that the welfare of animals that are used for teaching behavioural tasks for the purpose of neurocognitive research can be improved in a number of areas.

The first aspect relates to the preclusion of protocols that involve both fluid and food restriction. The NCad believes that a combination of fluid and food restriction results in too great an impairment of the welfare and health of the animal. In practice, this currently does not occur in the Netherlands and the NCad indeed recommends that this combination should not be permitted.

A second aspect relates to the facilitation of a dialogue between researchers and breeders in order to enable animals to grow up in an environment that is sufficiently enriched, in such a way that they learn to deal with complex/aversive situations from an early stage. As a result, the learning abilities of animals will be trained from an early age and the expectation is that they will be easier to train at a later stage.

A third aspect relates to training in their home cage (the familiar environment). By calling on established licensees to train animals in their home cage where possible, the stress related to the continuous displacement from and to the research cage can be decreased.

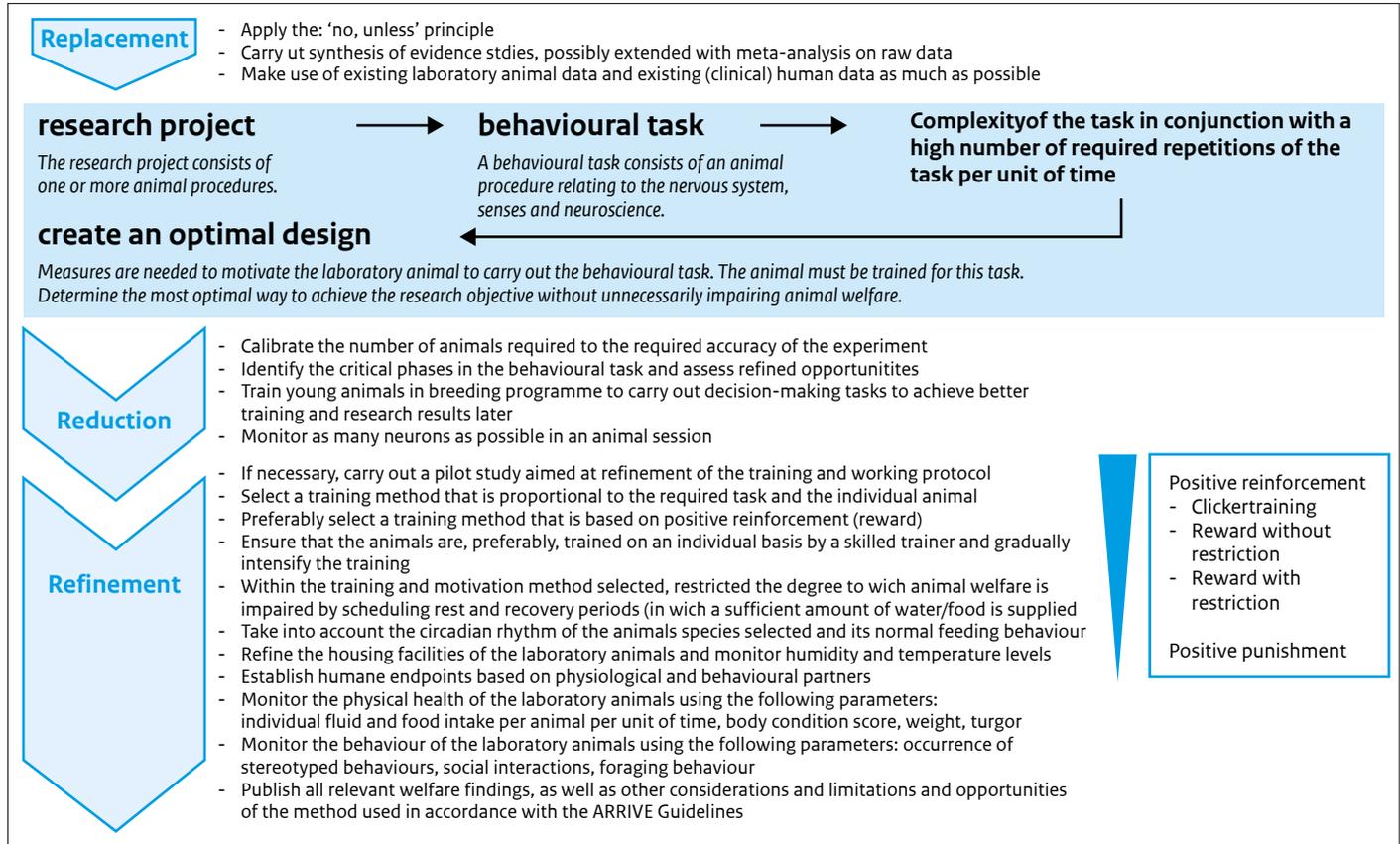
*Sub 5: Promote and ensure the knowledge of different motivation methods, including fluid or food restriction, among the parties involved. Allow the NVWA to oversee that this knowledge is ensured in practice.*

The NCad has found that the method of fluid and food restriction is familiar and common practice. The choice to use a restriction protocol seems virtually self-evident within neurocognitive research that uses animals to carry out behavioural tasks. The NCad believes that further refinement can be achieved by promoting and guaranteeing the knowledge of various motivation methods, including fluid and food restriction. By increasing the knowledge of other motivation methods, the choice for a restriction protocol will become less matter of course. Increasing knowledge on fluid or food restriction will allow a restriction protocol to be drawn up that is as refined as possible and which will have as little impact on the health and the welfare of the animals as possible.

In order to achieve this, the NCad recommends that the Minister appeal to researchers to publish their knowledge and experiences with regard to motivation methods. In addition, the NCad recommends that the Minister oblige the established licensees to conduct a Synthesis of Evidence, within the context of a project license application, into experiences relating to the advantages and disadvantages and effectiveness of motivation methods in relation to the level of discomfort and other options to carry out the research as refined as possible. The Synthesis of Evidence must be documented.

Furthermore, the NCad recommends that the Minister oblige the established licensees to provide sufficient clarification for their choice for a given motivation method and, as such, indicate why specific methods may or may not be applicable in that particular project. Specific aspects that must be addressed and substantiated in this regard relate to the training method, conditions and preconditions and, if restriction should appear necessary, the criteria governing the extent and duration of restriction. By clarifying and substantiating these aspects in the project license application, the Central Authority for Scientific Procedures on Animals (CCD) will be able to assess these aspects and they will be able to be monitored by the NVWA in the workplace. The figure (Figure 1) included in the opinion and the decision tree included in the framework may be useful in this regard.

Summarising Figure 1 framework/CoPs



# Appendices

## Appendix 1: Framework for best practices

This framework is an overarching document that can be applied to all animal species and is designed to act as a guideline for research in which the motivation of animals is required for the execution of a behavioural task. A highly common method within neurocognitive research, yet not limited thereto, is the restriction of fluid and/or food. This framework also outlines a number of other training methods. Researchers are required to make a reasoned choice for a particular training method, where, from an animal welfare perspective, in the event of equal effectiveness, methods of reward without restriction should be given preference over methods of reward involving restriction.

### Definition of terms

Methods that involve animals being motivated to carry out behavioural tasks by way of fluid or food restriction are categorised into a number of gradations according to the degree to which the amount of fluid or food is restricted and/or the duration within during which the fluid or food is made available to the animal. A number of concepts and definitions are used in these research methods. However, there is inconsistency in the interpretation of these concepts in the public debate on this subject, as well as in the literature and in existing Codes of Practice. The following section outlines what interpretations and definitions will be used for the

relevant concepts in this opinion and in the corresponding framework and the Codes of Practice.

- *Ad libitum fluid/food supply*: the supply of an unlimited amount of fluid or food of homogeneous quality and composition for a period of 24 hours a day, 7 days a week.
- *Restricted fluid/food supply*: the reduction of the **amount** of fluid or food made available to an animal and/or the **time units**<sup>12</sup> in which an animal is able to consume the fluid or food. Restriction may be applied in gradations, ranging from very limited to very severe restriction. Restriction may result in physiological changes, such as a decrease in body weight or decreased urine production. In cases of severe or long-term restriction, pathophysiological changes (physical and/or behavioural) may occur.

The terms restriction and deprivation are both used in the literature and in the field. Occasionally these concepts are used interchangeably, with the same definition, while on other occasions they are defined differently. Restriction is almost always interpreted as a reduced amount of fluid/food that is offered to the animal or a restriction of the duration during which the fluid/food is offered. Deprivation is sometimes used as a synonym for restriction, but is also defined as a total withdrawal of fluid/food (associated with a specific length of

time, e.g. 24 hours) or the omission of a specific nutrient, for example. Due to the various interpretations of the concept of deprivation, this opinion will use the term restriction.

Restriction may be necessary to sufficiently stimulate animals to carry out (complex/aversive)<sup>13</sup> behavioural tasks. The extent to which the animal has to be stimulated to carry out a particular task determines the extent and duration of the restriction applied by the researcher. The extent and duration of the restriction, in turn, determine the extent to which animal welfare may be impaired. This entails a delicate balance: too severe a restriction may impair the welfare of the animal to such an extent as to inhibit the stimulation of the animal to carry out the behavioural task, as it becomes lethargic (anhedonia) or overly tense (“over-motivation”), which adversely affects its learning ability. In such cases, an animal will no longer be capable of learning or carrying out the requested task. In extreme cases, the animal may even present symptoms of apathy. As such, restriction, dependent on the extent to which it is applied, may result in physical and/or behavioural changes such that this may result in a decrease in animal welfare.

A limited degree of restriction and the possibility of carrying out a task in order to obtain food may, however, have a positive impact on the animal’s welfare, both physically and mentally. For that reason, an optimal protocol, in which the intended scientific objectives are achieved with minimal infringement of the animal’s welfare, requires a carefully established balance between restriction, the animal’s needs and motivation.

## General

### *Selection of research method*

The NCad does not deem the means but rather the objective of the research crucial to selecting a research method, with the 3Rs as guiding principles. Research that involves the motivation of animals to perform a particular behavioural task must, in principle, apply a method without restriction, unless well-founded scientific arguments or compelling public interests have been demonstrated, or unless this would lead to a disproportionate increase in the number of laboratory animals being used. The selection of the research method must be well substantiated, with the foregoing aspects taken into consideration.

In order to guide this selection or decision process, a decision tree was included on page 26 which outlines the various decision points and options.

### *Animal species selection*

In general, neurocognitive research is carried out using rodents and primates (NHPs: non-human primates). In addition, as a general principle, the “lowest possible”, but nevertheless the most suitable animal species must be selected. Within this context, rodents are classified as lower animals than NHPs. The choice for NHPs is determined by a number of factors:

- Complexity of the task: the complexity of tasks that can be taught varies per animal species. The higher the animal species, the more complex the tasks will be that the animal is able to learn.<sup>14</sup>
- Translation to humans: the structure and functioning of the brain

in NHPs bears more similarities to that in and of humans than is the case with rodents. In addition, NHPs make eye movements and hand movements that are similar to those of humans.

In research into higher cognitive functions, the decision to use NHPs is considered.

### *Roles and responsibilities*

The roles of the various parties involved are laid down in the Experiments on Animals Act (Wod). Appendix 7 provides an outline of these roles and responsibilities, where possible specified according to research that requires animals to be motivated to carry out behavioural tasks within the context of neurocognitive research.

