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SCIENTIFIC OPINION

Guidance on the assessment criteria for studies evaluating the effectiveness of stunning interventions regarding animal protection at the time of killing[1]

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

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ABSTRACT

This guidance defines the assessment process and the criteria that will be applied by the Animal Health and Welfare Panel to studies on known new or modified legal stunning interventions to determine their suitability for further assessment. The criteria that need to be fulfilled are eligibility criteria, reporting quality criteria and methodological quality criteria. The eligibility criteria are based upon the legislation and previously published scientific data. They focus on the intervention and the outcomes of interest, i.e. immediate onset of unconsciousness and insensibility or absence of avoidable pain, distress and suffering until the loss of consciousness and sensibility, and duration of the unconsciousness and insensibility (until death). If a study fulfils the eligibility criteria, it will be assessed regarding a set of reporting quality criteria that are based on the REFLECT and the STROBE statements. As a final step in this first assessment phase, the methodological quality of the submitted study will be assessed. If the criteria regarding eligibility, reporting quality and methodological quality are fulfilled, a full assessment of the animal welfare implications of the proposed alternative stunning intervention, including both pre-stunning and stunning phases, and an evaluation of the quality, strength and external validity of the evidence presented would be carried out at the next level of the assessment. In the case that the criteria regarding eligibility and reporting quality and methodological quality are not fulfilled, the assessment report of the panel will highlight the shortcomings and indicate where improvements are required before the study can be assessed further. In addition to the assessment criteria, the guidance also specifies general aspects applicable to studies on stunning interventions that should be considered when studying the effectiveness of stunning interventions.

KEY WORDS

stunning, welfare, reporting guidelines

[1] On request from EFSA, Question No EFSA-Q-2013-00532, adopted on 26 November 2013.

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SUMMARY

The European Food Safety Authority (EFSA) asked the Animal Health and Welfare Panel (AHAW) to develop a guidance on the assessment criteria for studies evaluating the effectiveness of stunning interventions regarding animal protection at the time of killing.

The guidance defines the assessment process and the criteria that will be applied by the Animal Health and Welfare Panel to studies on known new or modified legal stunning interventions to determine their suitability for further assessment. The criteria that need to be fulfilled are eligibility criteria, reporting quality criteria and methodological quality criteria.

The eligibility criteria that must be fulfilled by submitted studies are based upon the legislation and previously published scientific data. They focus on the intervention and the outcomes of interest, i.e. immediate onset of unconsciousness and insensibility or absence of avoidable pain, distress and suffering until the loss of consciousness and sensibility, and duration of the unconsciousness and insensibility (until death). If a study fulfils the eligibility criteria, it will be assessed regarding a set of reporting quality criteria that are based on the REFLECT and the STROBE statements. As a final step in this first assessment phase, the methodological quality of the submitted study will be assessed.

If the criteria regarding eligibility, reporting quality and methodological quality are fulfilled, i.e. the study on the new or modified legal method provides sufficient detail regarding the intervention and the outcome to allow for a conclusion to be reached about the suitability (or lack thereof) of the intervention, a full assessment of the animal welfare implications of the proposed alternative stunning intervention, including both pre-stunning and stunning phases, and an evaluation of the quality, strength and external validity of the evidence presented would be carried out at the next level of the assessment. In the case that the criteria regarding eligibility and reporting quality and methodological quality are not fulfilled, i.e. the study does not provide sufficient detail regarding the intervention and the outcome to allow for a conclusion to be reached about its suitability (or lack thereof), the assessment report of the panel will highlight the shortcomings and indicate where improvements are required before the study can be assessed further.

In addition to the assessment criteria, the guidance also specifies general aspects applicable to studies on stunning interventions that should be considered when studying the effectiveness of stunning interventions.

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BACKGROUND AS PROVIDED BY EFSA

Council Regulation (EC) No 1099/2009 on the protection of animals at the time of killing 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". Annex I of the Regulation lists the stunning interventions and related specifications. Article 4 on stunning interventions 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". Furthermore, the methods referred to in Annex I which 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. Article 4 (2) of the Regulation allows the Commission to amend Annex I to this Regulation as to take account of 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.

Several studies assessing the efficacy of modified protocols of stunning interventions listed in Annex I or new stunning interventions have been submitted to the Commission who has requested EFSA's view on the studies, and it is likely that more studies of stunning intervention efficacy will be carried out and submitted to EFSA for assessment. Inconsistencies with reporting of intervention studies in the animal health area have been documented in the past and the lack of harmonization of designing and reporting intervention studies investigating stunning interventions' efficacy has been specifically identified as a drawback to assessing the proposed stunning interventions in previous EFSA opinions[4]. Therefore it is important to provide clear guidance to researchers on how these studies will be assessed by EFSA, i.e. what minimum eligibility criteria, reporting quality criteria and further study quality criteria need to be fulfilled for a given study so that it can be considered for assessment as a potential alternative to the stunning methods and related specifications listed in Council Regulation (EC) No 1099/2009.

TERMS OF REFERENCE AS PROVIDED BY EFSA

The European Food Safety Authority requests the Animal Health and Welfare Panel to develop a guidance document which defines the criteria against which studies evaluating the efficacy of stunning interventions regarding animal protection during stunning will be assessed.

The guidance should comprise a checklist of reporting quality criteria, eligibility criteria and further study quality criteria, accompanied with the scientific reasoning for each checklist item. It should also provide a description of the guidance development process and explain how studies will be evaluated. The guidance should cover mechanical, electrical and gas methods for the main livestock species (bovines, sheep, goats, pigs, poultry, and rabbits).

Work done on the critical appraisal of scientific studies by the Scientific Assessment Support Unit of EFSA should be considered during the preparation of the guidance document. A public consultation of the guidance document will also be made before adoption of the guidance in November 2013.

[4] Scientific Opinion on the electrical requirements for waterbath stunning equipment applicable for poultry. EFSA Journal 2012;10(6):2757 [80 pp.]. doi:10.2903/j.efsa.2012.2757

ASSESSMENT

1. Introduction

This guidance defines the assessment process and the criteria that will be applied to studies on known new or modified legal stunning interventions to determine their suitability for further assessment. The eligibility criteria are based upon the legal framework provided in Council Regulation (EC) No 1099/2009 and its Annex I. The scope of this guidance is limited to known new or modified legal stunning interventions and back-up stunning interventions used at slaughter; it does not cover interventions that are exclusively used for depopulation or for other forms of on-farm slaughter or killing (e.g. emergency killing interventions). For consistency with the legislation, the eligibility criteria defined in this guidance specify only the minimum requirements. In addition to eligibility criteria, the guidance also defines reporting quality and methodological quality criteria. Although detailed eligibility criteria for any possible intervention cannot be provided in this document, the intervention has to be reported in sufficient detail and the outcome eligibility criteria defined in this document must be fulfilled.

The criteria defined in this document apply only to the assessment of the stunning procedure itself and do not take into account pre-stunning phases and restraining methods applied, i.e. handling of the animal until its presentation for stunning. At this first level of assessment, only the documents that have been submitted by the European Commission for review by EFSA will be assessed. The outcome of the assessment outlined in this guidance applies only to whether the assessed study is adequate to be submitted to the next level of the assessment process: a full assessment of the animal welfare implications of the new or modified legal stunning intervention being considered, including both pre-stunning and stunning phases, and an evaluation of the quality, strength and external validity of the evidence presented (Figure 1) would be carried out at the next level of the assessment.

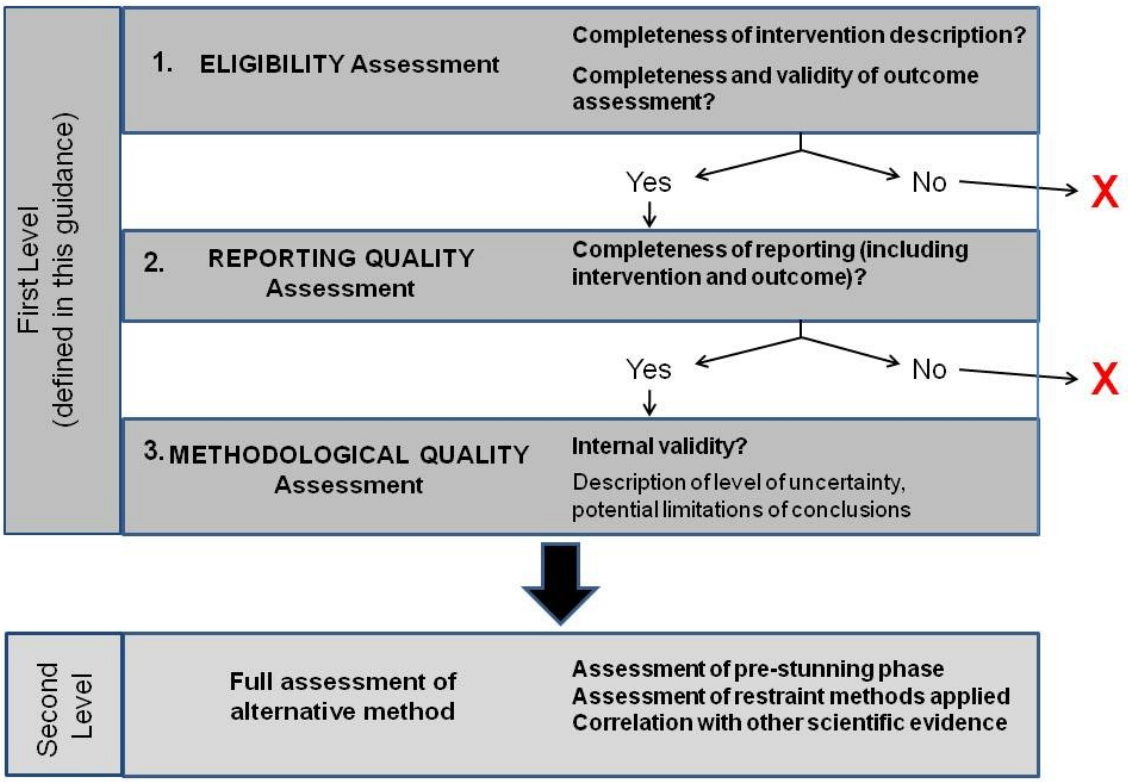

Figure 1: The approach to the assessment of studies evaluating alternative stunning interventions (X = exclusion of study from further assessment; in this case a description of the shortcomings and indications of improvements that are required before the study can be assessed further will be provided)

General aspects applicable to studies on stunning interventions

A number of general aspects that should be considered when studying the effectiveness of stunning interventions are outlined below.

