## SCIENTIFIC OPINION

# Scientific Opinion on the use of carbon dioxide for stunning rabbits[1]

**EFSA Panel on Animal Health and Welfare (AHAW)**[2, 3]

European Food Safety Authority (EFSA), Parma, Italy

This Scientific Opinion, published on 23 July 2013, replaces the earlier version published on 11 June 2013.*

## ABSTRACT

The Panel on Animal Health and Welfare was asked to deliver a scientific opinion on the use of carbon dioxide for stunning rabbits. Specifically, EFSA was asked to give its view on the findings of the study performed by the Polytechnic University of Valencia (Spain) and the Animal Technology Centre CITA-ITAVIA "Estudio sobre la valoración mediante parámetros técnicos y de manejo del sistema de aturdimiento con gas $CO_2$". As a first step, the type of study, critical variables, experimental design, data collection and analysis and reporting methods needed to supply scientific evidence that the use of $CO_2$ is an acceptable alternative for the stunning of rabbits were defined. These criteria were then applied to the study. The submitted study is not adequate for a full welfare assessment of the alternative method studied because it does not fulfil the eligibility criteria and the reporting quality criteria defined in this opinion. The shortcomings of the study have been highlighted to indicate where improvements are required. To be considered for a full assessment of the welfare implications of the use of high concentrations of $CO_2$ as a stunning method for rabbits, a study must meet the eligibility standards described herein. A full assessment of the welfare implications of the use of high concentrations of $CO_2$ as a stunning method for rabbits would need to take into account the restraining methods, the pre-stunning, and the stunning phases of the slaughter process and the correlation of the study findings with the results of other scientific evidence.



## KEY WORDS

carbon dioxide, stunning, rabbits, reporting criteria

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* Minor changes of editorial nature were made. The changes do not affect the overall conclusions of the opinion. To avoid confusion, the original version of the opinion has been removed from the website, but is available on request, as is a version showing all the changes made.

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## SUMMARY

Following a request from the European Commission, the Panel on Animal Health and Welfare (AHAW Panel) was asked to deliver a scientific opinion on the use of carbon dioxide for stunning rabbits. Specifically, EFSA was asked to give its view on the findings of the study performed by the Polytechnic University of Valencia (Spain) and the Animal Technology Centre CITA-ITAVIA "Estudio sobre la valoración mediante parámetros técnicos y de manejo del sistema de aturdimiento con gas $CO_2$".

As a first step, the type of study and data needed to supply scientific evidence that the use of $CO_2$ is an acceptable alternative for the stunning of rabbits were defined (TOR 2). These were then applied to the study submitted for review to assess the extent to which the use of $CO_2$ is an acceptable alternative for stunning rabbits, based on the submitted study (TOR 1).

EFSA assessed only the stunning procedure itself and did not take into account any pre-stunning phases. The outcome of the assessment in this opinion indicates only whether the submitted study is adequate for a full welfare assessment of the alternative method studied or not, whereas the quality and strength of scientific evidence will be assessed at the next stage.

**TOR 2: Definition of the type of study and data needed to supply scientific evidence that the use of $CO_2$ is an acceptable alternative for the stunning of rabbits**

The opinion defines the **eligibility criteria** of studies on alternative stunning methods that are based on the legal framework provided in Council Regulation (EC) No 1099/2009 and its Annex I. For consistency with the legislation, the eligibility criteria defined in this opinion specify only the minimum requirements. The minimum criteria that should be reported by studies on stunning methods to fully characterise the stunning intervention were defined to allow assessment of the alternative stunning method. Regarding the outcome measures, the onset and duration of unconsciousness and insensibility should be recorded and reported in all studies. If the onset of unconsciousness/insensibility achieved by the studied stunning intervention is not immediate, then the absence of pain, distress and suffering until the loss of consciousness/sensibility also has to be recorded and reported.

Regarding the **intervention**, stunning via high $CO_2$ concentrations, the legislation states that the key parameters to be provided are: carbon dioxide concentration, duration of exposure overall or just to peak concentration, maximum stun-to-stick/-kill interval(s) in the case of simple stunning, quality of the gas and temperature of the gas. Studies analysing (1) a modification of a currently permitted method, or (2) the application of high $CO_2$ concentrations in other species must report all of the legally required parameters. In order to ensure a comprehensive description of the applied stunning method, for some parameters additional information on several components of these parameters, which are in this opinion, need to be reported.

**Onset of unconsciousness and insensibility** is best demonstrated using electroencephalograms (EEGs). The reliable criteria to be employed during controlled environment studies are the presence of a profoundly suppressed or quiescent EEG and the abolition of evoked electrical activity in the brain, which is indicative of the brain's incapacity to receive and process external stimuli. Once the effectiveness of a given stunning method has been shown in controlled environment studies using EEGs, its effectiveness should also be studied in experiments under slaughterhouse conditions. Several indicators of recognising a successful stun that can be applied in slaughterhouse settings exist. In studies carried out under slaughterhouse conditions, the onset and the duration of unconsciousness and insensibility should be ascertained using the indicator that best detects unconsciousness and that has been shown to be correlated with EEGs in laboratory experiments. If different indicators are not in agreement, following from the precautionary principle and to benefit animal welfare, the one that indicates the longest time interval between application of the stunning intervention and onset of unconsciousness should be used.

If a stunning method does not induce immediate unconsciousness/insensibility, the **absence of pain, distress and suffering until the onset of unconsciousness/insensibility** should be assessed. Pain is a complex phenomenon and is very difficult to measure qualitatively and quantitatively owing to the absence of clear borders among pain, distress and suffering, as these states may not always be distinguishable in animals. At the moment, indirect animal-based measures of pain, distress and suffering have to be used as no direct tool is available to identify pain. Several examples of animal-based measures from the three response types (behavioural changes, physiological changes and neurological changes), which could be applied to observe changes in these responses, were identified. It is recommended that the animal-based measures are selected according to their relevance to the respective stunning intervention as shown by the available scientific knowledge of each measure's sensitivity and specificity. It has been further determined that two criteria/rules have to be fulfilled before a stunning method is considered not to induce pain, distress and suffering before the onset of unconsciousness and insensibility, these being that (1) animal-based measures from at least two different response types of the three response types presented above and relevant to the intervention/species must be indicative of absence of pain, distress and suffering before the onset of unconsciousness/insensibility, and that (2) these animal-based measures should be consistent at the level of the individual animal, depending upon the species and the coping strategies.

Studies in a controlled environment should determine the **duration of unconsciousness/insensibility** using EEGs as described for the determination of the onset of unconsciousness/insensibility. The maximal stun-to-stick/-kill time interval that guarantees unequivocal loss of consciousness/sensibility until the moment of death can be defined based on these results. The applicability of the stun-to-stick/-kill interval should then be analysed in commercial settings using indicators for recognising recovery of consciousness/sensibility that correlate with EEGs, as established in controlled environment studies. The selection of useful indicators will also depend upon the stunning method and the species involved and it is recommended that the indicator that is most sensitive to detect recovery be used.

For the definition of **reporting quality** criteria suitable existing reporting guidelines were identified and their criteria lists slightly modified to allow their use in the context of studies on stunning methods.

The **methodological quality** assessment focuses on the fulfilment of internal and external validity of the submitted study. Internal validity is reached when the study results reflect reality among the animals under study, whereas external validity is reached when the study results can reasonably be generalised to the broader reference population. It was decided to assess only the main biases affecting internal validity, namely confounding, selection bias and information bias, and only in the case that the submitted study fulfils the eligibility criteria.

**TOR 1: Assess the extent to which the use of $CO_2$ is an acceptable alternative for the stunning of rabbits based on the submitted study**

The review to assess the extent to which the use of $CO_2$ is an acceptable alternative for stunning rabbits, based on the submitted study, was carried out according to the criteria defined under TOR 2. The intervention is considered to be insufficiently described. The onset and the duration of unconsciousness were not assessed in the study. An assessment of whether pain, distress and suffering were present during the induction phase was not made. For these reasons, the eligibility criteria are not fulfilled. The study does also not fulfil the reporting quality criteria. As the study did not fulfil the eligibility criteria, the methodological quality of the study was not assessed. Therefore, the shortcomings have been highlighted to indicate where improvements would be required before the study could be submitted for a full assessment of the welfare implications of the use of high concentrations of $CO_2$ as a stunning method for rabbits, which would need to take into account both the pre-stunning and the stunning phases of the slaughter process and the correlation of the study findings with the results of other scientific evidence.

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## BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

Article 4 (2) of Council Regulation (EC) No 1099/2009 on the protection of animals at the time of killing allows the Commission to amend Annex I to this Regulation as to take into account scientific and technical progress on the basis of an opinion of the EFSA. Any such amendments shall ensure a level of animal welfare at least equivalent to that ensured by the existing methods.

