



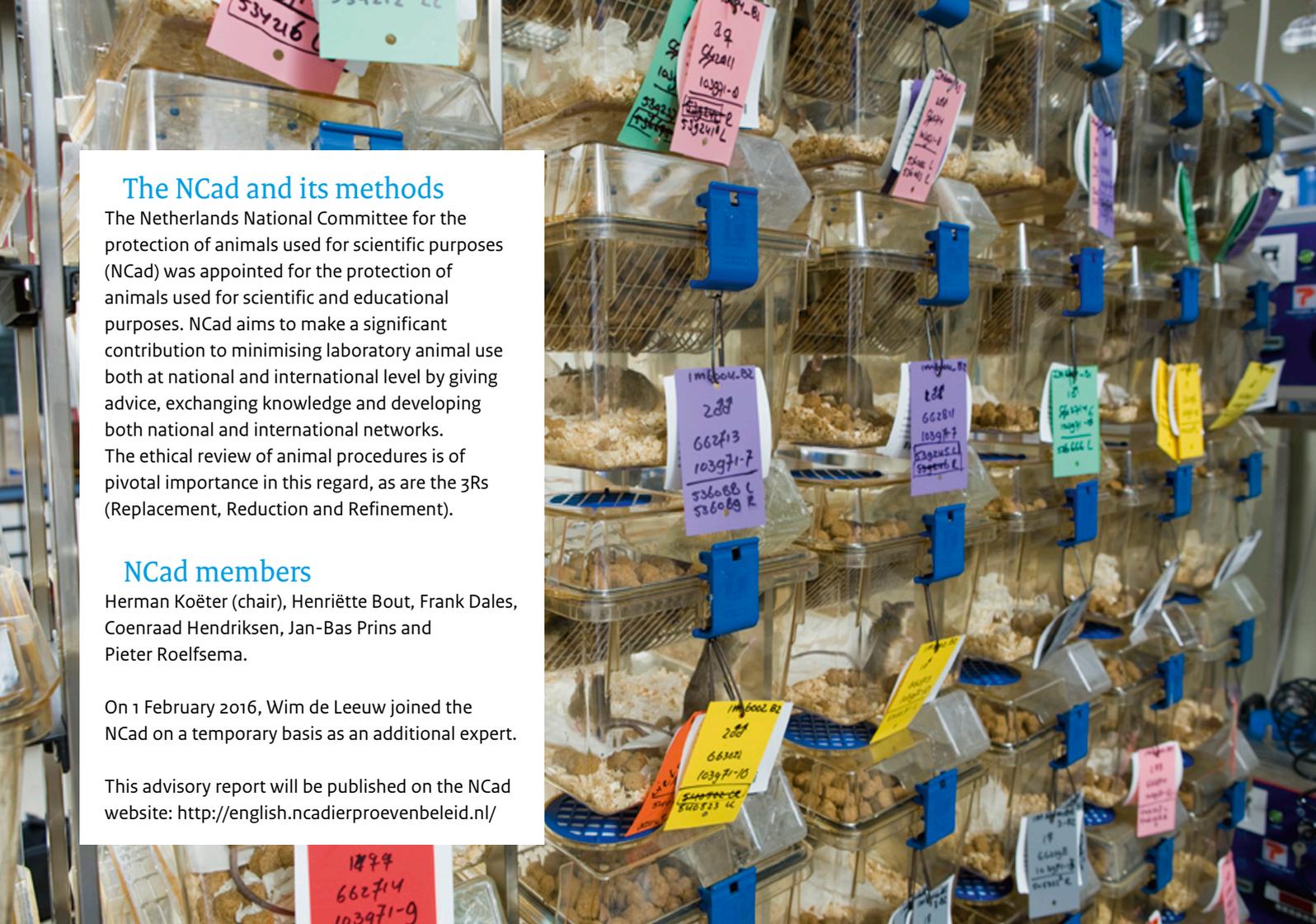
Netherlands National Committee
for the protection of animals
used for scientific purposes

Alternative methods for killing laboratory animals

*- for careful consideration in
structurally departing from the
prescribed methods*

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





The NCad and its methods

The Netherlands National Committee for the protection of animals used for scientific purposes (NCad) was appointed for the protection of animals used for scientific and educational purposes. NCad aims to make a significant contribution to minimising laboratory animal use both at national and international level by giving advice, exchanging knowledge and developing both national and international networks. The ethical review of animal procedures is of pivotal importance in this regard, as are the 3Rs (Replacement, Reduction and Refinement).

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This advisory report will be published on the NCad website: <http://english.ncadierproevenbeleid.nl/>

Summary

In September 2015, the Dutch Minister of Agriculture requested the NCad (Netherlands National Committee for the protection of animals used for scientific purposes) to advise on methods for killing laboratory animals that are considered to be at least as humane as the methods set out in European Directive 2010/63/EU. The Dutch Minister of Agriculture also asked for the Netherlands Food and Consumer Product Safety Authority (NVWA) to be given guidance in assessing such alternative methods of killing by providing elements that must comprise a scientific justification.

The Directive provides two possibilities for deviating from the prescribed methods of killing:

1. The purpose of the procedure cannot be achieved by the use of a method of killing set out in the Directive. The Central Authority for Scientific Procedures on Animals (CCD) can, on the basis of a scientific justification submitted by the applicant, decide to grant a project licence for a project in which a different method of killing is proposed than those set out in the Directive. The acceptance of such 'divergent' methods of killing is limited to the specific research project for which the licence is granted.
2. The other method of killing is considered to be at least as humane as the appropriate methods set out in the Directive. On behalf of the Minister, the NVWA can, on the basis of a scientific justification submitted by the applicant, grant the establishment licensee an exemption or dispensation for a *structural* (i.e. outside-the-project) use of the alternative method of killing.

The present opinion of the NCad focuses on the second option. At the same time, it may also offer guidance for the CCD, as, if a researcher opts for a divergent method of killing for scientific reasons, the CCD will review whether that method is also acceptable from an animal welfare perspective.

For the purpose of assessing whether an alternative method of killing is at least as humane with regard to the individual animal as the current legally permitted methods, the NCad advises using the following elements:

- *speed of loss of consciousness*
- *degree of pain, suffering and distress associated with (the entire experience relating to) the killing*

If it is intended to be used for groups of animals, the method of killing should be assessed on the basis of the individual animal within that group with the highest expected degree of pain, suffering and distress.

The NCad recommends performing the assessment of the alternative method of killing in the following way.

1. The applicant for an exemption or dispensation submits to the NVWA, on the basis of a Synthesis of Evidence evaluation, data (also from the literature) demonstrating that with regard to the two elements stated above, the method is at least as humane as the current prescribed methods. This analysis should be based on relevant (or as relevant as possible) measurable parameters for and clinical observations (such as regarding behaviour) of the animals to which the application relates. Experts can compare those data with the available data for the prescribed methods of killing.

2. If there are no data in the literature or a Synthesis of Evidence evaluation provides insufficient clarification for an assessment of the request for an exemption or dispensation, exploratory animal studies should be carried out in consultation with the NVWA (and after a project licence has been granted by the CCD), to add the missing data on the parameters relevant to welfare. The study (including 'negative' results) is required to be published in an open access, peer-reviewed scientific journal, in accordance with the ARRIVE Guidelines.
3. If the NVWA assesses favourably the data in the literature and a possible exploratory study, the NVWA can grant a dispensation for a defined period. The dispensation is granted subject to the condition that the applicant must first arrange for a scaled-up field trial to be conducted to ascertain the functionality of the alternative method of killing under the conditions that apply in practice (validate). As soon as the alternative method has been demonstrated to be at least as humane as the appropriate methods set out in the Directive, the NVWA should issue a generally applicable exemption for it.

Active sharing of data on methods of killing and alternative methods makes it possible for knowledge to be used effectively and the animal procedures to be Refined. The NCad recommends making centrally available the conditions for dispensation applied by the NVWA and data on the exemptions granted for alternative methods of killing, preferably in the data warehouse for laboratory animal use and 3R developments recommended previously by the NCad. Naturally, privacy protection and market positions should duly be taken into account. The Minister is also advised to promote knowledge sharing between the NVWA and CCD.

The NCad further recommends that the Animal Welfare Bodies (IvDs) should not only act in their legal role of sharing knowledge within the body concerned on humane methods of killing, refinement of these methods and related topics, but that they are also stimulated to share this knowledge between them. Licensees should, in turn, be aware of their obligation to have professionally competent employees.

The NCad has found that the rationale for the methods of killing set out in the annex (or text) of the European Directive is not clear. Therefore the NCad advises the Minister to propose that the evaluation of the Directive planned by the European Commission should also focus on the degree of scientific substantiation of the methods of killing currently prescribed in it. An evaluation of the degree to which the package of permitted methods of killing is adequate for the ways in which laboratory animals are currently used in practice should likewise be part of the evaluation of the Directive.