### **Animal welfare considerations**

The nature of the research and the competence of all parties involved in the implementation of the protocol in part determine the extent of discomfort. In order to guarantee animal welfare when these research methods are used, regular monitoring must take place of a number of key parameters related to physical health and behaviour. The findings must be recorded and assessed per individual animal. In the event of abnormalities, appropriate measures must be put in place to safeguard the welfare of the animals. Moreover, the documentation of the findings serves to build a dossier on the basis of which a welfare assessment can be drawn up that can lead to improvements in the research or in animal welfare. This should also result in a substantiated discomfort score at the end of the experiment for the benefit of the records. In addition, these notes are required for the

transfer of knowledge between the various stakeholders (researchers, analysts, biotechnicians, animal handlers, veterinarians). Finally, these notes are necessary to enable internal and external (NWWA) monitoring.

- Physical health parameters:
  - Fluid/food intake: daily
  - Condition of the animal:
    - (Deep-set) eyes, activity and coat grooming: daily; in addition, a more extensive observation during the weekly clean of the animal housing
    - Body condition score:<sup>15</sup> weekly, during cleaning activities or, if necessary, more frequently in the case of any deterioration in condition
  - Weight: registered at least weekly (rodents) or if the animal is removed from the cage and placed in the primate chair (NHP). More frequent monitoring may be required on indication of condition deterioration
  - On indication and if possible: urine production, specific weight, colour. In the event of any obvious deviations or on indication in combination with appropriate treatment or determinations of Humane Endpoint (HEP)
  - Dehydration (turgor): on indication
- Behavioural parameters:<sup>16</sup>
  - alertness
  - behavioural changes such as stereotypical behaviours, aggression and/or automutilation behaviour
  - social interaction within the group

- activity in the home cage
  - urine licking (in relation to NHPs)
  - body posture
- Housing parameters:<sup>17</sup>
    - humidity
    - temperature
    - light/dark rhythm

Access to fluid and food must meet the physiological and ethological needs of the animal as much as possible. This means meeting the nutritional, energetic and natural behavioural needs of the animal. The provisions in the CoPs Monitoring Welfare of Laboratory Animals issued by the Inspectorate for Health Protection, Commodities and Veterinary Public Health (predecessor to the NVWA; *Inspectie W&V: Inspectie Gezondheidsbescherming, Waren en Veterinaire Zaken*), if updated, constitute a guideline for welfare monitoring.<sup>18</sup>

## Humane endpoints

On the basis of the data collected through monitoring activities, a decision may be taken regarding whether it is necessary to intervene in the experiment. There are several types of possible intervention: treatment of an animal, modification of the degree of restriction, removing an animal from the procedure or killing the animal. The working protocol approved by the Animal Welfare Body should determine when an animal should be treated or whether the extent of restriction should be adapted or in the case of what severity or

combination of deviations (based on the foregoing parameters) a Humane Endpoint (HEP) can be established. In the event of a HEP, an animal must be removed from the procedure immediately or killed, depending on the severity of the deviation. If there are any doubts in this regard, the person referred to in Article 14 of the Experiments on Animals Act (WOD) or the Animal Welfare Body must be contacted immediately. These parties will determine whether – and if so what type of – intervention is required.

## Animal training

Animal training prior to their being used in the actual research must meet a number of requirements.

### Challenging animals to make choices

Experience has shown that animals that have grown up in an environment rich in stimuli which challenges them to make choices are frequently easier to train at a later stage. The best high-stimulus environment is created by providing (large-scale) group housing from a young age – social challenges and variation. For primates this means growing up in a breeding colony including family members. A high-stimulus environment can also potentially be created through the use of diet plates,<sup>19</sup> intelligence puzzles or other more complex types of environmental enrichment in addition to standard cage enrichment.

### *Uncooperative animals*

Should the experimental group include any animals that are difficult to train, then these animals may not be subjected to more stringent restriction than is outlined in the working protocol. In such cases, from an animal welfare perspective, the trainers must find other training methods/rewards that the animals respond to better, design the activity to be less complex/aversive or they must decide to remove these animals from the experiment.

### *Group versus individual*

Animals preferably should be trained individually and assessment take place on an individual basis regarding which training method/reward works best for that specific animal. A learning curve difference should be taken into account prior to an individual animal fulfilling a specific criterion (as such, an animal need not immediately be excluded if it demonstrates a slightly deficient learning curve). This, however, does not mean that this should result in the solitary housing of an animal during the training period.

### *Trainer requirements*

The trainer must be demonstrably qualified and must have knowledge of the species-specific behaviour and the needs of the animal species/strain they are working with. The trainer should have demonstrable knowledge of the various training methods and specifically of the training methods that are used. In addition, trainers are expected to keep their knowledge up to date.

### *Incorporating pilot study*

The execution of a Synthesis of Evidence<sup>20</sup> (SoE) is required prior to the study. The researcher and the Animal Welfare Body will subsequently discuss the information obtained from the SoE and the available knowledge and experience of the research group, taking into account the objective of the research, with a view to ascertaining whether it would make sense to incorporate a pilot prior to the actual research. This pilot could, for example, be used to explore whether a training method based on reward without restriction would be feasible within the framework of the research. If restriction is necessary, a pilot might be used to ascertain how the working protocol could be optimised. The pilot could be used to ascertain the minimum degree of restriction required to have a behavioural task be conducted with sufficient frequency.

### *Training structure*

The rate at which animals are able to learn varies per species, per strain and per individual animal. Therefore it is impossible to conceive a uniform training schedule that would function optimally for all animals. Gradual increase and build-up is crucial in any training method, allowing the animals to have time to learn what is required of them. If training is taking place using a restriction scheme, then this must be progressively increased, meaning that complex/aversive tasks should be taught in progressive steps. This also means that animals must initially be taught simple steps, such as getting used to the test design, using reward without restriction.<sup>21</sup> As the tasks become more extensive, food and fluid restriction can gradually be introduced. Based on a predetermined ad lib intake of fluid and/or food.

## Training methods

Animals are able to learn behavioural tasks through a variety of training methods. There is no generic best method, as the method depends on the research question. The section below provides a brief outline of some common training methods.

### *Positive reinforcement*

**Positive reinforcement training** (PRT) is usually used to teach a task.

This means that desired behaviour is rewarded with fluid or food and that undesired behaviour is ignored. This positive reinforcement will result in an increase in desired behaviour.

### **Reward without restriction**

In cases where the behavioural task permits, the animal will be trained without the use of restriction. Once the animal carries out its behavioural task, it is rewarded immediately, for example, with fruit juice, or with a “treat” (sucrose pellet; for macaques this may be a raisin or a peanut or, in any case, an item of food that is appealing compared to the daily diet). This method uses rewards that supplement the normal supply of fluid and food. This means that the animal will be saturated at a certain point and will no longer be prepared to carry out the task.

For that reason, the method is especially suited to tasks that are relatively simple and require little repetition. Reward without restriction is the preferred method for tasks that are less complex/aversive and as basic training for complex/aversive tasks.

### **Clicker training**

Positive reinforcement training may use a clicker (clicker training). During the acquisition phase<sup>22</sup> of clicker training, the animal is offered a reward at the same time or prior to a click (classical conditioning).

Over time, the animal will associate the sound of the clicker with receiving a reward, with the clicker acting as a bridge between the desired behaviour and the actual reward, which could be fluid or food (operant conditioning). As clicker training progresses, the click functions as a reward to the animal and a click need not necessarily be followed by a food reward every time. This will result in saturation occurring less quickly than in the case of animals subject to immediate reward without restriction. Clicker training can be combined both with a restriction regime as well as a regime without restriction.

### **Reward with restriction**

Once the task the animal must carry out becomes more complex/aversive, and more repetitions are required, training can be carried out using a restriction protocol. Here too the animal will be offered fluid or food immediately after it displays the desired behaviour (or carries out the behavioural task). However, the animal is restricted in the amount of fluid/food it is offered prior to the experiment. This makes the animal more thirsty or more hungry when the training (or task) starts and, as such, the animal will be more motivated to carry out its behavioural task than when it is not subject to restriction.

This method is currently widely used in teaching complex/aversive behavioural tasks and for tasks that require a large number of repetitions (for example, for over 100 repetitions). The Decision Tree and the Codes of Practice (*appendices 2 and 3*) outline the requirements for the execution of this training method.

#### *Punishing undesirable behaviour*

Instead of rewarding desired behaviour, trainers may also choose to punish undesirable behaviour, which is referred to as “positive punishment” in the literature.<sup>23</sup> In this instance, the animal experiences a certain negative consequence as soon as it exhibits undesirable behaviour (for example, an air puff or a light electric shock). “Positive punishment” is unpleasant, but generally not painful. The time-out is a variant of “positive punishment”, which involves undesirable behaviour punished with a time penalty. The subsequent trial of the task will, for example, begin half a second later, which prolongs the interval until the next opportunity of reward occurs. This variation of the punishment is used both for rodents and NHPs. As such, positive punishment can also be used in combination with positive reinforcement and restriction.

In summary, there are various training methods that can be used to teach animals behavioural tasks, either based on a system of rewarding desirable behaviour or based on a system of punishing undesirable behaviour, or a combination of the two.

As soon as an animal has learned the task and has mastered it, the trainers may choose to apply variable ratio and interval schedules:

#### *Variable ratio and interval schedules*

Variable ratio (VR) and variable interval (VI) schedules are methods of operant conditioning in which a reward is issued following a variable number of responses or following a response after a variable time interval. The absence of a fixed reward pattern results in a factor of unpredictability, which leads a stable and high response ratio. VR and VI can also be used regarding the nature of the rewards: by applying variation the animal is unable to predict which reward is to follow after correctly carrying out the task and will remain more motivated to carry out the task. The researcher is able to regulate the time, the amount and the nature of the reward. VR and VI can be used in all of the described training methods.

