Corrigenda to the Scientific Opinion on "Aspects of the biology and welfare of animals used for experimental and other scientific purposes"

In view of the different comments received to the Animal and Welfare Scientific Panel (AHAW) Scientific Opinion: "Aspects of the biology and welfare of animals used for experimental and other scientific purposes", the AHAW Panel decided, during the AHAW Plenary held on the 11th and 12th of December 2006, to adopt the following points of the Scientific Opinion:

SECTION 2 - Question on the sentience of invertebrate species, fetal and embryonic forms of both vertebrate and invertebrate species and on fetal and embryonic forms

Recommendation 3 (section 2.4, page 18, 19) - replace with:

"As a guideline, and because of the risk that even mammals *in utero* may sometimes be aware at times before parturition, when a procedure is performed on a fetus that is likely to produce pain in the newborn of that species, adequate anaesthesia and analgesia should be given provided. It should be noted that the administration of analgesia and anaesthesia may significantly increase the likelihood of fetal mortality. In the circumstance where no suitable anaesthetic or analgesic agents are available, procedures should not be carried out on such fetuses. When the procedure might cause a lasting inflammatory response that persists post-natally, protection should be given against pain and suffering."

The Categories (section 2.5, page 20) - should read:

- Category 1 - "The scientific evidence clearly indicates, either directly or by analogy with animals in the same taxonomic groups, that animals in those groups are able to experience pain and distress".
- Category 2 - "The scientific evidence clearly indicates, either directly or by analogy with animals in the same taxonomic groups that animals in those groups are NOT able to experience pain and distress".
- Category 3 - is correct.

SECTION 4 – Question on Humane methods of Euthanasia

Recommendation 1 (section 4.5.3, page 29) - should read:

"When using these techniques, cervical dislocation and decapitation, in some species, the necessary handling and restraint can be stressful for the animal and so they should first be anaesthetised to minimise distress and eliminate any subsequent pain, unless an exception can be justified on scientific grounds or the adverse effects of induction of unconsciousness would be greater than the adverse effects of killing without it."

SECTION 5 – Tables with the recommended methods for the humane killing of animals in the laboratory

Table 6 - Characteristics of methods for euthanasia of rabbits (page 39) - should read:

Rapid freezing – "Only for fetuses under **4g**."

The EFSA Journal (2005) 292, 1-46 - Opinion on the "Aspects of the biology and welfare of animals used for experimental and other scientific purposes"

Opinion of the Scientific Panel on Animal Health and Welfare on a request from the Commission related to

"Aspects of the biology and welfare of animals used for experimental and other scientific purposes"

EFSA-Q-2004-105

Adopted by the AHAW Panel on 14 November 2005

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Summary

EFSA was invited by the EU Commission to produce a scientific opinion concerning the "Revision of the Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes".

This scientific opinion was adopted by written procedure on the 14th November 2005, by the Scientific Panel on Animal Health and Welfare (AHAW) after its Plenary Meeting held on the 12th and 13th of October.

According to the mandate of EFSA, ethical, socio-economic, cultural and religious aspects are outside the scope of this opinion.

Summary of the Scientific Opinion for each of the three parts of the Mandate from the Commission:

1. Summary of the need for protection for invertebrates and fetuses and the criteria used (Questions 1 & 2)

   The Panel was asked to consider the scientific evidence for the sentience and capacity of all invertebrate species used for experimental purposes and of fetal and embryonic forms to "experience pain, suffering, distress or lasting harm". Indicators of an animal's capacity to experience suffering include long-term memory, plasticity of behaviour, complex learning and the possibility of experiencing pain. Some invertebrate species: (i) possess short and long term memory, (ii) exhibit complex learning such as social learning, conditioned suppression, discrimination and generalisation, reversal learning, (iii) show spatial awareness and form cognitive maps, (iv) show deception, (v) perform appropriately in operant studies to gain reinforcement or avoid punishment, (vi) possess receptors sensitive to noxious stimuli connected by nervous pathways to a central nervous system and brain centres, (vii) possess receptors for opioid substances, (viii) modify their responses to stimuli that would be painful for a human after having had analgesics, (ix) respond to stimuli that would be painful for a human in a manner so as to avoid or minimise damage to the body, (x) show an unwillingness to resubmit themselves to a painful procedure indicating that they can learn to associate apparently non-painful with apparently painful events. At a certain stage of development within an egg or the mother, the characteristics listed above may appear. Such information has been used in coming to conclusions about sentience.

   Cyclostomes (lampreys and hagfish) have a pain system similar to that of other fish and brains that do not differ much from those of some other fish. There is evidence that cephalopods have adrenal and pain systems, a relatively complex brain similar to many vertebrates, significant cognitive ability including good learning ability and memory retention especially in octopuses, individual temperaments, elaborate signalling and communication systems, especially in cuttlefish and squid that can show rapid emotional colour changes, may live in social groups and have complex social relationships. Nautiloids have many characters similar to those of other cephalopods, they can track other individuals, live for a long time and are active pelagic animals. The largest of decapod crustaceans are complex in behaviour and appear to have some degree of awareness. They have a pain system and considerable

learning ability. As a consequence of this evidence, it is concluded that cyclostomes, all Cephalopoda and decapod crustaceans fall into the same category of animals as those that are at present protected. Using similar arguments, the dramatic evidence of the sensory processing, analytical and prediction ability of salticid spiders provides evidence for awareness greater than in any other invertebrates except cephalopods but we have little evidence of a pain system so do not at present put these spiders in that same category. Free-swimming tunicates are also in this borderline area and social insects and amphioxus are close to it.

Whenever there is a significant risk that a mammalian fetus, or the fetus or embryo of an oviparous animal such as a bird, reptile, amphibian, fish or cephalopod, is for any length of time sufficiently aware that it will suffer or otherwise have poor welfare when a procedure is carried out on it within the uterus or egg, or after removal therefrom, such animals should be included in the list of protected animals. The stage of development at which this risk is sufficient for protection to be necessary is that at which the normal locomotion and sensory functioning of an individual independent of the egg or mother can occur. For air-breathing animals this time will not generally be later than that at which the fetus could survive unassisted outside the uterus or egg. For most vertebrate animals, the stage of development at which there is a risk of poor welfare when a procedure is carried out on them is the beginning of the last third of development within the egg or mother. For a fish, amphibian, cephalopod, or decapod it is when it is capable of feeding independently rather than being dependent on the food supply from the egg.

Precocial oviparous species, some of which are breathing at the time of hatching present much evidence of being aware before hatching and during the last days before hatching,

Even though the mammalian fetus can show physical responses to external stimuli, in some species perhaps for as much as the last third of their development, the weight of present evidence suggests that consciousness is inhibited in the fetus until it starts to breathe air. It is possible that in a mammalian fetus there might be transient episodes of increased oxygenation above the threshold required to support some aspects of consciousness. It is clear that there is a risk, perhaps a small risk, that any mammalian fetus may on occasion be affected by some experimental procedures in such a way that their welfare is poor, sometimes because they are suffering pain. If a mammalian fetus is removed from the mother and starts to breathe, its level of awareness will change to that typical of such animals after parturition. In addition, protection may need to be given against emotional states in pregnant mothers to safeguard subsequent behavioural modification and welfare of the offspring.