Research evaluating stunning interventions requires, as a first step, well-controlled studies conducted under laboratory conditions in order to characterize the animals' responses (unconsciousness, absence of pain) using the most sensitive and specific methods available (e.g. electroencephalography (EEG), blood samples) and to establish the correlations between these measurements and non-invasive animal based measures that can be applied in slaughterhouses (Figure 2). The second step, studies under slaughterhouse conditions (Figure 2, II), is intended to assess whether the results obtained in the laboratory studies can also be achieved in a slaughterhouse context. The eligibility criteria will be applied to both steps of the research on stunning interventions. Information obtained on other species can be used as an indication, but such species should be phylogenetically related or comparable to the species under investigation because coping strategies, pain thresholds and tolerances are species and individual specific.

Type	Conditions	Elements of research recommended
I. Proof of concept	Study under controlled laboratory conditions	A. Comprehensive record of stunning intervention and key parameters B. Assessment of onset and duration of unconsciousness by EEG or ECoG C. Assessment of absence of pain, distress and suffering using behavioural **and** either physiological **or** neurological animal-based measures D. Comprehensive record of outcome assessment E. Stunning without sticking to establish duration of unconsciousness achievable with simple stunning intervention
II. Ground truthing	Study under slaughterhouse conditons	F. Comprehensive record of stunning intervention and key parameters G. Assessment of onset and duration of unconsciousness using animal-based measures H. Assessment of absence of pain, distress and suffering using behavioural **and** either physiological **or** neurological animal-based measures I. Comprehensive record of outcome assessment J. Assessment of absence of pain, distress and sufferring during restraint/pre-stunning if it deviates from conventional methods and/or is potentially painful

Figure 2: Recommended approach for research on stunning interventions

It is important to note that in controlled environment studies EEG or electrocorticography (ECoG) should be used to demonstrate the effectiveness of a given stunning intervention (Figure 2, B). Indicators for recognising a successful stun should be applied in slaughterhouse settings, after their correlation with EEGs findings has been demonstrated in controlled environment studies (Figure 2, G).

For studies researching a new or modified legal simple stunning intervention, animals should be stunned without exsanguination (bleeding out by neck cutting (severing the carotid arteries) or sticking (severing the brachiocephalic trunk)) to establish the duration of unconsciousness achieved by the

stunning itself in proof-of-concept studies under controlled laboratory conditions (Figure 2, E). The experimental protocol must apply humane endpoints as specified in various international (e.g. http://www.animalethics.org.au/legislation/international) or European guidelines on the ethical use of animals in research (e.g. Directive 2010/63/EU[5]). In accordance with these guidelines, in the case of research on the long-term adverse effects of the stun experienced, the animals should be re-stunned and euthanized as soon as they regain consciousness. The research reported should cite the granting body and reference number for animal ethics approvals associated with the work within the methods of the document.
Studies on stunning interventions should explain, in detail, how and when the onset of unconsciousness and insensibility is measured (Figure 2, B, C, G, H). It is required that the methodology used in the determination of the onset and the duration of unconsciousness has previously been accepted in appropriate internationally recognised and stringently peer-reviewed journals, that data are provided at the individual animal level and that actions are taken to prevent the possibility of any kind of bias (see section 5). In the case of EEG (or ECoG), all parameters that are crucial to the assessment of the EEG data should be specified (e.g. the EEG recording electrode position on the skull or on the brain itself, the configuration of the electrode (transhemispheric or from the same hemisphere of the brain), the background noise filtration method employed in the data acquisition and analysis). In order to estimate quantitative changes apparent on the EEG (or ECoG), the method used to derive the transformations of EEG data must be described (Figure 2, B). In addition, the indicators used to assess recognition of unconsciousness should be relevant to that stunning intervention, based on the available scientific knowledge of each indicator's sensitivity and specificity.

In the methods section of the studies, it should be clearly explained how and when the animal-based measures were recorded and analysed (Figure 2, G, H, I). Furthermore, data should be provided at the individual animal level. Detailed experimental protocols should be provided to allow assessment of the limitations of the selected animal-based measures. For example, 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 can change its behavioural, physiological or autonomic responses. Therefore, selecting the combination of indicators to be used depends upon the design of the study and the test species. Accreditation to internationally recognised methods (e.g. International Standards Organisation) of data recording, acquisition and analysis should be clearly stated in the studies to facilitate effective assessments.

The scoring system applied to categorise/classify the animal-based measures should be clearly defined (Figure 2, G, H, I). It is essential that the observers making the measurements are carefully trained and that scoring systems are adapted to the species and the stunning conditions. If applicable, the observers assessing the outcomes should be blinded to the experimental groups (e.g. control and treatment).

For any intervention that does not lead to an immediate onset of unconsciousness and insensibility, the time to loss of consciousness from the beginning of the application of the stunning intervention, and signs of pain, distress and suffering until the onset of unconsciousness should be recorded in all animals and reported as individual animal-level data or mean or median and range and standard deviation or interquartile range (Figure 2, B, C, G, H).

It is recommended that the animal-based measures for pain, distress and suffering are examined under controlled laboratory conditions - for each animal undergoing the stunning procedure - during exposure of the animal to the procedure/apparatus without the actual stunning ("sham operation", providing a baseline result) and again during exposure of the animal to the full procedure/apparatus, including stunning (Figure 2, C, H). Comparison of the two observations differentiates between pain, distress and suffering due to the handling process versus pain, distress and suffering due to the

[5] 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. OJ L 276, 20,10,2010, p. 33-79.

stunning intervention itself (see section 3.2.2.). In the absence of avoidable pain, distress and suffering caused by the application of a stunning intervention, the response of animals exposed to the procedure/apparatus without the application of stunning (control or sham operation) should not be significantly different from the response of the animals exposed to the procedure/apparatus with stunning (treatment). It is, however, essential that the control/sham operation itself has not resulted in peak response levels in animals such that no further increases in response could be expected, within the physiological limits of the species/animal under investigation, owing to the additional avoidable pain, distress and suffering caused by the stunning intervention.

The assessment of pre-stunning handling associated with the proposed stunning intervention is beyond the scope of this guidance. However, if the pre-stunning handling of animals during the proposed intervention deviates considerably from that associated with the conventional process - and/or if it is possibly a source of pain, distress or suffering - then it is the responsibility of the researchers to provide scientific evidence that allows for an assessment of animal welfare (Figure 2, J). That assessment will be undertaken - at the next step in the process - following the criteria for assessing the absence of pain, distress and suffering specified in this guidance.

Information on all the preceding should be provided and will be assessed by the EFSA Panel on Animal Health and Welfare (AHAW), based upon scientific knowledge available at that time.

2. Approach

Building on previous EFSA scientific opinions,[6,7] the criteria regarding eligibility, reporting quality and methodological quality,,against which studies evaluating the effectiveness of stunning interventions regarding animal protection during killing will be assessed, are defined in this guidance document.

2.1. 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 states 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 of the Regulation 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 of the Regulation cause immediate onset of unconsciousness, with the exception of modified atmosphere methods.

The eligibility criteria that must be fulfilled by submitted studies are based upon the legislation and focus on the intervention and the outcome:

For the intervention:

> The key parameters described in the legislation as well as any others provided by experts on stunning interventions.

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;

[6] EFSA Journal 2013;11(6):3249, 40 pp.
[7] EFSA Journal 2013;11(6):3250, 33 pp.

AND

C. Duration of the unconsciousness and insensibility (until death).

To allow assessment of new or modified legal stunning interventions, the minimum criteria that fully define and characterise the stunning intervention were defined using previously published scientific data. Regarding measures of the outcome, the onset and duration of unconsciousness and insensibility should be recorded and reported. If the onset of unconsciousness and insensibility achieved by the stunning intervention is not immediate, then the absence of pain, distress and suffering until the loss of consciousness and sensibility must also be recorded and reported.

2.2. Reporting quality criteria

Inconsistencies in the reporting of scientific studies which make it difficult to assess and compare them have been identified in human and veterinary medicine (Schulz et al., 1994; Sargeant and O'Connor, 2013). Therefore, reporting guidelines designed to increase the transparency and comparability of conducting and reporting such scientific studies have been developed.[8] As these guidelines were not developed for application to studies on stunning interventions, parameters relevant to studies on stunning interventions were identified from the two most closely related guidelines, the REFLECT and the STROBE statements.[9,10] These parameters will be used as the basis for assessing the reporting quality of submitted studies. The decision over whether the overall reporting quality is sufficient will be based upon the judgment of the panel experts engaged to assess the submitted study.

2.3. Methodological quality criteria

The methodological quality of the submitted study will be assessed only if the eligibility and reporting quality criteria are met (Figure 1). In that case, the information provided in the study will be used to identify and assess possible biases (e.g. selection, attrition and performance bias) that might affect the study's internal validity. The assessment of methodological quality will be based upon the judgement of the panel experts engaged to assess the submitted study. It will be reported as a qualitative narrative, in the style of a peer review of a manuscript submitted for publication in a scholarly journal, and will describe the level of uncertainty surrounding the evidence presented in the study and the potential limitations of the conclusions in order to inform the next level of assessment.

Appraisal of a study's external validity (i.e. its applicability beyond the study population) requires that its results be assessed in the context of related studies. Since this guidance is applicable only to individual studies, assessing the external validity of those studies exceeds its mandate.

2.4. Possible outcomes of the assessment process outlined in this guidance

When all criteria regarding eligibility, reporting quality and methodological quality have been assessed, an overall conclusion will be provided. There are two possible outcomes of the assessment described in this guidance document:

- The criteria regarding eligibility, reporting quality and methodological quality are fulfilled.

This means that the study on the new or modified legal intervention provides sufficient detail regarding the intervention and the outcome to allow for a conclusion to be reached about the suitability (or lack thereof) of the intervention. In that case, a full assessment of the animal welfare implications of the proposed alternative stunning intervention, including both pre-stunning and stunning phases, and an evaluation of the quality, strength and external validity of the evidence presented would be carried out at the next level of the assessment (Figure 1).

[8] http://www.equator-network.org/
[9] http://www.reflect-statement.org/statement/
[10] http://www.strobe-statement.org/

- The criteria regarding eligibility, reporting quality or methodological quality are not fulfilled.