At present, the use of carbon dioxide is not allowed for stunning rabbits. The Commission has received a request from the Spanish authorities to allow the use of carbon dioxide as a method for stunning rabbits. This request is supported by a study (see attachment). Since the Spanish authorities acknowledge that further work is needed, they suggest allowing this method for a certain transitional period in order to make possible the collection of additional scientific data.

In order to reply to this request, the Commission would like to request the EFSA to review the scientific knowledge on the stunning of rabbits of this study.

## TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

The Commission therefore considers it opportune to request the EFSA to give an independent view on the use of carbon dioxide for stunning rabbits.

- The scope of this request is limited to the stunning of rabbits.
- The EFSA will give its view on the findings of the study performed by Polytechnic University of Valencia (Spain) and Animal Technology Centre CITA-ITAVIA "*Estudio sobre la valoración mediante parámetros técnicos y de manejo del sistema de aturdimiento con gas CO2*" with a focus on the following issues:

  − The extent to which the use of $CO_2$ is, in principle, an acceptable alternative for the stunning of rabbits compared to the welfare advantages/disadvantages related to other stunning methods used for rabbits under commercial conditions;

  − The extent to which the findings of the study are consistent with other sources of information;

  − Requirements possibly attached to the use of carbon dioxide for stunning rabbits, (minimum or maximum gas concentration, duration of exposure, stun-to-stick interval, quality of the gas, temperature of the gas, type of recording and maintenance etc.);

  − The extent to which the findings of study can be valid in the context of other rabbit slaughterhouses in the EU.

## ASSESSMENT

### 1. Introduction

Inhalation of carbon dioxide ($CO_2$) induces respiratory and metabolic acidosis, leading to neuronal inhibition via a pH reduction of the cerebrospinal fluid. The use of high $CO_2$ concentrations to stun animals is described in detail in previous EFSA opinions (2004, 2005 and 2006), but they do not specify its application in rabbits. On receipt of this mandate, its terms of reference (TORs) were discussed with the European Commission service and the following was agreed upon.

EFSA will assess the study submitted by the Spanish authorities with a focus on:

- TOR1 : the extent to which the use of $CO_2$ is an acceptable alternative for the stunning of rabbits based on the submitted study.
- TOR2 : the type of study and data needed to supply scientific evidence that the use of $CO_2$ is an acceptable alternative for the stunning of rabbits.

The term 'acceptable alternative' in this opinion is defined as an alternative stunning method that is at least as good as those listed in Council Regulation (EC) No 1099/2009. Specifically, the alternative procedure must induce immediate onset of unconsciousness/insensibility or absence of pain, distress and suffering until the onset of unconsciousness/insensibility and the animal must remain unconscious/insensible until death.

In this opinion, the moment that animals are exposed to $CO_2$ is considered as the start of the stunning phase. The pre-stunning handling and restraint methods are not considered in this opinion owing to the terms of reference. However, the implications of pre-stunning and restraint are very important for animal welfare and should be considered in a full welfare assessment of a stunning method for any given species.

The opinion defines eligibility criteria of studies on alternative stunning methods that are based on the legal framework provided in Council Regulation (EC) No 1099/2009 and its Annex I. For consistency with the legislation, the eligibility criteria defined in this opinion specify only the minimum requirements. The criteria concerning the outcome of the intervention are based on the legal definition of stunning and consequently focus on the onset and duration of unconsciousness and insensibility as well as the absence of pain, distress and suffering in case onset of unconsciousness and insensibility is not immediate. It is assumed that the criteria for using high concentrations of $CO_2$ as a stunning method for rabbits should be similar to the legal requirements described for the application of this stunning method in pigs and poultry, because the pain, distress and suffering caused by the restraining method or the pre-slaughter handling necessary for electrical stunning is thought to exceed the pain, distress and suffering caused by the inhalation of high concentrations of $CO_2$. In simple terms, there are welfare benefits of using $CO_2$ for stunning pigs and poultry.

EFSA assessed only the stunning procedure itself and did not take into account any pre-stunning phases. A full assessment of the welfare implications of the use of high concentrations of $CO_2$ as a stunning method for rabbits, which would need to take into account both pre-stunning and stunning phases of the slaughter process and the correlation of the study findings with the results of other scientific evidence, is beyond the scope of this mandate as the TORs are restricted to the assessment of the submitted study. The outcome of the assessment in this opinion indicates only whether the submitted study is adequate for a full welfare assessment of the alternative method studied, whereas the quality and strength of the scientific evidence will be assessed at the next stage.

This opinion is just the first step to providing guidance to the AHAW Panel for assessing studies examining alternative stunning methods. A document covering all stunning methods listed in Council Regulation (EC) No 1099/2009, with detailed guidance on assessing alternative stunning methods, will be generated and published in the near future.

## 2. Approach

The submitted study documents were assessed regarding fulfilment of eligibility criteria, reporting quality and methodological quality criteria. The criteria were first defined (fulfilment of TOR 2) and then applied to assess the submitted study with the objective of determining the extent to which the use of $CO_2$ is an acceptable alternative for the stunning of rabbits based on the submitted study (fulfilment of TOR1) (Figure 1). The assessment was first individually carried out by each working group member. The individual assessments were then discussed to reach a consensus on parameters where experts had initially had different opinions.

*Eligibility criteria*

Council Regulation (EC) No 1099/2009 defines "stunning" in Article 2(f) as "any intentionally induced process which causes loss of consciousness and sensibility without pain, including any process resulting in instantaneous death". Furthermore, Article 4 on stunning methods regulates that "animals shall only be killed after stunning in accordance with the methods and specific requirements related to the application of those methods set out in Annex I of the Regulation" and "that the loss of consciousness and sensibility shall be maintained until the death of the animal". The methods referred to in Annex I that do not result in instantaneous death shall be followed as quickly as possible by a procedure ensuring death such as bleeding, pithing, electrocution or prolonged exposure to anoxia. Most of the methods listed in Annex 1 cause immediate onset of unconsciousness, with the exception of controlled atmosphere- or gas-stunning methods. Eligibility criteria that need to be fulfilled by submitted studies were set based on the legislation and focused on the intervention and the outcome:

For the intervention:

The key parameters described in the legislation and provided by stunning experts

For the outcome:

A. Immediate onset of unconsciousness and insensibility **OR**
B. Absence of avoidable pain, distress and suffering until the loss of consciousness and sensibility **AND**
C. Duration of the unconsciousness and insensibility (until death)

The minimum criteria that should be reported by studies on stunning methods to fully characterise the stunning intervention were defined to allow assessment of the alternative stunning method. Regarding the outcome measures, the onset and duration of unconsciousness and insensibility should be recorded and reported in all studies. If the onset of unconsciousness/insensibility achieved by the studied stunning intervention is not immediate, then the absence of pain, distress and suffering until the loss of consciousness/sensibility also has to be recorded and reported.