When a procedure is performed on a fetus that is likely to produce pain in the newborn or newly-hatched of that species, adequate anaesthesia and analgesia should be given provided that the agents used do not significantly increase the likelihood of fetal mortality. In the circumstance where no suitable anaesthetic or analgesic agents are available, procedures should not be carried out on such fetuses. When the procedure might cause a lasting inflammatory response that persists post-natally, protection should be given against pain and suffering. A schedule of anaesthetics and analgesics that are suitable for use in pregnant animals, and fetuses should be prepared.

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2. Summary of the need for purpose breeding of animals and the criteria used (Question 3)

Species listed in Annex I to Directive 86/609/EEC are those that must be 'purpose bred' when used in experiments (unless a specific exemption has been obtained). The criteria for inclusion of species in Annex I have not been clearly defined and hence no information is available on why they were originally included. Therefore, the Commission has asked the EFSA to issue a scientific opinion on the scientific criteria that could be used to determine in which cases animals to be used in experiments should be purpose-bred and, based on these criteria, determine which species currently used in experiments meet these criteria.

It is the opinion of the AHAW panel that including a species as "purpose-bred" within Annex I will confer a considerable degree of assurance that animals of that species will be provided with suitable accommodation, welfare and care practices. As a consequence of health and colony management within breeding establishments, there can be improved confidence in the quality of the animal, resulting in improved science and a reduction in animal numbers required. Taking these factors in isolation, for the great majority of scientific investigations, there would be welfare and scientific merit in recommending that all animals used in scientific procedures be purpose-bred. However, before making such a recommendation, there are a number of other important factors that have to be considered. The consequences of inclusion of all species could, for example, result in loss of genetic diversity, the generation of large numbers of surplus animals and significant delays in scientific progress. A risk assessment approach has therefore been taken to this issue, with the group analysing the potential benefits for and the adverse consequences of the inclusion of each species in Annex I. Two issues have been considered: animal welfare and scientific quality. For each, three steps have been followed: identification of the hazards, exposure assessment and consequence assessment.

The criteria suggested by the Technical Expert Working Group (TEWG) organised by DG ENV (2003) have been considered and incorporated into an assessment process against which the inclusion of each of the commonly used laboratory species was reviewed. The criteria considered by the AHAW panel have been whether legislation already exists to protect animal welfare, genetically altered animals, health and genetic quality of animals, demand, extrapolation of results to farming or to wild populations and capture from the wild.

It is recommended that, wherever possible, animals used should be of a uniform standard so that there is good and effective control over the animals' genetic fidelity, microbial status, nutrition, socialisation to humans and other animals (e.g. ferrets, dogs and even rodents) and environment. Ideally all animals should be purpose bred but, in practice, some exceptions will be necessary. Exceptions should be made to purpose breeding when it is necessary for the research that a particular strain or breed is used, or that scientific progress would be unduly delayed providing that the scientific data resulting from such research were considered likely to be of good quality, i.e. the competent authorities should consider the potential adverse consequences for research should an exemption for the use of non-purpose bred animals be refused (86/609/EEC: Article 19(4)). Genetically altered animals (of all species) should be added to Annex I. The review of all the commonly used laboratory species has concluded that with the exception of quail (*Coturnix coturnix*) all the other species listed should continue to be

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purpose-bred and some further species should be added, namely: Chinese hamster (*Cricetus griseus*), Mongolian gerbils (*Meriones unguiculatus*), two *Xenopus* species (*X. laevis* and *X. tropicalis*) and two species of *Rana* (*R. temporaria* and *R. pipiens*).

3. Summary of humane methods of killing animals (Question 4)

Nearly all animals are killed at the end of a research project and it is important that this is done humanely i.e. causing as little suffering as possible for the animals concerned. The majority (85-90%) of animals used in research are small rodents however, of necessity (as we are trying to cover all methods for all animals), much of the Report deals with the methods for large animals. The Opinion of the scientific panel on AHAW is based on the Report annexed to this Opinion that presented recent data building on the three earlier authoritative reports on the humane killing of animals i.e.: 1) the Scientific Report related to welfare aspects of animal stunning and killing methods of the main commercial species of animals (EFSA, 2004, http://www.efsa.eu.int); 2) Close *et al.* 1996/1997 (endorsed by the EU for the humane killing of laboratory animals); and 3) the AVMA Report (2000) dealing with methods for all animals. The Opinion does not repeat what is already dealt with in detail in those reports but we have included a section dealing with new data for each method where applicable, and some conclusions and recommendations are retained. The Scientific Report and Opinion deal with the various technical ways of killing animals starting with electrical and mechanical methods, followed by gaseous and then injectable methods. The section on the use of gaseous agents is in some considerable detail as it is the subject of much new data, with more than 20 new papers in the past 10 years, many of them dealing with the commonest laboratory animals. The interpretation of this data has been varied. The recommended methods for each species are given in Tables 1 to 8 at the end of this section but, in general, we have adopted the recommendations given in the existing EU Guidance (Close *et al.*, 1996/97) except where stated. The AHAW panel suggested that these methods could be varied but only with a scientific justification and appropriate authority, i.e. the recommended methods represent the default position. We also address more general issues including ensuring death, training of personnel, killing animals for their tissues and oversight, the choice of method and when this might affect the scientific outcomes, and gathering information on methods used as well as their efficiency and effectiveness.

Key words

Animal research, experimental animals, animal welfare, invertebrate sentience, fetal sentience, purpose breeding, euthanasia.

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Table of Contents

Summary 2

Table of Contents 6

1. Terms of Reference 8

1.1. Background 8

1.2. Mandate 8

1.2.1. Question 1 on the sentience of invertebrate species, and fetal and embryonic forms of both vertebrate and invertebrate species 8

1.2.2. Question 2 on fetal and embryonic forms 9

1.2.3. Question 3 on purpose-bred animals 10

1.2.4. Question 4 on humane methods of euthanasia 11

1.3. Approach 11

2. QUESTION ON THE SENTIENCE OF INVERTEBRATE SPECIES, AND ON FETAL AND EMBRYONIC FORMS OF BOTH VERTEBRATE AND INVERTEBRATE SPECIES. 13