## Working protocol

In addition to the research objective and a substantiation of the elected procedure, the working protocol<sup>24</sup> must at least include the following information. This information may vary per animal species:

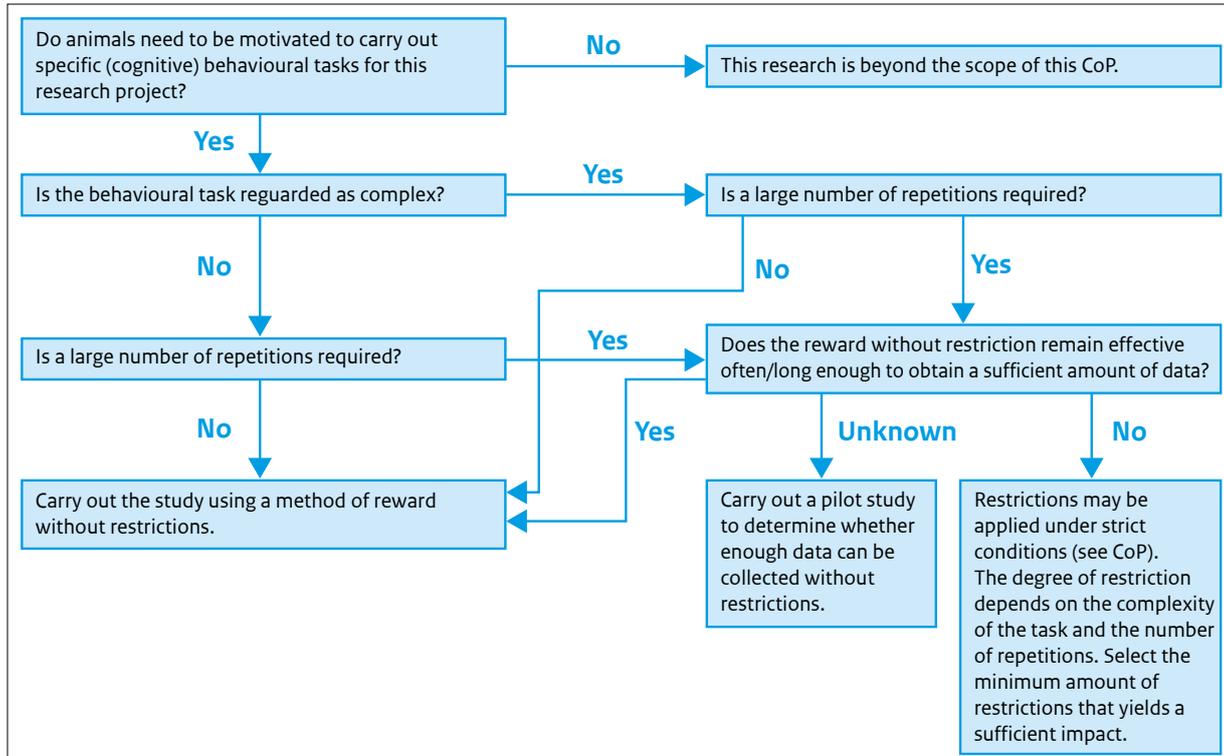
- duration of the research, starting from the training period
- species, strain, number of animals, age, sex, origin
- training of animals:
  - training method
  - training structure
  - selection of animals: age, sex, condition
- species-specific traits that should be taken into account (e.g. day/night rhythm, stress sensitivity)
- housing (with particular focus on group versus individual and cage enrichment)
- reduction: how to ensure that the minimum number of animals is used that is required in order to answer the scientific question
- refinement: a specific design of the protocol that limits discomfort to an unavoidable minimum
- restriction schedule if restriction is required
  - restriction schedule:
    - week regime
    - weekend regime
    - amounts per individual animal and dependency relationship, e.g. with body weight/natural intake per individual animal
  - minimum amount of fluid/food that is offered during training and during the experimental phase
  - times at which fluid/food is made available

- monitoring during the entire study (training, research)
  - in accordance with earlier description of “Welfare aspects” (fitness, body weight, etc.)
  - humane endpoints: criteria to adapt the protocol in the interim, suspend the procedure or prematurely terminate the procedure. Examples include:
    - criteria for maximum weight loss with regard to the reference weight<sup>25</sup>
    - nature and extent of physiological and behavioural changes
    - indication of dehydration
    - supplemental and experiment-specific endpoints
  - communication and agreements regarding monitoring
    - communication between researcher, appointed veterinarian, responsible biotechnician/animal handler and Animal Welfare Body (IvD)
    - monitoring times by supervisory bodies
  - reporting of monitoring findings
    - score sheets
    - welfare assessments

## Decision tree

All research must include a preliminary review of which method suits the purpose of the research best, taking into account the 3Rs. Researchers and assessors can use a decision tree<sup>26</sup> to establish which training method would best suit their research.

Decision tree



## Appendix 2: CoP for non-human primates

Neurocognitive research using non-human primates (NHPs) primarily use macaques, rhesus macaques in particular, and crab-eating macaques to a lesser extent. Marmosets are also occasionally used in research. This CoP is based on experiences with macaques.

If this type of research were to use another type of primate, a species-specific adaptation would be required of this CoP. Neurocognitive research conducted in the Netherlands only applies fluid restriction, not food restriction. There is no CoP for food restriction for NHPs.

### Guidelines for fluid restriction for NHPs

#### General:

1. During the drafting process of the project description and the working protocol in which fluid restriction is to take place, consultation will be required between the relevant researcher, the Animal Welfare Body (IvD) and an appointed veterinarian (and/or other relevant expert) regarding the design and the execution of the study, in particular, concerning the extent, duration and manner of fluid restriction, the training of the animals and the determination of decision points and criteria for interim adaptation, temporary suspension or premature termination of the protocol.
2. A project application must scientifically substantiate the researchers' choice for a motivation method (if fluid restriction is

selected) as well as provide the results of a Synthesis of Evidence.<sup>27</sup> It must also provide insight into the type of reward offered and method of dispensation.

3. The simultaneous application of fluid and food restriction is prohibited.
4. Pregnant animals may not be used for neurocognitive research in which fluid restriction is applied, unless explicitly permitted in the project license, following scientific substantiation and careful consideration and with an adapted degree of restriction.
5. In order to monitor the growth of the animal, the animal's weight progression is compared with the standardised growth curves for the species, sex and breeding line before and during the procedure, taking into consideration the percentile in which the animal is situated during the acclimatisation period. If the animal exhibits a sharply diverging growth curve and falls below 85% of its expected weight, then a decision must be made in consultation with the Animal Welfare Body/veterinarian regarding whether to (temporarily) remove the animal from the procedure or to limit restriction. In the event of a sharp drop in weight, a decision is made in consultation with the Animal Welfare Body and the person referred to in Article 14 (WOD) concerning what additional nutrition the animal is to receive and whether the procedure can be resumed.

6. The animals' living environment must be enriched in such a way that it encourages animals to make choices in their behaviour. This means that an animal must be housed in a social environment, unless solitary housing is permitted in the project license or is unavoidable for medical or social reasons. In such exceptional cases, the period of solitary housing must be kept to a minimum. In addition to a social environment, varying cage enrichment must also be offered to a sufficient extent.
7. An animal may carry out a task for a maximum duration per day that is outlined in the project license application, unless the animal is trained in the home cage and is free to determine when it wishes to carry out the task. If the animal is trained in a primate chair, the aim must be to have the animal seated in the chair for as short a time as possible. Each working protocol must make a reasoned case to the Animal Welfare Body regarding the maximum duration that the animal is allowed to spend on its task, also taking into account the consequent potential increase of the number of sessions required. Exceeding the length of time determined by the Animal Welfare Body (IvD) is only permitted following a revised recommendation based on an amendment to the working protocol. The Animal Welfare Body in such cases may not recommend that the animal be kept in the chair for longer than the maximum time laid down in the project license or, if not listed in the latter, in the corresponding project license application.
8. There should be enough time before the night period begins for the members of the cage to be re-introduced to one another after their "working day" (social housing). This also allows the health

and behaviour of the animals to be monitored. Sufficient time may vary for each pair. This may be determined in consultation with the responsible animal handler/behavioural expert.

#### *Training:*

9. Animals should have the opportunity to adjust to the schedule of restriction gradually and must demonstrably master their tasks before they are moved on to a higher level of training. The amount of time required for this will vary per species and per individual.
10. The use of a capture pole when training animals, for example, to ensure that the animals take their place in a primate chair, is only permitted if an agreement has been made with the Animal Welfare Body and/or external behavioural expert, and following approval by the NVWA, regarding what way the pole will be used if the animal cannot be trained in another way.

#### *Restriction protocol:*

11. The researcher responsible for the research must at all times, in consultation with the Animal Welfare Body, apply a type of restriction that is the least taxing for the animals and which still allows the research objective to be achieved. It is the task of the Central Authority for Scientific Procedures on Animals (CCD) to assess the reasons given for this choice.
12. There is a preference<sup>28</sup> for reward strategies that entail high-stimulation rewards, such as (watered down) fruit juice, which requires a minimum amount of restriction.

13. The degree of restriction is related to the number of required trials in combination with the complexity/aversiveness of the task. The lower number of trials required, the lower the degree of restriction is permitted.
14. Prior to a restriction protocol commencing, the body weight and the ad lib intake of fluid/food of the animal must be determined. Translated into metabolic weight (*please see Appendix 7*) this figure is used to determine the minimum daily fluid need of the animal. The actual supply of fluid will preferably be higher than this level and is determined on the basis of the results of the training.
15. The absolute minimum amount of fluid<sup>29</sup> that an animal must consume is 17 ml/kg<sup>30</sup> metabolic weight<sup>31</sup> per day. The running average over three days is at least 35 ml/kg metabolic body weight/day. For example: over a period of three days, an animal receives a total of 105 ml/kg metabolic weight. If an animal were to drink 17 ml/kg on Day 1 and 40 ml/kg on Day 2, then it must drink a further 48 ml/kg on Day 3 in order to arrive at a total of 105 ml/kg metabolic weight for those three days and a daily average of 35 ml/kg.

Animals that do not meet this minimum amount requirement during the procedure must be offered fluid in their home cage that same day, such that they still consume the daily minimum and the minimum running average for fluid. The aim should always be to have the animal drink more.

16. No uniform information could be obtained from the literature or consulted experts regarding young animals and the definition of “animals in a phase of growth” and the minimum volume of fluid to be provided. The fluid restriction protocols that are currently being applied lack scientific substantiation, making it impossible to provide a scientifically sound uniform opinion on the fluid needs of young animals. Due to the absence of a conclusive scientific substantiation, the everyday monitoring of the health and welfare of individual animals must be ensured by including a number of specific relevant observations in the restriction protocol, which must be reported and recorded daily. As a precaution, the minimum volume of fluid for young animals is set at 30 ml/kg of metabolic body weight per day, which is assumed to satisfy its physiological needs. The restriction protocol must be adapted as soon as the physiological, clinical and/or behavioural parameters begin to deviate.
17. Push days<sup>32</sup> are prohibited: if the animal succeeds at fewer trials than expected, the animal may not be (temporarily) subjected to more stringent restriction than stated in the working protocol. Push days constitute an infringement of animal welfare due to the fact that food and fluid is kept from the animal for too long.
18. When dispensing dry food, the fluid needs of the animal must be taken into account. As such, the combination of dry food with a type of fluid is preferred. Animals must be given fruit on a daily basis; the fluid intake from the fruit is not counted as part of the daily fluid intake.