This means that the study does not provide sufficient detail regarding the intervention and the outcome to allow for a conclusion to be reached about its suitability (or lack thereof). In that case, the assessment report would highlight the shortcomings and indicate where improvements are required before the study can be assessed further.

3. Eligibility criteria

3.1. Intervention

For studies researching new or modified stunning interventions, it needs to be demonstrated whether or not the intervention results in immediate unconsciousness and whether or not the stun is reversible (see section 3.2). In addition, the chances and the potential causes of failure need to be characterised.

3.1.1. Mechanical stunning interventions

3.1.1.1. Penetrative captive bolt

Penetrative captive bolt stunning is permitted in all species when the technical criteria described in Annex I of Council Regulation (EC) No 1099/2009 are fulfilled. When using the penetrative captive bolt, the legislative requirements prescribe severe and irreversible damage of the brain induced by the impact and penetration of the captive bolt.

The legislation states that the key parameters are shooting position and direction of the shot; appropriate velocity, length (after exiting the muzzle) and diameter of the bolt according to animal size and species; and maximum stun-to-stick/kill interval(s). Studies analysing a modification of a currently permitted method need to describe all of the legal key parameters. Some parameters are divided into several detailed components to ensure a comprehensive description of the applied stunning intervention (Table 1). In addition, the throughput rate should be specified where appropriate (e.g. studies under slaughterhouse conditions).

Table 1: Parameters to be provided when applying a mechanical stunning intervention based on penetrative captive bolt stunning, based on Annex I of Council Regulation (EC) No 1099/2009 and on further details of requirements as determined by the EFSA ad-hoc expert working group

Parameter	**Component**	**Description** (all specifications should be in internationally recognised units)
Position and direction of the shot	Restraining system	Describe how the animal and its head are restrained during the stunning procedure. Provide all information relevant to describing the restraining system used to facilitate accurate shooting.
	Position of captive bolt gun	Specify the topographical / anatomical position of the gun on the head (e.g. on the frontal bone), direction (directed towards the mouth or throat) and angle of firing (e.g. perpendicular to the frontal bone). Provide the distance between the muzzle of the gun and the skull surface at the intended bolt penetration site.
	Bolt penetration site	Specify the anatomical position of the penetration site - indicating the presence of any topographical features of the study population, such as the presence of horns or thick ridges on the skull, which may influence the selection of the shooting position, including any deviation from the intended penetration site.
Appropriate velocity, bolt length and diameter of the	Captive bolt gun characteristics	Provide details of the device including whether it is pneumatic or cartridge driven or spring operated, trigger operated or contact firing, and whether it uses a recessed bolt or a non-recessed bolt (i.e. the bolt is level with the

Parameter	Component	Description (all specifications should be in internationally recognised units)
bolt according to animal size and species		end of the gun muzzle). Provide details of the calibration method used for the assessment of the impact of the captive bolt
	Cartridge or compressed air specifications	The cartridges used are required to be appropriate for each species, based on the manufacturer's recommendations. Specify the cartridge calibre / grain / explosive content or the air pressure
	Bolt dimensions, mass and velocity	Specify the full bolt length (i.e. the length of the entire bolt) and its exit length (i.e. the length protruding from the barrel after firing, which is equal to the penetrating length) and the bolt diameter, bolt mass and bolt velocity at the time of impacting the skull. Describe the shape of the tip of the bolt (e.g. mushroom shaped, flat, curved with sharp edges)
	Type (e.g. beef or dairy cattle) and size of animal	The characteristics of the chosen captive bolt gun will vary depending on the type of animal it is used for. Therefore, provide details on the species, breed, age and weight of the animals in the study population.
	Equipment maintenance, cleaning and storage conditions	It is necessary that captive bolt guns are frequently cleaned and maintained in good working condition. The guns are fitted with several buffer rubber rings which regulate bolt penetration depth and recoil of the bolt into the barrel, and care should be taken to ensure that these rubber rings are maintained in good working condition. It is necessary to clean the expansion chamber frequently and in accordance with the manufacturer's instructions, to maximise the performance of the gun. If there is a build-up of carbon inside the gun, the bolt will fail to return fully to the primed position, which reduces the power of the next shot and the effectiveness of the stun. Stunner cartridges need to be stored in a dry and safe place. Therefore, provide details on the storage conditions, and the frequency of and time intervals between consecutive maintenance and cleaning of the equipment. Where manufacturer maintenance instructions are available, provide the details and how they were implemented
Maximum stun to stick/kill interval(s) [(a)]		Describe the maximum stun-to-stick/kill interval and the exsanguination method (blood vessels cut) that have been applied to guarantee non-recovery of consciousness and sensibility of the stunned animal until the onset of death (except for proof-of-concept studies where the duration of unconsciousness must be determined without sticking, or if the stunning intervention is proven to be irreversible). If the stunning intervention is shown to be reversible and pithing is applied as a killing method, the procedure should be described

[(a):] Provide information on mean or median and range and standard deviation or interquartile range of the detailed parameter.

3.1.1.2. Non-penetrative captive bolt

The non-penetrative captive bolt intervention of stunning is permitted for use in ruminants (of less than 10 kg live weight), poultry, rabbits and hares when the technical criteria described in Annex I of Council Regulation (EC) No 1099/2009 are fulfilled. When using the non-penetrative captive bolt intervention, the legislative requirements prescribe severe damage of the brain by the impact of a captive bolt without penetration and, in addition, fracture of the skull should be avoided.

The legislation states that the key parameters are shooting position and direction of the shot; appropriate velocity, diameter and shape of the bolt according to animal size and species; strength of the cartridge used; and maximum stun-to-stick/kill interval(s). Studies analysing a modification of a currently permitted method need to describe all of the legal key parameters. Some parameters are divided into several detailed components to ensure a comprehensive description of the applied stunning intervention (Table 2). In addition, the throughput rate should be specified where appropriate (e.g. field studies).

Table 2: Parameters to be provided when applying a mechanical stunning intervention based on non-penetrative captive bolt stunning, based on Annex I of Council Regulation (EC) No 1099/2009 and on further details of requirements as determined by the EFSA ad-hoc expert working group

Parameter	**Component**	**Description** (all specifications should be in internationally recognised units)
Position and direction of the shot	Restraining system	Describe how the animal and its head are restrained. Indicate how the head is restrained during the stunning procedure. Provide all information relevant to describing the restraining system used to facilitate accurate shooting
	Position of captive bolt gun	Specify the topographical / anatomical position of the gun on the head (e.g. on the frontal bone), direction (directed towards the mouth or throat) and angle of firing (e.g. perpendicular to the frontal bone). Provide the distance between the muzzle of the gun and the skull surface at the intended bolt penetration site
	Bolt impact site	Specify the anatomical position of the impact site indicating the presence of any topographical features of the study population, such as the presence of horns or thick ridges on the skull, which may influence the selection of the shooting position
Appropriate velocity, diameter and shape of the bolt according to animal size and species	Captive bolt gun characteristics	Provide details of the device, including whether it is pneumatic, cartridge driven, spring or trigger operated, or contact firing, and whether it is a recessed bolt or non-recessed bolt (i.e, bolt is level with the end of the gun muzzle). Provide details of the calibration method used for the assessment of the impact of the captive bolt
	Cartridge or compressed air specifications	Specify the strength of the cartridge (see below) or the air pressure
	Bolt dimensions, mass and velocity	Specify the bolt diameter (including the diameter of the bolt head), size, shape, mass and velocity at the time of impacting the skull
	Type and size of animal (e.g. beef or dairy cattle) and size of animal	The characteristics of the chosen captive bolt gun will vary depending on the type of animal it is used for. Therefore, provide details on the species, breed, age and weight of the animals in the study population
	Equipment maintenance, cleaning and storage conditions	It is necessary that captive bolt guns are frequently cleaned and maintained in good working condition. The guns are fitted with several buffer rubber rings which regulate bolt impact and recoil, and care should be taken to ensure that these rubber rings are maintained in good working condition. It is necessary to clean the expansion chamber frequently and in accordance with the manufacturer's instructions, to maximise the performance of the gun. If there is a build-up of carbon inside the gun the bolt fails to return fully to the primed position, which reduces the power of the next shot and the effectiveness of the stun. Stunner cartridges need to be stored in a dry and safe place. Therefore, provide details on the storage conditions, and the frequency of and time intervals between consecutive maintenance and cleaning of the

Parameter	Component	Description (all specifications should be in internationally recognised units)
		equipment. Where manufacturer maintenance instructions are available, provide the details and how they were implemented
Strength of the cartridge used		The cartridges used are required to be appropriate for each species, based on manufacturer's recommendations. Specify the cartridge strength described by calibre/ grain/ explosive content, using internationally recognised units
Maximum stun to stick/kill interval(s) [(a)]		Describe the maximum stun-to-stick/kill interval and the exsanguination method (blood vessels cut) that have been applied to guarantee non-recovery of consciousness and sensibility of the stunned animal until the moment of death (except for proof-of-concept studies where the duration of unconsciousness must be determined without sticking)

[(a)] Provide information on mean or median and range and standard deviation or interquartile range of the detailed parameter.

3.1.2. Electrical stunning interventions

3.1.2.1. Head-only and head-to-body stunning

Head-only and head-to-body electrical stunning are permitted in all species when the technical criteria described in Annex I of Council Regulation (EC) No 1099/2009 are fulfilled. When using head-only electrical stunning, the legislative requirements prescribe that the brain should be exposed to a current generating generalised epileptiform activity in the EEG; the electrodes should span the brain of the animal and be adapted to its size. For head-to-body electrical stunning, the electrodes should span the brain and heart leading to generalised epileptiform activity in the EEG and fibrillation or stopping of the heart. Head-to-body electrical stunning can be applied using one or multiple current cycles provided that the animals have been rendered unconscious by the first cycle. The legislation states that the key parameters to be provided are minimum current, minimum voltage, maximum frequency, minimum time of exposure, maximum stun-to-stick/kill interval(s), frequency of calibration of the equipment, optimisation of the current flow, prevention of electrical shocks before stunning and position and contact surface area of electrodes. Studies analysing a modification of a currently permitted method need to describe all of the legal key parameters. Some parameters are divided into several detailed components to ensure a comprehensive description of the applied stunning intervention (Table 3). In addition, the throughput rate should be specified where appropriate (e.g. field studies).