Keywords

Method of killing, euthanasia, laboratory animals, humane

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1. Introduction

Animal procedures are performed for a wide variety of purposes, including biomedical research and education. In 2014 Dutch establishment licensees performed 621,027 procedures on 563,769 animals. Most animals which are bred and/or are used for animal procedures die or are killed as part of the procedure or because they constitute a surplus. *Zo doende*, the annual review of animal procedures and laboratory animals published by the Netherlands Food and Consumer Product Safety Authority (NVWA) contains data on the number of animals involved. The figures stated below are derived from *Zo doende 2014*¹.

Possible times of death or killing

In the context of laboratory animal use, animals may die or be killed at various times (figure 1):

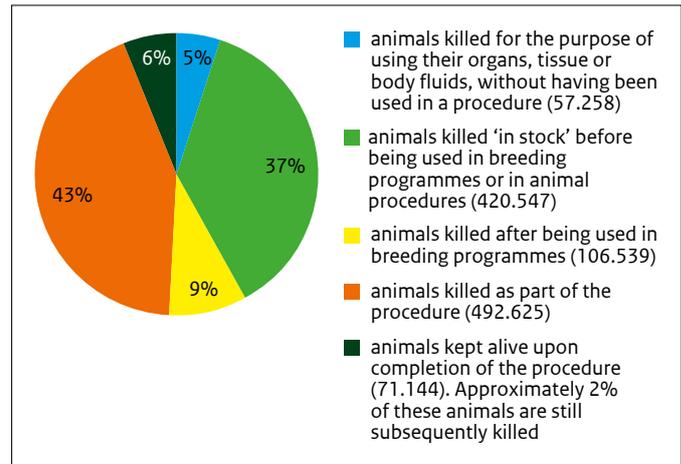
- Without having been used in the procedure: this group comprises animals which, after weaning, are killed ‘in stock’ⁱⁱ before being used in breeding programmes or in animal procedures (420,547 animals in 2014). They may, for instance, have been unsuitable for the procedure due to their unwanted gender or genotype. Animals killed before weaning are not included in the registration statistics.
- After use in breeding programmes: this concerns animals killed ‘in stock’ after being used in breeding programmes (106,539 animals in 2014).
- During the procedure: a large number of animals are killed (or die) as part of the procedure. As an example, when the research purpose has been fulfilled, body material from the animals may be needed to conduct additional research or because the humane endpointⁱⁱⁱ

has been reached (492,625 animals in 2014). This group includes laboratory animals which, without any prior activity, are killed merely for the purpose of using their organs, tissue or body fluids (57,258 in 2014).

- Kept alive upon completion of the procedure, but still killed later: animals which are initially kept alive upon completion of the procedure, but are subsequently killed because they were unable to be reused or rehomed^{iv} (approximately 2% of the 71,144 animals that were kept alive in 2014).

Figure 1. The fate of laboratory animals which are bred and/or used.

Source: ‘*Zo doende 2014*’, NVWA



Statutory provisions concerning the killing of laboratory animals

The responsible use of laboratory animals is based on the principle of the 3Rs - Replacement, Reduction and Refinement in animal procedures. The guiding principle in the killing of laboratory animals is the 'R' for Refinement, aimed at alleviating distress for the animals and/or optimising their welfare. This imposes a duty on all parties concerned to ensure they choose, substantiate and perform methods of killing laboratory animals with due care. The Experiments on Animals Act (*Wet op de dierproeven*)^v stipulates in Article 13c that laboratory animals should be killed by a competent person, and that this must be performed in a manner that minimises animal pain, suffering and distress. Moreover, an appropriate method of killing must be used as specified in Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes.^{vi} The relevant provisions and Annex IV of the Directive are included in Appendices 1 and 2 respectively.

The provisions set out in Article 13c of the Experiments on Animals Act apply to animals located on the premises of an establishment licensee, but equally apply, for instance, to animals captured in the wild for the purpose of biological field studies and during or after capture are found to be wounded or in poor health. Article 10f(4) 4 of the Act stipulates that these animals must be examined by a vet or another competent person and that measures must be taken to minimise animal suffering. There may also be situations in which an animal has to be killed for animal welfare, public health, public security, animal health or environmental reasons in an emergency situation. The animals may in that case be killed outside of the establishment licensee by someone other than the competent person described in this Act.

Furthermore, a method of killing may be used other than the prescribed methods. In such an emergency situation any practicable and available method of killing may be regarded as a more humane approach than 'doing nothing'. For this reason, isolated 'emergency killings' as described in Article 13c(4) fall outside the scope of this opinion. However, the NCad believes that in emergency situations the most humane method, appropriate for the situation, should also be sought.

Departing from the prescribed methods of killing

The European Directive provides two options for departing from the specified methods of killing in a project incorporating animal procedures. Each option has its own procedure and its own decision-making body.

1. There is scientific justification that the purpose of the animal procedure cannot be achieved by the methods set out in the Directive. The Central Authority for Scientific Procedures on Animals (CCD, Competent Authority) may in that case decide to grant a project licence for a project which proposes another (possibly less humane) method of killing than those included in the Directive. Consequently, in such cases the 'divergent' method of killing is always linked to the specific, licensed research project.
2. Based on scientific justification, the divergent method of killing is considered to be at least as humane as the appropriate methods specified in the Directive. On behalf of the Minister, the NVWA may grant the relevant establishment licensee an exemption or dispensation for the *structural* (i.e. outside-the-project) use of the divergent method of killing.

Given that the implementation of alternative methods of killing can have a considerable impact on animal welfare, the exemptions and dispensations granted in any European Member State must be reported to the European Commission on an annual basis. The European Commission publishes this information and moreover may use it for further harmonisation and inclusion in the future evaluation of the Directive.

The NCad's present opinion focuses on the second option, and may also serve as guidance for decision-making by the CCD. If a researcher opts for a 'divergent' method of killing for scientific reasons, the CCD will review whether this method is acceptable also from an animal welfare perspective.

2. Request for opinion

In order to grant an exemption or dispensation for using a method of killing not specified in Annex IV, the scientific justification must be closely assessed and all aspects carefully weighed. In her letter of 25 September 2015 the Minister of Agriculture requested the NCad to provide guidance on the careful assessment of the scientific justification and to deliver an opinion on the following issues:

1. What elements should a scientific justification contain, having regard to Article 13c(3) of the Experiments on Animals Act,^{vii} in order to depart from the methods of killing specified in Annex IV of the European Directive?
2. What scientifically justified methods of killing in generally accepted practice can the NCad recommend, which based on scientific grounds have proven to be as humane as the methods of killing set out in Annex IV of the Directive?

The key concept in this request for opinion is the term ‘humane’ in the context of ‘humane method of killing’. On the basis of Article 13c(1) of the Experiments on Animals Act, the NCad applies the following definition in its advisory report: ‘the killing of an animal, causing the animal no, to minimum pain, suffering and distress’. The ‘at least as humane’ method of killing is also referred to as ‘alternative method of killing’ in this advisory report.

The guiding principle of the NCad’s advisory report is the welfare of the individual animal. Furthermore, in its advisory report the NCad focuses primarily on specifying the elements and criteria which the scientific justification must fulfil. The NVWA is responsible for

reviewing the application of these elements in concrete assessment situations.

Since the entry into force of the Experiments on Animals Act in 2014, to this end one exemption or dispensation application for an alternative method of killing was submitted to the NVWA, and a number of other methods of killing are currently being discussed. On making enquiries among members of the Professional Group of Animal Welfare Officers (*Beroepsgroep Proefdierdeskundigen*), in November 2015 the NCad found that there was no reason to expect many more dispensation applications for alternative methods of killing in the short term.

In response to the request for opinion, the NCad carried out a narrative review and consulted various experts, including members of the ‘National Committees’ in other European Member States, representatives of the assessment authorities concerned (the NVWA and the CCD), members of the Professional Group of Animal Welfare Officers, and various social groups. Based on the results, in this report the NCad has formulated recommendations concerning the focus areas in assessing exemption or dispensation applications for alternative methods of killing. A current list of alternative methods of killing considered to be humane would have a very limited period of validity in the light of the current scientific developments and discussions, and would require a more comprehensive, systematic literature review.

3. Advisory report

Concerning the assessment of the alternative methods of killing, the NCad advises the Minister of Agriculture as follows:

1. Ensure that the NVWA adopts the following elements in assessing whether an alternative method of killing an individual animal is at least as humane as the currently legally permitted methods:
 - *speed of loss of consciousness*
 - *degree of pain, suffering and distress (the entire experience relating to) the killing*Where the method of killing is intended to be used for groups of animals, it should be assessed on the basis of the individual animal within that group with the highest expected degree of pain, suffering and distress.
2. Ensure that the applicant for an exemption or dispensation submits to the NVWA, on the basis of a Synthesis of Evidence evaluation, data (also from the literature search) demonstrating that with regard to the two elements stated above, the method is at least as humane as the currently legally permitted methods. This analysis should be based on relevant (or as relevant as possible) measurable parameters for, and clinical observations (including behavioural) of the specific animal species, strain, gender, age, weight class and possibly the animal's condition, to which the application relates.
3. If there are no data in the literature or a Synthesis of Evidence provides insufficient clarification for an assessment of the exemption or dispensation application, an exploratory animal study should be carried out in consultation with the NVWA (and after a project licence has been granted by the CCD), to add the missing data on the parameters relevant to welfare. The study

(including 'negative' results) is required to be published in an open access peer-reviewed scientific journal, in accordance with the ARRIVE Guidelines^{viii};

4. If the NVWA assesses favourably the literature data and a possible exploratory study, the NVWA can grant a dispensation for a defined period. Stipulate that the NVWA includes as a condition for granting the dispensation that the applicant must first arrange for a scaled-up field trial to be conducted to ascertain the functionality of the alternative method of killing under the conditions that apply in practice (validate). As soon as the alternative method has been demonstrated to be at least as humane as the appropriate methods set out in the Directive, the NVWA should issue a generally applicable exemption for it.
5. Advocate that the degree of scientific justification of the methods of killing currently prescribed in the Directive should be addressed during the evaluation of Directive 2010/63/EU planned by the European Commission. An evaluation of the degree to which the package of permitted methods of killing is adequate for the ways in which laboratory animals are currently used in practice should form part of the above evaluation.