19. As soon as it is clear that the animal has ceased carrying out a task<sup>33</sup> or is making too many errors, the procedure must be halted and this must be recorded in the welfare log. If, at that point, the animal has not yet consumed its daily minimum volume in fluid or food during the procedure, this must be provided until the animal has consumed its minimum amount. If the animal has consumed the minimum amount of fluid or food during the procedure, it can keep working as it will be possible to consume more fluid or food.
  20. If an animal begins to make too many errors, it is highly likely that the task is too difficult for the animal. In such cases, the supply of fluid may not be lowered to a level lower than is laid down in the working protocol. It may be determined, in consultation with the veterinarian or another sufficiently qualified expert and the Animal Welfare Body, whether the supply of fluid must be elevated in order to counter (initial) deviant behaviour or whether the task should be simplified.
- Monitoring health and behaviour*
21. Animals that are used in a fluid restriction protocol must be evaluated and assessed on a daily basis for potential changes in their health and behaviour. In the event of deviations, appropriate measures must be put in place to ensure the welfare of the animals. The findings must be recorded in the welfare log. The assessment, in any case, focuses on the behaviour, activity, alertness and the weight of the animals on the days that the animals are working.
  22. All information about the animals, procedure and the monitoring findings should be present or retrievable in the proximity of the animals.
  23. The duration and degree of the fluid restriction must be laid down in the working protocol, as well as any information on the training, key responsibilities and endpoints.
  24. If an animal falls ill during the procedure or is recovering from an illness or operation, or if the animal exhibits deviant behaviour, the animal will only be subjected to restriction once it has fully recovered. Whether or not the fluid restriction regime should be adapted is determined in consultation with the veterinarian. An animal that is ill at the start of the experiment or procedure, or is recovering from an illness or operation, or exhibits deviating behaviour, may only be used in the research – and be subjected to restriction – once it has fully recovered and has been released by the veterinarian or another competent person that is not involved in the research.
  25. It is undesirable to allow animals that have been subjected to a restriction protocol to suddenly have unlimited access to fluid, since this could lead to excessive intake. An animal should be offered no more than the individually determined *ad lib* intake established beforehand. In the event of extreme circumstances (e.g. dehydration) an animal must be taken out of the procedure for a long period of time. Even in the case of short-term recovery periods and periods during which the animal is not participating in training, the researchers must ensure gradual transitions; on

occasion a permanent – albeit light – restriction compared to voluntary intake is desirable (this must be assessed individually for each animal). In the case of short-term interruptions (weekends), restriction may be maintained, or the supply of fluid may be increased slightly. On both weekend days, an animal must at least receive the absolute minimum amount of food/fluid.

26. The parties responsible for the monitoring of the welfare of the animals must be demonstrably competent and qualified and must be able to recognise any deviations that indicate physical or behavioural changes.



## Appendix 3: CoP for Rodents

Research that involves rodents uses both rats and mice. There are physiological and behavioural differences not only between mice and rats, but also between the different strains of mice and rats, as well as between individuals within a certain strain. Researchers must be aware of the specific traits (fluid and food intake, behavioural, physical) of the specific breeding lines/strains/individuals they are working with. Neurocognitive research conducted in the Netherlands uses fluid and food restriction in research involving mice and food restriction in research involving rats. Fully substantiated values for the minimum fluid volume per kg of body weight per day for rats are not available.

### Guidelines for fluid or food restriction in rodents

#### General:

1. During the drafting process of the project description and the working protocol, in which fluid or food restriction is to take place, consultation is required between the responsible researcher and the Animal Welfare Body (IvD) regarding the design and the execution of the study, in particular concerning the extent, duration and manner of fluid and food restriction, the training of the animals and the determination of decision points and criteria for either interim adaptation, temporary suspension or premature termination of the protocol.
2. A project license application must scientifically substantiate the choice for a motivation method if fluid or food restriction is selected, as well as provide the results of a Synthesis of Evidence. It must also provide insight into the type of reward offered and method of dispensation.
3. The simultaneous application of fluid and food restriction is prohibited.
4. Pregnant animals may not be used for research in which fluid or food restriction is applied, unless explicitly permitted in the project license, following explicit scientific substantiation and careful consideration, and with an adapted degree of restriction.
5. The animals' living environment must be enriched in such a way that it encourages animals to make choices in their behaviour. This may mean offering an alternating variety of cage enrichment. In addition to cage enrichment, the animals must also be given a type of social enrichment.

*Training:*

6. Animals must have the opportunity to gradually adapt to the schedule of restriction and the fact that fluid/food can be obtained by conducting a type of task. The amount of time required for this will vary per species and per individual.
7. The individual weight of an animal must be determined prior to the protocol commencing. In the case of animals still in a phase of growth, a relevant control group/historical data pool must be used (growth curves of the suppliers are not suitable for this purpose due to the effect of the specific feed, the microbiological status of the animal facility, etc. on the growth curve). Correction may take place for the observation that animals on ad lib intake keep gaining weight after their growth period (fattening) by levelling off the control growth curve from adult age onward (approximately 2-3 months for mice and approximately 6 months for rats).

*Restriction protocol:*

8. The researcher responsible for the research must at all times apply a type of restriction that is the least taxing on the animals and which still allows the research objective to be achieved.
9. In protocols that use conditioning, the best type of reward and the best supply method must be ascertained based on the SoE or based on a pilot study. If necessary, a pilot study must be conducted beforehand.

10. The following absolute lower daily intake limits (i.e. this minimum must be provided every day) shall apply to animals (adult, not pregnant) subject to restriction:

	<b>Rats</b>	<b>Mice</b>
<b>Fluid restriction</b> <sup>34</sup>	No reliable values found	25 ml/kg/day
<b>Food restriction</b>	The amount of food that results in the animal not falling below the threshold of 85% of its body weight based on the growth curve of the relevant strain/ individual animal	The amount of food that results in the animal not falling below the threshold of 85% of its body weight based on the growth curve of the relevant strain/ individual animal

A higher supply must always be established, in consultation with the Animal Welfare Body, than is merely sufficient for the procedure, based on the strain used and dependent on the need of the animals. If the research uses the amounts such as are listed in the table above, then the necessity of such a choice must be substantiated in the project license application. The Animal Welfare Body is unable to recommend that an animal be given less than the amounts outlined in the table above.

- a. Animals that do not fulfil this established fluid intake requirement during the procedure, must be offered fluid that same day (in a familiar environment, such as the home cage or a cage specifically designed for that purpose), such that they still consume the required minimum daily amount of fluid.

- b. Animals that do not fulfil this established food intake requirement during the procedure, must be offered food that same day (in a familiar environment, such as the home cage or a cage specifically designed for that purpose), such that they still consume the required minimum daily amount of food.
11. Push days are prohibited: if the animal succeeds at fewer trials than expected, the animal may not be subjected to (temporary) more stringent restriction than is determined in the working protocol. Push days constitute an infringement of animal welfare due to the fact that fluid or food is kept from the animal for too long.
12. As soon as an animal ceases to carry out a task,<sup>35</sup> the session<sup>36</sup> must be halted. If, at that point, the animal has not yet consumed its daily minimum volume in fluid or food during the procedure, this must be provided until the animal has consumed this minimum amount. If the animal has consumed the minimum amount of fluid or food during the procedure, yet continues working, it must be given the opportunity to consume more fluid or food as a result.
13. Animals are rewarded using high-stimulant rewards, such as sucrose/saccharose pellets, sucrose/saccharose water or baby milk. The nature of what constitutes a highly stimulating reward may vary per specific animal or breed. The objective is to choose a reward that entails optimal fluid/food intake, which in some cases may be water.
14. The degree of restriction is tailored to the number of trials required, possibly in conjunction with the complexity/aversiveness of a task. The shorter in duration the task (possibly in relation to the complexity/aversiveness of a task), the less the animal may be restricted. No restriction is applied in cases of a complex/aversive task that need only be carried out a small number of times. *(please see decision tree on page 26)*
15. If the animal does not understand the task or is not inclined to perform the task, the complexity/aversiveness of the task must be reduced. In such cases, the fluid or food supply may not be lowered. In consultation with the person referred to in Article 14 of the Experiments on Animals Act (Wod, Article 25: designated veterinarian (2010/63/EU) and the Animal Welfare Body (IvD), it must be determined whether the fluid/food supply must be increased to counter (initial) deviating behaviour.
- Monitoring health and behaviour:*
16. Animals that are used in a fluid or food restriction protocol must be evaluated and assessed on a daily basis for potential clinical and behavioural changes. The findings must be recorded in the welfare log. Part of the assessment includes the monitoring/scoring of the animals' alertness, activity and physical condition. In addition, animals should be weighed regularly<sup>37</sup> and should be assessed for signs of dehydration.
17. All information about the animals, procedure and the monitoring findings and a description of individual monitoring deviations should be present or retrievable in the proximity of the animals.

18. The duration and the degree of the fluid or food restriction must be laid down in the working protocol, as well as any information on the training, key responsibilities, decision points and criteria for either the interim adaptation, suspension or premature termination of the protocol.
19. In order to monitor the growth of the animal, the animal's weight progression is compared with the standardised growth curves for the species, sex and breeding line before and during the procedure, taking into consideration the percentile in which the animal is situated during the acclimatisation period. Given that animals no longer grow in the case of ad lib intake but do grow fatter once they reach adult age, the growth curve may be levelled off at a maximum value after they have reached an adult age. If the animal exhibits a sharp drop or a sharply diverging growth curve and falls below the 85% threshold of its expected body weight, the animal must be (temporarily) removed from the procedure or its level of restriction must be limited. In the event of a sharp drop, in consultation with the Animal Welfare Body (IvD) and/or veterinarian, it must be determined how the animal should be treated (this may be laid down in the working protocol) and whether the procedure may be resumed. A sharp drop may also constitute a reason for a Humane Endpoint.
20. If the animal falls ill during the procedure or is recovering from an illness, or if the animal exhibits sharply deviating behaviour, a decision is made, in consultation with the Animal Welfare Body and/or veterinarian, whether the fluid or food regime should be adapted. An animal that is ill at the start of the experiment or procedure, or is recovering from an illness or operation, or exhibits deviating behaviour, may only be used in the research – and be subjected to restriction – once it has fully recovered. It is undesirable to allow animals that have been subjected to a restriction protocol to suddenly have unlimited access to fluid or food. Particularly with regard to fluid restriction this may result in excessive intake. An animal should be supplied with no more than the average voluntary fluid intake of the species (age, weight). Even in the case of short-term recovery periods and periods during which the animal is not participating in training, the researchers must ensure gradual transitions and a permanent, albeit light, restriction compared to voluntary intake. Restriction may be maintained during short-term interruptions (weekends) or the supply of fluid/food may be increased slightly (e.g. in the case of social housing where the supply is distributed per cage, to ensure that every animal consumes a sufficient amount). On both weekend days, an animal must at least receive the absolute minimum amount of fluid/food.
21. The parties responsible for the monitoring of the welfare of the animals must be demonstrably competent and qualified and must be able to recognise any deviations that indicate physical or behavioural changes.
22. If animals are required to undergo an operation during behavioural training, it is not recommended that animals be put on ad lib fluid and food intake and then be placed under restriction following their recovery. This is to avoid any major fluctuations in the animals' weight. The recovery period following an operation may



vary per species, type of operation and per individual. This will be monitored based on the clinical observation of the recovery.

#### Species-specific key areas of focus:

- Rodents live according to another circadian rhythm than humans. Given that their day and night rhythm is the reverse of that of humans, this must be taken into account in the research design. The restriction of animals during their active period will have a more significant physiological impact than any restriction of animals during their non-active period and may affect their welfare.<sup>38</sup> The behavioural task is carried out during their active period and any deviation from this should be substantiated in the project license application.
- Cage enrichment: an exercise wheel (or other movement stimulator) in conjunction with food restriction may result in caloric malnutrition and behavioural abnormalities (e.g stereotypy and disruption of the circadian rhythm) in rodents. In the event of sharp weight loss and behavioural abnormalities, the exercise wheel (or other movement stimulator) must be removed from the cage. (*Also see point 19*).