2.1. Memory and Learning in Invertebrates 13

2.2. Nociception and Pain in Invertebrates 13

2.3. Non-vertebrate groups 14

2.3.1. Cyclostomes (lampreys and hagfish). 14

2.3.2. Amphioxus 14

2.3.3. Tunicate 14

2.3.4. Hemichordata such as Balanoglossus 15

2.3.5. Cephalopods (octopods, squid, cuttlefish, nautiloids) 15

2.3.6. Land gastropods 15

2.3.7. Tectibranch and nudibranch molluscs 16

2.3.8. Social insects 16

2.3.9. Other insects 16

2.3.10. Spiders, especially jumping spiders 16

2.3.11. Decapod crustaceans (lobsters, crabs, prawns etc.) 16

2.3.12. Isopods (woodlice and marine species) 17

2.3.13. Other phyla (e.g. Annelida, Echinodermata, Platyhelminthes, and Nematoda) 17

2.4. Fetal and embryonic animals which might be protected 17

2.5. Implications for the definition of a “protected animal” 19

3. QUESTION ON PURPOSE-BRED ANIMALS 21

3.1. Key criteria to be considered for being purpose bred and inclusion in Annex I: 21

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3.2. Conclusions and Recommendations 21
4. QUESTION ON HUMANE METHODS OF EUTHANASIA **25**
4.1. Reasons for euthanasia: 25
4.1.1. Scientific reasons 25
4.2. Education, training and competence of those carrying out humane killing: 26
4.3. Killing animals for their tissues: 26
4.4. Gathering information 27
4.5. Methods of euthanasia 27
4.5.1. Electrical stunning 27
4.5.2. Mechanical stunning methods 28
4.5.3. Mechanical disruption of tissues (Neck dislocation, decapitation, maceration) 28
4.5.4. Physical methods 29
4.5.5. Gaseous methods 30
4.6. Humane killing of cephalopods, cyclostom es, decapods (if accepted) 32
5. Tables with the recommended methods for the humane killing of animals in laboratory. ..**34**
6. DOCUMENTATION PROVIDED TO EFSA **42**
6.1. REFERENCES 42
7. AHAW Scientific Panel Members **43**
8. ACKNOWLEDGEMENTS **46**

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1. Terms of Reference

1.1. Background

Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes provides for controls of the use of laboratory animals, it sets minimum standards for housing and care as well as for the training of personnel handling animals and supervising the experiments.

Since 1986, important progress has been made in science and new techniques are now available, such as use of transgenic animals, xenotransplantation and cloning. These require specific attention, which the current Directive does not provide. Nor is the use of animals with a higher degree of neurophysiological sensitivity such as non-human primates specifically regulated. Therefore, Directorate-General Environment (DG ENV) has started revising the Directive.

The revision addresses issues such as compulsory authorisation of all experiments, inspections, severity classification, harm-benefit analysis and compulsory ethical review. Also specific problems relating to the use and acquisition of non-human primates will be tackled.

In 2002, as part of the preparatory work for the revision, DG ENV requested the opinion of the Scientific Committee on Animal Health and Animal Welfare, SCAHAW, on the welfare of non-human primates used in experiments. This Opinion, adopted by SCAHAW on 17 December 2002, was made available to the TEWG. The Opinion already provides some information especially concerning the requirements for purpose-bred animals and the question on gestation for non-human primates.

In 2003, DG ENV organised a Technical Expert Working Group (TEWG) to collect scientific and technical background information for the revision. The experts from Member States, Acceding Countries (which are now the new Member States), industry, science and academia as well as from animal welfare organisations worked through a set of questions prepared by DG ENV. The results of the TEWG provide an important input for the revision of the Directive. However, the TEWG highlighted four specific questions requiring further scientific input. These questions are detailed below. The final reports of the TEWG are provided as background documents.

1.2. Mandate

1.2.1. *Question 1 on the sentience of invertebrate species, and fetal and embryonic forms of both vertebrate and invertebrate species*

1.2.1.1. Detailed background on invertebrate species

The following definitions are applied in the current Directive:

"'animal' unless otherwise qualified, means any live non-human vertebrate, including free-living larval and/or reproducing larval forms…"

"'experiment' means any use of an animal for experimental or other scientific purposes which may cause it pain, suffering, distress or lasting harm, including

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any course of action intended, or liable, to result in the birth of an animal in any such condition, but excluding the least painful methods accepted in modern practice (*i.e.* 'humane' methods) of killing or marking an animal"

The TEWGs and other experts recommended to enlarge the scope to include **invertebrate species** provided there is sufficient scientific evidence as to their sentience and capacity to "experience pain, suffering, distress or lasting harm". Certain species of invertebrates are already included in the national legislation of some countries, both within and outside the EU (*e.g.* UK, some Scandinavian countries, Australia Capital Territories, New Zealand). The UK currently only includes *Octopus vulgaris* in its national legislation but is considering the inclusion of additional cephalopod species.

1.2.1.2. Terms of reference of question 1

In view of the above, the Commission asks the European Food Safety Authority to issue a scientific opinion on:

- the sentience and capacity to "experience pain, suffering, distress or lasting harm" of all invertebrate species used for experimental purposes.

1.2.2. *Question 2 on fetal and embryonic forms*

1.2.2.1. Detailed background on fetal and embryonic forms

The definition of 'animal' in the current Directive excludes fetal or embryonic forms.

According to TEWG and other experts, fetal and embryonic forms should be brought under the scope of the Directive in case there is enough scientific evidence on their capacity to "experience pain, distress or lasting harm".

Some Member States have included in their national legislation such forms beyond a certain stage of pregnancy. A criterion for determining the appropriate stage of pregnancy may be the development of the cerebral cortex and when it reaches a stage at which it can register sensory experiences.

The view of several members of the TEWG was that a time limit of half way through the gestation period should be used, at least for all large mammalian species other than rodents. This was based on data relating to sheep and non-human primates whilst providing for a 'safety margin' with regard to the ability of fetuses/embryos of these species to feel pain. However, the TEWG could not reach a consensus on when a rodent fetus or new-born may be capable of suffering, although they suggested that the final 20% of pregnancy may be appropriate for rodent and poultry species.

1.2.2.2. Terms of reference of question 2

In view of the above, the Commission asks the European Food Safety Authority to issue a scientific opinion on:

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- The stage of gestation after which the fetus/embryo of the species in question is assumed to be capable of "experiencing pain, suffering, distress or lasting harm",

- whether a generic rule for a cut-off point for the advancement of gestation can be indicated for those species where insufficient scientific data exist to establish a species-specific cut-off point.

1.2.3. *Question 3 on purpose-bred animals*

1.2.3.1. Detailed background on purpose-bred animals

Species listed in Annex I to Directive 86/609/EEC are those that must be 'purpose bred' when used in experiments (unless a specific exemption has been obtained). The criteria for inclusion of species in Annex I have not been clearly defined and no information is available on why the various species were originally included.

For example, mini-pigs which have become a widely-used laboratory species, obtained from commercial suppliers where they are bred in a controlled environment similar to that to be experienced at user facilities. According to the TEWG, their inclusion in Annex I would therefore appear logical and in the interest of sound principles of scientific research and welfare. Other species to be considered for inclusion could be ferrets and some hamster species in addition to *Mesocricetus auratus*. Conversely, the current inclusion of quail (*Coturnix coturnix*) should be re-considered.

The TEWG proposed multiple criteria as a basis for species inclusion into Annex I, such as:

- numbers of animals required for procedures;

- the type of procedures (*e.g.* farm animal studies/population studies);

- animal welfare aspects;

- practical and commercial aspects of establishing breeding;

- disease-free requirements;

- specific animal welfare aspects such as social deprivation, confinement and other aspects of sudden involuntary changes of living environment (use of pet or stray animals as experimental animals.)