To effectively utilise data and knowledge of methods of killing, alternative or otherwise, knowledge must be actively shared. In this context, the NCad advises the Minister of Agriculture as follows:

6. Make the conditions for dispensation applied by the NVWA centrally available as well as data on the exemptions granted for alternative methods of killing, thereby taking account of the protection of privacy and market positions, preferably in the data warehouse for laboratory animal use and 3R-developments

recommended by the NCad^{ix}. Encourage the NVWA and CCD to share knowledge.

7. The Animal Welfare Bodies (IvDs) have a legal duty to share knowledge within the body concerned of humane methods of killing, refinement of these methods and related topics. Encourage these bodies to also share this knowledge between them.

4. Substantiation of the advisory report

The recommendations set out in the previous section are substantiated in this chapter. Each recommendation is elaborated in a separate section.

4.1 Elements to be considered in assessing an alternative method of killing

The choice of an appropriate, humane method of killing an animal (of a certain age, animal species, strain, weight and condition) in a research setting under certain field conditions (such as individual animals versus groups, and inside versus outside) is based on aspects relating to animal welfare, the animal procedure and/or implementation.

Animal welfare: the entire experience of the individual animal must be taken into consideration, such as the following aspects:

- stress arising from catching, handling and securing an animal;
- stress arising from transferring an animal from its own cage to a new environment;
- stress arising from putting unfamiliar animals together;
- pain, suffering and distress linked to the practical implementation of the killing technique:
 - the irritating or otherwise aversive characteristics of administering a euthanasia drug;
 - the method of administering the euthanasia drug or the method of killing;
 - speed of loss of consciousness (slowly losing consciousness is regarded as particularly stressful for prey animals);

- observing and experiencing the killing of the same animal species.
- the degree of irreversibility and effectiveness (risk of failure and the associated risks for animal welfare).

Animal procedure: this concerns the compatibility of the method of killing with the purpose of the research, which looks at the impact of the method of killing used on:

- the relevant organs and tissues (histopathology);
- other clinical and biochemical factors.

Implementation: this concerns the degree to which a method of killing in the broadest possible sense 'can be implemented', from the implementer's perspective:

- the ease of learning and implementing the technique with confidence;
- aesthetics and psychological stress on the implementer's part;
- safety of the implementer and the environment;
- availability of the method and the economic feasibility;
- risk of failure.

Annex IV of the Directive is based largely on an opinion^x of the European Food Safety Authority (EFSA), in which methods of killing are set out by animal species, which on the grounds of scientific knowledge and from an animal welfare perspective are considered to be the most appropriate. Ethical, socio-economic, cultural and religious aspects have not been taken into account, nor have factors relating to the safety of the employees concerned. The NCad notes that it is not clear what the underlying arguments are for including the methods of killing in the Annex (or text). The selection of

prescribed methods would seem to be the resultant of the common denominator (current practice) in long-standing international practice.

Elements of a humane method of killing

Given that the 3R principle promotes the development and use of more humane methods of killing, in the NCad's view it will suffice to establish that an alternative method of killing is at least as humane as the prescribed methods. The NCad considers two elements important in the scientific justification of the alternative method of killing:

1. speed of loss of consciousness
2. degree of pain, suffering and distress (the entire experience relating to) the killing

A structured literature review (Syntheses of Evidence), pilot projects and relevant opinions can offer a solid basis for the scientific opinion on whether the alternative method of killing 'scores' at least as well on these elements as the prescribed methods.

Other European Member States likewise seem to have embraced a similar approach. In November 2015 the NCad consulted its sister National Committees in Europe on the exemptions or dispensations which had been issued in the respective Member States until that date and the underlying scientific justification. The results of this consultation are provided in Appendix 5. Here too, in most cases the two elements stated above constituted the core of the justification.

4.2 The use of literature data for the scientific justification

The scientific justification for the alternative method of killing should be based on a Synthesis of Evidence evaluation. To this end the applicant will need to make a critical and careful selection from the available sources, and provide a good research design accompanied by a clear and complete description of the research design and the results obtained.

Similarly, the relevant parameters (measurable parameters and clinical and behavioural observations) in the scientific justification should be carefully selected and evaluated. These parameters must provide clarity on how humane the alternative method of killing is by offering insight into the speed of the loss of consciousness and the degree of pain, suffering and distress associated with the method of killing. Experts can subsequently compare these data with the available data on the prescribed methods.

A number of parameters that may be useful in determining the speed of the loss of consciousness and the degree of pain, suffering and distress are set out below. For references, please see Appendix 6 (Sources).

Speed of loss of consciousness

- In order to measure the speed of loss of consciousness for a new or existing method of killing, a neurophysiological parameter must at least be used, such as measuring spontaneous brain activity using an EEG. Because the relationship between the EEG and the loss of

consciousness is only partially known, a flat line EEG should be used as the criterion for the absence of brain activity. In other words: 'the speed of loss of consciousness' is defined as the time starting from the administration of the method of killing until the EEG shows a flat line.

- Somatic-evoked potentials (SEPs) can be used for rats, but possibly also for other animal species, to obtain a more detailed measurement of neurophysiological activity.
- In applying a method of killing in day-to-day practice, fairly simple behavioural observations (reflex tests) can be used as a parameter for the loss of consciousness and irreversibility. In order to establish the various stages of loss of consciousness until death, it is important to carry out sequential reflex tests. It is important to test reflexes that are located in the closest proximity to the brain, such as the eyelid reflex, the swallowing reflex (in fish) and jaw tension. The withdrawal reflex (for instance after squeezing a rat's middle toe) affects the spinal level and is an adequate first reflex to measure. However, the loss of consciousness must be corroborated by measuring a reflex linked more closely to the cerebral cortex or brain. At brainstem level, the heart rate and respiratory rate are usable parameters, which, like blood pressure, can also be measured by telemetry during the development and evaluation of an alternative method of killing.

Pain, suffering and distress

- It is not an easy task to identify the degree of pain, suffering and distress objectively. During the development and evaluation of alternative methods of killing, the aversiveness of adult animals to certain gases (an overdose used as a method of killing) can, for

instance, be determined in preference tests and by repeated dose studies, in which the strength of the behavioural response is measured after administering a second dose against the first. The emotional response (including the response to confrontation with the killing of the same animal species in the same space and/or at hearing or smelling distance) can similarly be measured on the basis of behavioural observations.

- Ultrasonic vocalisations are reliable signs of pain, suffering and distress and can be determined comparatively easily. The *degree* of pain, suffering and distress which these signs represent are more difficult to identify objectively.
- Furthermore, immediate early genes (proteins such as c-Fos) are useful biomarkers for activation of the stress system.
- The behaviour of very young animals (neonates) differs from that of adult animals and shows rapid development in the early days after birth. In the early stages of life the nervous system is still immature and these animals may therefore react differently to a stimulus. This development and the absence of an ethogram showing normal, and pain and stress behaviour in neonatal animals makes it difficult to evaluate pain, suffering and distress in young animals.
- Due to the wide individual variation, it will not suffice to use measurements of stress hormones as the sole parameter.

It can be concluded from the above that it will not suffice to use only one parameter in the scientific justification. Moreover, there may be large differences between animal species, strains, age groups and even individual animals. Environmental factors also have a major influence on measurements of the various parameters. In this light, it is important that in the scientific justification, the applicant for an

exemption or dispensation for an alternative method of killing incorporates literature data relating to parameters which are as relevant as possible to the animals in their particular situation. For pragmatic reasons, in many cases it will be necessary to disregard any strain differences between genetically modified animals with the same background or, where methods of killing fish are concerned and to lesser extent birds, to categorise on the basis of groups of animal species. However, the report must clearly state what the specifications of the animals were and what the considerations were in the justification.