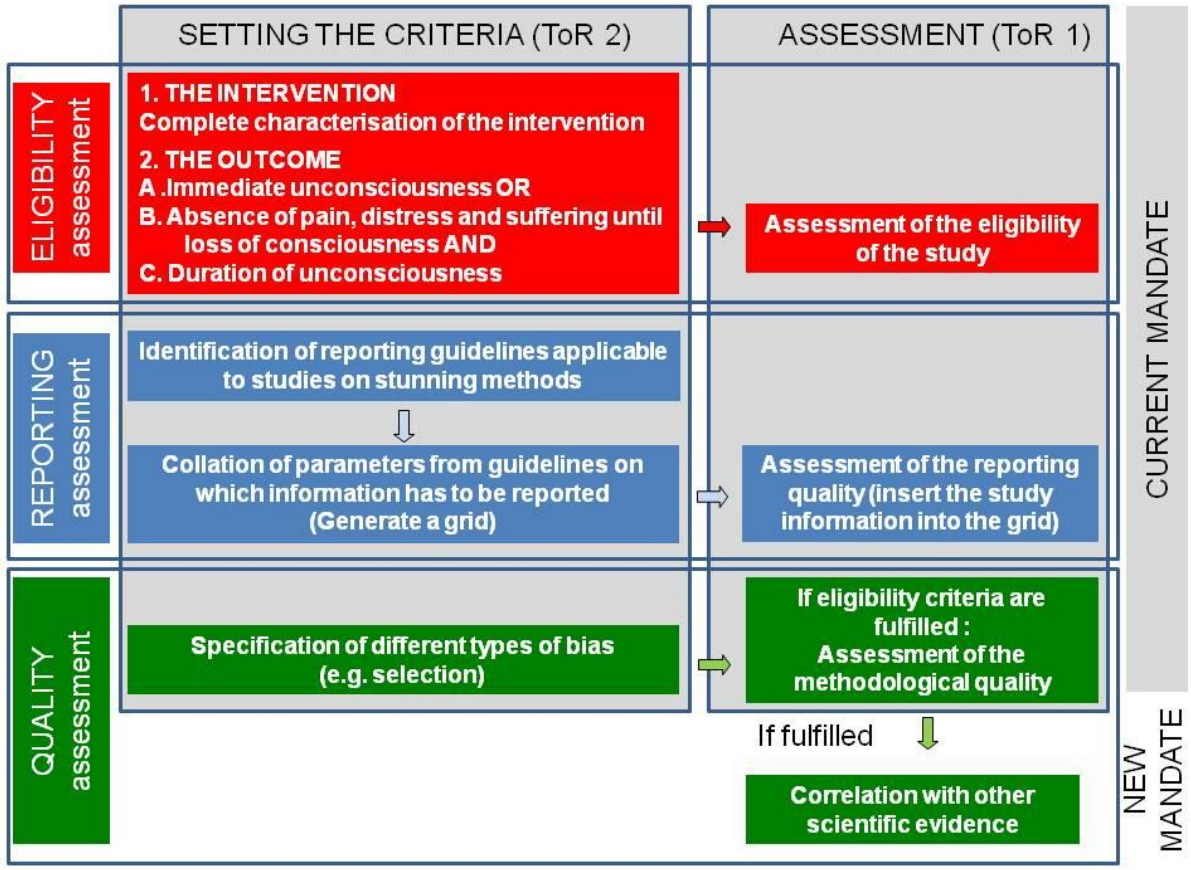


**Figure 1:** The approach of the mandate.

*Reporting quality*

Inconsistencies in the reporting of scientific studies have been identified in the fields of both human and veterinary medicine. Therefore, reporting guidelines designed to increase the transparency of conducting and reporting such scientific studies have been developed by various groups in the past. As these guidelines were not developed to be applied specifically to studies on stunning methods, the two most relevant guidelines were identified. Both guidelines were screened and the relevant parameters in relation to studies on stunning methods were listed and later used as the basis for assessing the reporting quality of the submitted studies.

*Quality assessment*

The methodological quality of the submitted studies is assessed only if the eligibility criteria are fulfilled. At this stage, the presence of biases affecting internal validity is assessed: confounding, selection and information bias.

An analysis of the external validity of the results of the submitted studies, including comparing them with other available scientific evidence will be performed only if all the requirements of the previous steps (assessment of eligibility criteria, reporting quality criteria and methodological quality criteria) of the assessment have been met by the submitted study. However, this analysis is beyond the time frame of the current mandate and will be performed only if the European Commission provides a new mandate for that task.

Furthermore, results obtained under controlled laboratory conditions need to be confirmed in a range of slaughterhouse conditions. Therefore, analysis of alternative stunning methods requires a first phase of the study under controlled (laboratory) conditions to analyse the animals' responses (unconsciousness, absence of pain, distress and suffering) using the most sensitive and specific

methods and to find a correlation with non-invasive parameters that can be applied during the second phase of the study in slaughterhouses. The eligibility criteria should be applied to both phases of the study. Information obtained in other species can be used as an indication, but should be confirmed in the species under investigation because coping strategies, pain thresholds and tolerances are species and individual specific.

*Possible conclusions*
When all criteria regarding eligibility, reporting quality and methodological quality have been assessed individually, an overall conclusion is provided. There are two possible overall conclusions of the assessment made in this opinion:

- All the criteria regarding eligibility, the reporting quality and the methodological quality are fulfilled and the results are conclusive.

  This means that the study on the alternative method provides sufficient detail regarding the intervention and the outcome with conclusive results allowing to conclude that it does not induce pain, distress and suffering until the onset of unconsciousness/insensibility and that unconsciousness/insensibility lasts sufficiently long to cover the stun-to-stick interval and onset of brain death through loss of blood.

  In consequence, the study could be further assessed in the context of additional scientific evidence, taking account of both the pre-stunning and stunning phases and the restraint methods of the slaughter process, under a new mandate.

- Not all the criteria regarding eligibility, reporting quality and methodological quality are fulfilled or the results of the submitted study are inconclusive.

  This means that the study does not provide sufficient detail regarding the intervention and the outcome and/or the results are inconclusive as to whether it does not induce pain, distress or suffering until the onset of unconsciousness/insensibility and whether unconsciousness/insensibility lasts sufficiently long to cover the stun-to-stick interval and onset of brain death through loss of blood.

  In consequence, the assessment would highlight the shortcomings to indicate where improvements are required before the study can be further assessed in the context of additional scientific evidence and taking account of both the pre-stunning and stunning phases and restraint methods of the slaughter process.

## 3. Eligibility criteria

As described in section 2, the requirements specified in this section are based on the definition of stunning laid down by Council Regulation (EC) No 1099/2009[4] on the protection of animals at the time of killing and are applied as eligibility criteria for assessing studies in this opinion.

### 3.1. Specification of eligibility criteria

#### 3.1.1. Intervention

At the moment, stunning via high $CO_2$ concentrations is permitted in pigs, mustelids, chinchillas and poultry, except for ducks and geese, when the technical criteria described in Annex I of Council Regulation (EC) No 1099/2009 are fulfilled. The legislative requirements depend on the purposes (slaughter or depopulation) and the species. The method may be used in pits, tunnels, containers or previously sealed buildings. The legislation states that the key parameters to be provided are: carbon dioxide concentration, duration of exposure overall or just to peak concentration, maximum stun-to-

[4] COUNCIL REGULATION (EC) No 1099/2009 of 24 September 2009 on the protection of animals at the time of killing. OJ L 303, 18.11.2009, p. 1-30.

stick/-kill interval(s) in the case of simple stunning, quality of the gas and temperature of the gas. Studies analysing (1) a modification of a currently permitted method, or (2) the application of high $CO_2$ concentrations in other species must report all of the legally required parameters. Some parameters are subdivided into several components to ensure a comprehensive description of the applied stunning method (Table 1). The animals should also be exposed to the maximum concentration as soon as possible to achieve a rapid induction of unconsciousness.

For studies researching a new or modified simple stunning method, animals should be stunned without sticking to establish the duration of unconsciousness achieved by the stunning itself in proof-of-concept studies under controlled laboratory conditions. The experimental protocol should consider humane endpoints and, therefore, in the case of the long-term adverse effects of the stun experienced, the animal should be re-stunned and bled as soon as it regains consciousness.

**Table 1:** Parameters to be provided when applying a stunning method based on high $CO_2$ concentrations, based on Annex I of Council Regulation (EC) No 1099/2009 and further specifications of components of the parameters

| **Parameter** | **Component** | **Description** |
|---|---|---|
| $CO_2$ concentration | Lowest $CO_2$ concentration[a] | Specify the initial $CO_2$ concentration to which animals are exposed at the initiation of the stunning (at first contact with the modified atmosphere) |
| | Targeted $CO_2$ concentration(s) [a] | Specify the targeted $CO_2$ concentration used to stun the animals. If animals are exposed to $CO_2$ in a step-wise manner in a pre-filled chamber system, several $CO_2$ target concentrations could be applied |
| | Highest $CO_2$ concentration [a] | Specify the final/highest $CO_2$ concentration to which animals are exposed |
| | $CO_2$ concentration gradient | The $CO_2$ concentration is likely not to be homogeneous in a stunning device as $CO_2$ has a higher density than air. For a pre-filled chamber-system, $CO_2$ gradients in the stunning device have to be described in detail (e.g. every 50 cm in height). In the case in which gas is added to a chamber containing animals, specify the gas flow rate (l/min) and the chamber volume (l)<br>If animals are exposed to $CO_2$ in a step-wise manner in a pre-filled chamber system, the concentrations at each step and the duration of the exposure to each concentration and the transition time between each step must be reported |
| | Animal stocking density | Specify the animal density during the $CO_2$ exposure phase |
| | Monitoring | Describe how, where and when the $CO_2$ concentration was monitored |
| Duration of intervention[5] | Time to reach exposure of animal to targeted $CO_2$ concentration [a] | Report the time elapsing until animals are exposed to the targeted $CO_2$ concentration<br>If animals are exposed to $CO_2$ in a step-wise manner in a pre-filled chamber system, the concentrations at each step and the duration of the exposure to each concentration and the transition time between each step must be reported |
| | Total duration of targeted $CO_2$ exposure [a] | Report the total duration of exposure of animals to the targeted $CO_2$<br>If animals are exposed to $CO_2$ in a step-wise manner in a pre-filled chamber system, the concentrations at each step and the duration of the exposure to each concentration and the transition time between each step must be reported |
| Maximum stun-to-stick/-kill interval(s) [a,b] | | Describe the maximum stun-to-stick/-kill interval that has been applied to guarantee unconsciousness/insensibility of the stunned animal until the moment of death (except for |

[5] Referring to the legal parameter 'duration of exposure'.

| Parameter | Component | Description |
|---|---|---|
| | | proof-of-concept studies in which the duration of unconsciousness must be determined without sticking) |
| Quality of the gas | $CO_2$ source | Specify the source of the $CO_2$. |
| | Gas composition of the atmosphere | Clarify if $CO_2$ was applied in an air atmosphere or if other gases (e.g. $O_2$) were added. If other gases were added in addition to $CO_2$, provide information on their concentration (in accordance with the key parameter "$CO_2$ concentration"). |
| | Humidity | Report the humidity of the gas inside the chamber. |
| Temperature of the gas | | Specify the temperature of the gas inside the chamber that was used |

[a]Provide information on mean or median and range and standard deviation or interquartile range of the detailed parameter.
[b]In the case of simple stunning.