1.2.3.2. Terms of reference of question 3

In view of the above, the Commission asks the European Food Safety Authority to issue a scientific opinion on:

- the scientific criteria that could be used to determine in which cases animals to be used in experiments should be purpose-bred, in order to safeguard *inter alia* animal welfare, using the proposal of the TEWG. The proposed criteria should also take into account other factors such as current and future needs, practicability or any specific scientific requirements.

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- Based on these criteria, determine which species currently used in experiments meet these criteria.

1.2.4. *Question 4 on humane methods of euthanasia*

1.2.4.1. Detailed background on humane methods of euthanasia

Some experimental animals are only bred to be euthanised for the purpose of using their tissues and/or organs, e.g. in the development and application of *in vitro* methods. To ensure highest possible animal welfare standards in the EU, it needs to be defined which methods of killing are scientifically the most humane and appropriate for different species of experimental animals.

1.2.4.2. Terms of reference of question 4

In view of the above, the Commission asks the European Food Safety Authority to issue a scientific opinion on:

- the methods of euthanasia which could, on the basis of current scientific knowledge and respecting good animal welfare, be justified as being the most appropriate per type of species.

- To specify these methods and their suitability for different species most commonly used in experiments.

1.3. Approach

This Scientific opinion is a scientific assessment of the needs for a revision of the Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes. It has been based on the Scientific Report accepted by the EFSA AHAW Panel. In drafting this Scientific Opinion, the panel did not take into consideration any ethical, socio-economic, human safety, cultural or religious aspect of the topic, the emphasis has been to look at the scientific evidence and to interpret that in the light of the terms of reference.

The three working groups (WGs) were set up to address these questions with relevant experts being appointed as members.

This scientific opinion comprises 3 parts / Chapters in response to the 4 questions posed by the Commission (see Section 1.2). Questions 1 and 2 overlapped in scope essentially dealing with sentience of both fetal forms and invertebrates, and are addressed in *Chapter 2*. Questions 3 and 4 remain separate and as they are given in the mandate. They cover purpose breeding of animals (*Chapter 3*), and euthanasia of the commonly used species (*Chapter 4*). It was decided that if in Chapter 2, some species were to be recommended to receive protection, then the report and opinion should also address the question of whether they should be purpose bred in Chapter 3, and how they could be humanely killed in Chapter 4.

A full assessment and the risk profiles can be found in the Scientific Report, published on the EFSA web site, which were drafted by three Working Groups set up by the AHAW Panel.

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The Tables 1-8, at the end of the Opinion are taken from Close *et al.* 1996, 1997 and have been modified according to the Scientific Report and update the EU recommendations on humane methods of killing protected animals.

As part of the approach by EFSA two Stakeholders consultation meetings were held on 18th February and the 31st August 2005. At the first meeting Stakeholders were asked to comment on the mandate from the Commission and on the proposed method working. Stakeholders were asked to propose scientific experts, not organisational representatives, that EFSA could call on for help in the working groups (WGs), and to provide any background scientific papers that the WGs might find useful. The suggestions made were very helpful. The scientific experts were selected by EFSA on the basis that they had made a significant contribution to the topic under review in the past 5 years or, where there was no or little scientific data, that they had relevant and appropriate experience. A draft of the Scientific Report (including the proposed recommendations) was sent out on the 28th July for the Stakeholders to seek comments from their members in time for the meeting on the 31st August. At that meeting views were sought from the Stakeholders on the draft Report and the WG's conclusions and recommendations. After Aug 31st Stakeholders were given another 7 days to reconsider their views in the light of the responses from other Stakeholders to make a written response to EFSA on their final views. These views were then considered by the WGs in their preparation of their final Report.

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2. QUESTION ON THE SENTIENCE OF INVERTEBRATE SPECIES, AND ON FETAL AND EMBRYONIC FORMS OF BOTH VERTEBRATE AND INVERTEBRATE SPECIES.

All invertebrate animals were considered and our recommendations propose some groups as "protected animals".

2.1. Memory and Learning in Invertebrates

Conclusion: The memory and learning of invertebrates has been widely investigated. It has been shown that invertebrates are capable of learning in several ways very similar to vertebrates: for example, slugs are capable of first- and second-order conditioning, blocking, one-trial associative learning and appetitive learning (Yamada *et al.*, 1992). In a comprehensive review of invertebrate learning and memory, Carew and Sahley (1986, p. 473) were so impressed by the learning capabilities of invertebrates they were moved to write -

"In fact, the higher-order features of learning seen in some invertebrates (notably bees and *Limax*) rivals that commonly observed in such star performers in the vertebrate laboratory as pigeons, rats, and rabbits."

2.2. Nociception and Pain in Invertebrates

Summary: In respect to brain and nervous complexity, there is no doubt that invertebrates have simpler nervous systems than vertebrates, but does this mean they are unable to suffer? The cerebral cortex is thought to be the seat of consciousness in humans (Smith and Boyd 1991). In fact, pain and suffering are sometimes defined in terms of neural activity in the cerebrum, which makes it a rather circular argument to then dismiss the possibility of invertebrates being capable of suffering because they lack such a structure. It is possible that other structures, as yet undetermined, within the brain or elsewhere fulfil a similar function to the cerebrum in terms of processing information related to suffering. Analogous yet disparate structures have evolved throughout the animal kingdom. For example, the compound eye of some invertebrates is strikingly different in form from the mammalian eye, yet they both achieve the same function - they allow the animal to perceive light. Parts of the nervous system of invertebrates that are not the anterior brain are capable of controlling breathing, movement and learning (e.g. octopuses, cockroaches). Possibly, areas of invertebrate nervous tissue have evolved abilities analogous to the cerebrum of mammals and give these animals the capacity to suffer. Above all, we should remember that absence of evidence is not evidence of absence.

Conclusion 1: It is often suggested that indicators of an animal's capacity to experience suffering include long-term memory, plasticity of behaviour, and 'higher' learning. Many invertebrate species:

- Possess short and long term memory;
- Exhibit higher learning such as social learning, conditioned suppression, discrimination and generalisation, reversal learning;

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- Show great spatial awareness and form cognitive maps (possibly indicating self-awareness);
- Appear to show deception (possibly indicating they possess a theory of mind);
- Perform appropriately in operant studies to operate a manipulandum or change the environment in some way to gain reinforcement or avoid punishment.

Conclusion 2: Regarding the possibility of invertebrates experiencing pain, many invertebrate species:

- possess receptors sensitive to noxious stimuli connected by nervous pathways to a central nervous system;
- possess brain centres;
- possess nervous pathways connecting the nociceptive system to the brain centres;
- possess receptors for opioid substances;
- after having had analgesics, modify their responses to stimuli that would be painful for a human;
- respond to stimuli that would be painful for a human in a functionally similar manner (that is, respond so as to avoid or minimise damage to the body);
- show behavioural responses that persist and show an unwillingness to resubmit to a painful procedure; they can learn to associate apparently non-painful with apparently painful events.

2.3. Non-vertebrate groups

2.3.1. *Cyclostomes (lampreys and hagfish).*

Conclusion: Cyclostomes have a pain system similar to that of other fish and brains which do not differ much from those of some other fish.

Recommendation: Cyclostomes should be in Category 1 (see Section 2.5) and so receive protection.