Killing very young animals

Killing very young (foetal and neonatal) rats and mice requires closer attention and a different approach than killing adult animals. The nervous and respiratory system of these animals is still immature and a neonatal rat or mouse may display brain activity even in a hypoxic or anoxic state. Killing with the aid of CO₂ is an extremely slow process in these animals and may possibly be accompanied by pain, and therefore is debatable. When killing foetuses, the method of killing used on the mother animal affects the foetuses. This effect should be taken into account in considering the method of killing foetuses. As long as the foetuses have not yet grown fur, hypothermia of the foetus may be an appropriate method of sedation and killing, which can moreover be carried out effectively in practice although it will need to be determined scientifically how such a protocol should be designed to minimise pain, suffering and distress. Lastly, submerging in liquid nitrogen is considered an extremely fast and effective method of killing neonatal (furless) rats and mice. From an aesthetic point of view, some implementers have emotional difficulty

in implementing this method, yet it is deemed a very humane method from an animal welfare perspective.

The influence of environmental factors

Laboratory animal anaesthesiology has shown that animals lose consciousness more calmly with a lower dose of anaesthetic when administered in a familiar, relatively stimulus-deprived environment. The NCad therefore advises that researchers and Animal Welfare Bodies endeavour to ensure that animals lose consciousness where possible in their own cages and to change as little as possible in the environment prior to killing them. This includes avoiding interruptions of the circadian rhythm. When animals that live in a laboratory in a reversed day/night rhythm are taken from a darkened animal facility to a light treatment room for anaesthesia or euthanasia, this will lead to a more stressful experience and a higher dose will be required to achieve rapid loss of consciousness.

Killing after appropriate sedation

The prescribed methods of killing do not apply to animals which have already lost consciousness and are certain to remain unconscious until they die. Using appropriate sedation, combined with analgesics, before killing the animal can widen the options of the methods of killing to be used, for instance with methods that would be accompanied by pain without prior anaesthetisation. A general consideration is whether after surgery under anaesthesia an animal should awaken or should be killed under sedation. In the latter case, the animal will be spared from any pain, suffering and distress due to awakening from anaesthesia and post-operative pain. As the pain, suffering and distress associated with the entire experience of killing

must be taken into account in the justification of alternative methods of killing, in the case of killing animals which have already been sedated, only the speed of the sedation method (speed of loss of consciousness) and the pain and distress accompanying the procedure, should be taken into consideration.

It should be borne in mind that previously sedated animals can experience more stress if they are anaesthetised once again. In the practical implementation, account must self-evidently also be taken of the aspects relating to the laboratory animal and the implementer in considering methods of killing.

4.3 Carrying out exploratory animal studies for the scientific justification

If there are no literature data or a Synthesis of Evidence offers insufficient insight into the relevant parameters and observations, an exploratory animal study incorporating the alternative method of killing should be carried out in consultation with the NVWA. This study, the project proposal for which will be assessed by the CCD for the purpose of granting a licence, should take the issues stated in Section 4.2 into account. It is important to involve experts, for instance in the field of neurophysiology and behavioural biology (perhaps even international experts), to ensure that the trial is properly set up, and in order to draw the right conclusions and acquire support for the results from the professional field.

In the context of sharing knowledge, the findings (including the 'negative results') of any exploratory study of alternative methods of killing should be shared with the NVWA and published in an open

access peer-reviewed scientific journal in line with the ARRIVE Guidelines. The assessment of the results of the study by the NVWA will, however, be carried out in parallel with the publication process and therefore may not be delayed by any deferral relating to acceptance of the article and peer review. The knowledge acquired can also be made available in the Netherlands through the Platform of Animal Welfare Bodies (IvD Platform, in the process of formation), the Dutch journal *Biotechniek* and the datawarehouse recommended by the NCad.

4.4 Performing a field trial

As discussed earlier in Section 4.1, the choice of an appropriate, humane method of killing is based on aspects relating to animal welfare, the laboratory animal and the implementer. The functionality of the alternative method of killing on the premises of the relevant establishment licensee (the applicant), and possibly other establishments, can be established by performing a field trial.

Multiple parameters are measured in the exploratory study in connection with loss of consciousness and pain, suffering and distress. Before practicable parameters, such as reflex tests and other clinical and behavioural parameters, can be relied on in day-to-day practice, they must first be tested (validated). Consequently, an important component of the field trial is to examine whether those 'basic' parameters still produce the same 'scores' when scaling up the method to field conditions.

The NCad advises the NVWA to grant a dispensation for the maximum standard duration when it favourably assesses the literature data and

any exploratory study. Performing a field trial should be included as a condition for the dispensation, and the findings must at least be shared with the NVWA. A second important condition is that the dispensation may be revoked, if the particular alternative method of killing is no longer considered to be 'at least as humane' based on new knowledge and scientific publications.

Competence of the implementer

The humane implementation of a method of killing depends on the competence of the implementer and the reliability of the required equipment. Licensees should be aware of their responsibility for competent employees and must fulfil it. The European Directive stipulates in Article 23(2c) that staff must be adequately trained and educated before they may kill animals. They must be supervised in the performance of their tasks until they have demonstrated the requisite competence.

In English, this phase of deeper learning is aptly summarised by the trinity of skill, confidence and practice. The implementer should not only be trained in the skill but should also be able to perform it with confidence and undergo regular training in the skill.

The above can only be guaranteed if there is openness among the implementers (in many cases the biotechnician or research analyst) and the Animal Welfare Body and/or the researcher about any aesthetic, emotional or other personal objections against implementing a specific method of killing. After all, the implementer's preferred method will not automatically be the best method in terms of animal welfare. The guiding principle is the

welfare of the individual animal, but the implementer must nonetheless be prevented from hesitating when performing a procedure because this can equally lead to unnecessary pain, suffering or distress in the animal.

In addition, the competent implementation of the method of killing in the context of a field trial should also be established by means of a spot check, such as observing the performance of a basic post mortem examination. This is set out in the framework^{x1} for the education and training of individuals involved in animal procedures, under the European Directive.

Risk of failure to kill an animal

In the practical implementation of an alternative method of killing it is vital to eliminate all factors that could cause the killing of an animal to fail. A method which could prove to have a higher risk of failure in practice constitutes a risk in terms of animal welfare.

Killing large numbers of animals

The consideration of methods for routinely killing large numbers of animals simultaneously, for instance in the context of breeding transgenic animals, cleansing infected colonies, or minimising the standard number of animals in breeding programmes, requires particular attention from both the establishment licensee and the NVWA. In considering methods of killing, including those for extremely large numbers of animals, the pursuit of efficiency must not compromise the welfare of the individual animal. This aspect comes into play in new automated systems, in which several cages of adult animals can be simultaneously filled with CO₂ based on a preset

programme. Such systems fulfil the aim of killing animals in their own cages and a rapid death with minimum pain, suffering and distress. A matter for attention here is to ensure and establish death.

In assessing alternative methods for killing several animals simultaneously or shortly after each other, consideration must be given to any differences in the degree of pain, suffering and distress among animals in that group. The NCad recommends that the method, when used for groups of animals, be assessed on the basis of the individual animal within that group with the highest expected degree of pain, suffering and distress.

A key aspect of the practical application of any method of killing, irrespective of whether it is a prescribed or alternative method, is establishing death. The death of every animal killed must be established, even when using automated systems. The Directive provides guidance on this in Annex IV (Appendix 2).

4.5 Substantiation of the prescribed methods of killing in 2010/63/EU

As stated earlier in Section 4.1, the NCad has noted that it is not clear what the underlying arguments are for including the methods of killing in the Directive.

The NCad therefore recommends that the Minister of Agriculture ensures that scientific justification of the prescribed methods of killing be addressed during the planned evaluation of the Directive by the European Commission. Authorised methods should be

systematically examined and tested on the basis of appropriate and validated authorisation criteria, the primary factor being that the method must be at least as humane. A further aspect that should be evaluated is whether the package of authorised methods sufficiently covers the use of laboratory animals in current practice.

4.6 Making available data on exemptions and dispensations granted for alternative methods of killing

For the purpose of sharing knowledge with stakeholders in the field of animal experimentation, particularly on the more humane alternative methods of killing, the NCad wishes to advise the Minister of Agriculture that the dispensation conditions applied by the NVWA and the data on the exemptions granted by the NVWA for alternative methods of killing be made centrally available. Obviously the protection of company-sensitive and personal data will be taken into account. The recommended data warehouse for laboratory animal use and 3R-developments should preferably be used for this purpose as it will also contain the NVWA's registration data on animal procedures.

4.7 Sharing knowledge within and between the established licensees' organisations

Established licensees are required to submit an application for an exemption or dispensation for an alternative method of killing to the NVWA. A possible dispensation will be granted to a specific establishment for a specific group of animals. However, other organisations (and the animals they kill) should also be able to

benefit from the knowledge acquired. Particularly where more humane alternative methods of killing are concerned, they should be promoted according to the Experiments on Animals Act. The at least as humane alternative methods of killing which could replace one or more of the prescribed methods of killing may be authorised by the NVWA, on the basis of Article 13c(3) of the Act, by way of a general exemption (for national application).

Moreover, the availability of data on the dispensation conditions applied by the NVWA and the exemptions granted, as recommended in Section 4.6, offers other establishment licensees the opportunity to submit an application. It should be examined whether there are sufficient reasons and possibilities for making available more humane alternative methods of killing based on a Code of Practice.