Table 3: Parameters to be provided when applying a stunning intervention based on head-only and head-to-body electrical stunning, based on Annex I of Council Regulation (EC) No 1099/2009 and on further details of requirements as determined by the EFSA ad-hoc expert working group

Parameter	Component	Description (all specifications should be in internationally recognised units)
Minimum current (A or mA)	Current type	The electrical current used to stun animals can be sine or square wave alternating current (bipolar or biphasic) or pulsed direct current (monopolar or monophasic). Define the current type used
	Waveform	The waveform of the current used for stunning animals varies widely and includes clipped or rectified sine or square waves. The proportion of clipping also varies widely. Define the waveform used, including the proportion of clippings, and report the mark-space ratio when pulsed direct current is used. If multiple frequencies and waveforms are used, describe them
	Minimum current [(a)]	Specify the minimum current (A or mA) to which animals are exposed. Explain how this value was obtained. Normally, when using sine wave alternating current the minimum current will be expressed as the root mean square current. When a pulsed direct current is used, the minimum will be expressed as the average current. Describe how the minimum

Parameter	Component	Description (all specifications should be in internationally recognised units)
		current was calculated. In a multiple-cycle method of a head-to-body stunning system, details should be provided for each cycle
	Latency [(a)]	Specify how soon the minimum current was reached after the intervention was applied to the animal. In a multiple-cycle method of a head-to-body stunning system, details should be provided for each cycle
Minimum voltage (V)	Exposed minimum voltage (V) [(a)]	Specify the minimum voltage (V) to which animals are exposed. Explain how this value was measured (e.g. peak voltage, peak-peak voltage, root mean square voltage or average voltage). Root mean square voltage is the recommended description of the exposed minimum voltage. In a multiple-cycle method of a head-to-body stunning system, details should be provided for each cycle
	Delivered minimum voltage (V) [(a)]	According to Ohm's law, the amount of voltage required to deliver 1 A will depend upon the electrical resistance in the pathways, which in turn is determined by several factors. Describe how the stunning equipment was set up to deliver the minimum current level to the animal. In a multiple-cycle method of a head-to-body stunning system, details should be provided for each cycle. Describe how the pre-set constant current was applied (e.g. variable voltage/constant current stunner)
Maximum frequency (Hz)	Maximum frequency (Hz)	If applicable, define the maximum frequency (Hz) applied to the animal. In a multiple-cycle method of head-to-body stunning system, details should be provided for each cycle.
	Minimum frequency (Hz)	If applicable, define the minimum frequency (Hz) applied to the animal. In a multiple-cycle method of a head-to-body stunning system, details should be provided for each cycle
Minimum time exposure [(a)]		Define the minimum duration of electrical exposure applied to the animals. In a multiple-cycle method of a head-to-body stunning system, details should be provided for each cycle
Maximum stun-to-stick-/kill interval(s) [(a),(b)]		Describe the maximum stun-to-stick/kill interval and the exsanguination method (blood vessels cut) that have been applied to guarantee unconsciousness and insensibility of the stunned animal until the moment of death (except for proof-of-concept studies where the duration of unconsciousness must be determined without sticking)
Frequency of calibration of the equipment		Provide information on the method used for, and the time intervals between, consecutive calibrations of the equipment
Optimisation of the current flow	Electrode characteristics	The form of the stunning tongs or electrodes and the material are important to overcome the resistance in the pathway. Provide a description of the electrode (form/shape, presence and description of spikes (depth of penetration), wetting)
	Electrode appearance	The condition (e.g. corroded) and cleanliness (fat and wool cover, carbonisation of dirt) of stunning electrodes contribute to the electrical resistance. Electrodes should be cleaned regularly using a wire brush to prevent build-up of materials. Describe the appearance of the electrodes as well as the method used to clean them between use on individual animals.
	Animal restraining	Describe how animals are restrained
Prevention of electrical shocks before stunning		Explain how the animals are protected from inadvertent, unintentional electrical shocks immediately before the stunning intervention is initiated. For instance, the stunning electrodes could be placed firmly without slipping and held with uniform pressure throughout the duration of stunning to ensure that the current flows uninterruptedly
Position and contact surface area of electrodes	Position of the electrodes	Specify the topographical anatomical position where the electrodes are attached to the animal and the method to hold electrodes in place during the intervention. Placement and application of electrodes should be described and validated
	Type of electrode	Provide information on the type of electrodes used (e.g. tong, wand, etc.)

Parameter	Component	Description (all specifications should be in internationally recognised units)
	Animal skin condition	The amount of wool/hair/feathers covering the head at the site of stunning electrode position is critical as the electrical resistance increases with the increasing amount of wool, etc. Provide a description of the study population in relation to the wool/hair/feather cover, and cleanliness of the coat (e.g. clipped or not, breed, wet/dry head)

(a) Provide information on mean or median and range and standard deviation or interquartile range.
(b) In case of simple stunning.

3.1.2.2. Electrical waterbath stunning

Electrical waterbath stunning is permitted for use in poultry when the technical criteria described in Annex I of Council Regulation (EC) No 1099/2009 are fulfilled. The legislative requirements prescribe that the entire body should be exposed to a current generating generalised epileptiform activity in the EEG and possibly fibrillation or stopping of the heart. A study researching modified electrical parameters of waterbath stunning should record the intervention applied to individual animals.

The legislation states that the key parameters are minimum current, minimum voltage, maximum frequency, frequency of calibration of the equipment, prevention of electrical shocks before stunning, minimising pain at shackling, optimisation of the current flow, maximum shackle duration before the waterbath, minimum time of exposure for each animal, immersion of the birds up to the base of the wings and maximum stun-to-stick/kill interval(s) for frequency(ies) over 50 Hz. Studies analysing a modification of a currently permitted method need to describe all of the legal key parameters. Some parameters are divided into several detailed components to ensure a comprehensive description of the applied stunning intervention (Table 4). In addition, the throughput rate should be specified where appropriate (e.g. field studies).

Table 4: Parameters to be provided when applying a stunning intervention based on electrical waterbath stunning, based on Annex I of Council Regulation (EC) No 1099/2009 and on further details of requirements as determined by the EFSA ad-hoc expert working group

Parameter	Component	Description (all specifications should be in internationally recognised units)
Minimum current (A or mA)	Current type	The electrical current used to stun birds can be sine wave or square wave alternating current (bipolar or biphasic) or pulsed direct current (monopolar or monophasic). Define the current type used
	Waveform	The waveform of the current used for stunning birds varies widely and includes clipped or rectified sine or square waves. The proportion of clipping also varies widely. Define the waveform used including the proportion of clippings and report the mark-space ratio, when pulsed direct current (DC) is used
	Minimum current (a)	Specify the minimum current (A or mA) to which birds are exposed. Explain how this value was obtained. Normally, when using sine wave alternating current, the minimum current will be expressed as the root mean square current. When a pulsed direct current is used, the minimum will be expressed as the average current. Describe how the minimum current was calculated

Parameter	**Component**		**Description** (all specifications should be in internationally recognised units)
Minimum voltage (V)	Exposed minimum voltage (V) [(a)]		Specify the minimum voltage (V) to which birds are exposed. Explain how this value was measured (e.g. peak voltage, peak-peak voltage, root mean square voltage or average voltage). Root mean square voltage is the recommended description of the exposed minimum voltage when using sine wave alternating current. When a pulsed direct current is used, the minimum will be expressed as the average voltage. Describe how the minimum voltage was calculated
	Delivered minimum voltage (V) [(a)]		According to Ohm's law, the amount of voltage required to deliver a pre-set (chosen) current will depend upon the electrical resistance in the pathways, which in turn is determined by several factors. Describe how the stunning equipment was setup to deliver the minimum current level to each bird
Maximum frequency (Hz)	Maximum frequency (Hz)		Define the maximum frequency (Hz) applied to the birds when a combination(s) of different frequencies is used
	Minimum frequency (Hz)		Define the minimum frequency (Hz) applied to the birds when a combination(s) of different frequencies is used
Frequency of calibration of the equipment			Provide information on the method used for, and the time intervals between, consecutive calibrations of the equipment
Prevention of electrical shocks before stunning			Explain how the birds are protected from inadvertent, unintentional electrical shocks immediately before the stunning intervention is initiated. For example, there should be sufficient delay between shackling and stunning to provide time for the birds to stop wing flapping, as wing flapping predisposes poultry to receiving pre-stun electric shocks. Other measures are also known to reduce or stop wing flapping
Minimising pain at shackling			The size and shape of the shackles should be appropriate to the size of the legs of poultry, such that secure electrical contact is provided without causing avoidable pain. The method of shackling should be such that it minimises the potential for joint dislocation and fractures through careful handling and good shackle design. Describe the measures taken to minimise pain during shackling of the birds
Optimisation of the current flow	Shackles	Wetting the leg-shackle contact area	Wetting shackles prior to hanging live birds reduces electrical resistance and improves contact between the legs and the shackle. Specify if this procedure was performed
		Contact with earth bar	There should be secure and uninterrupted contact between the shackle and the earth bar. Explain how contact between the shackle and the earth bar was ensured during the stunning procedure

Parameter	Component	Description (all specifications should be in internationally recognised units)
	Waterbath and electrode characteristics	The electrodes in waterbath stunners should extend to the full length of the waterbath. Provide a description of the dimensions of the waterbath and electrodes
	Water conductivity	Food-grade salt, at least 0.1 % weight/volume, should be added to the fresh water bath to improve electrical conductivity, where appropriate. Specify if this procedure was performed and the salt concentration applied
	Electricity source characteristics	The variation in the amount of current delivered to each bird can be overcome by the use of a constant current stunner that would ensure delivery of a pre-set current to the birds in a waterbath. Specify whether the waterbath stunners are supplied with a constant current or a constant voltage source
	Electrical resistance/impedance	According to Ohm's law, each bird in a multiple-bird waterbath will receive a current inversely proportional to the electrical resistance or impedance in the pathway. Electrical impedance will vary between different species/sizes of birds and the degree of leg keratinisation. Provide details on the species, breed, age, sex, weight and cleanliness of the birds
Maximum shackle duration before the waterbath [(b)]		Poultry should be hung on the shackle line for as short a time as possible. The maximum time interval between shackling and stunning should not exceed the legally prescribed duration of one minute for chickens and two minutes for turkeys, ducks and geese. Specify the time interval between shackling of the bird and stunning
Minimum time of exposure for each bird [(a)]		State the number of birds in the waterbath at any one time and the minimum duration of exposure to the electrical current applied to each bird
Immersion of the birds up to the base of the wings		The height of the waterbath should be adjusted according to the size of the poultry, to ensure at least complete immersion of the birds' heads in the water or, preferably, immersion up to the base of the wings. Specify the immersion depth and describe measures taken to minimise variation in depth of immersion
Maximum stun-to-stick/kill interval(s) for frequency over 50 Hz [(a), (b)]		Describe the maximum stun-to-stick/kill interval and the exsanguination method (blood vessels cut) that have been applied to guarantee unconsciousness and insensibility of the stunned bird until the moment of death (except for proof-of-concept studies where the duration of unconsciousness must be determined without sticking)

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

3.1.3. Modified atmosphere stunning interventions

3.1.3.1. Carbon dioxide (CO_2) at high concentrations and CO_2 in two phases

Exposure to 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 intervention may be used in pits, tunnels, containers or previously sealed buildings. The legislation states that the key parameters to be provided are CO_2 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.