2.3.2. *Amphioxus*

Conclusion: In general, insufficient is known about whether amphioxus are able to experience pain and distress

Recommendation: Given our present state of knowledge amphioxus should be in Category 3 (see Section 2.5) and not receive protection at present.

2.3.3. *Tunicate*

Conclusion: Free swimming larval forms and pelagic adult tunicates show responses which may indicate complex processing of stimuli but little information on this topic

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is available. The free-swimming adult and larval tunicates are similar in form and in some aspects of behaviour to amphibian tadpoles but most are smaller.

Recommendation: Given our present state of knowledge tunicates should be in Category 3 (see Section 2.5) and not receive protection at present.

2.3.4. *Hemichordata such as Balanoglossus*

Conclusion: *Balanoglossus,* the acorn worm, lives on the bottom in marine environments. There is no indication from its behaviour that it has any sophisticated brain function.

Recommendation: Given our present state of knowledge *Balanoglossus* should be in Category 2 (see Section 2.5) and not receive protection.

2.3.5. *Cephalopods (octopods, squid, cuttlefish, nautiloids)*

Conclusion: There is evidence that cephalopods have a nervous system and relatively complex brain similar to many vertebrates, and sufficient in structure and functioning for them to experience pain. Notably, they release adrenal hormones in response to situations that would elicit pain and distress in humans, they can experience and learn to avoid pain and distress such as avoiding electric shocks, they have nociceptors in their skin, they have significant cognitive ability including good learning ability and memory retention, and they display individual temperaments since some individuals can be consistently inclined towards avoidance rather than active involvement. Most work on learning ability has been carried out in the non-social but visually very competent *Octopus vulgaris*. All squid, cuttlefish and octopods (coleoid cephalopods) studied have a similar ability to sense and learn to avoid painful stimuli, and many are more complex and more likely to experience pain and distress than *O. vulgaris*. Learning is involved in most signalling and the most elaborate signalling and communication systems occur in cuttlefish and squid that can show rapid emotional colour changes and responses to these. Indeed many of these animals live in social groups and hence may have levels of cognitive ability like those of vertebrates that have complex social relationships. Nautiloids have less complex behaviour than coleoid cephalopods and much less is known about their learning ability. They use odour discrimination to find mates and respond to and track other individuals of their own species (Basil 2001, 2002) but little is known about their pain system and it is not clear whether they are as capable of suffering as other cephalopods. However, they live for a long time and are active pelagic animals so we cannot be sure about their level of awareness.

Recommendation: All cephalopods should be in Category 1 (see Section 2.5) and so receive protection.

2.3.6. *Land gastropods*

Conclusion: Snails and slugs can show quite complex learning but the relatively slow locomotion of most of them does not enable them to show rapid escape responses, except for localised movements like eye withdrawal. The case for a substantial degree of awareness would appear to be weak.

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Recommendation: Given our present state of knowledge land gastropods should be in Category 2 (see Section 2.5) and not receive protection

2.3.7. *Tectibranch and nudibranch molluscs*

Conclusion: The most active marine gastropod molluscs are the tectibranchs, such as *Aplysia* and some of the nudibranchs (sea slugs). Much research has been carried out on the nervous system of *Aplysia* and it relatives. Evidence of learning and flexibility of behaviour is considerable but there are also studies showing very rigid responses. Nudibranchs appear to be less flexible than some tectibranchs.

Recommendation: Given our present state of knowledge tectibranch and nudibranch molluscs should be in Category 2 (see Section 2.5) and not receive protection.

2.3.8. *Social insects*

Conclusion: The social ants and bees, and to a lesser extent the wasps and termites, show considerable learning ability and complex social behaviour. There is evidence of inflexibility in their behaviour but the trend in recent research has been to find more flexibility. The small size of the brain does not mean poor function as the nerve cells are very small. A case might be made for some bees and ants to be as complex as much larger animals. They might be aware to some extent but we have little evidence of a pain system.

Recommendation: Given our present state of knowledge social insects should be in Category 3 (see Section 2.5) and not receive protection

2.3.9. *Other insects*

Conclusion: There is a difference in complexity of behaviour between the social and non-social insects. However, learning is clearly possible in these animals. There is little evidence of awareness but few people have looked for it.

Recommendation: Given our present state of knowledge other insects should be in Category 2 (see Section 2.5) and not receive protection.

2.3.10. *Spiders, especially jumping spiders*

Conclusion: In recent years, dramatic evidence has been produced of the sensory processing, analytical and prediction ability of salticid spiders. The eyes are large and complex and although the brain is composed of a relatively small number of cells, the level of processing is considerable and sophisticated, if rather slow. Evidence for awareness is greater than in any other invertebrates except cephalopods but we have little evidence of a pain system.

Recommendation: Given our present state of knowledge spiders should be in Category 3 (see Section 2.5) and not receive protection at present.

2.3.11. *Decapod crustaceans (lobsters, crabs, prawns etc.)*

Conclusion: The largest of these animals are complex in behaviour and appear to have some degree of awareness. They have a pain system and considerable learning

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ability. Little evidence is available for many decapods, especially small species. However, where sub-groups of the decapods, such as the prawns, have large species which have been studied in detail they seem to have a similar level of complexity to those described for crabs and lobsters.

Recommendation: All decapods should be in Category 1 (see Section 2.5) and so receive protection.

2.3.12. ***Isopods (woodlice and marine species)***

Conclusion: Learning is clearly possible in these animals and some of them live socially. The degree of complexity of functioning is lower than that of the larger decapods or many insects and spiders.

Recommendation: Given our present state of knowledge isopods should be in Category 2 (see Section 2.5) and not receive protection.

2.3.13. ***Other phyla (e.g. Annelida, Echinodermata, Platyhelminthes, and Nematoda)*** not described above, as well as other Classes, have been considered but are not thought to need protection and therefore have all been placed in Category 2

2.4. Fetal and embryonic animals which might be protected

Summary: Even though the mammalian fetus can show physical responses to external stimuli, the weight of present evidence suggests that consciousness does not occur in the fetus until it is delivered and starts to breathe air. However, events *in utero* can influence the behaviour of the individual once it is born, and some of those effects could be important to its subsequent welfare. Precocial oviparous species present much evidence of being conscious at hatching, and during the last days before hatching.

Conclusions

1. Precocial oviparous species, some of which are breathing at the time of hatching present much evidence of being aware before hatching and during the last days before hatching, perhaps for as much as the last third of their development. They are often capable of independent life if removed from the egg during the last few days before hatching. Altricial oviparous species and species with larval forms do not develop awareness until a later age. For all oviparous species and especially for the many precocial species there is a high risk that fetuses in the egg during the last part of incubation will be affected by some experimental procedures in such a way that their welfare is poor, sometimes because they are suffering pain.

2. Even though the mammalian fetus can show physical responses to external stimuli, the weight of present evidence suggests that consciousness is not the normal state in the fetus until it is delivered and starts to breathe air.

3. It is possible that in a mammalian fetus there might be transient episodes of increased oxygenation above the threshold required to support some aspects of consciousness. We have insufficient knowledge to conclude whether or not this occurs in all, or even any, fetuses. It is clear that there is a risk, perhaps a small risk, that any mammalian

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fetus may on occasion be affected by some experimental procedures in such a way that their welfare is poor, sometimes because they are suffering pain.