## Appendix 4: Recommendations arising from the consultation of community groups

On 26 January 2017, the public consultation was conducted in Utrecht. The following organisations were present at this consultation:

- Animal Rights
- Association of Laboratory Animal Science Professionals (Biotechnische Vereniging)
- Biomedical Primate Research Centre (BPRC)
- DEC Consult
- Dutch Society for the Protection of Animals (Dierenbescherming)
- Donders Instituut Radboud MC
- Dutch Brain Council
- Animal Welfare Body Erasmus MC
- Platform of Animal Welfare Bodies (IvD Platform)
- Janssen Pharmaceutica NV
- Katholieke Universiteit Leuven
- Netherlands Federation of University Medical Centres
- NVDEC
- PETA
- Three R's Alternative Initiating Network (TRAIN)
- Willy van Heumenfonds/Stichting Stimuleringsfonds Alternatieven voor Dierproeven

The NCad derived recommendations from the audio recordings of the meeting, which were then presented to the organisations in question for approval. In addition, all participating organisations were given the opportunity to submit additional input in writing.

Below is a list of the recommendations for each subject that were approved by the organisations present, and for each one, indication is made of whether or not the recommendation in question was included in the NCad's advice. If it was not included, then a brief reason for this is given.

### Definitions:

#### Netherlands Federation of University Medical Centres

A more species-specific approach will have to be taken. Body weight is very important to physiology, because the metabolism per gram of an animal is much higher in a small animal than in a large animal. The focus should be on the supply of water/food (something done by humans) rather than on the intake (something done by the animals). Be accurate in the language – water, fluid and liquid are all used randomly and interchangeably in the presentation, yet do not always come down to the same thing.

*Duly noted*

## NVDEC

The scientific literature uses the term “controlled water supply”. The correct Dutch translation of this term is “gereguleerde waterverstrekking”. Well-defined concepts are crucial, however must not be canonised. Their complex meaning may lead to more confusion rather than clarity. The terms are even used inconsistently and incorrectly in the literature at times. The following elements should in any case be specified in the definitions:

Food withdrawal	Food restriction	Both concepts
Start and end times of the withdrawal	Amount of food per animal per unit of time	Start and end time of training/procedure
Frequency with which this withdrawal is applied	Times at which food is offered	Transition of dark-light period
Key principle: outside of the withdrawal period, food is available ad libitum	Nutritional ratios in the food	Transition of light-dark period
	On which days of the week is restriction applied if not continuous?	Housing: individual or social
	What is the target body weight (% compared to weight for ad libitum feeding)	

*Duly noted*

## DEC Consult

There are enormous differences between the various strains of mice. As such the level of the species is too high an aggregation level to serve as a frame of reference in this instance.

What exactly is meant by natural homeostasis? It is not just about the amount of water the animals consume, but also we should also take into account the time of day at which it happens. If the animal is given enough water, but at the wrong time of day, this can have significant consequences, however this would then fit within that natural homeostasis.

The “allowed to consume” section is very vague. What is “allowed”? What is the reference framework? If we assume an ad libitum reference framework, then we could ask ourselves whether that reference framework is appropriate. There is evidence that animals start drinking more than they actually want out of sheer boredom. The idea is to decrease something – but in comparison to what? Setting that reference framework is paramount. At present, that reference framework is not clear in any of the current definitions. Perhaps we should be making assumptions based on the individual animal, however should we than base it on what the individual animal drinks if it has access to a bottle of water in its cage for an entire day? Is that natural homeostasis? No. In fact, we could do away with all the definitions. There is still another term: access to water.

*Duly noted*

### **Dutch Society for the Protection of Animals**

Where should the definitions be situated in the end result? The definitions should be defined as clearly as possible, whereas currently they can be interpreted in every which way.

*Included in the opinion: yes.*

### **Dutch Brain Council**

Non-contaminated can be removed from the definition. Are we only talking about the amount of food or including the quality of the food – for example, by leaving out a specific nutrient? The definitions should be more uniform.

*Included in the opinion: yes.*

### **Animal Welfare Body Erasmus MC**

All four definitions are currently problematic. Standard water intake does not need to be defined. The best thing to do would be to leave it at ad libitum and compare everything with that.

Controlled water intake currently ends with the word restricted, which is unfortunate. It now looks as if no controlled and restricted water intake can take place.

Natural homeostasis is also problematic. In practice, these animals will anyway experience some weight loss compared to the ad libitum feeding. If the ad libitum state is regarded as natural homeostasis, all procedures that are conducted in practice would fall outside of that. The definition of water deprivation is not in accordance with the literature in English, where deprivation is described as total withdrawal of fluid, even if it is only for a six-hour period and if there is no impact on the animal.

The reference framework we need is that of the animal's health. If we were to use normal water intake as a reference framework, that throws up endless discussions on what normal water intake actually is.

*Duly noted*

### **Willy van Heumenfonds/Stichting Stimuleringsfonds Alternatieven voor Dierproeven**

Homeostasis is not a standard aspect that is incapable of variation: it's something dynamic and adaptable. Rats are able to be deprived of water for 24 hours without any problems, because they are able to adapt by way of a number of homeostatic mechanisms. So this is a tricky concept to be using here. The definition "long-term" is used. When is something long-term? An hour, a day, a week? This is also a problematic term. In fact, everything that is not ad libitum should be regarded as a controlled supply of food or water. The only thing one can debate in that instance is the extent to which the researcher controls that supply. The researcher is the deciding actor. The only debate in this case is "how long?" and "what kind of pattern?"

*Duly noted*

### **Platform of Animal Welfare Bodies (IvD Platform)**

The terms currently contained in the definitions, such as intake, fluid, and homeostasis can cause confusion. Primates that are subject to deprivation potentially can start drinking their own urine. Urine is a fluid, the animal is conducting the intake itself – is this a case of homeostasis? Granted, it is an undesirable behavioural expression, yet it would fit within this definition. If we were to replace the term intake by supply, the problem would be solved.

*Duly noted*

## Animal welfare

### Animal Rights

At the moment, the main focus is on the animal's physiological state, but what is missing is the animal's mental state, which is an equally important aspect of the animal's health. It is not only about ensuring that the animal receives a sufficient amount of water; if it has had to work very hard to get, then this will have a certain impact on its behaviour. If everyone would only get 80% of what they normally eat, they would be very unhappy.

*Included in the opinion: yes.*

Inspection reports have shown that there are serious issues surrounding neurological research in primates. Primates have been known to drink their own urine and there are other welfare issues in play.

*Included in the opinion: yes, by setting minimum amounts for fluid intake and by setting requirements for enrichment.*

Neurological research involves wounds to the skulls of primates, which frequently do not heal properly. Is this a result of the restriction due to the fact that they consume an insufficient amount of water?

*Included in the opinion: no, the NCad has found no evidence to that effect.*

### NVDEC

The objective of the restriction is to affect the mental state of the animal: the purpose is to motivate the animal to do something. Animals that receive a limited amount of food or water will exhibit all the signs of stress. The key issue is how severe that stress is allowed to be. This is difficult to quantify and measure. As long as in doing so

the minimal physiological needs of the animal are met throughout the entire circadian cycle the related occurring stress should not be such that it would constitute an impairment of the animal's welfare. Food can be restricted in all sorts of ways. For rats this can take place using a diet board, where pellets of feed are hidden inside a wooden cross. The rats must subsequently make an effort to extract the food. This results in the animals falling to 80% of their normal body weight and their requiring no further restriction to be stimulated. The diet board was only recently validated for a number of rat strains and, as such, is not yet widely applicable.

*Included in the opinion: yes.*

Small animals have a high metabolic rate and little storage of nutrients, which requires them to have access to food very regularly. After 4 hours of food withdrawal, their metabolic rate will drop dramatically and the animal will enter into a type of brief hibernation. This is how they save energy. After 16 hours, the animal will recover quite suddenly, resulting in it being able to make one last attempt to find food. This mechanism requires a great deal of the physiological adaptability of the mouse and may have a significant impact on the results of the procedures. The mental state of the animals in such cases is far from ideal. Driven by hunger, these animals will not learn well, they will make many mistakes and respond impulsively. Key areas of focus for food restriction in rodents:

- Timing: withdrawal during the active dark period will have a larger impact on welfare and physiology.
- Duration: food withdrawal may not be imposed for too long. The withdrawal period must align with the training/the procedure.
- Smart adaptation of the circadian rhythm: reverse circadian rhythm

or switch from light to dark period at noon and from dark to light at midnight.

*Included in the opinion: yes.*

#### **Netherlands Federation of University Medical Centres**

A wide range of behavioural and welfare studies have shown that animals find food far more interesting when they have had to work for it, for example, through solving a puzzle. This means that we would have to take a highly nuanced look at what would be effective to assess what kind of behavioural impact is triggered. Even repetitive behaviour requires a critical examination of what is happening – whether it is a type of compulsive behaviour (e.g. stereotypy) or whether it is learned, often context-dependent, behaviour that keeps repeating itself once the reward follows (e.g. a dog that sits in front of the door barking as soon as someone grabs the leash).

*Duly noted*

Animals can be trained using positive reinforcers and negative reinforcers. Initially withdrawing food ensures that food becomes a positive reinforcer (reward). Researchers should take care that the learning capacity of the animal has not been affected if it has not eaten or drunk for a period of time. Too drastic a withdrawal of food or water would be an unnecessary impairment of the animal's welfare. Nevertheless, the literature shows that animals that are subject to food or water restriction are healthier, live longer and are better at learning and remembering. So there is a grey area within which a certain level of restriction improves the performance of the animal, also benefiting it in terms of health and life expectancy.

*Included in the opinion: yes.*

#### **Willy van Heumenfonds/Stichting Stimuleringsfonds Alternatieven voor Dierproeven:**

The controlled supply of food and water can have highly significant welfare benefits. It results in the foraging behaviour of the animal being accompanied by a positive reward of the success of its own behaviour. Animals become euphoric once the food appears. There are a number of great studies by Kelly Lambert in which she has the animals working for food, for example. While the animals did have a lower body weight, they were healthier, more robust and resistant to stress – in short, many positive things. The starting principle of having ad libitum food and water continually available, in fact, is unnatural and for many species will result in boredom and obesity.

*Included in the opinion: yes.*

#### **Dutch Society for the Protection of Animals**

How does it relate to the outcome and predictability of your research results? How far do you go in affecting behaviour by affecting the physiology of a living creature and how does this compare and relate to the outcome of your research? How do you weigh one against the other? Not every researcher will take the time to examine every individual animal in every test. There should be objectively quantifiable indicators. In addition, researchers will need help, however much knowledge and expertise they already have. There should be guidelines on which researchers all agree, otherwise this type of research simply should not be carried out.

*Included in the opinion: yes.*

### **DEC Consult**

Providing 80% food is better for animals than ad libitum. Plus, animals enjoy it and develop less stereotypical behaviour when they have to work. The training itself can also be regarded as a type of cage enrichment. It is, however, very essential that the point at which the animal ceases to enjoy it and this adversely affects its physiology be clearly defined.

*Included in the opinion: partly, the NCad recognises that ad libitum feeding may have a negative effect on animal welfare.*

Should an animal have access to water during a procedure and if it were to drink its daily requirement there but afterwards be returned to its cage and be given dry food to eat, then, at that point, it would not have any water at a time when water is needed. In this case, an animal would formally have consumed its daily amount of water, but at the wrong time and in the wrong context. The criterion should be: when do circumstances become unpleasant for the animal?

*Included in the opinion: yes.*

In mice, it is very easy to take the step to simply maintain an ordinary circadian rhythm. It is surprising how easily researchers opt to carry out these types of things for animals kept in standard housing. And it is clear that animals are far less able to cope with this than when you implement this during their active period.