The use of CO_2 in two phases is allowed only for poultry for slaughter, depopulation and other situations. The intervention consists of a successive exposure of conscious animals to a gas mixture containing up to 40% by volume of CO_2 in the air, followed, when animals have lost consciousness, by a higher concentration of CO_2. The key parameters specified by the legislation are CO_2 concentration, duration of exposure, quality of the gas and temperature of the gas. Currently, also multi-stage CO_2 systems are being used in EU poultry slaughterhouses and further developments may be made in this area.

Studies analysing (1) a modification of a currently permitted method or (2) the application of high CO_2 concentrations or of CO_2 in two phases 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 intervention (Table 5). The animals should also be exposed to the maximum concentration as soon as possible to achieve a rapid induction of unconsciousness.

Table 5: Parameters to be provided when applying a stunning intervention based on high CO_2 concentrations or CO_2 in two/multiple phases, based on Annex I of Council Regulation (EC) No 1099/2009 and on further details of requirements as determined by the EFSA ad-hoc expert working group

Parameter	**Component**	**Description** (all specifications should be in internationally recognised units)
CO_2 concentration	Initial CO_2 concentration [(a)]	Specify the initial CO_2 concentration to which animals are exposed at the initiation of 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
	Final 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 not likely 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, depending on the system). In cases where gas is added to a chamber containing animals, specify the gas flow rate (l/min) and the chamber volume (l) If animals are exposed to CO_2 in a step-wise manner in a pre-filled chamber system, the concentrations at each step, the duration of the exposure to each concentration and the transition time between each step must be reported
	Animal stocking density and type	Specify the animal density (number and kg/m^2) during the CO_2 exposure phase and report the species, breed and age of animals
	Monitoring	Describe how, where and when the CO_2 concentration was monitored, in order to ensure that the animals continuously

Parameter	Component	Description (all specifications should be in internationally recognised units)
		inhale the recommended gas mixture (e.g. above the animal's head while standing at the first stop position and at the bottom position in a paternoster system (Ferris wheel type), or above the animal's head while standing at the first stop position and at the position the gondola reaches after 10 sec in a dip-lift system). Monitoring equipment should be calibrated using appropriate gases. The calibration methods applied should be reported
Duration of intervention[11]	Time to reach targeted CO_2 concentration [(a)]	Report the time elapsing until animals are exposed to the targeted CO_2 concentration. If animals are exposed to CO_2 in a step-wise manner in a pre-filled chamber system, the concentrations at each step, and 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 concentration. If animals are exposed to CO_2 in a step-wise manner in a pre-filled chamber system, the concentrations at each step, and 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 and exsanguination method (blood vessels cut) that have been applied to guarantee unconsciousness and insensibility of the stunned animal until the moment of death (except for 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" above)
	Humidity and temperature	Report how and when humidity of the gas and temperature inside the chamber were monitored and, if needed, adjusted
Temperature of the gas		Specify the temperature of the gas used at the point of entry in the chamber and the average temperature of the gas mixture (after the gas has been mixed with air atmosphere) inside the chamber

[(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.

3.1.3.2. CO_2 associated with inert gases

Exposure to CO_2 associated with inert gases is a stunning/killing intervention currently allowed for pigs and poultry for the purpose of slaughter, depopulation and other situations. This intervention consists of a direct or progressive exposure of conscious animals to a gas mixture containing up to 40% CO_2 associated with inert gases leading to anoxia. The intervention may be used for slaughter purposes in pits or tunnels. Moreover, this intervention is considered to be a simple stunning procedure for pigs if the duration of exposure to at least 30% CO_2 is of less than seven minutes. It is a simple stunning procedure for poultry if the overall duration of exposure to at least 30% CO_2 is of less than three minutes. The critical factors involved in the induction of unconsciousness in animals is the concentration of CO_2 (hypercapnia) and residual oxygen (hypoxia) levels. The key parameters specified by the legislation are CO_2 concentration, duration of exposure, maximum stun-to-stick/kill

[11] Referring to the legal parameter 'duration of exposure'

interval(s) in the case of simple stunning, quality of the gas, temperature of the gas and oxygen (O_2) concentration. Some parameters are subdivided into several components to ensure a comprehensive description of the applied stunning intervention (Table 6).

Table 6: Parameters to be provided when applying a stunning intervention based on CO_2 associated with inert gases, based on Annex I of Council Regulation (EC) No 1099/2009 and on further details of requirements as determined by the EFSA ad-hoc expert working group

Parameter	**Component**	**Description** (all specifications should be in internationally recognised units)
Inert gases	Type of inert gases used to create the atmosphere	Specify the gases that were used to create the atmosphere
CO_2 and O_2 concentration	Initial CO_2 and O_2 concentration [(a)]	Specify the initial CO_2 and O_2 concentration in the gas mixture to which animals are exposed at the initiation of stunning (at first contact with the modified atmosphere)
	Targeted CO_2 and O_2 concentration(s) [(a)]	Specify the targeted CO_2 and O_2 concentration in the gas mixture used to stun the animals. If animals are exposed to the gas mixture in a step-wise manner in a pre-filled chamber system, several CO_2 and inert gas target concentrations could be applied
	Final CO_2 and O_2 concentration [(a)]	Specify the final/highest CO_2 and final O_2 concentration in the gas mixture to which animals are exposed
	CO_2 and O_2 concentration gradient	The CO_2 and O_2 concentration in the atmosphere should be maintained uniformly; if there are any variations in the composition of the atmosphere, these should be described. If a multi-stage system with a different gas composition in each stage is used, these should be clearly described for each stage. Conditions described for two-stage or multi-stage CO_2 stunning apply here
	Animal stocking density	Specify the animal density (number and kg/m^2) during the gas mixture exposure phase and report the species, breed and age of animals
	Monitoring	Describe how, where and when the CO_2 and O_2 concentration were monitored. Monitoring equipment should be calibrated using appropriate gases. The calibration methods applied should be reported
Duration of intervention[12]	Time to reach targeted CO_2 and O_2 concentration[a]	Report the time elapsing until animals are exposed to the targeted CO_2 and O_2 concentrations. If animals are exposed to the gas mixture in a step-wise manner in a pre-filled chamber system, the concentrations at each step, the duration of the exposure to each concentration and the transition time between each step must be reported
	Total duration of targeted CO_2 and O_2 exposure [(a)]	Report the total duration of exposure of animals to the targeted gas mixture

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

Parameter	Component	Description (all specifications should be in internationally recognised units)
		If animals are exposed to the gas mixture in a multi-stage manner in a pre-filled chamber system, the concentrations at each step, 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) [(b)]		Describe the maximum stun-to-stick/kill interval and the exsanguination method (blood vessels cut) that have been applied to guarantee unconsciousness and insensibility of the stunned animal until the moment of death (except for proof-of-concept studies where the duration of unconsciousness must be determined without sticking)
Quality of the gas	CO_2 and inert gases source	Specify the source of the CO_2 and inert gases
	Humidity and temperature	Report how and when humidity and temperature were monitored and, if needed, adjusted
Temperature of the gases		Specify the temperature of the gas used at the point of entry in the chamber and the average temperature of the gas mixture (after the gas has been mixed with air atmosphere) inside the chamber

[(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.

3.1.3.3. Inert gases

Exposure to inert gases is allowed for stunning / killing pigs and poultry for slaughter, depopulation and other situations. It consists of a direct or progressive exposure of conscious animals to an inert gas mixture such as argon (Ar) or nitrogen (N_2) leading to anoxia. The intervention may be used in pits or tunnels. It is a simple stunning intervention in the case of the slaughter of pigs and also of poultry, if the duration of exposure to anoxia is less than three minutes. The key parameters described by the legislation are O_2 concentration, duration of exposure, quality of the gas, maximum stun-to-stick/kill interval(s) in the case of simple stunning, and temperature of the gas. Some parameters are subdivided into several components to ensure a comprehensive description of the applied stunning intervention (Table 7).

Table 7: Parameters to be provided when applying a stunning method based on inert gases, based on Annex I of Council Regulation (EC) No 1099/2009 and on further details of requirements as determined by the EFSA ad-hoc expert working group

Parameter	Component	Description (all specifications should be in internationally recognised units)
Inert gases	Type of inert gases (Nitrogen, Argon, Helium)	Specify the gas or gases that are part of the modified atmosphere
	Concentration of inert gases [(a)]	Specify their concentration expressed by volume of residual oxygen
Oxygen concentration	Initial inert gases or oxygen concentration [(a)]	Specify the initial inert gases or oxygen concentration to which animals are exposed at the initiation of the stunning (at first contact with the modified atmosphere)
	Targeted inert gases or oxygen concentration(s)[a]	Specify the targeted oxygen concentration used to stun the animals. If animals are

Parameter	Component	Description (all specifications should be in internationally recognised units)
		exposed to the gas mixture in a multi-stage manner in a pre-filled chamber system, several O_2 target concentrations could be applied
	Final inert gas or oxygen concentration [(a)]	Specify the final/highest inert gas or oxygen concentration to which animals are exposed
	Inert gas or oxygen concentration gradient	The inert gas or O_2 concentration in the atmosphere should be maintained uniformly; if there are any variations in the composition of the atmosphere, these should be described. If a multi-stage system with a different gas composition in each stage is used, the compositions at each stage should be clearly described. Conditions described for two- or multistage CO_2 stunning apply here
	Animal stocking density	Specify the animal density (number and kg/m^2) during the phase of exposure to the modified atmosphere and report the species, breed and age of animals
	Monitoring	Describe how, where and when the inert gas concentration was monitored. Monitoring equipment should be calibrated using appropriate gases. The calibration methods applied should be reported
Duration of intervention[13]	Time to reach targeted inert gas or residual O_2 concentration [(a)]	Report the time elapsing until animals are exposed to the targeted inert gas or oxygen concentration. If animals are exposed to the modified atmosphere in a multi-stage manner in a pre-filled chamber system, the concentrations at each step, the duration of the exposure to each concentration and the transition time between each step must be reported
	Total duration of targeted inert gases or residual O_2 exposure [(a)]	Report the total duration of exposure of animals to the targeted gas mixture. If animals are exposed to the modified atmosphere in a multi-stage manner in a pre-filled chamber system, the concentrations at each step, 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) [(b)]		Describe the maximum stun-to-stick/kill interval and exsanguination method (blood vessels cut) that have been applied to guarantee unconsciousness and insensibility of the stunned animal until the moment of death (except for proof-of-concept studies where the duration of unconsciousness must be determined without sticking)
Quality of the inert gas	Source	Specify the source of the inert gases
	Humidity and temperature	Report how and when humidity and

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

Parameter	Component	Description (all specifications should be in internationally recognised units)
		temperature were monitored and, if needed, adjusted
Temperature of the gases		Specify the temperature of the gas used at the point of entry in the chamber and the average temperature of the gas mixture (after the gas has been mixed with air atmosphere) inside the chamber

(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.