4. If a mammalian fetus is removed from the mother and starts to breathe, its level of awareness will change to that typical of such animals after parturition.

5. Emotional stresses experienced by a pregnant mother mammal can influence the behaviour of the offspring after it is born and some of those effects could be important to the offspring's subsequent welfare. It may be that the effects are mediated via nutrition or other means from the mother or it may be that the fetus experiences these effects directly.

6. The fetus in oviparous species, especially those which are precocial, can react to and learn from experiences received during the last few days of incubation.

7. For most vertebrate animals and cephalopods, the stage of development at which there is little risk of poor welfare when a procedure is carried out on them is the beginning of the last third of development during incubation or pregnancy. Before that time the risk to animal welfare is not thought to be significant. For some species this may be earlier but we have not been able to compile a database of species and fetal forms at which some form of protection was assessed as being necessary.

8. For fish, amphibians and cephalopods which develop in water, functioning has many similarities to that of adult fish once they start to feed independently rather than being dependent on the food supply from the egg.

9. The protection of the animals recommended to be included as a protected animal in Chapter 2 poses practical problems during the early stages of their development when they will be microscopic.

Recommendations

1. Whenever there is a significant risk that a mammalian fetus or the fetus or embryo of an oviparous animal such as a bird, reptile, amphibian, fish or cephalopod is for any length of time sufficiently aware that it will suffer or otherwise have poor welfare when a procedure is carried out on it within the uterus or egg, such animals should receive protection. The stage of development at which this risk is sufficient for protection to be necessary is that at which the normal locomotion and sensory functioning of an individual independent of the egg or mother can occur. For air-breathing animals this time will not generally be later than that at which the fetus could survive unassisted outside the uterus or egg.

2. Once a fetus is removed from the uterus or egg, if it is capable of breathing such animals should receive protection.

3. As a guideline, and because of the risk that even mammals in utero may sometimes be aware at times before parturition, when a procedure is performed on a fetus that is likely to produce pain in the newborn of that species, adequate anaesthesia and analgesia should be given provided that the agents used do not significantly increase the likelihood of fetal mortality. In the circumstance where no suitable anaesthetic or analgesic agents are available, procedures should not be carried out on such fetuses.

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When the procedure might cause a lasting inflammatory response that persists post-natally, protection should be given against pain and suffering.

4. A schedule of anaesthetics and analgesics that are suitable for use in pregnant animals, oxygenated fetuses and newborn animals should be prepared.

5. Protection against pain and distress during any procedures that might cause these, should be given to any precocial birds or reptiles, for example domestic chicks, that are breathing before hatching.

6. In order to avoid the risk that a fetus, whether it is developing in the mother or in an egg outside the mother, will be affected by some experimental procedures in such a way that its welfare is poor, sometimes because it is suffering pain, it should receive protection if it is in the last third of its development during incubation or pregnancy. This recommendation should be taken together with those above in order that any species at an appropriate stage of development will be protected.

7. Protection may need to be given against emotional states in pregnant mothers to safeguard subsequent behavioural modification and welfare of the offspring. This needs to be considered on a case-by-case basis.

8. In order to avoid the risk that a fish, amphibians, cephalopods or decapods will be affected by some experimental procedures in such a way that its welfare is poor, sometimes because it is suffering pain, it should be included in the list of protected animals receive protection if it is capable of feeding independently rather than being dependent on the food supply from the egg. This food supply is carried around by young fish etc. after emerging from the egg but the young animal is not independent of it for some time. The point of development at which complex function is possible is predicted well by independent feeding.

2.5. Implications for the definition of a "protected animal"

While the principal reason for the existence of legislation is to harmonise the implementation of the Three Rs of Replacement, Reduction and Refinement. This would imply that it is important to define the term "protected animal" and other animal forms which are to be protected during experimental and other research work.

When experiments are carried out in vivo (literally meaning scientific procedures involving a living animal with its whole body systems intact) a key point is whether the animal is able to experience pain and distress and other forms of suffering. The inclusion, therefore, of invertebrates and fetal forms from certain stages of gestation, as well as vertebrates, based on the information given in Chapter 2, is essential information for risk management. The WG have tried to give guidance on that issue with the criteria used to do so. The use of terms such as free-living, capable of independent feeding etc are fraught with difficulties as they do not allow all animals forms at all stages of development to be clearly distinguished on the basis if their ability to experience pain, distress etc. There are however, some worthwhile analogies that can be made, so that more complex forms are more likely to be sentient than simple forms i.e. independent feeders are more likely to be sentient than sessile free living forms,

The WG is proposing therefore, that three categories be established.

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Category 1 - The scientific evidence clearly indicates that those groups of animals are able to experience pain and distress, or the evidence, either directly or by analogy with animals in the same taxonomic group(s), are able to experience pain and distress.

Category 2 - The scientific evidence clearly indicates that those groups of animals are NOT able to experience pain and distress, or the evidence, either directly or by analogy with animals in the same taxonomic group(s), are unable to experience pain and distress.

Category 3 - Some scientific evidence exists that those groups of animals are able to experience pain and distress, either directly or by analogy with animals in the same taxonomic group(s), but it is not enough to make a reasonable risk assessment on their sentience to place them in either Category 1 or 2.

Any such categorisation of animals and their forms will need updating as scientific knowledge accumulates.

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3. QUESTION ON PURPOSE-BRED ANIMALS

Including a species as "purpose-bred" within Annex I will confer a considerable degree of assurance that animals of that species will be provided with suitable accommodation, welfare and care practices. As a consequence of health and colony management within breeding establishments, there can be improved confidence in the quality of the animal, resulting in improved science and a reduction in animal numbers required. Taking these factors in isolation, for the great majority of scientific investigations, there would be welfare and scientific merit in recommending that all animals used in scientific procedures be purpose-bred. Before making such a recommendation, there are a number of other important factors that have to be considered and there will have to be exceptions to this in some areas of research e.g. studies into the normal biology of a species, commercial strains and veterinary clinical research. The consequences of inclusion of all species could, for example, result in loss of genetic diversity, the generation of large numbers of surplus animals and significant delays in scientific progress, breeding wild animals in captivity could be detrimental to their health and welfare.

A risk assessment approach has therefore been taken to this issue, with the group analysing the potential benefits and adverse consequences of inclusion of each species in Annex I.

3.1. Key criteria to be considered for being purpose bred and inclusion in Annex I:

1. Other legislation already protecting animal welfare - Absence of any relevant animal welfare legislation is a reasonable criterion for inclusion into Annex I.

2. Genetically altered animals - Welfare requirements for GAA are more likely to be met if purpose bred.

3. Health and genetic fidelity of animals - Animals that are purpose bred are likely to be of high health status and genetic fidelity.

4. Demand - Species with low or widely fluctuating demands are reasons for not including in the Annex I.

5. Extrapolation of results to farming or to wild populations - Species primarily used in studies where the data are extrapolated, for example, to commercial farming production, or ecological studies in wild animals, is a reason for not including them in Annex I.