*Included in the opinion: yes.*

### **PETA**

There are myriad relationships between restriction, on the one hand, and the welfare of animals on the other hand. For that reason, the Association of Primate Veterinarians in the United States has condemned this type of research.

*Duly noted*

### **TRAIN**

Water restriction is worse than food restriction.

*Duly noted*

Delayed wound healing could rather be attributed to stress caused by the deprivation, than by the deprivation itself.

*Duly noted*

Reversing the circadian rhythm is not even necessary if the researchers apply an effective feeding schedule whereby the animals do not have to actively search for food the entire night but do have an empty stomach in the morning.

*Included in the opinion: yes.*

### **Animal Welfare Body Erasmus MC**

For rodents, food restriction is worse than fluid restriction.

*Duly noted*

### **Platform of Animal Welfare Bodies (IvD Platform)**

If you restrict rodents during the night-time, this leads to a sharp increase in foraging behaviour, meaning that they burn an additional amount of calories. Such animals will generally lose 10% of their body weight by morning and a post-mortem will show that their liver will have almost completely disappeared.

*Duly noted*

### **Donders Instituut Radboud MC**

Researchers are open to receiving help. We agree with the debate regarding the difficulty of determining a benchmark of what constitutes a healthy animal and what a change in homeostasis means. We also agree with the view that considerations must be made from the perspective of the animal regarding whether water or food deprivation can lead to undesirable effects.

*Duly noted*

Another training method uses sucrose pellets or flavour pellets to reward animals. Give animals something tastier than their normal food. This already makes rats sufficiently motivated to work and does not require deprivation. It may also be possible to motivate animals without water/food restrictions if researchers take a more thoughtful approach to dealing with the animals' natural rhythm (more food/water intake during dark phase than light phase) or by challenging the animal by hiding food or enriching the cage.

*Included in the opinion: yes.*

We can support the proposal that states that researchers must first look for an alternative to motivate animals, with food/water deprivation being a Plan B. Finding other alternatives need not be a detailed study costing animals/time/money, but hopefully this process can be supported by the pooling of the knowledge/experience available in the Netherlands.

*Included in the opinion: yes.*

### **Training and selection of animals:**

#### **BPRC**

Teaching and training the animal is a crucial aspect. What happens to the animal from the moment it has to learn that something is changing in its normal situation? And how can we ensure that the animal carries out a learning task optimally with as little impairment of its welfare as possible? This is an often forgotten aspect.

We should consider things from the perspective of the animal, certainly in the case of primates. Choosing which animals are suitable for participation in such procedures is also very important.

*Included in the opinion: yes.*

### DEC Consult

As a researcher, you can choose how big a step you want an animal to make in a single session. And how do you motivate it for this? As a researcher, you even have the option of selecting a specific strategy. You might take three weeks to teach it in small steps, or you might be in a hurry and apply more stringent deprivation – this might motivate the animal even more and lead to you making that crucial step sooner. Everything so very much depends on the choices an individual researcher or a group makes in that regard. There are situations in which the animal need not be deprived at all, because it will carry out the test anyway. And that means you may have to establish that in neurocognitive research, deprivation should be Plan B. It means first trying to establish whether you can succeed without any form of deprivation. This is also related to how the training is structured. It is also incredibly important that there is complete transparency. The supervisory bodies should be right on top of things and be given the opportunity to guide practices.

*Included in the opinion: yes.*

The first part, the training, is the most important part, as well as doing things in a standardised way by one and the same person or a very small group of people. And if at a certain point you can then safely say “this animal has been trained” and it enters the procedure, you may have avoided a lot of trouble.

*Included in the opinion: yes.*

### Janssen Pharmaceutica NV

Belgium uses another system that involves local ethics committees and local review of protocols. This means that the parties involved in assessing the protocols visit the workplace more often and are physically present during the procedures to see how the animals are faring.

*Duly noted*

In my opinion it is crucial to apply restriction when training rodents for neurocognitive research. It makes them more motivated to find food in the setup of the procedure. We have observed that rodents perform the task with pleasure, virtually waiting in their home cage for the moment they can get started.

*Included in the opinion: yes.*

### NVDEC

For mice and rats, a lot could be gained if the Animal Welfare Bodies (IvDs) simply had the knowledge that is available at the moment, which is actually not very much – not a lot of knowledge is required. There are a number of guidelines that can be used to make a tremendous stride in the right direction to ensure deprivation takes place more effectively than in general. This is something the IvDs should be aware of. The deprivation protocol should be clearly set out in writing and the Platform of Animal Welfare Bodies should make an effort to put this issue on the agenda of the IvDs.

*Included in the opinion: yes.*

## Types of deprivation:

### Biotechnische Vereniging

One type of deprivation that has not been addressed yet at all, but of which we could wonder what type of impact it might have on animal welfare, is social isolation. Is this included in the Code of Practice?

*Included in the opinion: yes.*

The more enriched the animals grow up, the more they are capable of. Having animals grow up in an impoverished environment, in fact, also constitutes a type of deprivation, namely deprivation of cage enrichment.

*Included in the opinion: yes.*

### Animal Rights

Sleep deprivation is another type of deprivation.

*Included in the opinion: yes.*

### DEC Consult

There have been a number of very intriguing developments in recent years. Animals have previously been kept in relatively spartan cages, where there is nothing at all to do. Subsequently they are made to carry out numerous complicated tasks within the context of a study – and are unable to do so very well. Many animals drop out because they are not able to learn effectively. There are, however, increasing numbers of articles that assert that those young animals should be raised in an enriched environment, in order for them to learn how to solve problems from the very beginning, citing some examples, and referring to standardised enriched cages. It has been shown that these

types of animals are significantly more successful at neurocognitive experiments and that these experiments thus are accompanied by much less discomfort. As such, this aspect of specifically selecting animals, which already happens in the case of primates, should also be done for rats and mice.

*Included in the opinion: yes.*

## Other remarks:

### Dutch Society for the Protection of Animals

How is the international context put to use? It is vital that knowledge and best practices are exchanged on an international level.

*Included in the opinion: yes.*

### Biotechnische Vereniging

Please include the following in the opinion: each researcher in their research application, within the context of the 3Rs policy, should indicate whether they have considered methods of refinement or replacement. The box for the 3Rs can be used to require the researcher to indicate: have other methods than deprivation been considered, such as reward? If so, which methods have been considered and why have they not been deemed suitable?

*Included in the opinion: yes.*

### Dutch Brain Council

The researcher should be encouraged to reward the animals with rewards other than food or water. The animals should be challenged to do something they enjoy or find appealing.

*Included in the opinion: yes.*

### Animal Rights

Water restriction in conjunction with the surgical procedures and long-term animal procedures or experiments cause stress, anxiety and pain in animals. As such, we are in favour of a ban on water restriction applied to primates in neurological research.

*Duly noted*

### Katholieke Universiteit Leuven

There is a clear difference between rodents and NHPs, which must be taken into account in the Code of Practice. A CoP should be workable, even for primates that are required to perform highly complex tasks and which require a high number of trials. In addition, the CoP must be clear and specific: the concepts and definitions that can currently be found in the literature and which were also represented into the definitions are highly plastic and open to interpretation (in both directions), for example, “a normal physiological condition”: what does that mean? Specific circumstances should also be taken into account: if the animals (NHPs) only eat dry food, they would need more water than if their diet included a lot of fruit. Time of day is a key element with regard to rodents. Finally, the transition of ad libitum feeding to restriction and vice versa should also be addressed.

*Included in the opinion: yes.*

### Written input in addition to input provided during consultation of community groups

#### PETA

- The use of non-human primates in scientific research should be abandoned immediately: alternatives are available. The use of non-human primates for scientific purposes is ethically unacceptable given the cognitive and emotional abilities of these animals. Standard laboratory housing does not meet the ethological requirements of non-human primates. Although there are neurological similarities between humans and non-human primates, small deviations in such complex systems lead to major functional discrepancies and inequalities. As such, the use of non-human primates has not significantly contributed to the development of treatments to human diseases.

*Duly noted*

- In neurocognitive research, non-human primates are subject to considerable suffering due to invasive and demanding procedures. In single-neuron tracing studies, a metal plate is screwed to the skull to immobilise the heads of the animals. Infections as a result of this surgical procedure are a frequent problem. For that reason, animals are kept in isolation and deprived of social interaction. Humane alternatives are available for this type of research. Humane single-neuron studies have yielded valuable insights in this regard.

*Duly noted*

- Food and fluid restriction are applied as a method to coerce the animal to carry out a specific task. Serious welfare issues are associated with food and fluid restriction which could affect both the physical and mental health of the animal and which could also endanger the objective of the research.

*Included in the opinion: yes.*

- The problems regarding animal welfare in food and fluid restriction are as follows:
  - the risk of nutritional imbalance
  - weight loss (or reduced growth in young animals)
  - the aversive experience of hunger/thirst
  - fluid-restricted animals are less able to cope with stress, caused by illness

*Included in the opinion: yes.*

- Experiences with hunger or thirst are not desirable. Animals work in order to avoid their ending up in such an aversive state. In rhesus macaques undergoing a 5-day or 7-day fluid restriction protocol, behavioural changes will occur that can indicate a reduction in animal welfare, such as stereotypical behaviour.

*Duly noted*

- The individual housing of non-human primates for restriction to be carried out results in the animals not only being denied food/fluid, but also being denied essential social contact.

*Included in the opinion: yes.*

- Alternative research methods:
  - preferred reward: if the reward is appealing enough, the animal will carry out the task without requiring restriction. Given that this is an individual preference, “preference tests” may be carried out prior to the research, in order for the best rewards to be

determined. Variation in rewards will also ensure that the animals’ level of motivation remains high.

- variable-ratio schedules: the number of responses does not determine when the reward follows. Not knowing when a reward is to follow will ensure that the animals stay motivated and yield more consistent response rates.

Conditioned reinforcers: a reward for carrying out the task is referred to as the primary reinforcer. A conditioned reinforcer is associated with the primary reinforcer and thereby becomes a reinforcer in itself. Every now and then the conditioned reinforcer can be used without the primary reinforcer.

*Included in the opinion: yes.*

#### NVDEC

For mice and rats, a lot could be gained if the Animal Welfare Bodies (IvDs) simply had the knowledge that is available at the moment, which is actually not very much – not a lot of knowledge is required. There are a number of guidelines that can be used to make a tremendous stride in the right direction to ensure deprivation takes place more effectively than it currently generally is. The IvDs should be aware of these guidelines and I would like to call on the Platform of Animal Welfare Bodies to ensure that this issue is put on the agenda.

*Included in the opinion: yes.*

# Appendix 5: Body Condition Score for rodents

## Description of procedure:

Determining the Body Condition Score (BCS) in rodents is a non-invasive method to assess the health of the animals. BCS has a range of 1 (emaciated) to 5 (obese). An expected BCS of 1 or 2 requires intervention in the protocol (IACUC).

The BCS is determined by way of a visual and physical examination of the animal. The physical examination is carried out by gently holding the mouse by the base of tail and moving a finger across the sacroiliac joint. The figures below illustrate which findings correspond to which score. Scores must be documented per animal.

## References

Ullman-Culleré, M.H., Foltz, C.J., (1999). Body Condition Scoring: a rapid and accurate method for assessing health status in mice. *Lab. Animal Science*; Vol 49 (3) 319-323.