### 3.1.2. Outcome

#### 3.1.2.1. Onset of unconsciousness and insensibility

The EFSA Scientific Report of the Scientific Panel for Animal Health and Welfare on a request from the Commission related to welfare aspects of animal stunning and killing methods concludes that stunning and stunning/killing methods should ideally induce an immediate (e.g. in less than one second) and unequivocal loss of consciousness and sensibility. Induction of unconsciousness and insensibility is best demonstrated using EEGs (EFSA, 2004). EEGs or electrocorticograms (ECoGs) are widely used to record the spontaneous and evoked electrical activity in the brain to ascertain the state of consciousness and sensibility following stunning. It is acceptable that studies on alternative stunning methods assess only the onset of unconsciousness as this state is always accompanied by the onset of insensibility. This is based on the animal welfare concern that not all insensible animals are necessarily unconscious, for example analgesia rather than unconsciousness induced by gas mixtures (Raj et al., 1990) and also the insensibility (analgesia) lasts longer than the unconsciousness induced by head-only electrical stunning (Velarde et al., 2002).

The neuronal basis of consciousness with regard to stunning is presented in detail in the EFSA (2004) report. The normal functioning of neurons in the thalamus and cerebral cortex is accepted as a necessary condition for perceptual processes and consciousness. Therefore, stunning methods should disrupt the depolarised state of neurons in the brain and thereby render animals unconscious and insensible. The extent of disruption caused by a stunning method can be measured using electroencephalography, which is normally recorded from the surface of the cerebral cortex. The amplitude and frequency of activity seen in the EEG is related to the degree of synchronisation of activity of neurons.

Animals are rendered gradually unconscious and insensible during exposure to gas mixtures, and the animals may show signs of different stages of anaesthesia as seen in clinical veterinary practice. In general, the different stages of anaesthesia include (1) excitement (voluntary and involuntary), (2) anaesthesia (light, medium and deep), (3) respiratory and cardiovascular depression, and finally (4) death. The stage of voluntary excitement may not be seen in animals when the induction of unconsciousness is smooth and non-aversive. However, the rate of induction of unconsciousness, hence the duration of different stages of anaesthesia, during exposure of animals to a gas mixture may vary and depends mainly upon the concentration of the gas. For example, the rate of induction of unconsciousness will be slow during exposure to 30 % by volume of $CO_2$ in air when compared with exposure to 80 % by volume of $CO_2$ in air. Animals may show signs of pain, distress and suffering caused by the inhalation of a high concentration of $CO_2$ or breathlessness caused by the inhalation of $CO_2$. In addition, inhalation of $CO_2$ stimulates nerve endings in the nasal epithelium which induces sniff-like aspiration reflexes (EFSA, 2005). Some scientists interpreted the animals' reaction during the induction phase as a part of the excitation phase, whereas some others interpreted it as a response to pain caused by the inhalation of the gas.

EEG signatures correlated with loss of consciousness are reported in humans (e.g. Gandelman-Marton and Neufeld, 2012; Purdon et al., 2013) and different animals, but can depend on how unconsciousness is induced, for example on whether electrical, mechanical or modified atmosphere stunning is used (e.g. Raj et al., 1992a, 1992b, 1992c, 1998; EFSA, 2004; Gerritzen et al., 2004, 2006; Benson et al., 2012a, 2012b).

Exposure of animals to gas mixtures leads to loss of consciousness and sensibility due to the inhibition of brain function, as evidenced from the abolition of spontaneous and evoked electrical activity. The physiological brain mechanisms associated with the induction of unconsciousness and insensibility and the EEG manifestations appear to be common to all terrestrial vertebrate animals. The survival time of different regions of the brain and the spinal cord to the effects of gas mixtures may vary. EEG patterns indicating unconsciousness and insensibility following stunning by high carbon dioxide concentrations are as follows. When animals are exposed to $CO_2$, there is a transition period during which conscious EEG patterns change to unconscious EEG patterns, but EEG pattern interpretation is subjective. Therefore, the reliable criteria to be employed during controlled environment studies are:

- Profoundly suppressed or quiescent EEG. This is indicative of a complete loss of spontaneous brain activity or a reduction of EEG total power content to less than 10 % of the pre-stun EEG power content and occurs after exposure to gas mixtures (Rodríguez et al., 2008; Llonch, 2013).
- Abolition of evoked electrical activity in the brain (somatosensory evoked potentials, auditory evoked potentials or flash visual evoked potentials), which is indicative of the brain's incapacity to receive and process external stimuli (Raj et. al., 1997; Martoft, 2002; Rodríguez et al., 2008).

It is important to note that, once the effectiveness of a given stunning method has been shown in controlled environment studies using EEGs, its effectiveness should also be studied in experiments under slaughterhouse conditions. Indicators for recognising a successful stun (see next paragraph) should be applied in slaughterhouse settings, after their correlation with EEGs has been shown in controlled environment studies.

A list with indicators for recognition of a successful stun in different species with gas mixtures is provided in EFSA's 2004 opinion. No specifications have been made for rabbits in particular (EFSA, 2005) as the list of indicators available in the 2004 opinion can be applied to rabbits as well. However, literature concerning gas stunning of rabbits suggests that the earliest observable visible indicator of onset of unconsciousness and insensibility is the loss of posture in animals followed by onset of convulsions (Llonch et al., 2012b). Studies in poultry and pigs concerning welfare suggest that it may not always be possible to determine the time to loss of posture as animals start to convulse before they lose posture (Raj et al., 1997; Rodríguez et al., 2008). Rabbits show tonic immobility during the induction phase of exposure to high concentrations of $CO_2$ (Llonch et al., 2012b); this could be interpreted as evidence for stressful induction of unconsciousness with high concentrations of $CO_2$. However, as exposure to a gas mixture continues, these convulsions stop, leading to a completely relaxed body. There is also a suppression of respiration, which can be evidenced from progressively declining rate and depth of breathing, resulting in complete cessation of any respiratory activity, including gagging. Llonch et al. (2012b) reported respiratory distress in rabbits when exposed to 90 % $CO_2$. Other indicators of effective gas stunning include dilated pupils, absence of palpebral, corneal and pupillary reflexes and absence of response to painful stimuli such as nose pricking. In conclusion, in studies carried out under slaughterhouse conditions, the onset and the duration of unconsciousness and insensibility should be ascertained using the indicator that best detects unconsciousness and that has been shown to be correlated with EEGs in laboratory experiments. If different indicators are not in agreement, following on from the precautionary principle and to benefit animal welfare, the one that indicates the longest time interval between application of the stunning intervention and onset of unconsciousness should be used. Studies on alternative stunning methods should explain in detail how and when the onset of unconsciousness and insensibility is measured. It is recommended that the

methods used have previously been published in peer-reviewed journals, that data are provided at the individual animal level and that actions are taken to prevent the possibility of bias (see section 5) as much as possible. In the case of EEGs, all parameters crucial for assessment of the electroencephalography data should be specified (e.g. the electrode position on the skull or on the brain itself, the configuration of the electrode (transhemispheric or from the same hemisphere of the brain)). In order to estimate quantitative changes occurring in the EEG, the method used to derive the transformations of electroencephalography signals must be described. In addition, the indicators used to assess recognition of a successful stun should be relevant to the respective stunning intervention, based on the available scientific knowledge of each indicator's sensitivity and specificity. Furthermore, the scoring system applied to categorise/classify the signs should be clearly defined. It is essential that the observers making the measurements of the signs have been carefully trained and that scoring systems are adapted to the species and the stunning conditions. Information on all these aspects should be provided and will be assessed by the AHAW Panel, based on scientific knowledge available at that time.