The Animal Welfare Bodies (IvDs) have a legal duty to share knowledge within the institute concerned of animal welfare matters, including humane methods of killing, refinement of these methods and related topics. It is essential that the Animal Welfare Bodies take on this role. Furthermore, the Animal Welfare Bodies should, in the interests of animal welfare, be encouraged to share this knowledge with each other through the national Platform of Animal Welfare Bodies (IvD Platform, in the process of formation).

5. Appendices

Appendix 1: Relevant provisions on methods of killing in European Directive 2010/63/EU

Article 6

Methods of killing

1. Member States shall ensure that animals are killed with minimum pain, suffering and distress.
2. Member States shall ensure that animals are killed in the establishment of a breeder, supplier or user, by a competent person. However, in the case of a field study an animal may be killed by a competent person outside of an establishment.
3. In relation to the animals covered by Annex IV, the appropriate method of killing as set out in that Annex shall be used.
4. Competent authorities may grant exemptions from the requirement in paragraph 3:
 - (a) to allow the use of another method provided that, on the basis of scientific evidence, the method is considered to be at least as humane; or
 - (b) when, on the basis of scientific justification, the purpose of the procedure cannot be achieved by the use of a method of killing set out in Annex IV.
5. Paragraphs 2 and 3 shall not apply where an animal has to be killed in emergency circumstances for animal-welfare, public-health, public-security, animal-health or environmental reasons.

Appendix 2: Annex IV to European Directive 2010/63/EU

Methods of killing animals

1. In the process of killing animals, methods listed in the table below shall be used.

Methods other than those listed in the table may be used:

- (a) on unconscious animals, providing the animal does not regain consciousness before death;
- (b) on animals used in agricultural research, when the aim of the project requires that the animals are kept under similar conditions to those under which commercial farm animals are kept; these animals may be killed in accordance with the requirements laid down in Annex I to Council Regulation (EC) No 1099/2009 of 24 September 2009 on the protection of animals at the time of killing⁽¹⁾.

2. The killing of animals shall be completed by one of the following methods:

- (a) confirmation of permanent cessation of the circulation;
- (b) destruction of the brain;
- (c) dislocation of the neck;
- (d) exsanguination; or
- (e) confirmation of the onset of *rigor mortis*.

(1) OJ L 303, 18.11.2009, p. 1.

3. Table

Animals-remarks/ methods	Fish	Amphibians	Reptiles	Birds	Rodents	Rabbits	Dogs, cats, ferrets and foxes	Large mammals	Non-human primates
Anaesthetic overdose	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)
Captive bolt	-	-	(2)	-	-	+	-	+	-
Carbon dioxide	-	-	-	+	(3)	-	-	-	-
Cervical dislocation	-	-	-	(4)	(5)	(6)	-	-	-
Concussion/percussive blow to the head	+	+	+	(7)	(8)	(9)	(10)	-	-
Decapitation	-	-	-	(11)	(12)	-	-	-	-
Electrical stunning	(13)	(13)	-	(13)	-	(13)	(13)	(13)	-
Inert gases (Ar, N ₂)	-	-	-	+	+	-	-	(14)	-
Shooting with a free bullet with appropriate rifles, guns and ammunition	-	-	(15)	-	-	-	(16)	(15)	-

Requirements

1. Shall, where appropriate, be used with prior sedation.
2. Only to be used on large reptiles.
3. Only to be used in gradual fill. Not to be used for foetal and neonate rodents.
4. Only to be used for birds under 1 kg.
Birds over 250 g shall be sedated.
5. Only to be used for rodents under 1 kg.
Rodents over 150 g shall be sedated.
6. Only to be used for rabbits under 1 kg.
Rabbits over 150 g shall be sedated.
7. Only to be used for birds under 5 kg.
8. Only to be used for rodents under 1 kg.
9. Only to be used for rabbits under 5 kg.
10. Only to be used on neonates.
11. Only to be used for birds under 250 g.
12. Only to be used if other methods are not possible.
13. Specialised equipment required.
14. Only to be used on pigs.
15. Only to be used in field conditions by experienced marksmen.
16. Only to be used in field conditions by experienced marksmen when other methods are not possible.

Appendix 3: Relevant provisions on methods of killing in the Experiments on Animals Act (Wet op de dierproeven - Wod)

Article 13c

1. Animals will be killed in the breeder's, supplier's or user's establishment by a competent person in a manner that minimises animal pain, suffering and distress. However, in the case of a field study an animal may be killed by a competent person outside the breeder's, supplier's or user's establishment.
2. An appropriate method of killing as set out in the Directive will be used to kill the animals specified in the Directive.
3. Contrary to paragraph 2, a project licence may be granted for a project involving the use of a method of killing animals which is stated as inappropriate in the Directive, if there is scientific justification that the purpose of the animal procedure cannot be achieved using the methods set out in the Directive. Furthermore, the Minister may grant an exemption or dispensation from paragraph 2, if based on scientific justification the divergent method of killing is considered to be at least as humane as the appropriate methods set out in the Directive.
4. If an animal has to be killed in an emergency situation for animal welfare, public health, public security, animal health or environmental reasons, this may be carried out outside of the breeder's, supplier's or user's establishment by someone other than a competent person. In this case the second and third paragraphs do not apply.

Appendix 4: Recommendations arising from the consultation of community groups

During a meeting held in The Hague on 24 March 2016, the following groups representing the Societal Expert Group for Animal Procedures and Alternatives (*Maatschappelijke Expertgroep Dierproeven en Alternatieven*), provided input for this advisory report: Radboud university medical center, the Dutch Association for Laboratory Animal Science (NVP), the Netherlands Federation of University Medical Centres (NFU), the Netherlands Food and Consumer Product Safety Authority (NVWA), Wil Research, the Platform of Animal Welfare Bodies (IvD Platform), the Animal Ethics Committee of the Royal Netherlands Academy of Arts and Sciences (DEC KNAW), VU Amsterdam/VUmc, the Professional Group of Animal Welfare Officers (*Beroepsgroep Proefdierdeskundigen*), and Three R's Alternatives Initiating Network (TRAIN).

The NCad distilled recommendations from the recordings made of the meeting and subsequently submitted the recommendations for agreement and for any further clarification to the representative of the relevant group. The recommendations as approved by the participating groups are set out below by topic, indicating whether the relevant recommendation is included in the NCad's advisory report. A brief explanation has been provided where a recommendation has not been included.

Elements for the scientific justification of alternative methods of killing

<p>Radboud university medical center (Radboud umc)</p>	<ul style="list-style-type: none"> • In determining measurable parameters and behavioural observations, it is important to make a distinction between adult and neonatal animals. You can induce hypoxia/anoxia in neonates, although brain activity (EEG) will not have ceased at that moment. This occurs more rapidly in adult animals than in neonates. In this sense neonatal rodents are similar to frogs and toads. This means that you should look for a solution to ensure that brain activity rapidly decreases. Hypothermia might be a practicable solution (included in the advisory report). • There is a limit to establishing whether an animal has lost consciousness or is dead. Hold a discussion on whether an EEG would be a meaningful measurement method (included in the advisory report). • The requirement that animals be ‘killed in their own cages’ is unfeasible for killing very large numbers of animals (included in the advisory report). • The Directive offers the option of using other methods for killing appropriately sedated animals. Examine, apart from methods of killing, what we believe to be an acceptable method of sedation (included in the advisory report). • Examine the possibility of a ‘two-step methodology’, in which mainly neonatal rats or mice are first sedated (using CO₂ or hypothermia for instance) and then killed by freezing them. Determine what sedation methods are acceptable for neonates and the associated prioritisation criteria (included in the advisory report).
<p>Netherlands Federation of University Medical Centres (NFU)</p>	<ul style="list-style-type: none"> • Include the implementer’s safety as a criterion in the assessment of alternative methods of killing (included in the advisory report). • Be aware that under the Directive killing animals outside the procedure is the same as during the procedure. However, the Directive also addresses situations in which ‘distressed’ animals have to be killed. In some cases, not all the usual measures will be available and immediate action must be taken from an animal welfare perspective. We recommend that an alternative, i.e. improvised, method used in this context be interpreted as the most humane (included in the advisory report). • Consider the context of the animal before killing it. This will avoid additional stress arising from putting together unfamiliar animals that are to be killed (included in the advisory report). • Consider in the assessment criteria for the method of killing whether it is sufficiently practicable so that it can be learned properly and applied with confidence and a successful result (even if it is not a daily routine) (included in the advisory report). • Include as a criterion for a good method of killing how it is experienced by the implementer or others, people should not be unnecessarily emotionally burdened (included in the advisory report). • The scientific substantiation of alternative methods of killing should focus on the rapid and painless loss of consciousness. As this is not always measurable in practice, other relevant parameters are also needed in order to establish death (particularly heart activity). In addition, other clinical observations, including behaviour, are important (included in the advisory report). • Attention should also be paid to the simultaneous killing of larger numbers of animals in a responsible manner, where efficiency is an important factor but should not comprise animal welfare (included in the advisory report).