Hickman, D.L., Swan, M., (2010). Use of a body condition score technique to assess health status in a rat model of polycystic kidney disease. *J Am Assoc Lab Anim Sci.* 49(2):155-9.

The Institutional Animal Care and Use Committee (IACUC);  
Body Condition Scoring of mice

<http://www.iauc.ucsf.edu/Policies/awSPBodyCondScore.asp>

Figure 2: Body Condition Score for mice

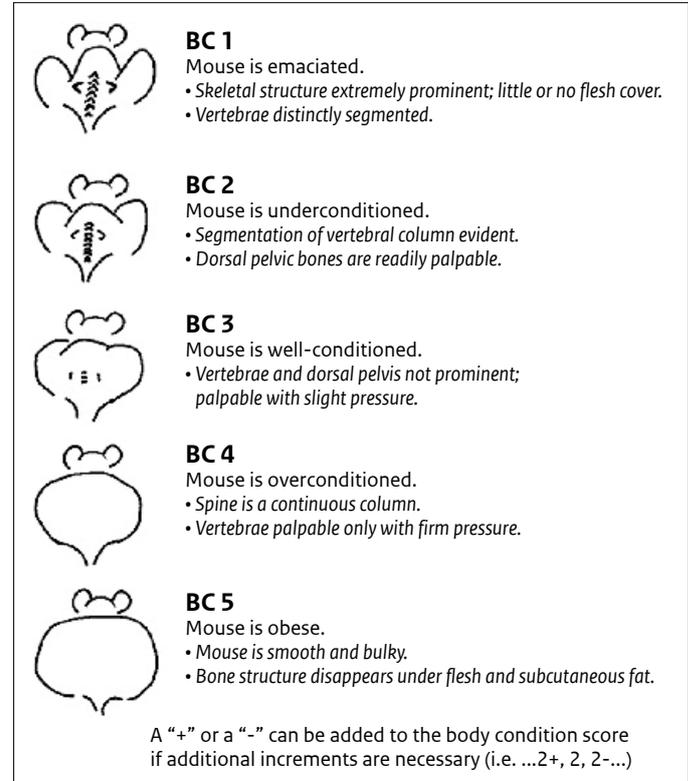


Figure 3: Body Condition Score for rats



**BC 1**

- Rat is emaciated.
- Segmentation of vertebral column prominent if not visible.
  - Little or no flesh cover over dorsal pelvis. Pins prominent if not visible.
  - Segmentation of caudal vertebrae prominent.



**BC 2**

- Rat is underconditioned.
- Segmentation of vertebral column prominent.
  - Thin flesh cover over dorsal pelvis, little subcutaneous fat. Pins easily palpable.
  - Thin flesh cover over caudal vertebrae, segmentation palpable with slight pressure.



**BC 3**

- Rat is well-conditioned.
- Segmentation of vertebral column easily palpable.
  - Moderate subcutaneous fat store over pelvis. Pins easily palpable with slight pressure.
  - Moderate fat store around tail base, caudal vertebrae may be palpable but not segmented.



**BC 4**

- Rat is overconditioned.
- Segmentation of vertebral column palpable with slight pressure.
  - Thick subcutaneous fat store over dorsal pelvis. Pins of pelvis palpable with firm pressure.
  - Thick fat store over tail base, caudal vertebrae not palpable.



**BC 5**

- Rat is obese.
- Segmentation of vertebral column palpable with firm pressure; may be a continuous column.
  - Thick subcutaneous fat store over dorsal pelvis. Pins of pelvis not palpable with firm pressure.
  - Thick fat store over tail base, caudal vertebrae not palpable.

## Appendix 6: Roles and responsibilities

### *Research*

The researcher holds primary responsibility for the research and its execution. The researcher is responsible for drafting a design for a research model for which the Animal Welfare Body (IvD) must be contacted prior to submission of the project application, in order to arrive at the most refined research design possible. The choices made by the researcher must be well substantiated. In addition, the researcher must have knowledge of the specific animal species/strain he/she is working with as well as of the various training methods. After a license has been issued for the project by the Central Authority for Scientific Procedures on Animals (CCD), the researcher must draw up a working protocol, which is aligned with the Animal Welfare Body and the designated veterinarian or other relevant expert.

### *Biotechnician/animal handler*

Animal training is preferably always carried out by the same trainer. This benefits animal welfare as well as the research, given that the animals need not become accustomed to a variety of trainers, each with their own style and preferences. In cases where the trainer is not the researcher, the responsible party may advise the researcher in the selection of a training method. The trainer will at all times be responsible for informing the researcher regarding any observed abnormalities in the animals' health and/or behaviour.

### *Veterinarian or other relevant expert*

The veterinarian or other relevant expert, such as an ethologist, (hereinafter: the expert), is responsible for monitoring the health and the behaviour of the animals, both prior to, during, and after the training and the research has been completed. The expert will liaise with the Animal Welfare Body and in consultation with the researcher determines whether the humane endpoint has been reached and is responsible for estimating the (cumulative) discomfort of the training and the research. The expert also contributes to determining the optimal method of welfare monitoring. The expert is responsible for advising the Animal Welfare Body.

### *Role of the Animal Welfare Body (IvD)*

The researcher is responsible for drawing up a project proposal or work plan, which should be based on the 3Rs as much as possible, given the research objective. This plan is subsequently aligned with the Animal Welfare Body, which includes establishing when the endpoints will have been achieved and the extent of (cumulative) discomfort the training and the research may cause. The Animal Welfare Body is also responsible for advising the Animal Ethics Committee (DEC). The Animal Welfare Body is in charge of monitoring animal welfare for the duration of the research and verifies whether the implementation of the study is in accordance with the project license.

### *Role of the Animal Ethics Committee (DEC)*

The Animal Ethics Committee is responsible for the ethical assessment of a project proposal for which a license has been requested from the Central Authority for Scientific Procedures on Animals (CCD). The DEC weighs the various interests such as the utility, necessity and feasibility of the research project, on the one hand, and the potential of the 3Rs and the discomfort inflicted on the animals on the other hand. The DEC is responsible for advising the Central Authority for Scientific Procedures on Animals.

### *Role of the Central Authority for Scientific Procedures on Animals (CCD)*

The Central Authority for Scientific Procedures on Animals is responsible for evaluating the assessment made by the Animal Ethics Committee regarding the ethical and social accountability and the availability of alternatives, and determines whether the assessment is consistent and in line with similar project applications. The Central Authority ultimately decides whether a license will be granted for the project.

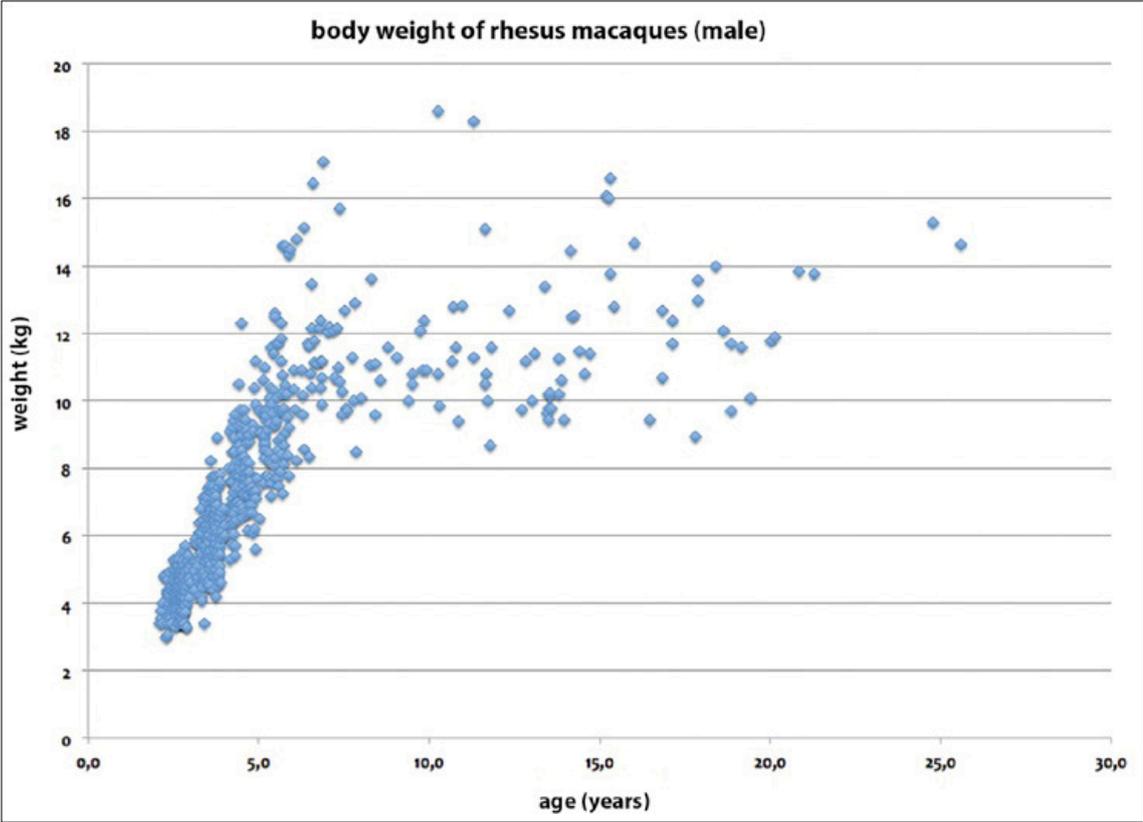
## Appendix 7: Conversion table for body weight and metabolic weight

The table below represents the relationship between body weight (BW) and metabolic body weight (Mb BW). Metabolic weight can be calculated using the following formula:

Metabolic weight (in kg) = Weight (in kg)  $\wedge$  0.75

Body weight (in kg)	Metabolic weight (in kg)
0.025	0.063
0.03	0.072
0.05	0.106
0.075	0.143
0.1	0.178
0.25	0.354
0.5	0.595
0.75	0.806
1	1
2	1.682
3	2.280
4	2.828
5	3.344
6	3.834
7	4.303
8	4.757
9	5.196
10	5.623

# Appendix 8: Growth curve for male rhesus macaques



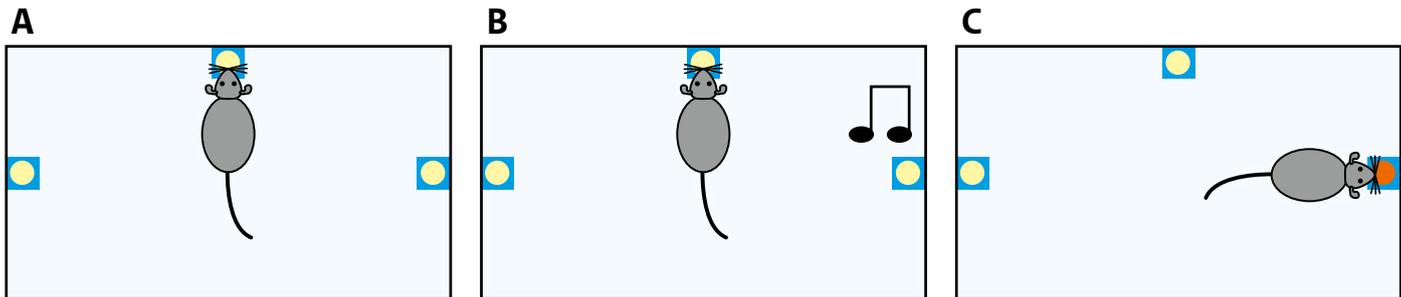
Source: BPRC (2017)

## Appendix 9: Examples of behavioural tasks in rodents and NHPs

Fundamental and applied scientific research make a significant contribution to our understanding of biological processes and the causes of diseases. Brain disorders are a growing problem in society, in part the result of an ageing population and the corresponding increase in neurological diseases, including Alzheimer's disease. Many scientific and medical questions and issues in relation to brain research as yet remain unsolved. For that reason, animal procedures will still be necessary for the foreseeable future. Yet the suffering of laboratory animals must be kept to a minimum and researchers must aim to achieve the replacement, reduction and refinement of animal procedures.