6. Capture from the wild - Capturing a species from the wild for use in a laboratory is a major welfare concern and is, therefore, an important criterion for inclusion of the species in Annex I. Purpose breeding primates may in some cases be the only alternative source to capture in the wild.

3.2. Conclusions and Recommendations

Specific conclusions and recommendations with regard to species where changes might be made to their particular purpose bred status are given in the Tables from the Scientific Report (Appendices 1 - 7). See below.

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Conclusion 1: Purpose-breeding is considered to be an important measure of producing high quality animals for research, to minimise inter-animal variability thus reducing the overall number required, and to promote improved welfare for the animals as well as the scientific outcomes. Therefore, the most appropriate animals in most cases will be purpose bred.

Recommendation 1: For most areas of research it is appropriate that the animals used should be of a uniform standard so that there is good and effective controls over the animals' genetic fidelity, microbial status, nutrition, socialisation to humans and other animals (e.g. ferrets, dogs and even rodents) and environment. The most appropriate animals should be used for research. In most cases, these will be purpose bred. The use of non-purpose breed animals will require appropriate justification.

Conclusion 2: Purpose breeding some species of animals that are not frequently used, or that are needed for a narrow area of research, or whose demand fluctuates widely, or that are protected by other legislation, or that have long gestation periods, could all result in difficulties in obtaining suitable animals for research programmes. At best this could delay scientific progress and could result in the abandonment of some research programmes.

Recommendation 2: Exceptions should be made to purpose breeding when it is necessary for the research that a particular strain or breed is used, or that scientific progress would be unduly delayed providing that the scientific data resulting from such research was of good quality, i.e. the competent authorities should consider the potential adverse consequences for research should an exemption for the use of non-purpose bred animals be refused (Council Directive 86/609/EEC: Article 19(4)).

Conclusion 3: Welfare requirements for genetically altered animals are more likely to be met if they are purpose bred.

Recommendation 3: Genetically altered animals should be purpose-bred unless an exemption is authorised by the Competent Authority. An exemption should only be approved where good evidence is provided that any genetic alteration does not cause the animals pain, suffering, distress or lasting harm, and is unlikely to cause such suffering in subsequent generations.

Conclusion 4: The process of genetic alteration can produce, either intentional adverse effects, or as an unexpected consequence of the alteration produce unexpected adverse effects, both of which require that animals are provided with specialist husbandry and care. Failure to provide appropriate accommodation and care practices could adversely affect animal welfare and scientific outcomes.

Recommendation 4: Genetically altered animals of all protected species and forms should be added to Annex I but can be exempted if it is shown that there are, or likely to be, no serious adverse effects on the animals in their future environment and the way they are used (e.g. future breeding programmes).

Conclusion 5: Because the welfare of the animals and the scientific validity of the data are inextricably linked with good quality care and husbandry of animals it is important that all those who come into contact with the animals are adequately educated, trained and skilled on an ongoing basis. This is more likely to happen when animals are purpose bred.

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Recommendation 5: In registered breeding and supplying establishments personnel should be properly trained and only competent staff should be given responsibility for the care and husbandry of animals.

Conclusion 6: Inclusion of a species in Annex I requires that animals will be purpose-bred for research purposes. The inclusion of such an Annex is considered to have welfare and scientific benefits. The review of all the commonly used laboratory species has concluded that with the exception of quail (*Coturnix coturnix*) all the other species listed should continue to be purpose-bred. The review also concluded that some further species should be added.

Recommendation 6: The criteria for purpose bred animals and the current guidelines on accommodation and care included in Annex II (and any revision) which is expected in the future to be revised to reflect the revised Appendix A of Council of Europe Convention (1986) ETS 123 should apply irrespective of the origin of the experimental animals. In making this recommendation it is appreciated that in practice not all establishments will at present meet these criteria, but nonetheless all establishments should be strongly encouraged to make progress towards these in a timely manner.

Conclusions in relation to specific species used in research

Hamsters

Conclusion 7: Syrian hamsters are the most commonly used of all the 'hamster types' and, at present, are included in Annex I. However, from an analysis of scientific papers through PUBMED, Chinese hamsters are also commonly used, and only very few European and Djungarian hamsters.

Arguments against inclusion of all hamster species: The small numbers of European and Djungarian hamsters used would make difficulties to match supply and demand leading to delays in scientific programmes

Arguments for inclusion of all hamster species: It would be likely that there would be an improved and more uniform health quality. Moreover no other welfare legislation exists.

Recommendation 7: Retain Syrian hamsters and include Chinese hamsters. No compelling need to include any other hamster species.

Gerbils

Conclusion 8: The commonest gerbil used in research is the Mongolian *(Meriones unguiculatus)* which is not in Annex I.

Arguments against inclusion: Difficulties to match supply and demand that may lead to some delays in scientific programmes;

Arguments for inclusion: Better and more uniform health quality; improved accommodation leading to reduced behavioural abnormalities; no other suitable welfare legislation

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Recommendation 8: To include Mongolian gerbils in Annex I *(Meriones unguiculatus*).

Quail

Conclusion 9:

Arguments for inclusion: There may possibly be better protection for quail if listed in Annex I, through improved accommodation and care practices.

Arguments against inclusion: Small numbers of *Coturnix coturnix* used. Few breeding establishments – difficult to match supply and demand.

Recommendation 9: There is no compelling need to retain *Coturnix coturnix*, nor to include any other species of quail.

Xenopus species (laevis and tropicalis), Rana species (temporaria and pipiens)

Conclusion 10:

Arguments against inclusion: Wide range of species but for many species only small numbers are used. Production of the less commonly used species, *e.g.* newts, salamanders (including axolotls) may not be practicably viable due to the very small numbers used. The purpose breeding of *Xenopus laevis* and *tropicalis* may prove to have economies of scale that make it viable. Potentially high cull rates, difficulties to match supply and demand leading to delays in scientific programmes, lack of information on husbandry and care practices.

Arguments for inclusion: better and more uniform health quality, increasing numbers of some species, no other welfare legislation, elimination of zoonotic diseases, no animals taken from wild.

Recommendation 10: *Xenopus* species (*laevis* and *tropicalis*) and *Rana* (*Rana temporaria* and *R. pipiens*) should be purpose bred.

Invertebrates such as cephalopods, cyclostomes, decapods.

Conclusion 11: The recommendation from Chapter 2 is for these phyla to receive protection during experimental work due to their potential to experience pain and distress.

Recommendation 11*:* If the recommendations put forward in Chapter 2 are accepted, there is no compelling need to include any of these invertebrate species, at the moment, in those to be purpose bred.

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4. QUESTION ON HUMANE METHODS OF EUTHANASIA

4.1. Reasons for euthanasia:

The reasons for killing animals have also to be considered, as some methods may cause more pain and distress than others. For example, breeding more animals than are required simply to have them available on demand, and then killing those that have not been used. This is especially true for animals that have a painful harmful defect caused for example by a genetic alteration. Sometimes killing of surplus is inevitable as in the breeding of some transgenic or mutant animals as only a particular genotype is wanted, and uses cannot be found for the surplus animals. On other occasions, breeding strategies can avoid having to kill such large numbers, but can also increase the numbers that have to be killed due to a balance between inducing adverse effects in all animals as opposed to just some. Archiving (freezing down) rodent strains that are currently unwanted is a way of reducing the number of animals to be culled, as is accurately forecasting the number of animals to be used.