#### 3.1.2.2. Absence of pain, distress and suffering until the onset of unconsciousness and insensibility

If a stunning method does not induce immediate unconsciousness/insensibility, the absence of pain, distress and suffering until the onset of unconsciousness/insensibility should be assessed. Pain is a complex phenomenon and is very difficult to measure qualitatively and quantitatively owing to the absence of clear borders among pain, distress and suffering, as these states may not always be distinguishable in animals. At the moment, indirect animal-based measures of pain, distress and suffering have to be used because no direct tool is available to identify pain. In addition, thresholds for pain, distress and suffering can be different among animals within and between species. Inherent concealing of pain in animals has been reported (Underwood, 2002). Several definitions of pain are frequently reported in the scientific literature (e.g. Zimmermann, 1986; IASP, 1994; Molony, 1997; Broom, 2001; OIE, 2012). Kavaliers (1988), based on the International Association for the Study of Pain 1979 definition, suggested that for non-humans, pain is an aversive sensory experience caused by actual or potential injury that elicits protective motor and vegetative reactions, results in learned avoidance and may modify species-specific behaviour, including social behaviour. Although there are more recent definitions, this one is considered to be appropriate for this opinion. Previous EFSA opinions and scientific papers focus on assessing three "response types" for the evaluation of pain: behavioural changes, physiological changes and neurological changes.

Groups of animal-based measures that could be applied to observe changes in these responses were identified, based on previous EFSA opinions, an expert report and a scientific review of the field of pain assessment in animals (EFSA, 2005; Le Neindre et al., 2009; Landa, 2012). As no specific indicator is available for pain, combinations of animal-based measures for pain, distress and suffering are used as a proxy for pain. Seven "groups of animal-based measures" associated with pain, distress and suffering during the induction of unconsciousness and insensibility are presented: vocalisations; posture and movements; general behaviour; hormone concentrations; blood metabolites; automatic responses; and brain activity. Some research papers that describe the use of a particular animal-based measure to assess pain are included as examples, but the list is not exhaustive.

**Table 2:** Overview of response types and animal-based measures associated with pain, distress or suffering during the induction of unconsciousness and insensibility

| Response type | Groups of animal-based measures | Example | References |
|---|---|---|---|
| Behaviour | Vocalisations | e.g. number and duration, intensity, spectral components | EFSA 2005; Le Neindre et al., 2009; Atkinson et al., 2012; Landa, 2012; Llonch et al., 2012a, 2012b, 2013 |
| | Postures and movements | e.g. kicking, tail flicking, avoidance | Jongman et al., 2000; EFSA, 2005; McKeegan et al., 2006; Gerritzen et al., 2007; Velarde et al., 2007; Kirkden et al., 2008; Svendsen et al., 2008; Dalmau et al., 2010; Atkinson et al., 2012; Landa, 2012; Llonch et al., 2012a, 2012b, 2013 |
| | General behaviour | e.g. agitation, freezing | EFSA 2005; Landa, 2012 |
| Physiological response | Hormone concentrations | e.g. HPA axis: cortisol, ACTH; sympathetic system: adrenaline, noradrenaline | Mellor et al., 2000; EFSA, 2005; Le Neindre et al., 2009; Coetzee et al., 2010; Landa, 2012 |
| | Blood metabolites | e.g. glucose, lactate, free fatty acids | EFSA, 2005; Vogel et al., 2011; Landa 2012; Mota-Rojas et al., 2012 |
| | Autonomic responses | e.g. heart rate, blood pressure, respiratory rate, body temperature, dilatation of the pupil, sweating | Martoft et al., 2001; EFSA 2005; Gerritzen et al., 2007; Rodriguez et al., 2008; Svendsen et al., 2008; Dalmau et al., 2010; Le Neindre et al., 2009; McKeegan et al., 2011; Atkinson et al., 2012; Landa, 2012; Llonch et al., 2012a, 2012b, 2013 |
| Neurological response | Brain activity | e.g. EEG, ECoG | Raj et al., 1998; Martoft et al., 2001; Murrell et al., 2003; EFSA, 2005; Gibson et al., 2009; Johnson et al., 2012; Llonch et al., 2012a, 2012b, 2013 |

ACTH, adrenocorticotrophic hormone; HPA, hypothalamic–pituitary–adrenal.

Studies on alternative stunning methods should assess at least animal-based measures from behavioural, physiological and neurological response types (see Table 2) using methods previously published in peer-reviewed journals, and data should be provided at the individual animal level. In the methods section of the studies, it should be explained how and when the animal-based measures were performed and analysed. It is recommended that the animal-based measures are examined under experimental conditions - for each animal undergoing the stunning procedure - (1) during exposure of the animal to the procedure/apparatus without the actual stunning (providing a baseline result), and again (2) during exposure of the animal to the full procedure/apparatus including the stunning act. Comparison of the two observations differentiates between pain, distress and suffering due to the handling process vs pain, distress and suffering due to the stunning itself. Animals may be acclimatised or sensitised to the new procedure apparatus in the second operation, depending upon the species, the circumstances and the severity of pain, distress and suffering. In the event of a high pre-stun response, additional experiments with an adjusted experimental design should be sought to enable a more critical evaluation of the stunning itself. Making pre- and post-stunning observations on the same animal reduces the risk of selection bias. The scoring system of the measure should be clearly defined. The uniformity of high scores among the animals exposed to the stunning intervention (as evidenced by a low standard deviation of the response) is an indication of the presence of pain, distress and suffering. The greater the variance, the more plausible is the argument that it is a matter of the individual animal's response (EFSA, 2005). On the other hand, highly variable animal responses could also indicate inconsistent effects of the alternative stunning method. The various animal-based measures should be examined independently from each other and in all animals in the study population.

It is recommended that the animal-based measures are selected according to their relevance to the respective stunning intervention as shown by the available scientific knowledge of each measure's sensitivity and specificity. Detailed experimental protocols should be provided to allow assessment of the limitations of the selected animal-based measures. For instance, animals connected to measuring equipment may behave differently, the effect of the sampling procedure or the latency of a physiological response could influence the results obtained with physiological parameters, and exposure of an animal to a new environment could change its autonomic responses. Therefore, the combination of indicators to be used depends on the design of the study and the animal species.

Animal-based measures to identify pain, distress and suffering are often subjective and have a relatively low specificity and/or sensitivity (EFSA, 2005; Le Neindre et al., 2009). Therefore, two criteria/rules have to be fulfilled before a stunning method is considered not to induce pain, distress and suffering before the onset of unconsciousness and insensibility:

- Animal-based measures from at least two different response types out of three response types presented in Table 2 relevant to the intervention/species (e.g. behavioural and physiological) must be indicative of absence of pain, distress and suffering before the onset of unconsciousness/insensibility. This means that these animal-based measures should not be significantly different when the response of the animals exposed to the procedure/apparatus without the stunning act is compared with their response following exposure to the procedure/apparatus including the stunning act, provided that the pain and distress responses are not already maximum before the actual stunning.
- In general, these animal-based measures should be consistent at the level of the individual animal, depending upon the species and the coping strategies (that is, consistent with respect to their interpretation).

Finally, it is essential that the observers making the measurements have been carefully trained and that scoring systems are adapted to the species and the stunning conditions. Information on all these aspects should be provided and will be assessed by the AHAW Panel, based on scientific knowledge available at that time.

3.1.2.3. Duration of the unconsciousness and insensibility

Council Regulation (EC) No 1099/2009 states that unconsciousness/insensibility induced by stunning should last until the moment of death. Studies in a controlled environment should determine the duration of unconsciousness/insensibility using EEGs as described in section 3.1.2.1. Based upon the results obtained (e.g. the shortest time to recovery of consciousness observed minus 2 SD), the maximal stun-to-stick/-kill time interval can be defined that guarantees unequivocal loss of consciousness/sensibility until the moment of death (EFSA, 2004). The applicability of the stun-to-stick/-kill interval should then be analysed in commercial settings using indicators for recognising recovery of consciousness/sensibility that correlate with EEG measurement as established in controlled environment studies. The selection of useful indicators will also depend upon the stunning method and the species involved. It is acceptable that studies on alternative stunning methods assess only the duration of unconsciousness as this will always precede the recovery of sensibility.

The duration of unconsciousness and insensibility induced with a gas mixture depends upon factors such as the duration of exposure and the atmosphere composition (Raj et al., 1998; Martoft et al., 2003; Rodriguez et al., 2008; Dalmau et al., 2010; Llonch et al., 2012a, 2012b). A prolonged exposure to gas mixtures would be necessary to prevent recovery of consciousness and sensibility during shackling, hoisting, sticking and bleeding. Under batch or group stunning situations, the duration of unconsciousness and insensibility becomes more critical because the time interval between the end of exposure to a gas mixture and sticking would be considerably longer for the last animal in a group.

Indicators of recovery of consciousness after stunning are listed in EFSA's 2004 opinion, but their sequence depends on the stunning method. Recovery of spontaneous breathing is considered to be the earliest indicator of recovery of consciousness, which may begin as regular gagging (a brainstem reflex of forced/laboured breathing through the mouth) in a recumbent animal. These gagging movements gradually lead to resumption of rhythmic breathing. There is a lack of information on the correlation of EEGs and the sequence or the time to recovery of other indicators of consciousness, such as pupillary, palpebral or corneal reflex. However, the return of corneal reflex has been used to recognise recovery of consciousness in pigs under slaughterhouse conditions (EFSA, 2004). Animals begin to vocalise before attempting to regain posture owing to metabolic acidosis caused by the inhalation of carbon dioxide, which is expected to delay the return of skeletal muscle tone. In conclusion, it is recommended that the indicator that is most sensitive in detecting recovery be used.