3.1.3.4. Low atmosphere pressure

The low atmosphere pressure stunning (LAPS) is a stunning system whereby animals are rendered unconscious prior to slaughter by gradually reducing the oxygen tension in the atmosphere to achieve a progressive hypoxia. The induction of unconsciousness with LAPS is not instantaneous. This stunning intervention is currently not approved for use in the EU. Therefore, no parameters are defined by Council Regulation (EC) No 1099/2009. The parameters and components listed in Table 8 have been defined by experts on stunning methods consulted during the preparation of this guidance.

Table 8: Parameters to be provided when applying a stunning intervention based on low atmosphere pressure as determined by the EFSA ad-hoc expert working group

Parameter	Component	Description (all specifications should be in internationally recognised units)
Animal density	Animal species/ age/ type and stocking density (number/m^2 and kg of body weight/ m^2)	Specify the animal density in the crate or containers during the decompression
Duration of intervention[14]	Time to achieve the target pressures and corresponding partial pressure of oxygen in a single-phase system or multi-phase system (a)	Report the time elapsing until animals are exposed to the targeted pressure and corresponding partial pressure of oxygen. Report the duration of exposure to the target pressure and the corresponding partial pressure of oxygen. If animals are exposed to a multi-stage system, report the target pressure in each stage, the duration of the exposure to each step as well as the transition time between each step
Rate of decompression	Time/pressure treatment graphic representation	Describe the rate at which pressure changes are achieved in the chamber through a time/pressure curve. If decompression is achieved in more than one step, the profile for each step should be described. Re-pressurisation of the chamber prior to opening of door should be described and any incidence of birds surviving the treatment should be reported
Rate of changes in partial pressure of oxygen	Time/partial pressure of oxygen treatment graphic representation	Describe the rate at which partial pressure of oxygen changes in the chamber in relation to the rate of decompression.

[14] Referring to the legal parameter 'duration of exposure' of other stunning methods.

Parameter	Component	Description (all specifications should be in internationally recognised units)
		If decompression is achieved in more than one step, the profile for each step should be described
Temperature/ humidity/ illumination of the chamber		Specify the temperature and humidity profile inside the chamber. Specify the light source if present
Maximum stun-to-stick/kill interval(s) [(b)]		Describe the maximum stun-to-stick/kill interval and the exsanguination method (blood vessel cut) that have been applied to guarantee unconsciousness and insensibility of the stunned animal until the moment of death (except for proof-of-concept studies where the duration of unconsciousness must be determined without sticking). Report the stun- to-stick/kill interval(s) for the last animal stuck that did not recover consciousness in a group stunning situation
Calibration of the LAPS equipment and monitoring system		Describe how the decompression procedure was controlled and how and with which frequency the equipment was calibrated. The monitoring equipment should be regularly calibrated. The calibration methods applied should be reported

[(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.

3.2. Outcome

3.2.1. Onset of unconsciousness and insensibility

The EFSA Scientific Report of the Scientific Panel for Animal Health and Welfare on welfare aspects of animal stunning and killing methods, prepared on a request from the Commission, concludes that stunning and stunning/killing methods should ideally induce an immediate and unequivocal loss of consciousness and sensibility (EFSA, 2004).

The neuronal basis of consciousness with regard to stunning is presented in detail in the EFSA report on welfare aspects of the main systems of stunning and killing the main commercial species of animals (EFSA, 2004). The normal functioning of neurons in the thalamus and cerebral cortex is accepted as a necessary condition for perceptual processes and consciousness. Therefore, stunning interventions should disrupt the neuronal function and thereby render animals unconscious and insensible. The extent of disruption caused by a stunning intervention and the induction of unconsciousness and insensibility are best demonstrated using EEGs (EFSA, 2004). EEG or ECoG is widely used to record the spontaneous and evoked (somatosensory, visual and auditory) electrical activity in the brain to ascertain the state of consciousness and sensibility following stunning. It is acceptable that studies on alternative stunning interventions 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 animals insensible to pain are necessarily unconscious, for example, analgesia rather than unconsciousness can be induced by gas mixtures (Raj and Gregory, 1990), and also the insensibility (analgesia) lasts longer than the unconsciousness induced by head-only electrical stunning (Velarde et al., 2002). 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, e.g. on whether electrical, mechanical or modified atmosphere

stunning is used (e.g. Raj et al., 1992, 1998; Cook et al., 1995, 1996, EFSA, 2004; Gerritzen et al., 2004, 2006; Benson et al., 2012a, b).

Established stunning methods induce unique brain states that are incompatible with the persistence of consciousness. These altered brain states are associated with certain behavioural patterns and physical reflexes which are referred to as animal-based indicators. The correlation between EEG evidence of unconsciousness and animal-based indicators is characterized for established stunning methods, permitting the use of animal-based indicators as proxies for unconsciousness.

3.2.1.1. Mechanical stunning

Penetrative and non-penetrative captive bolt guns are the most commonly used mechanical stunning interventions for rendering animals unconscious and insensible prior to slaughter. In the EU Slaughter Regulation 1099/2009, the use of non-penetrative captive bolt guns is restricted to ruminants weighing up to 10 kg; however, no such restriction applies to penetrative captive bolts. Captive bolt stunning induces immediate loss of consciousness and sensibility in animals through concussion of the brain upon the impact of the bolt on the skull. Penetrative captive bolts also induce structural damage to the brain, and severe damage to the brain stem can result in death in animals. The neurophysiological basis of brain concussion and the consequences of structural damage occurring to different regions of the brain are well documented in the scientific literature (EFSA, 2004).

Induction of unconsciousness and insensibility by captive bolt stunning can be ascertained in the laboratory by studying EEG activity:

- Induction of brain concussion can be recognised from the predominance of less than 4 Hz high-amplitude (slow wave) EEG activity.
- The slow wave activity is followed by a quiescent EEG owing to severe brain injury caused by the penetrative bolt.
- Somatosensory, visual or auditory evoked responses or potentials in the brain are abolished immediately after captive bolt stunning and also during the manifestation of slow waves and quiescent EEG.

In mammals, successful induction of brain concussion leads to immediate collapse of the animal accompanied with apnoea (absence of breathing), onset of tonic seizure, which can be recognised from the occurrence of arched back and legs flexed under the body, and fixed eyes. The tonic seizure lasts for several seconds, leading to the loss of muscle tone, which can be recognised from drooping ears, relaxed jaw, protruding tongue and limp tail and legs, especially when the animals have been shackled and hoisted on to the overhead bleeding rail. Additionally, palpebral, corneal and pupillary reflexes and response to external stimuli including pain (e.g. nose prick) are also abolished during the period of unconsciousness and insensibility.

Ineffective or unsuccessful captive bolt stunning in mammals can be recognised from the absence of immediate collapse and onset of tonic seizure, and animals may also vocalise in extreme cases. Rotation of the eye ball, including nystagmus is also a sign of ineffective or poor captive bolt stunning. The ineffectively stunned animal may collapse partially, but retain some muscle tone and, as a consequence, attempt to regain posture, i.e. stand upright again. Ineffectively stunned animals and those recovering consciousness will show positive eye reflexes (palpebral, corneal and pupillary), or violent kicking, especially of hind legs. Head righting (attempt to raise head) after stunning and body arching during bleeding are also signs of ineffective stunning or recovery of consciousness.

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. An

overview of indicators that can be applied during slaughter at different key stages can be found in EFSA (2013).

In laboratory experiments on penetrative captive bolt stunning interventions, a sagittal sectioning of the skull should be performed on all animals to assess and report the trajectory of the bolt and the damage incurred in different brain sections and the effectiveness of the stun. Brain concussion induced by penetrative captive bolt stunning is accompanied by haemorrhages due to rupture of the blood vessels at the site of entry of the bolt, in the sub-arachnoid space and at the base of the brain.

For non-penetrative captive bolt applications, the incidence of skull fractures needs to be reported.

3.2.1.2. Electrical stunning

Electrical stunning interventions are considered to result in immediate onset of unconsciousness and insensibility. The electrical stunning of animals with a current of sufficient magnitude and duration leads to long-lasting strong depolarisation of the cell membrane leading to generalised epilepsy (e.g. grand mal epilepsy). The generalised epilepsy is followed by a period of quiescence in the EEG, which is referred to as "spreading depression" and occurs as a result of hyperpolarisation. When these two EEG manifestations occur after electrical stunning, the animals are considered to be unconscious and insensible (EFSA, 2004). The evoked electrical activity (somatosensory, visual and auditory) in the brain is also abolished during the manifestation of epileptiform activity and quiescent EEG. Therefore, in laboratory condition studies, unconsciousness and insensibility can be ascertained by the following EEG patterns:

- Induction of a generalised epileptiform activity in the brain, which can be recognised from the predominance of 8–13 Hz high-amplitude EEG activity, followed by a quiescent EEG.

OR

- An immediate onset of a quiescent EEG.

OR

- No somatosensory, visual or auditory evoked responses or potentials in the brain immediately after the stunning.