Elements for the scientific justification of alternative methods of killing

Platform of Animal Welfare Bodies (IvD Platform)

- The humane nature of a method of killing largely depends on the level of competence of the person killing the animal. The method of killing preferred by the implementer is not always in the animal's interests. The desire to anaesthetise the animal first, particularly if the animal has already been previously sedated by an anaesthetic gas, is extremely stressful for the animal, sometimes even more stressful than the method of killing. The implementer should not allow his or her own preference to take precedence over animal welfare and should refrain from unnecessarily applying additional procedures. Restraint should therefore be exercised in using sedation as an opportunity to apply other methods of killing (included in the advisory report).
- Competence is at least as important as capacity, ensure sufficient training. In terms of competent implementation, certain other, scientifically justified, methods of killing should be able to be applied. The competence of the implementer of the method of killing entails not only that this person effectively kills the animal, but carries it out in the appropriate manner (included in the advisory report).
- We recommend that researchers examine the list of authorised methods of killing to determine which methods should be abandoned because they interfere with the purpose of the research. If, after doing so, there are no methods left, other methods should be sought that may in fact be appropriate (included in the advisory report).
- Losing consciousness as quickly as possible is an aim that cannot be achieved with CO₂. We would probably be better off with a slow fill method. Nowadays there are automated CO₂ systems for killing several cages of animals simultaneously. The advantage of such a system is that it runs through a preset programme until the end and the implementer cannot interfere with the process (included in the advisory report).
- When using hypothermia it is important to make a distinction between warm and cold-blooded animals. Hypothermia is used as standard for zebra fish; the fish are placed in a cooled facility and sedation is instantaneous. Gradual hypothermia can be used for neonatal rats, thereby ensuring that the animal is gradually supercooled (not only on one side). Submerging in liquid nitrogen is the quickest way of supercooling, but meets several practical objections (included in the advisory report).
- As for measurable parameters for loss of consciousness: an EEG measures brain activity, not the degree of consciousness. Where the scientific justification is concerned, it is important to determine which brain activity is measured and to what extent this relates to consciousness (included in the advisory report).
- Concerning the killing of larger animals, such as dogs, in current practice animals must be sedated in their own cage first and then taken in a sedated condition to the room where they will ultimately be euthanised (included in the advisory report).

Elements for the scientific justification of alternative methods of killing

Three R's Alternatives Initiating Network (TRAIN)

- Consider mechanical methods of killing, such as decapitation, also for birds and cold-blooded animals. Ample literature is available on this topic due, among other things, to discussions on ritual slaughter. In this context, reference is often made to an old and now outdated article asserting that after decapitation a rat's brain would still remain active for some time. Regrettably, the European Directive has failed to take into account that brain death in fact already occurs within a second (included in the advisory report).
- The aspects which are important in justifying the method of killing can be categorised as animal welfare-related, procedure-related, and implementer-related (included in the advisory report).
- Animal welfare: alternative methods of killing should induce rapid loss of consciousness and be accompanied by minimum pain and stress. Slow loss of consciousness is highly stressful for adult prey animals. When using hypothermia, it is essential to take measures to prevent the animal from freezing solid (pain) (not included in the advisory report, this relates to implementation).
- Animal procedure: the method of killing must not interfere with the procedure (included in the advisory report).
- Implementer: the method of killing must be practicable (aesthetically acceptable) for the implementer. Should the implementer hesitate during the procedure, this will cause the animal additional pain, suffering and stress. Many staff and students prefer euthanasia by gas or injection to mechanical methods. After further questioning, we found that this was primarily because they were more comfortable with this method. This is not always in the interests of animal welfare (included in the advisory report).
- A conflict can arise between animal welfare and the aesthetic aspect of a method of killing. Killing animals with 100% CO₂ is a visually unpleasant experience. However, the convulsions occur after loss of consciousness; at that moment the EEG is already completely flat. If you add oxygen (slow/gradual fill) convulsions also occur, but the brain continues to be active for a longer period. Nevertheless, gradual fill with CO₂ was included in the law. The middle ground may have been opted for here. It still is important to take into account what the implementer thinks of the method. A method should not be so repulsive that people reject it flat out. When using CO₂ as a method of killing animals in their own cage, in which the cage is slowly filled with CO₂, the degree of distress, at least for rats, decreases significantly. This is inadequately reflected in the Directive (included in the advisory report).
- Numerous researchers, particularly those conducting brain research, have no desire to use anaesthesia before killing an animal because they do not want to contaminate their measurements with a sedative. If we can sedate animals first, we will have more options in terms of methods of killing, with respect to painful methods for instance (included in the advisory report).
- Anaesthetic gas, such as CO₂, is not an effective sedation method for neonates. It takes too long. Depending on the purpose of the research following euthanasia, an option may be to sedate the animal by inducing hypothermia and then killing it by freezing it. Hypothermia can only be used in animals that have not yet grown fur. Reflex tests have revealed that neonatal rats with induced hypothermia, lose consciousness very quickly. Examine whether reflex tests provide sufficient justification for this. The death of neonates can be ensured by decapitating the animals after cooling (included in the advisory report).
- Use the available measurable parameters and clinical observations. It may be better to use somatic-evoked potentials (SEPs) rather than an EEG because SEPs provide a more detailed picture, where an EEG no longer provides any information. Examine whether there is a difference between animal species relating to the use of an EEG and SEPs. An EEG is used as standard for dogs and cats (included in the advisory report).

Elements for the scientific justification of alternative methods of killing

	<ul style="list-style-type: none"> • Ensuring (completion) and establishing death are essential aspects in implementing any humane method of killing, particularly when using automated systems (included in the advisory report). • While it is more difficult to kill large numbers of animals in their own cage in practice, animal welfare should nonetheless serve as the guiding principle (included in the advisory report). • Killing animals in the proximity of animals of the same species must be avoided. In decapitation the smell of blood and the noise of ultrasonic vocalisations causes stress among animals of the same species. The relationship between humans and animals plays a significant role in killing dogs in the animal research laboratory. If you sedate a dog in the presence of other dogs, the decisive factor is the signal the dog gives to the other animals. Key aspects are: a trusted person, calmness and competence. Transportation and the unfamiliar environment were found to create more stress for farm animals than watching animals of the same species being slaughtered (included in the advisory report).
Wil Research	<ul style="list-style-type: none"> • An additional aspect that is important in justifying the methods of killing is the assessment of the loss of consciousness (and establishing death), especially in neonates and foetuses (included in the advisory report). • Examine whether there is a difference between adult and foetuses/neonatal animals in EEG and evoked potential (included in the advisory report).
Professional Group of Animal Welfare officers	<ul style="list-style-type: none"> • An alternative method of killing should, in principle, cause the animal to lose consciousness rapidly, but if there is no stress, it may also take longer (included in the advisory report). • If the neonates have not yet been born by natural delivery, it is important to take into account how the mother was killed. This affects the pups' welfare (included in the advisory report). • There is no compelling scientific justification opposing the killing of rats and mice (sedated exsanguination) in the proximity of animals of the same species (not included in the advisory report, this view was refuted during the community consultation). • The European Directive refers to 'appropriate sedation'. Examine whether we can establish that hypothermia is an appropriate form of sedation (demonstrably effective) (included in the advisory report). • The effectiveness of a method of killing is a highly important aspect. Include consideration of the risk of failure. If we, for instance, feel that concussion is a good method for a guinea pig, develop a validated system for this purpose (included in the advisory report).

Elements for the scientific justification of alternative methods of killing

<p>Dutch Association for Laboratory Animal Science (NVP)</p>	<ul style="list-style-type: none"> • Public acceptance is another important aspect in the choice of a method of killing. While generally accepted methods are used in veterinary practice, they are not stated in the European Directive (included in the advisory report). • Refrain from imposing too many additional restrictions on the authorised methods specified in the table, even though they may not be the best methods in all cases. Take a critical look, but exercise restraint in declaring legally permitted methods ‘unsuitable’ (included in the advisory report). • The number of animals to be killed does not constitute a criterion for the choice of a method of killing (included in the advisory report). • The guiding principle of preferably not killing animals in the proximity of animals of the same species stems from issues in abattoirs. This is a good guiding principle, given that there probably also are signals which we as human beings do not pick up, but which do have an impact on stress among animals of the same species (included in the advisory report).
<p>VU Amsterdam/ VUmc (VU/VUmc)</p>	<ul style="list-style-type: none"> • While the method of killing should be acceptable to the implementer, minimum stress for the animal and rapid loss of consciousness should be the guiding principle (included in the advisory report). • From the perspective of rapid loss of consciousness, the preference is for decapitation or cervical dislocation, without sedation carried out by a competent person (included in the advisory report). • Prior sedation based only on the employee’s preference is debatable (included in the advisory report).
<p>Animal Ethics Committee of the Royal Netherlands Academy of Arts and Sciences (DEC KNAW)</p>	<ul style="list-style-type: none"> • Explain how the prescribed methods were authorised. How are these methods validated, not just for ad hoc cases but ultimately for inclusion in the table in the Directive? If you follow the discussion on CO₂, for instance, you notice that the scientific justification seems to be fairly random (included in the advisory report). • Many people find the ‘violent’ nature of mechanical methods of killing, such as decapitation and concussion, repulsive. It feels almost inhuman. Employees may have a moral right to refrain from using these methods because they will need to act contrary to their principles. Yet, from an animal welfare perspective, they may very well be the preferred methods (included in the advisory report).