Studies on alternative stunning methods should explain in detail how and when the duration of unconsciousness and insensibility is measured (protocol used, e.g. scoring system). The indicators used for determining the state (onset of unconsciousness and recovery of consciousness) should be clearly defined in the study report. It is recommended that the methods used have previously been published in peer-reviewed journals, that data are provided at the individual animal level and that actions are taken to prevent the possibility of bias as much as possible. In case of EEGs, all parameters crucial for assessment of the electroencephalography data should be specified (the electrode position on the skull or on the brain itself, the configuration of the electrode (transhemispheric or from the same hemisphere of the brain)). In order to estimate quantitative changes occurring in the EEG, the method used to derive the transformations of electroencephalography signals must be described. In addition, the indicators used to assess recognition of recovery of consciousness after stunning should be relevant to the respective stunning intervention based on the available scientific knowledge. Furthermore, the scoring system applied to categorise/classify the indicators should be clearly defined. It is essential that the observers making the measurements of the indicators have been carefully trained and that scoring systems are adapted to the species and the stunning conditions. Information on all these aspects should be provided and will be assessed by the AHAW Panel, based on the scientific knowledge available at that time.

## 3.2. Assessment of the eligibility of the submitted study

An assessment of all the eligibility criteria, defined in section 3.1, was performed, and detailed information is provided in Appendix A.

### 3.2.1. Intervention

The reporting of the intervention lacks detailed information regarding several key components of the parameters listed in Annex I of Council Regulation (EC) No 1099/2009; some essential components of the parameters are not reported at all. It is not clear if the reported $CO_2$ concentrations represent the target or the maximum $CO_2$ concentrations. There is no clarity on whether a $CO_2$ concentration gradient is or could be present or not. No information is provided on animal stocking density and how, where and when the $CO_2$ concentration was monitored. The time to reach exposure of animals to the targeted $CO_2$ concentration is not given, the exposure procedure is not clear and the (probably total) duration of the exposure cycle is provided without any specification of the exposure time at the targeted $CO_2$ concentration. No information is provided on the quality and the temperature of the gas used in the study. For these reasons, the intervention is considered to be insufficiently described.

### 3.2.2. Outcome

#### 3.2.2.1. Onset of unconsciousness and insensibility

The onset of unconsciousness and insensibility was ascertained by EEG. No signs of recognition of the onset of unconsciousness and insensibility are studied and reported. Therefore, it is considered that the onset of unconsciousness was not assessed in the study.

#### 3.2.2.2. Absence of pain, distress and suffering

The "Materials and methods" section of the submitted study states that at the time at which the animal presented, vocalisations, changes of posture and movements and autonomic responses were measured, but they were not measured during the phase of induction of unconsciousness and insensibility. In addition, neither is a detailed description given on how these measurements were performed nor what the definition of a positive result was. Furthermore, no data on vocalisations are reported in the "results" section. For these reasons, an assessment of whether pain, distress or suffering were present during the induction phase is not possible.

#### 3.2.2.3. Duration of unconsciousness

The duration of unconsciousness and insensibility was not measured by EEG. Several indicators of recovery of consciousness after stunning were examined in the submitted study. However, information is lacking regarding how these indicators were examined. Therefore, it is considered that the duration of unconsciousness was not adequately assessed in the study.

## 4. Reporting assessment

### 4.1. Identification of reporting guidelines applicable to studies on stunning methods

Studies on alternative stunning methods should analyse equivalence to the requirements prescribed in Council Regulation (EC) No 1099/2009: induction of immediate onset of unconsciousness/insensibility or absence of pain, distress and suffering until the onset of unconsciousness/insensibility and the duration of unconsciousness/insensibility until death. Several study designs could be applied. At the moment, several guidelines are available on reporting of randomised controlled and observational studies[6], but none of these guidelines can be applied directly to studies on stunning methods. The REFLECT[7] statement and the STROBE[8] statement were identified as the most suitable guidelines that could be applied to studies on stunning methods. The REFLECT statement is a reporting guideline for randomised controlled trials in animals. The STROBE statement is a reporting guideline for observational studies on humans but can be readily adapted to animals.

[6] http://www.equator-network.org/
[7] http://www.reflect-statement.org/statement/
[8] http://www.strobe-statement.org/

Collation of parameters from guidelines on which information has to be reported:

A checklist that could be applied to studies on stunning methods should be generated, taking into account the specificities related to the design of randomised controlled trials or observational studies. However, this could not be done within the time frame of this mandate. As preparatory work before generating such a checklist, all of the parameters from the checklist of the REFLECT and the STROBE statements were listed and reviewed. The parameters dealing with information that could be valuable in assessing the reporting quality of studies on stunning methods are briefly described in Table 3. The description of the parameters was modified in some cases to allow their use in the context of studies on stunning methods.

**Table 3:** Parameters used to assess the reporting quality of studies on stunning methods, per section of the study report

| Parameter | Description |
|---|---|
| *Introduction* | |
| Background and rationale | Explain the scientific background and rationale for the investigation being reported |
| Objective | Describe the specific objectives and hypotheses. Clearly state primary and secondary objectives (if applicable) |
| *Materials and methods* | |
| Study population | Give characteristics of the study population (species, breed, animal type (e.g. dairy or beef cattle), and weight) and potential confounders (health status, fasting, water deprivation, husbandry system); indicate the number of animals with missing data for each variable of interest |
| Number of animals (sample size) | How was the sample size determined and, when applicable, explanation of any interim analyses and stopping rules. Experimental/intervention units must be described and information on whether true replication was done is needed |
| Intervention | Precise details of the interventions intended for each group, how and when interventions were actually administered. In addition, specifications of the requirements for the stunning method are provided in section 3.1.1 |
| Outcome | Clearly define all primary outcomes (onset of unconsciousness/insensibility, absence of pain, distress and suffering and duration of unconsciousness/insensibility) and ancillary outcomes (e.g. heart beat, tail flicking). Report category boundaries when continuous variables were categorised. Specifications of the requirements for the assessment of unconsciousness and insensibility as well as absence of pain, distress and suffering are provided in section 3.1.2.1–3.1.2.3 |
| Bias and confounding | Describe any efforts to address potential sources of bias that are relevant to the study design and could affect the internal and external validity of the study. Concerning external validity, report methods to control for sampling bias. Was any comparison made between the reference population and animals under study? Concerning internal validity, report methods to control for selection bias, information bias and confounding. These may include random allocation, matching, blocking stratification for randomised controlled trials, and multivariable analytical methods. |
| Blinding (masking) | Specify if blinding was performed or not. If done, describe who was blinded (e.g. the data collector, the data analyst) as well as how and when it was done. If the process was different for outcomes, clarify per outcome (e.g. behaviour data was blinded but electroencephalography data were not) |
| Statistical methods | Describe all statistical methods used to summarise the data and test the hypotheses, including those used to control for confounding; include information about data transformations. Describe any methods used to examine subgroups and interactions; Explain how missing data were addressed. Guidance can be found in Lang (2013) |
| *Results* | |
| Numbers analysed | Basic information about the distribution of important confounders and effect modifiers in the each study group (age, weight, sex). If variables are |

| Parameter | Description |
|---|---|
| | continuous provide means (SD) if normally distributed, if not provide medians and interpercentile ranges, ranges, or both. Report the upper and lower boundaries of interpercentile ranges and the minimum and maximum values of ranges, numbers of study units (denominator) in each group included in each analysis and whether the analysis was by "intention-to-treat". State the results in absolute numbers when feasible (e.g. 10/20, not 50 %). |
| Outcomes and estimations | For each outcome, report a summary of results for each group (although it is recommended that data are made available at individual animal level, at least in studies performed in a controlled environment); give unadjusted estimates and their precision (e.g. 95 % confidence interval) and, if applicable, confounder-adjusted estimates and number. If the design includes non-independent observations, ensure variance components are reported. Make clear which confounders were adjusted for |
| Adverse events | Describe all important adverse events or side effects in each intervention group and report the number of adverse events in each group and indicate if they appear prior to or after unconsciousness is reached. For example, in the case of electrical stunning, high electrical resistance could cause overheating of the stunning electrodes, leading to poor stunning as well as burn marks on the skin |
| Ancillary analyses | Report the outcome of any other analyses performed, including subgroup analyses and adjusted analyses, indicating those pre-specified and those exploratory |
| *Discussion* | |
| Key results and interpretation | Summarise key results with reference to study objectives; provide a well-founded interpretation of results considering objectives and limitations, taking into account sources of potential bias or imprecision, multiplicity of analyses, results from similar studies, and other relevant evidence |
| External validation | Discuss the potential for external validation of the study results (e.g. applicability of the stunning method in slaughterhouses in different Member States) |
| *Other* | |
| Funding | Give the source of funding and the role of the funders for the submitted study. |

### 4.2. Assessment of the reporting quality based on the selected parameters

An assessment of all the reporting quality criteria, defined in section 4.1, were performed and detailed information is provided in Appendix B.