Generalised epileptiform activity induced by head-only or head-to-body stunning results in the immediate collapse of the animal and the occurrence of tonic seizures, which can be used as behavioural indicators (depending on the slaughter process). Head-only electrical stunning-induced tonic seizure leads to clonic seizure. On the other hand, head-to-body stunning-induced tonic seizure may be very short and the clonic seizure will be absent or will present with only very mild muscle activity, owing to cardiac fibrillation in animals. The occurrence of tonic seizure after the application of the electric current followed by apnoea, or lack of response to painful stimuli, can be used together to recognise effective electrical stunning (as monitoring points) under slaughterhouse conditions. However, under the head-only stunning situation, the animal has the capacity to recover consciousness during clonic seizure, i.e. to resume breathing. Seizures can also be induced by currents below the level needed to induce epileptiform activity in the brain/ unconsciousness. Electro-immobilisation prevents the animal from presenting tonic/clonic seizures and from showing signs of consciousness, including reactions to painful stimuli. For these reasons, for studies carried out under slaughterhouse conditions, it is necessary to assess the effectiveness of electrical stunning by using the following sequence of indicators to be sure that the animal is unconscious and insensible:

1. Presence of tonic seizures after removal of the current;

AND

2. Apnoea during tonic and clonic seizures.

Indicators of failed stunning are escape behaviour, often with prolonged purposeful vocalisation, absence of the typical tonic or clonic muscle activity; resumption of rhythmic breathing, during and after the current application, or righting attempts after the current application. If the eyeball is able to focus and follow stimuli from the surroundings, the animal is conscious (EFSA, 2004).

3.2.1.3. Modified atmosphere stunning including low atmosphere pressure stunning

Animals are rendered unconscious and insensible gradually 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 comprise (1) muscle jerk (voluntary and involuntary excitation), (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, and hence the duration of the different stages of anaesthesia, during the 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 or breathlessness caused by the inhalation of CO_2. The higher the CO_2 concentration, the more aversive is the inhalation. In addition, inhalation of CO_2 stimulates nerve endings in the nasal epithelium, which induces sniff-like aspiration reflexes (EFSA, 2005). Some scientists have interpreted the animals' reaction during the induction phase as a part of the involuntary excitation phase, whereas others have interpreted it as a voluntary response to pain, distress and suffering caused by the inhalation of the gas.

Exposure of animals to gas mixtures leads to loss of consciousness and sensibility owing to the inhibition of brain function, as evidenced by 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. When animals are exposed to gas mixtures, there is a transition period during which conscious EEG patterns change to unconscious EEG patterns, but EEG pattern interpretation is subjective.

In addition, changes in the EEG patterns seem to vary depending upon the composition of the gas mixture and between mammals (e.g. pigs) and birds (e.g. chickens). For example, inert gases inducing loss of consciousness through anoxia result in hypersynchronisation of the brain electrical activity as evidenced by the appearance of slow waves (high-amplitude, low-frequency activity) in the EEGs of mammals, leading to quiescent EEGs. In poultry, however, only quiescent EEGs occurred without the manifestation of slow waves. Exposure of mammals and poultry to high concentrations of CO_2 inducing loss of consciousness through hypercapnia results in profoundly suppressed EEGs. Exposure of mammals to a mixture of CO_2 and inert gases inducing loss of consciousness through hypercapnic hypoxia results in different EEG manifestations, depending upon the residual O_2levels in the gas mixture, i.e. slow waves in some and suppression in the EEG of others. On the other hand, hypercapnic hypoxia in poultry seems to result in profoundly suppressed or quiescent EEGs. Nevertheless, brain evoked potentials are abolished to the appearance of slow waves in the EEGs or during the occurrence of profoundly suppressed or quiescent EEGs. Therefore, it is recommended that abolition of evoked electrical activity in the brain should be used as an indicator of unconsciousness when EEG manifestations are ambiguous.

Exposure of animals to LAPS is analogous, in physiological terms, to simulated exposure to high altitudes, and, if the partial pressure is low enough, is expected to produce loss of consciousness and sensibility via hypoxia. Hypoxia inhibits brain function, as evidenced by the gradual depression leading to 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 hypoxia may vary. When animals are exposed to low atmosphere pressure, there is a transition period during which conscious EEG patterns change to unconscious EEG patterns, but EEG pattern interpretation is subjective. Loss of consciousness through hypoxia results in hyper synchronisation of the brain electrical activity as evidenced by the appearance of slow waves (high-amplitude, low-frequency activity) in the EEGs of mammals, leading to quiescent EEGs. In poultry, however, only quiescent EEGs occur without the manifestation of slow waves. Nevertheless, brain evoked potentials are abolished to the appearance of slow waves in the EEGs or during the occurrence of profoundly suppressed or quiescent EEGs. Therefore, it is recommended that abolition of evoked electrical activity in the brain should be used as an indicator of unconsciousness when EEG manifestations are ambiguous.

Therefore, the reliable criteria to be employed during controlled laboratory studies are:

- Appearance of slow waves (high amplitude, low frequency (less than 4 Hz)) in EEG activity during exposure of mammals to anoxic gas mixtures (Raj et al., 1997).
- Profoundly suppressed or quiescent EEGs in mammals and poultry. 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 high concentrations of CO_2 or to gas mixtures (Raj et al., 1998; Rodríguez et al., 2008; Llonch et al., 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 et al., 2002; Rodríguez et al., 2008).

In addition to EEG evidence, arterial partial pressure of blood oxygen or pulse oximetry could be used as direct measures of hypoxia in animals. Evidence should be provided showing that reported values are not compatible with persistence of consciousness.

A list with indicators for recognition of a successful stun in different species after exposure to hypoxic atmospheres using gas mixtures is provided in previous EFSA opinions (EFSA, 2004, 2013). Studies in poultry and pigs concerning welfare suggest that the loss of posture is the earliest behavioural sign of the onset of unconsciousness; however, it may not always be possible to determine the time to loss of posture, as animals start muscle jerks before or in conjunction with loss of posture depending upon the rate of induction of hypoxia/ anoxia (Raj et al., 1997; Rodríguez et al., 2008).

Other indicators of effective stunning include dilated pupils, absence of palpebral, corneal and pupillary reflexes, apnoea, relaxed body / lack of muscle tone 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 the application of the stunning intervention and the onset of unconsciousness should be used.

3.2.2. Absence of pain, distress and suffering until the loss of consciousness and sensibility

If a stunning intervention does not induce immediate unconsciousness and insensibility, the absence of pain, distress and suffering until the onset of unconsciousness and insensibility should be assessed. Loss of consciousness during exposure to modified atmospheres is not immediate and animals may experience pain, distress and suffering. For example, pain might be elicited by the irritation of the

nasal mucosal membranes and lungs, where the presence of CO_2-sensitive chemoreceptors has been described, or as a result of respiratory distress causing hyperventilation and a sense of breathlessness (Raj and Gregory, 1995, 1996; Raj, 1996; Fedde et al., 2002; Velarde et al., 2007). Pain is a complex phenomenon and is very difficult to measure qualitatively and quantitatively owing to the absence of clear borders between pain, distress and suffering, as these states may not always be distinguishable in animals. Currently, indirect animal-based measures of pain, distress and suffering have to be used as no direct tool is available to identify them. In addition, thresholds for pain, distress and suffering can be different between 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 and Kent, 1997; Broom, 2001; OIE, 2012). Kavaliers (1988), based on the International Association for the Study of Pain (IASP) definition of 1979, 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 guidance document.

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 in Table 9: 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, distress and suffering are included as examples, but the list is not exhaustive. Behavioural, physiological and neurological responses to pain, distress and suffering can be different between animals within and between species.

Table 9: Overview of response types and animal-based measures associated with pain, distress and 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, b, 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, b, 2013
	General behaviour	e.g. agitation, freezing, retreat attempts, escape attempts	EFSA 2005; Velarde et al., 2007; Dalmau et al., 2010; Landa, 2012
Physiological response	Hormone concentrations	e.g. Hypothalamic-pituitary-adrenal axis: corticosteroids, Adrenocorticotrophic hormone; 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, lactate dehydrogenase	EFSA, 2005; Vogel et al., 2011; Landa 2012; Mota-Rojas et al., 2012
	Autonomic responses	e.g. heart rate and heart rate variability, blood pressure, respiratory rate, body temperature	Martoft et al., 2001; EFSA ,2005; Gerritzen et al., 2007; von Borell et al. 2007; Rodriguez et al., 2008; Svendsen et al., 2008; Le Neindre et al., 2009; Dalmau et al., 2010; McKeegan et al., 2011; Atkinson et al., 2012; Landa, 2012; Llonch et al., 2012a, b, 2013
Neurological response	Brain activity	e.g. EEG, ECoG	Gibson et al., 2009

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 intervention is considered not to induce pain, distress and suffering before the onset of unconsciousness and insensibility:

- Animal-based measures from the behaviour response type AND animal-based measures from at least one of the two additional response types presented in Table 9 (i.e. physiological or neurological response) relevant to the intervention/species, which must be indicative of the absence of pain, distress and suffering before the onset of unconsciousness and insensibility. This means that these animal-based measures should not be significantly different between the appropriate control and treatment groups. In this regard, in the absence of pain, distress and suffering caused by the application of a stunning intervention, the response of animals exposed to the procedure/apparatus without the application of stunning (control or sham operation) should not be significantly different from the response of the animals exposed to the procedure/apparatus with stunning (treatment). The possibility that the control/sham operation itself has not resulted in a maximum response in animals - such that no further increases in response could occur owing to the additional pain and distress caused by the stunning intervention - should be demonstrated.

- 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).

3.2.3. Duration of unconsciousness and insensibility

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

In general, animals are considered to be unconscious as long as the altered brain states, as recognised from the profound changes in EEGs, that are unique to the intervention and are established as being incompatible with the persistence of consciousness, are demonstrated immediately after the intervention. When changes occurring in the spontaneous EEGs are ambiguous, abolition of evoked electrical activity in the brain (somatosensory, visual or auditory evoked potentials) can be used as an indicator of unconsciousness. Recovery of spontaneous or evoked electrical activity in the brain can also be used to ascertain the time to recovery of consciousness in animals following the application of reversible stunning. In this regard, the time to the return of total EEG power content (voltage squared) to 10% or more of the pre-stun level has been used as an indicator of consciousness. The time to the recovery of spontaneous activity has been reported to coincide with the time to the recovery of evoked activity in the brain.