Focus areas for alternative methods of killing

<p>Radboud university medical center (Radboud umc)</p>	<ul style="list-style-type: none"> • The humane killing of neonatal rats and mice should be addressed. The methods of killing these animals prescribed in the European Directive are insufficiently scientifically justified (included in the advisory report). • It is positive that a scientific justification will be required and we will be pleased to collaborate in researching humane methods for killing neonatal rats and mice (included in the advisory report).
<p>Netherlands Federation of University Medical Centres (NFU)</p>	<ul style="list-style-type: none"> • Verify whether the methods of killing prescribed by the European Directive are adequate for all situations; there are gaps particularly where the killing of individual birds and very young rodents (foetuses and neonates) is concerned. If there are more humane alternatives, they should be added to the list (included in the advisory report). • Examine the feasibility of an alternative method for killing individual birds. This involves administering a barbiturate overdose by an injection through the foramen magnum (hole in the back of the head), directly into the brain. This method is easy to carry out (the foramen is easy to locate and the injection requires minimum fixation of the animal). Moreover, it is very quick and effective (included in the advisory report).
<p>Platform of Animal Welfare Bodies (IvD Platform)</p>	<ul style="list-style-type: none"> • Not only are the methods of killing prescribed in the European Directive inadequate where birds and very young rodents are concerned, but there are no guidelines for killing pregnant animals and rodents (mother animal and foetuses) and newborn pups (included in the advisory report).
<p>Three R's Alternatives Initiating Network (TRAIN)</p>	<ul style="list-style-type: none"> • Clove oil can be added to water as a sedative for fish/zebra fish (instead of MS222) (included in the advisory report). • CO (carbon monoxide) is in fact the most humane method of killing, but there are objections against its use, for reasons of the user's safety (OHS). It must be possible to design devices that use CO safely, with monitoring systems to protect users (Included in the advisory report)
<p>Professional Group of Animal Welfare officers</p>	<ul style="list-style-type: none"> • Under the US Directives, hypothermia is an authorised method for killing neonatal rodents. Examine whether this method, although slow for neonatal animals, might be acceptable. CO₂ causes agitation in neonates before they lose consciousness. Hypothermia does not have this effect and moreover more rapidly leads to the effective loss of consciousness. Examine what the priority elements are in the justification and determine which measurable parameters and clinical observations will be linked to this (included in the advisory report).
<p>VU Amsterdam/ VUmc (VU/VUmc)</p>	<ul style="list-style-type: none"> • Examine the side effects of barbiturates (included in the advisory report). • Examine the side effects of killing through CO₂ + O₂ or CO₂ only (included in the advisory report).

Other recommendations

<p>Radboud university medical center (Radboud umc)</p>	<ul style="list-style-type: none"> • The section on methods of killing in the European Directive is based on an EFSA report. This report, and the parties who contributed to it, can serve as input for a scientific justification. However, it is incomplete because the options described in the Directive - particularly methods for killing neonate rodents - have not been scientifically justified (included in the advisory report). • Advocate, following on from the FELASA proposal, that a possible new training programme in 'killing laboratory animals' be set up not only for 'former Article 12 students', but also for external candidates (not included in the advisory report, this relates to implementation). • The United Kingdom has designated persons for killing laboratory animals. Examine what requirements they must meet in terms of demonstrable competence (not included in the advisory report, this relates to implementation).
<p>Netherlands Federation of University Medical Centres (NFU)</p>	<ul style="list-style-type: none"> • Verify whether there are best practices in methods of killing that depart from current practice (the prescribed methods of killing) (included in the advisory report).
<p>Platform of Animal Welfare Bodies (IvD Platform)</p>	<ul style="list-style-type: none"> • Clarify the term 'small laboratory animals' where Dutch laws and regulations state that vocationally trained (MBO-level) laboratory animal carers may kill 'small laboratory animals'. At present, this can still be interpreted as 'animals belonging to small animal species or small (young) animals of any animals species, or even the smallest animals (piglets) within the total group of animals (farm animals) in a facility' (not included in the advisory report, this relates to implementation). • Incorporate the new publication 'The end of animal life...' by Elsbeth Stassen and Frank Meijboom in the preparation of this advisory report (included in the advisory report). • In fur farming, CO (carbon monoxide) is used as a method of killing. This application is feasible because an installation in the outside air is used, which minimises the risk to human beings (included in the advisory report).
<p>Three R's Alternatives Initiating Network (TRAIN)</p>	<ul style="list-style-type: none"> • In a report by a European Commission Working Party published in 1997 all methods of killing in that period were ranked and well-substantiated (in order of practicability). Use this as a guideline (included in the advisory report). • It is vital to ensure that implementers of methods of killing are trained. They must demonstrate that they are competent. Managers should respect the fact that some employees are opposed to decapitating animals. In the past, during the training programme candidates were taught how to use mechanical methods of killing, such as concussion in guinea pigs. This no longer is the case. Although it is a highly effective method, it is no longer applied. Someone must verify whether staff are capable of carrying out a method, including mentally, and whether they are sufficiently proficient in the technique. They must be given sufficient practice in using the method on anaesthetised and dead animals (included in the advisory report). • Where applicable, take the animals killed in the field study into consideration (included in the advisory report). • A specific microwave technique was applied during brain research, in which the brain was instantaneously fixated, halting the metabolic processes in the brain. However, this requires the animal to be secured in a restraining device to place it in the exact spot for the beam and this gives rise to stress. The effect of stress was not recognised as such in the past, but it is today. For this reason, this method should be regarded as obsolete (not included in the report, relates to implementation).

Other recommendations

Wil Research	<ul style="list-style-type: none">• The Directive does not clearly state the administration route that should be used for substances. An overdose of barbiturates often is administered parenterally in animals, but is administered orally in children for the purpose of sedation. It is unclear whether barbiturates may be administered orally in lab animals and whether it is authorised (included in the advisory report).
Professional Group of Animal Welfare officers	<ul style="list-style-type: none">• FELASA recommended a specific course on 'Killing laboratory animals', not only for inclusion in the regular curriculum of the former Article 12 training programme, but also for other 'external' participants. This has not been adopted in the Netherlands to date, yet the training programme 'Killing laboratory animals' should preferably be made available to external participants as well (not included in the advisory report, this relates to implementation).
Dutch Association for Laboratory Animal Science (NVP)	<ul style="list-style-type: none">• Based on all the experience acquired in practice, it may ultimately be worthwhile setting out best practice methods of killing in association with the Animal Welfare bodies (included in the advisory report).• In the light of prudent implementation and in the context of the law, the guiding principle is that candidates must have undergone specific training in killing animals. Please therefore exercise considerable restraint in granting dispensation for persons who do not meet this requirement (not included in the advisory report, relates to implementation).

Appendix 5: Experience gained by European Members States in using divergent methods of killing

Country	Departure from the prescribed methods of killing	Method of killing	Aspects	Elements of justification stated by the respondent
Denmark	Yes	Focused microwave irradiation (MWR) in brain research conducted in rats and mice	Animal welfare	Speed of death (<1 sec.) Pain, suffering and distress
			Animal procedure	Superior fixation method for brain tissue
Ireland	Yes	Rapid hypothermia in zebra fish	Animal welfare	Speed of killing Signs of distress
			Animal procedure	Histopathological changes
			Implementer	Minor risk of failure
				Easy to perform
Poland	Yes	Intraperitoneal injection with pentothal (or veterinary mixture Morbital) in rats and mice	Animal welfare	Pain Rapid loss of consciousness (< 1 min.) Degree of agitation
			Animal procedure	Possibility of collecting blood with minimum change in stress hormone levels
			Implementer	Easy for a trained person to perform
			Republic of Latvia	No
Belgium	No			
Hungary	No			
Luxembourg	No			
Sweden *	No			
Finland **	No			

- * *The respondent in Sweden reported that they had departed from the prescribed methods of killing a number of times because they were unable to achieve the purpose of the animal procedure with the methods set out in the Directive. This entailed killing fish using hypothermia (followed by exsanguination or not), killing fish on dry ice after anaesthesia by concussion, killing fish in fish nets (max. 18 hours) and killing starlings (after anaesthesia by concussion) by heart compression until breathing and the heart beat stops, and then placing the birds on dry ice.*
- ** *The respondent in Finland also reported that they had departed from the prescribed methods of killing a number of times because they were unable to achieve the purpose of the animal procedure with the methods set out in the Directive. This entailed killing small rodents by using focused microwave, and killing zebra fish using hypothermia.*

Appendix 6: Sources

American Veterinary Medical Association (AVMA), *AVMA Guidelines for the Euthanasia of Animals: 2013 Edition*. 2013

Canadian Council on Animal Care (CCAC), *CCAC guidelines on: euthanasia of animals used in science*. 2010

Close B, Banister K, Baumans V, Bernoth E-M, et al., *Recommendations for euthanasia of experimental animals: Part 2*, Report of a Working Party, *Laboratory Animals* 31:1-32, 1997