The study has several shortcomings in the description of materials and methods, as well as in the reporting and discussion of the results. No information on the study population's breed or weight, on potential confounders or the number of animals with missing data is provided. It is not explained how the sample size was determined, nor is it specified what the experimental/intervention unit is. There are no true replicates in the experiments as all of the animals were from the same source population and were not allocated to the controls and treatments in a truly random manner (meaning that they are not statistically independent units). No efforts are described to assess potential sources of bias, including confounding. There is no indication whether blinding was applied or not. The study contains statements regarding differences between groups without performing statistical analysis of the data. The number of animals included in each analysis was not specified and data were not presented in absolute numbers. There is no information on important confounders. A summary of results is provided only for some of the parameters measured. Some outcomes measures mentioned in the "Materials and methods" section are not documented in the "Results and discussion" section and vice versa. There is no indication that adverse effects were assessed and there is insufficient reference between the key results of the study and the study objective. The conclusions are reported as statements without mentioning the key result, its limitations, potential bias or other relevant evidence and without providing clear suggestions on further external validation of the results obtained. The role of the funding organisation is not specified. For these reasons, the study does not fulfil the reporting quality criteria.

## 5. Quality assessment

The methodological quality criteria focus on elements in the report that allow the assessment of the internal and external validity of the submitted study. Internal validity is reached when the study results reflect reality among the animals under study, whereas external validity is reached when the study results are reasonably generalised to the broader reference population. The main biases affecting internal validity are confounding, selection bias and information bias (Rothman, 2002). The most relevant bias affecting external validity is sampling bias. It is assumed that a high-quality study is conducted in such a way that these biases are minimised. Assessment of other parameters that might be related to the methodological quality of a study could not be considered owing to the short deadline of the mandate.

### 5.1. Specification of different types of potential biases impacting on internal validity

#### 5.1.1. Confounding

Confounding can be described as the mixing together of the effects of two or more factors. It is present when the observed measure of association between a given exposure/intervention factor and an outcome becomes biased owing to the effects of one or more extraneous factors. Confounding can be controlled in the study design, for example by matching, or during data analysis by stratification or adjusting (Dohoo et al., 2010).

#### 5.1.2. Selection bias

Selection bias arises in studies that compare two or more groups, such as an intervention versus a control. If the way in which study subjects selected to go into the different groups creates groups that differ in other characteristics, then the estimate of the effect of the intervention made will be potentially confounded. For instance, in experimental conditions, it is recommended that, for methods not inducing immediate unconsciousness, the animal-based measure for pain, distress and suffering is analysed for each animal undergoing the stunning procedure twice: first without the stunning act (gives the baseline result per animal) and afterwards with the stunning act.

#### 5.1.3. Information bias

Information bias is a collective term for misclassification bias and measurement bias and arises from incorrectly classifying or measuring the study subject's exposure, extraneous factors and/or outcome status. It can alter the magnitude and the direction of estimates of association and can affect different measures of association differently. Misclassification bias results from assigning study individuals into incorrect categories because of errors in classifying exposure, outcome or both, whereas measurement bias results from errors in measuring quantitative factors, e.g. owing to a lack of accuracy or a lack of precision (Dohoo et al., 2010).

### 5.2. Specification of different types of potential biases impacting on external validity

#### 5.2.1. Sampling bias

Where study subjects systematically differ from those to whom the results are likely to be applied, a study is described as having a sampling bias (e.g. a study may have used only heavy animals but the method is intended to be used later on animals with a broad weight range). It essentially relates to definitions of and relationships between the reference population (to which one wishes to generalise), the target population (from which one is sampling) and the eligible or study population (those eventually enrolled).

Assessment of this criterion is beyond of the scope of this mandate.

### 5.3. Quality assessment of the internal validity of the submitted study

As the study did not fulfil the eligibility criteria, the methodological quality of the study was not assessed.

## CONCLUSIONS AND RECOMMENDATIONS

### CONCLUSIONS

*Conclusions on TOR 1*

- Regarding fulfilment of the eligibility criteria it is concluded that:
    - The intervention is considered to be insufficiently described.
    - The onset and the duration of unconsciousness were not assessed in the study.
    - An assessment of whether pain, distress and suffering were present during the induction phase was not done.
- Regarding fulfilment of the reporting criteria it is concluded that:
    - The study does not fulfil the reporting criteria.
- Regarding fulfilment of the quality criteria it is concluded that:
    - As the study did not fulfil the eligibility criteria, the methodological quality of the study was not assessed.

### RECOMMENDATIONS

*Recommendations on TOR 1*

- Further studies on the use of $CO_2$ as an acceptable alternative for the stunning of rabbits are needed, which should include the eligibility criteria set out in this opinion.

*Recommendations on TOR 2*

- As a follow-up action, a document covering all stunning methods types listed in Council Regulation (EC) No 1099/2009 with detailed guidance on assessing alternative stunning methods is proposed.
- Alternative stunning methods should be first studied under controlled (laboratory) conditions to analyse the animals' responses (unconsciousness, absence of pain, distress and suffering) using the most sensitive and specific methods and to find a correlation with non-invasive parameters that can be applied during the second phase of the study in slaughterhouses. In a second step, the results obtained under controlled laboratory conditions need to be confirmed under a range of slaughterhouse conditions.
- The criteria for eligibility, reporting quality and study quality defined in this document should be applied to studies carried out under controlled (laboratory) conditions as well as to studies carried out under slaughterhouse conditions.
- Information obtained in other species can be used as an indication, but should be confirmed in the species under investigation because coping strategies, pain thresholds and tolerances are species and individual specific.
- For studies researching a new or modified stunning method, animals should be stunned without sticking to establish the duration of unconsciousness achieved by the stunning itself in proof-of-concept studies under controlled laboratory conditions.

- The criteria to be employed to ascertain the onset and the duration of unconsciousness and insensibility during controlled environment studies are profoundly suppressed or quiescent EEG and abolition of evoked electrical activity in the brain.
- In studies carried out under slaughterhouse conditions, the onset and the duration of unconsciousness and insensibility should be ascertained using the indicator that best detects unconsciousness and that has been shown to be correlated with EEGs in laboratory experiments. If different indicators are not in agreement, following on from the precautionary principle and to benefit animal welfare, the one that indicates the longest time interval between application of the stunning intervention and onset of unconsciousness should be used.
- As no specific indicator is available for pain, combinations of animal-based measures for pain, distress and suffering should be used as a proxy for pain, selected according to their relevance to the respective stunning intervention as shown by the available scientific knowledge of each measure's sensitivity and specificity.
- Studies on alternative stunning methods should assess at least animal-based measures from behavioural, physiological and neurological response types, using methods previously published in peer-reviewed journals.
- The animal-based measures should be examined under experimental conditions - for each animal undergoing the stunning procedure - first during exposure of the animal to the procedure/apparatus without the actual stunning (providing a baseline result) and again during exposure of the animal to the full procedure/apparatus including the stunning act.
- Animal-based measures from at least two different response types (behavioural, physiological, neurological responses) relevant to the intervention/species must be indicative of absence of pain, distress and suffering before the onset of unconsciousness/insensibility, and these negative animal-based measures should be strongly correlated at the level of the individual animal to be able to conclude on the absence of pain, distress and suffering.
- Data reported in studies on alternative stunning methods should be provided at the individual animal level.