Indicators of recovery of consciousness after stunning are listed in EFSA scientific opinions (EFSA, 2004, 2013), but their sequence depends on the stunning intervention. 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, return of corneal reflex has been used to recognise recovery of consciousness under slaughterhouse conditions (EFSA, 2004). In conclusion, it is recommended that the indicator that is most sensitive in detecting recovery be used. Indicators that can be measured at different stages during slaughter can be found in EFSA (2013).

4. Reporting quality

Studies on alternative stunning interventions should analyse equivalence to the requirements prescribed in Council Regulation (EC) No 1099/2009: induction of immediate onset of unconsciousness and insensibility or the absence of pain, distress and suffering until the onset of unconsciousness and insensibility and the duration of unconsciousness and insensibility until death. Several study designs could be applied. The REFLECT statement and the STROBE statement were identified as the most suitable guidelines that could be applied to studies on stunning interventions. 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. All of the parameters from the checklist of the REFLECT and the STROBE statements were reviewed and, in some cases, modified to allow their use in the context of studies on stunning interventions (Table 10).

Table 10: Parameters used to assess the reporting quality of studies on stunning interventions, 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 the 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, give an 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 intervention are provided in section 3.1
Outcome	Clearly define all primary outcomes (onset of unconsciousness and insensibility, absence of pain, distress and suffering, and duration of unconsciousness and 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 3.2
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 the methods used to control for sampling bias. Was any comparison made between the reference population and animals under study? Concerning internal validity, report the methods used 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 it was done, describe who was

Parameter	Description
	blinded (e.g. the data collector, the data analyst) as well as how it was done (e.g. when it started and when it ceased). If the process was different for outcomes, clarify this per outcome (e.g. behaviour data were blinded but electroencephalography data were not)
Statistical methods	Describe all statistical methods used to summarise the data and to 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 and Altman (2013)
Results	
Numbers analysed	Provide basic information about the distribution of important confounders and effect modifiers in each study group (age, weight, sex). If variables are continuous provide means (and standard deviation) 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 out of 20, not 50 %)
Outcomes and estimations	For each outcome, report a summary of the 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 the 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 before 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 which are pre-specified and those which are exploratory
Discussion	
Key results and interpretation	Summarise the key results with reference to the 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 validity	Discuss the potential for external validity of the study results (e.g. applicability of the stunning intervention in slaughterhouses in different Member States or whether study results can be extrapolated beyond the study population)
Other	
Funding	Give the source of funding and the role of the funders for the submitted study. State any potential conflicts of interest

The reporting quality of a study submitted for assessment will be evaluated against each of these criteria. However, the decision over whether the overall reporting quality is sufficient will be based upon the judgment of the panel experts that have been engaged to assess the submitted study.

5. Methodological quality

The methodological quality of a research study can be determined by assessing its precision and its internal and external validity. These elements are related to the extent to which the study's design, implementation, data acquisition, analysis and interpretation of results (1) minimise systematic errors (biases) that compromise the study's internal validity; (2) minimise random errors that reduce the

precision of the measurements made in the study; and (3) allow broad applicability of the results beyond any single study (i.e. external validity). The methodological quality criteria assessment of this guidance focuses on elements in the report that allow the assessment of the internal validity of the submitted study.

Appraisal of a study's external validity (i.e. its applicability beyond the study population) requires that its results be compared with those of related studies. As this guidance is only applicable to individual studies, assessing the external validity of those studies exceeds its mandate.

EFSA has embarked on various initiatives aimed at improving the quality of reporting and standardising the process for assessing the strength of the evidence used as a basis of risk assessments. Currently, a guidance document on statistical reporting is being prepared. In addition, a series of quality assessment checklists, called Critical Appraisal Tools (CATs), which are applicable to different study types used in the agri-food public health domain, are being developed. To date, EFSA has issued CATs to support and harmonise the evaluation of Randomised Controlled Trials and of Systematic Reviews for Intervention. These resources will provide guidance for the next level of assessment, where a full assessment of the animal welfare implications of the proposed alternative stunning intervention, including both pre-stunning and stunning phases, and an evaluation of the quality, strength and external validity of the evidence presented will be carried out.

5.1. Parameters to be considered when assessing methodological quality

The first level of assessment of studies evaluating alternative stunning interventions described in this guidance focuses on the assessment of three types of bias and confounding. In this guidance, the terminology and the definitions of biases provided in the Cochrane Handbook (Higgins and Green, 2011) have been adopted.

5.1.1. Selection bias

As defined in the Cochrane Handbook, "*systematic differences between baseline characteristics of the groups that are compared*" constitute selection bias. For studies assessing the effectiveness of alternative stunning interventions, selection bias would be present if the allocation of animals to treatment and control groups does not follow the same rules or if systematic differences between characteristics of animals allocated to treatment and control groups exist.

5.1.2. Attrition bias

The Cochrane Handbook defines attrition bias as "systematic differences between groups in withdrawals from a study". For example, if animals with certain characteristics are withdrawn differentially from treatment or control groups, this could be attrition bias.

5.1.3. Performance bias

The Cochrane Handbook defines performance bias as "*systematic differences between groups in the care that is provided, or in exposure to factors other than the interventions of interest*". For example, if the observers are aware of details of the intervention and that awareness differentially affects their handling of the treatment and control groups, this could be performance bias.

5.1.4. Confounding

Confounding is bias arising from the co-occurrence or mixing of the effects of extraneous factors - referred to as confounders - with the main effect(s) of interest in a study. In practice, studies assessing the effectiveness of alternative stunning interventions should consider the possibility that the variables that they are measuring as indicators of stun effectiveness are confounded by other variables that are correlated with some aspect of the intervention that is not the source of the stun itself.

Parameter	Description
Selection bias	Assess whether systematic differences between characteristics of animals allocated to treatment and control groups exist
Attrition bias	Assess whether the characteristics of the animals withdrawn from the study/analysis differ systematically between control and treatment groups
Performance bias	Assess whether the observers were blinded to the details of the intervention or whether differential handling (that could affect comparisons between treatments and control) might have occurred
Confounding	Assess whether confounding has been addressed properly

5.2. Evaluating the methodological quality

The assessment of methodological quality will be based upon the judgement of the panel experts engaged to assess the submitted study. It will be reported as a qualitative narrative in the style of a peer review of a manuscript submitted for publication in a scholarly journal. The assessment will focus on the level of uncertainty surrounding the evidence presented in the study and the potential limitations of the conclusions in order to inform the next level of assessment.

RECOMMENDATIONS

The criteria for eligibility, reporting quality and study quality, as well as the general aspects applicable to studies on stunning interventions defined in this guidance, should be applied to studies carried out under both controlled laboratory conditions and under slaughterhouse conditions.

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GLOSSARY AND ABBREVIATIONS

Adverse events	A detrimental outcome measured in a study of an intervention that may or may not have been caused by the intervention.
Attrition bias	Systematic differences between comparison groups in withdrawals from a study.
Bias	Systematic deviation of a measurement from the 'true' value leading to either an over- or underestimation of the treatment effect. Bias can originate from many different sources, such as allocation of subjects, measurement, interpretation, publication and review of data.
Blinding (masking)	Blinding or masking is the process used in epidemiological studies and clinical trials in which the observers and the subjects have no knowledge as to which treatments subjects are assigned to. This is done in order to minimise bias occurring in the subject response and outcome measurement. In single-blind studies only the subjects are blind to their allocations, whilst in double-blind studies both observers and subjects are ignorant of the treatment allocations.
Confounding	The bias arising from the co-occurrence or mixing of the effects of extraneous factors - referred to as confounders - with the main effect(s) of interest in a study.
External validity	Refers to the extent to which a study's results provide a correct basis for generalisation beyond the setting of the study and the particular subjects studied. It implies the applicability of the results of a study to another group or population.
Information bias	A bias that occurs during data collection. The most frequent information bias is misclassification bias, which is present, when the detection of the exposure status (exposure identification bias) and/or the outcome assessment (outcome identification bias) is biased, i.e. exposed/diseased individuals are classified as non-exposed/non-diseased and vice versa. A common source of misclassification is the inaccuracy of diagnostic tests.
Intervention	An intervention will generally be a therapeutic procedure such as treatment with a pharmaceutical agent, surgery, a dietary supplement, a dietary change or psychotherapy. Some other interventions are less obvious, such as early detection (screening), patient educational materials, or legislation. The key characteristic is that a person or their environment is manipulated in the hope of benefiting that person.
Objective	Describes the scope of the study and the specific hypotheses to be verified. Depending on the study primary and secondary objectives could be defined.
Outcome	An outcome is an indicator/variable measured in a subject or biological sample to assess the safety, efficacy or other objective of a trial.
Paternoster system	The paternoster system works continuously with gondolas (or cradles) like a Ferris wheel, where pigs are lowered successively into the maximum carbon dioxide concentration at the bottom of the pit with stops during the procedure through an increasing carbon dioxide gradient as live pigs enter or unconscious pigs leave the gondolas for shackling. The number of pigs contained within each gondola varies according to the model and age of the system; older models have space to accommodate 1 to 3 pigs, whereas newer ones can take up to 6-8 pigs. The size of the chamber, size of the individual cradle, and number of pigs per cradle can be

	varied according to the throughput rates.
Performance Bias	Systematic differences between intervention groups in care provided apart from the intervention being evaluated.
Pithing	The laceration of the central nervous tissue and spinal cord by means of an elongated rod-shaped instrument introduced into the cranial cavity.
Sample size	Number of units selected to enter the trial.
Sampling bias	A bias in which a sample is collected in such a way that some members of the target population are less likely to be included than others.
Selection bias	Systematic differences between comparison groups in prognosis or responsiveness to treatment.
Sensibility	The ability to perceive stimuli.
Stunning intervention	An intervention that is applied to an animal to stun it. Stunning interventions include the stunning methods listed in Annex I of Council Regulation (EC) No 1099/2009 and modified or new interventions that aim at stunning animals.
Randomization	A process of allocating participants to treatment or control groups within a controlled trial by using a random mechanism, such as coin toss, random number table, or computer-generated random numbers.
Unconsciousness	A state of unawareness (loss of consciousness) in which there is temporary or permanent damage to brain function and the individual is unable to respond to normal stimuli, including pain.

ABBREVIATIONS

CAT	Critical Appraisal Tool
CO_2	Carbon dioxide
ECG	Electrocardiogram/ electrocardiography
ECoG	Electrocorticogram/ electrocorticography
EEG	Electroencephalogram/ electroencephalography
O_2	Oxygen
TOR	Term of reference