European Food Safety Authority (EFSA), *Opinion of the Scientific Panel on Animal Health and Welfare on a request from the Commission related to "Aspects of the biology and welfare of animals used for experimental and other scientific purposes"*. *The EFSA Journal* 292; 1-46, 2005

Golledge H, *Good practice for carbon dioxide euthanasia of rodents*, Report of the 2006 RSPCA/UFAW Rodent Welfare Group meeting, *Lab Animal* 37(5):219-220, 2008

Hawkins P, Playle L, Golledge H, Leach M, et al, *Newcastle Consensus Meeting on Carbon Dioxide Euthanasia of Laboratory Animals*. 2006

Hurst JL, West RS, *Taming anxiety in laboratory mice*. *Nature Methods* 7(10):825-841, 2010

Jager H, *Newcastle Consensus Meeting 2013 over euthanasie van proefdieren*. *Biotechniek* 52/5, 2013

Meijboom FLM, Stassen EN, *The end of animal life: a start for ethical debate*. Ethical and societal considerations on killing animals, Wageningen Academic Publishers, 2016

Mohan Raj AB, Leach MC, Morton DB, *Carbon dioxide for euthanasia of laboratory animals*, *Comparative Medicine* 54(5):470-471, 2004

Moody CM, Makowska IJ, Weary DM, *Testing three measures of mouse insensibility following induction with isoflurane or carbon dioxide gas for a more humane euthanasia*, *Applied Animal Behaviour Science* 163:183-187, 2015

Nederlandse Voedsel- en Warenautoriteit (NVWA), *Zo doende 2014 – Jaaroverzicht dierproeven en proefdieren van de Nederlandse Voedsel- en Warenautoriteit*, 2016

Niel L, Weary DM, *Behavioural responses of rats to gradual-fill carbon dioxide euthanasia and reduced oxygen concentrations*, *Applied Animal Behaviour Science* 100:295-308, 2006

Pecaut MJ, Smith AL, Jones TA, Gridley DS, *Modification of immunologic and hematologic variables by method of CO₂ euthanasia*, *Comparative Medicine* 50(6):595-602, 2000

Pritchett K, Corrow D, Stockwell J, Smith A, *Euthanasia of neonatal mice with carbon dioxide*, *Comparative Medicine* 55(3):275-281, 2005

Reed B, Hawkins P, Latham N, Westwood K, et al, *Report of the 2006 RSPCA/UFAW Rodent Welfare Group meeting*. *Lab Animal* 37 (5): 216-222, 2008

Research Animals Department RSPCA, *Good Practice for humane killing*, supplementary resources for members of local ethical review processes, 1st edition, 2011

Sectie Dierproeven Veterinaire Hoofdinspectie van de volksgezondheid en Nederlandse Vereniging voor proefdierkunde, *Euthanasie van proefdieren – evaluatie van enkele in Nederland veel toegepaste methoden*, 1993

Sharp J, Azar T, Lawson D, *Comparison of carbon dioxide, argon, and nitrogen for inducing unconsciousness or euthanasia of rats*, *Journal of the American Association for Laboratory Animal Science* 45(2):21-25, 2006

Thomas AA, Flecknell PA, Golledge HDR, *Combining nitrous oxide with carbon dioxide decreases the time to loss of consciousness during euthanasia in mice – refinement of animal welfare?*, *PLoS ONE* 7(3):e32290, 2012

Valentine H1, Williams WO, Maurer KJ, *Sedation or inhalant anesthesia before euthanasia with CO₂ does not reduce behavioral or physiologic signs of pain and stress in mice*, *Journal of the American Association for Laboratory Animal Science* 51(1):50-7, 2012

Wallace J, *Attitudes to rodent euthanasia techniques*, *ALN Magazine*, <http://www.alnmag.com/articles/2008/11/attitudes-rodent-euthanasia-techniques>, 2008

Ziemann AE, Allen JE, Dahdaleh NS, Drebot II, et al., *The amygdala is a chemosensor that detects carbon dioxide and acidosis to elicit fear behaviour*, *Cell* 139(5):1012-1021, 2009

Footnotes

- ⁱ *Zo doende* is the annual review of animal procedures and laboratory animals published by the Netherlands Food and Consumer Product Safety Authority (NVWA) <https://www.rijksoverheid.nl/documenten/jaarverslagen/2016/03/01/zo-doende-2014>.
- ⁱⁱ The advisory report on ‘Genetically modified animals killed in stock’ which the NCad submitted to the Minister of Agriculture in October 2015 contains recommendations on reducing the number of genetically modified animals that ‘die or are killed in stock’, mainly fish and mice. <http://www.ncadierproevenbeleid.nl/documenten/rapport/2015/11/26/advise-stock-animals>
- ⁱⁱⁱ The website www.Humane-endpoints.info describes a humane endpoint as ‘the earliest indicator in an animal experiment of potential pain and/or distress that, within the context of the scientific endpoints to be met, can be used to avoid or limit pain and/or distress by taking actions such as humane killing or terminating or alleviating the pain and distress’ (Hendriksen and Morton, 1999). The researcher defines the humane endpoint and the measures to be taken for the relevant animal procedure in the project application and the work protocol. A humane endpoint:
- Does not necessarily mean the humane killing of the animal, but could also result in interventions to alleviate the stressful/painful experimental procedure (e.g. performing surgery) or providing analgesics.
- Is not necessarily based on clinical signs but could also start from pre-clinical signs or from physiological or molecular biomarkers predictive of pain/distress later on in the disease process.
 - Should be balanced against the scientific endpoints to be met. Thus, pain and distress might be intrinsic to a certain experimental model (e.g. arthritis). However, in this case the humane endpoint should never be beyond the scientific endpoint.
 - Should never be beyond the level of moral justification.
- ^{iv} In 2014, 71,144 animal procedures were performed, in which the animals were kept alive upon completion of the procedure. Subject to certain conditions, such animals may be reused in animal procedures or in education (6,319 animals in 2014, mainly in education). In some cases, however, animals may be rehomed upon completion of the procedure. In its March 2016 advisory report on the ‘Rehoming of former laboratory animals’, the NCad provides recommendations and Codes of Practice (CoP) for rehoming former laboratory animals, mainly dogs, cats and non-human primates. <http://english.ncadierproevenbeleid.nl/documents/publications/16/7/19/adoption-of-former-laboratory-animals>
- ^v The Experiments on Animals Act: (Wet op de dierproeven) (Dutch version): <http://wetten.overheid.nl/BWBR0003081/>
- ^{vi} Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes: <http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32010L0063>

^{vii} Article 13c(3) of the Experiments on Animals Act: Contrary to paragraph 2, a project licence may be granted for a project involving the use of a method of killing animals which is stated as inappropriate in the Directive, if there is scientific justification that the purpose of the animal procedure cannot be achieved using the methods set out in the Directive. Furthermore, the Minister may grant an exemption or dispensation from paragraph 2, if based on scientific justification the divergent method of killing is considered to be at least as humane as the appropriate methods set out in the Directive. <http://wetten.overheid.nl/BWBR0003081/>

^{viii} Animal Research: Reporting In Vivo Experiments (ARRIVE) of the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) in the UK aims to improve publications on animal procedures and to minimise unnecessary animal procedures. <http://www.nc3rs.org.uk/arrive-guidelines>

^{ix} The NCad considers openness and transparency about animal procedures and the possibilities of replacing, reducing and refining such procedures (3Rs) of vital importance. Data on laboratory animal use and the possibilities of the 3Rs are often concealed, fragmented or simply unavailable. In its advisory report on 'Indicators, management and utilisation of data for monitoring laboratory animal use and 3R alternatives' (Parts 1 and 2), the NCad recommends that the accessibility of data on the use of laboratory animals and the possibilities of the 3Rs be improved by creating a data warehouse for animal procedures and 3R developments. Making new insights and facts accessible will contribute to the more focused development and application of the available

alternatives and, consequently result in fewer animal procedures.
Section 1: <http://www.ncadierproevenbeleid.nl/adviezen-ncad/documenten/rapport/2015/11/1/ncad-advies-dataopslag>
Section 2: <http://www.ncadierproevenbeleid.nl/adviezen-ncad/documenten/rapport/2016/5/17/ncad-advies-data-deel-2>

^x Opinion of the Scientific Panel on Animal Health and Welfare on a request from the Commission related to 'Aspects of the biology and welfare of animals used for experimental and other scientific purposes': http://ec.europa.eu/environment/chemicals/lab_animals/pdf/efsa_opinion.pdf

^{xi} National Competent Authorities for the implementation of Directive 2010/63/EU on the protection of animals used for scientific purposes - A working document on the development of a common education and training framework to fulfil the requirements under the Directive http://ec.europa.eu/environment/chemicals/lab_animals/pdf/Endorsed_E-T.pdf

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The NCad was appointed for the protection of animals used for
scientific purposes and for education.

The NCad achieves visible improvements in the Replacement,
Reduction and Refinement (3Rs) of animal procedures and the
ethical review thereof in order to minimise the use of laboratory
animals, both nationally and internationally. The ethical review
of animal procedures is of pivotal importance in this regard,
as are the 3Rs.