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## APPENDIX A. ASSESSMENT OF THE ELIGIBILITY CRITERIA

**Table 4:** Information provided by the submitted study in relation to the intervention

| Parameter | Component[a] | Information provided in the submitted study | Fulfilment criterion (yes or no) |
|---|---|---|---|
| $CO_2$ concentration | Lowest $CO_2$ concentration[a] | Slaughterhouse A and B use 100 % and 69 % $CO_2$, respectively. However, it is not clear if these concentrations represent the minimum $CO_2$ concentrations | No |
| | Targeted $CO_2$ concentration(s) [a] | Slaughterhouse A and B use 100 % and 69 % $CO_2$, respectively. However, it is not clear if these concentrations represent the targeted $CO_2$ concentrations | No |
| | Highest $CO_2$ concentration [a] | Slaughterhouse A and B use 100 % and 69 % $CO_2$, respectively. However, it is not clear if these concentrations represent the maximum $CO_2$ concentrations | No |
| | $CO_2$ concentration gradient | There is no clarity whether a $CO_2$ concentration gradient is or could be present or not | No |
| | Animal stocking density | No information provided | No |
| | Monitoring | No information provided | No |
| Duration of intervention | Time to reach exposure of animal to targeted $CO_2$ concentration [a] | No information provided | No |
| | Total duration of targeted $CO_2$ exposure [a] | The exposure procedure is not clear. The (probably total) duration of the exposure cycle is provided (45 seconds in slaughterhouse A; between 95 and 115 seconds in slaughterhouse B) without specification of the exposure time at the targeted $CO_2$ concentration | No |
| Maximum stun-to-stick/-kill interval(s) [a,b] | | The reported time between stunning and slaughter of the last animal is $153.8 \pm 11.2$ seconds (slaughterhouse A) and $64.3 \pm 9.2$ seconds (slaughterhouse B). However, there are no electroencephalography data assessing the duration of unconsciousness/insensibility after the stunning intervention | Yes |
| Quality of the gas | $CO_2$ source | No information provided | No |
| | Gas composition of the atmosphere | No information provided | No |
| | Humidity | No information provided | No |
| Temperature of the gas | | No information provided | No |

[a]Provide information on mean or median and range and standard deviation or interquartile range of the detailed parameter.
[b]In the case of simple stunning.

**Table 5:** Information provided by the submitted study in relation to the onset of unconsciousness and insensibility

| | **Information provided in the submitted study** | **Is the induction of unconsciousness/insensibility addressed adequately? (yes, no or not possible to assess)** |
|---|---|---|
| EEG | Not applied | No |
| Indicator(s) to detect onset of unconsciousness/insensibility | No signs of recognition of a successful stun to determine the onset of unconsciousness/insensibility are analysed | No |

**Table 6:** Information provided by the submitted study in relation to animal-based measures (ABMs) associated with pain, distress and suffering during the induction of unconsciousness and insensibility

| **Response type** | **Groups of ABMs** | **Information provided in the submitted study** | **Do the ABMs suggest pain, distress and suffering? (yes, no or not possible to assess)** |
|---|---|---|---|
| Behaviour | Vocalisations | The "Materials and methods" section of the submitted study states that the time at which the animal presented vocalisations was measured. However, neither a detailed description on how and when this measurement was performed nor the definition of a positive result is given. In addition, there are no data reported in the "Results" section | No assessment possible |
| | Postures and movements | The "Materials and methods" section of the submitted study states that the time at which the animal "raised its head" or "stood up on all fours" were measured. However, neither a detailed description on how and when these measurements were performed nor the definition of a positive result is given | No assessment possible |
| | General behaviour | No information provided | No assessment possible |
| Physiological response | Hormone concentrations | No information provided | No assessment possible |
| | Blood metabolites | No information provided | No assessment possible |
| | Autonomic responses | The "Materials and methods" section indicates the measurement of the following autonomic responses: "presence/absence of heartbeat", "time of recovery of heartbeat", "time of loss of heartbeat", "time at which the animal attempted to breathe again", "time at which the animal presented a respiration rate" and "time at which the animal began blinking normally". However, neither a detailed description on how and when these measurements were performed nor the definition of a positive result is given | No assessment possible |
| Neurological response | Brain activity | No information provided | No assessment possible |

**Table 7:** Information provided by the submitted study in relation to the duration of unconsciousness and insensibility

| | **Information provided in the submitted study** | **Is the duration of unconsciousness/insensibility addressed adequately? (yes, no or not possible to assess)** |
|---|---|---|
| EEG | Not applied | No |
| Indicator(s) to detect duration of unconsciousness/ insensibility | No signs of duration of unconsciousness/insensibility are analysed. | Not possible to assess |

## APPENDIX B. REPORTING ASSESSMENT

**Table 8:** Assessment of the reporting quality parameters by the submitted study; NA: not applicable.

| Parameter | Information provided in the submitted study | Fulfilment criterion (yes or no) |
|---|---|---|
| *Introduction* | | |
| Background and rationale | The study explains that $CO_2$ stunning of rabbits is not permitted at the moment, although it was before January 2009 | Yes |
| Objective | The objective was to assess the effectiveness of $CO_2$ stunning in two commercial slaughterhouses | Yes |
| *Materials and methods* | | |
| Study population | Information on the study population (breed and weight), potential confounders and the number of animals with missing data is not provided | No |
| Number of animals (sample size) | In each slaughterhouse, 180 rabbits are assessed throughout the slaughterhouse working day. It is not explained how the sample size was determined, nor is it specified what was the experimental/intervention unit. There were no true replicates in the experiments as all of the animals were from the same source population and were not allocated to the controls and treatments in a truly random manner (meaning that they are not statistically independent units) | No |
| Intervention | See Table 4 | See Table 4 |
| Outcome | See Tables 5, 6 and 7 | See Tables 5, 6 and 7 |
| Bias and randomisation | No efforts are described to assess potential sources of bias or to control for confounding | No |
| Blinding (masking) | There is no indication of whether blinding was applied or not | No |
| Statistical methods | The study contains statements regarding differences between groups without performing statistical analysis of the data. For instance, comparisons between the two slaughterhouses were made for "recovery rates" and "percentage of rabbits that did not recover a heartbeat at all". In addition, a difference between the moment of sticking of stunned animals and the moment they recover from stunning was stated | No |
| *Results* | | |
| Numbers analysed | The number of animals included in each analysis was not specified and data were not presented in absolute numbers. There is no information on important confounders | No |
| Outcomes and estimations | A summary of results is provided for some parameters (e.g. "time at which the animal presented a respiration rate" and "time at which the animal raised its head"). There are some outcomes mentioned in the "Materials and methods" section that are not documented in the "Results and discussion" section (e.g. presence/absence of heartbeat, vocalisations) and vice versa (e.g. "percentage of animals that did/did not recover a heartbeat") | No |

| | | |
|---|---|---|
| Adverse events | There is no indication that adverse effects were assessed | No |
| Ancillary analyses | Although the "Materials and methods" section states that "the aim was not to draw comparisons", some comparisons within (e.g. time of recovery versus time of sticking) and between (e.g. recovery time) the two slaughterhouses were made | No |
| *Discussion* | | |
| Key results and interpretation | The authors draw the following conclusions in relation to $CO_2$ gas stunning :<br>"In both of the slaughterhouses studied, all the animals arrived at slaughter fully stunned"<br>" A $CO_2$ concentration of 100 % results in irreversible stunning of almost all of the animals"<br>"It is not possible to assess what happens during the stunning process within the tunnels: given the animals' aversion to $CO_2$ a more precise study of the interior of the tunnels would be necessary"<br>"Tests of times and concentrations need to be carried out in order to fully explain the effects of $CO_2$ stunning on rabbits"<br>There is insufficient reference between the key results of the study and the study objective. The conclusions are reported more as statements without mentioning the key result, its limitations, potential bias or other relevant evidence | No |
| Validation | The study states that "tests of times and concentrations need to be carried out in order to fully explain the effects of $CO_2$ stunning on rabbits", without clearly specifying the new evidence brought to this research field by the submitted study and without providing clear suggestions on further external validation of the results obtained | No |
| *Other* | | |
| Funding | INTERCUN is mentioned as the funder but its role is not specified | No |

## APPENDIX C. QUALITY ASSESSMENT

The quality assessment was not carried out, as the study did not fulfil the eligibility quality criteria.

## GLOSSARY

| | |
|---|---|
| Adverse event | Any observation in animals that is unfavourable and unintended and occurs after the intervention |
| Immediate unconsciousness | Induce immediate (e.g. in less than one second) and unequivocal loss of consciousness and sensibility |
| Insensibility | An animal can be presumed to be insensible when it does not show any reflexes or reactions to stimulus such as sound, odour, light or physical contact |
| Maximum stun-to-stick/-kill interval(s) | This is the legal parameter describing the time interval between the end of the stunning and the moment of killing by any method (e.g. sticking, neck cutting) |
| Simple stunning | Stunning methods that do not result in instantaneous death |
| Stunning | Means any intentionally induced process which causes loss of consciousness and sensibility without pain, including any process resulting in instantaneous death |
| True replicate | This means that more than one (statistically independent) experimental or observational unit was subjected to the same treatment. Each unit with the same treatment is called a replicate. True replication permits the estimation of variability within a treatment. Without estimating variability within treatments, it is impossible to do statistical inference, hence most models for statistical inference require true replication |
| Unconsciousness | This is a state of unawareness (loss of consciousness) in which there is temporary or permanent impairment of brain function and the individual is unable to respond to normal stimuli, including pain |

## ABBREVIATIONS

| | |
|---|---|
| $CO_2$ | Carbon dioxide |
| ECG | Electrocardiogram |
| ECoG | Electrocorticogram |
| EEG | Electroencephalogram |
| TOR | Term of reference |