### Example of a behavioural task for rodents

Figure 4: Example of rodent behavioural task

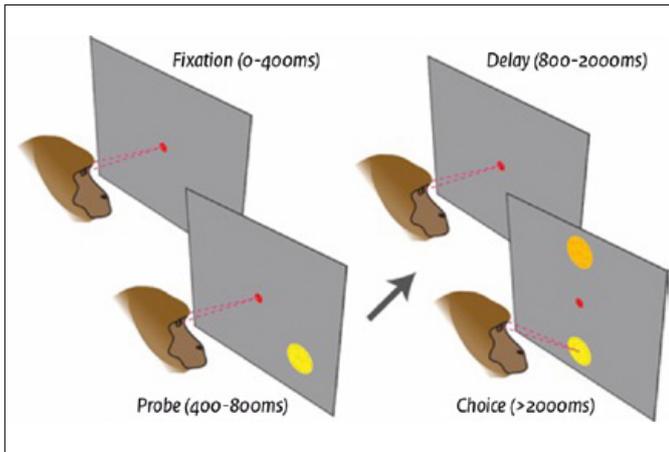


In this hypothetical example, a rodent (mouse or rat) has been trained to initiate a trial by sticking its muzzle into a bowl in the centre of the procedure setup (A). During the early training phase, the animal receives a reward for learning this behaviour, however in later training phases this reward may be replaced by a reward for the final task (see C). Then a sound is played (B). This stimulus can be offered either on the left or on the right (on the right in this example). Subsequently, every time the animal puts its muzzle in the bowl corresponding to the sound (right-hand bowl), it is rewarded with a fluid or food reward (C). If the animal would have chosen to go to the left-hand bowl, this would be an incorrect response and it would receive no reward. After this the trial is complete and the animal is able to return to the central bowl to initiate a new trial.

This task is designed to test the hearing of the rodent. This can be used for various types of research, for example, in research into hearing loss during ageing. For example, it would also be possible to use this procedure to test a method against hearing loss. The task can also be used to learn how the brain processes auditory information, for example, by combining the task with measurements of brain activity. Depending on the specific research question, the animal will have to carry out dozens to hundreds of trials per session to answer the question.

Description of a typical task for an NHP:

Figure 5: example of a behavioural task in NHPs



An example of a common task is the “delayed match to sample” task. This task is widely used to conduct research into perception and memory. For example, in research into making a cortical prosthetic to help blind patients this task is used to find out exactly what a primate sees when the visual cortex in the brain is stimulated.

The final version of the task takes place as follows: the primate focuses its eyes (“fixates”) on a small red dot on a screen. It must keep fixating throughout the entire task, except when it must give an answer. After 400 ms a probe stimulus (the yellow circle) appears elsewhere on the screen. The primate must keep fixating and so must keep his eyes on the red dot – if his eyes focus on the probe (the yellow circle) the trial ends. After 800 ms the probe (the yellow circle) disappears and there is a waiting period (delay) between 1–2 s. After this delay, two new circles appear, one with the colour of the probe (yellow) and another with another colour (light brown). The primate must make an eye movement to the circle which has the same colour as the colour of the probe (yellow circle). If it picks the correct circle, it receives a reward – if not, it continues with the next trial. The degree of difficulty of the task depends on the length of the delay and the perceptual difference between the two choices.

This is a difficult task and it will take 3–6 months to train an inexperienced primate to carry out this task accurately. Teaching the primate to keep fixating as the probe (yellow circle) appears is the element that takes a lot of time. The task is broken down into many small steps that the primate must go through each time during the training programme. The objective is to ensure that the primate achieves a success rate between 75–90% for each step. If the primate performs successfully, the task is made more difficult by the next step.

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# Endnotes

- <sup>1</sup> European Directive 2010/63/EU, Annex VIII, Part III
- <sup>2</sup> See also: “Happy animals make good science” (Poole, T., 1997)
- <sup>3</sup> Utrecht University, “Dierenwelzijn” [Animal Welfare]. <https://www.uu.nl/organisatie/faculteit-diergeneeskunde/dierenwelzijn>
- <sup>4</sup> Letter to Parliament 18 December 2015: Progress of recommendations for non-human primates
- <sup>5</sup> European Directive 2010/63/EU, Article 33:  
Member States shall, as far as the care and accommodation of animals is concerned, ensure that:
  - a) all animals are provided with accommodation, an environment, food, water and care which are appropriate to their health and well-being;
  - b) any restrictions on the extent to which an animal can satisfy its physiological and ethological needs are kept to a minimum.
- <sup>6</sup> Deprivation in this legislation is defined as total restriction of fluid or food for more than 24 hours. This is different from the procedures that are outlined in this opinion, where restriction of fluid or food is always for less than 24 hours and is not absolute.
- <sup>7</sup> In this case, another distinction is made between the terms deprivation and restriction than is used by the NCad in this opinion.
- <sup>8</sup> Fluid restriction often results in a reduced intake of food.
- <sup>9</sup> See also: NCad opinion “Synthesis of Evidence” (published in March 2016). SoE is the synthesis of relevant knowledge and expertise. This is vital to substantiating the choice of the most relevant research model. SoE is an umbrella term and can take various forms, such as narrative reviews (descriptive literature review), systematic reviews (SR), the use of databases, the use of expert panels, or combinations thereof. Link: <https://www.ncadierproevenbeleid.nl/adviezen-ncad/documenten/rapport/2016/5/17/ncad-advies-soe>
- <sup>10</sup> See NCad opinion on “Transition to non-animal research methods – On opportunities for the phasing out of animal procedures and the promotion of innovation without laboratory animals” (2016).
- <sup>11</sup> This means that desired behaviour is rewarded with fluid or food and that undesired behaviour is ignored. This positive reinforcement will result in an increase in desired behaviour.
- <sup>12</sup> The time units are so short so as to inhibit the animal from fulfilling its normal fluid/food needs.
- <sup>13</sup> Please see Appendix 9 of this opinion for specific examples.
- <sup>14</sup> (Royal Netherlands Academy of Arts and Sciences, 2014) (Scientific Committee on Health Environmental and Emerging Risks, 2017)
- <sup>15</sup> For rodents, please see Appendix 5 “Body Condition Score Rodents”
- <sup>16</sup> Knowledge of the normal species-specific behaviour of an animal is required in order to monitor the behaviour of an animal. The following resources may be used: <https://www.humane-endpoints.info/nl> or [www.ncjrs.uk/macacques](http://www.ncjrs.uk/macacques) or <http://pin.primate.wisc.edu> or [marmosetcare.org](http://marmosetcare.org)
- <sup>17</sup> In cases of fluid restriction, changes in humidity/temperature may have a physiological impact on the animal. As such, during animal procedures involving fluid restriction, these housing parameters require additional attention.
- <sup>18</sup> Inspectorate for Health Protection, Commodities and Veterinary Public Health (2000). Code of Practice for Monitoring Welfare of Laboratory Animals
- <sup>19</sup> In this regard, the material of the plates must be taken into account – certain types of wood splinter easily.
- <sup>20</sup> See also: NCad opinion “Synthesis of Evidence” (published in March 2016). SoE is the synthesis of relevant knowledge and expertise. This is vital to substantiating the choice of the most relevant research model. SoE is an umbrella term and can take various forms, such as narrative reviews (descriptive literature review), systematic reviews (SR), the use of databases, the use of expert panels, or combinations thereof.

- <sup>21</sup> This may work better the opposite way for certain protocols. Using a mild form of restriction, and following the learning of certain (good) behaviour, restriction may be scrapped or substituted by a sucrose\* reward without restriction. Initially working with a reward, such as sucrose (because the animal is not subject to restriction), and subsequently switching to a reward of a regular food pellet (without restriction) may result in frustration and/or negative association.  
\*Note that offering of food to a non-restricted animal does not necessarily serve as a reward.
- <sup>22</sup> Learning and imprinting of the method relies on creating an association between hearing a click and receiving a reward.
- <sup>23</sup> Positive punishment: positive means that something is offered to the animal; punishment indicates something unpleasant. This means that this method involves something unpleasant being offered as soon as the animal exhibits undesirable behaviour.
- <sup>24</sup> This refers to the protocol that is to be used in the workplace
- <sup>25</sup> See Codes of Practice to determine reference weight
- <sup>26</sup> The decision tree refers to a high number of repetitions: whether a high number of repetitions occur in the study will depend on the research and experimental design and must be substantiated in the project application.
- <sup>27</sup> See also: NCad opinion "Synthesis of Evidence" (published in March 2016). SoE is the synthesis of relevant knowledge and expertise. This is vital to substantiating the choice of the most relevant research model. SoE is an umbrella term and can take various forms, such as narrative reviews (descriptive literature review), systematic reviews (SR), the use of databases, the use of expert panels, or combinations thereof.
- <sup>28</sup> The term "preference" in this context and in the CoPs refers to the fact that any deviations from this preference must be explicitly substantiated in the project license application.
- <sup>29</sup> This minimum amount does not include the fluid obtained from fruit that contains fluid.
- <sup>30</sup> In this type of restriction regime there is no physiological damage either in the short or long term. *Gedragsveranderingen zijn marginaal* [Behavioural changes are minimal] (Gray et al., 2016) (Prescott et al., 2010).
- <sup>31</sup> Metabolic weight is partly determined by the minimum amount of energy required for the functioning of tissues and body processes. Metabolic weight: = body weight  $^{\wedge} 0.75$  See Appendix 8 for a conversion chart between metabolic weight and body weight.
- <sup>32</sup> Push days are days on which the animal is restricted more stringently (for example, 24 hours of total restriction of fluid/food) than normal in order to motivate the animal to a greater extent.
- <sup>33</sup> The animal may cease activity for a variety of reasons, such as due to fatigue, but also due to saturation or for a completely different reason.
- <sup>34</sup> This relates to water.
- <sup>35</sup> The animal may cease activity for a variety of reasons, such as due to fatigue, but also due to saturation or for a completely different reason.
- <sup>36</sup> The term sessions refers to the behavioural task the animal should perform at that time.
- <sup>37</sup> This should take place at least once a week and more frequently upon indication.
- <sup>38</sup> <https://norecopa.no/media/6351/food-deprivation.pdf>













## With thanks to the following experts

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In order to respond to this request for an opinion, the NCad appointed two ad hoc working groups consisting of experts from the field, with the request that they draw up a Code of Practice for rodents and non-human primates. The experts consulted are not co-authors of this NCad opinion and their views on certain matters may differ from those presented by the NCad in this opinion and in the Codes of Practice.

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