Recommendation: One way in which any poor welfare during euthanasia could be avoided is to not have to kill animals in the first place. Therefore, the production of animals should be carefully considered so that an avoidable surplus is not generated.

4.1.1. *Scientific reasons*

Occasionally, after considering all available methods, animals may have to be killed using methods that do not meet the animal welfare criteria set out for a humane method of killing for scientific reasons e.g. using some of the recognised methods may interfere with the scientific outcome. In a choice between two or more methods of humane killing, pilot studies may be carried out to determine the method that is most suitable for the scientific purpose and for the animals concerned. This may not always be the traditional method as new methods come along, or more information is gained on old methods questioning its humaneness, or its impact on the animal, its scientific validity and, therefore, its suitability. If animals are killed using less than ideal methods then that should be justified and taken into account when carrying out the harm (cost) benefit analysis. Some methods are listed in the report that cannot be considered humane, and are identified as such. For others, where there is a lack of information, that is addressed in future research.

Because the numbers of animal killed at any one time can range from one to several hundred, the method should be appropriate to dealing with both ends of the scale, again with the minimum distress to the animals as well as to the human operators.

Recommendation 1: In a choice between two or more methods of humane killing, the scientist should choose the most appropriate and humane but where this is not known pilot studies should be carried out.

As all methods have a margin of error it is important that death is confirmed, and if necessary ensured by the use of a method, such as exsanguination, freezing, or some physical insult that results in an irreversible destruction of the brain or central nervous system, or permanent cessation of the heart.

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Recommendation 2: The death of an animal should be confirmed by a method that results in an irreversible destruction of the brain or permanent cessation of the heart.

4.2. Education, training and competence of those carrying out humane killing:

It is important that those carrying out such methods of killing are suitably trained and are deemed competent in that method (Council of Europe 1993). As nearly all methods require an element of restraint, it is equally important that they are competent in handling animals humanely.

The attitude of persons carrying out humane killing is important as over-sensitivity or a lack of care is more likely to result in poor welfare for the animals concerned. Killing animals in research establishments has been described as a kind of "initiation right" for animal care staff, and appropriate help and guidance should be available to guide young persons who are asked to do it (Arluke 1993, 1996). If senior staff members treat animals without sufficient respect, habits which lead to poor welfare may be formed in younger staff members. No-one should be coerced to kill animals, so scientists and others should be sensitive to the fact that those looking after animals did not enter this area of work to kill them; it is seen as an unavoidable, unpleasant aspect of animal care in research.

Recommendation 1: The humane killing of animals for *in vitro* and *ex vivo* research should be addressed so that persons carrying out such work are trained and competent.

Recommendation 2: A training plan should be drawn up, particularly for the use of physical methods that require a measure of manual skill, such as cervical dislocation or concussion, should incorporate a progression from the use of freshly killed animals, to anaesthetised animals, before going on to kill conscious animals. In that way there is less chance of poor welfare and poor scientific outcome due to poor technique.

4.3. Killing animals for their tissues:

Killing animals to retrieve tissues for *in vitro* work is outside the existing EU Directive (86/609/EEC), but such a use of animals is included in some countries (e.g. The Netherlands, Germany), and the number of animals used is counted giving an indication of the level of *in vitro* research by the scientific community. By including those animals killed for their tissues, the total annual number of animals used in research in those countries increased by 10 to 15%. Even though this use of animals is outside the Directive, there is EU and other national guidance on the ways by which animals should be humanely killed under laboratory conditions. Consequently, at present, research work involving killing animals by a recognised and approved method would permit, for example, researchers to kill 100 chimpanzees or dogs for a research purpose, without a licence, without oversight, and without any ethical or scientific approval. As death can be considered to be a lasting harm, it is debatable as to what level of licensing and scrutiny is required, and whether killing should be classified as a regulated procedure. In that case, animals killed for their tissues would receive the same level of care during euthanasia as an experimental animal and the staff would receive appropriate training and be certified competence as for any regulated procedure. Killing sick or injured stock animals could be exempted or encompassed.

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Opinion: The humane killing of animals for *in vitro* and *ex vivo* research that, at present, is outside the Directive could cause public concern in regard to the species, the numbers and the competence of those carrying out the killing.

4.4. Gathering information

In order to know how often poor welfare occurs during euthanasia, we need to have quality control procedures and document when things go wrong and why, and what measures have been taken to stop it happening again. It is also important to know how often the method is used successfully so that an overall picture can be gained. This will then inform future risk assessments. At present this sort of information is not available, as it is in abattoirs in some countries.

Recommendation: Information should be collected on methods of euthanasia, e.g. their success rate in terms of an efficient and effective kill and the reasons for failure.

4.5. Methods of euthanasia

General comments applying to all methods:

The WG suggested that the recommended methods can be varied but only with a scientific justification and appropriate authority, i.e. the recommended methods represent the default position.

When pregnant animals are killed, the fetuses should be allowed to die *in utero* before being removed, unless they are required for scientific reasons, in which case they should be considered as neonates and killed by another method that is appropriate for the species and that causes a minimum of pain and distress.

4.5.1. *Electrical stunning*

Conclusions: Electrical methods, at present, are only used for farm animal species.

Equipment needs to be well maintained to function well.

The outcome depends on many variables including the equipment and the current delivered and also on the particular physical characteristics of the animal that might affect the effectiveness of the method.

Recommendations: Head-only electrical stunning and-head-body killing can be recommended for the following adult species: rabbits, horses, donkeys and cross-bred equidae, pigs, goats, sheep, cattle and birds. Head-body stunning is recommended for fish. After electrical stunning an animal may recover with the consequence that it needs to be exsanguinated to be killed (or another method e.g. cooling down for fish). The unborn fetus will be killed by exsanguination or the cessation of blood supply due to heart failure of the pregnant dam.

Future Research: At present, there is considerable interest in the development in the electrical stunning of fish species. Since electrical techniques are easy to apply it may be worthwhile developing these methods for reptiles and amphibians.

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The criteria used to determine a loss of consciousness in amphibia, reptiles, some fish species, and possible some invertebrates are not well known and should be investigated.

4.5.2. *Mechanical stunning methods*

Conclusions: The penetrating captive bolt is an effective method of euthanasia for use in slaughterhouses and in research given adequate facilities in those species of animals in which the captive bolt has been specifically designed.

The equipment needs to be well maintained to function well.

Percussion stunning can be used for several species, however, there may be some doubts about effective stunning and killing in some animals. When correctly performed a concussive blow is very effective for smaller animals with ossified skulls, but it requires skill, confidence and practice (EFSA 2004).

Handling and restraint for concussive methods will cause some distress as the animal will be restrained in an unnatural position.

Recommendations: Concussive methods should not be used on animals with skulls that are not completely ossified or the sutures have not fused.

Future Research (probably depends on species): Water jet and air jet techniques and may be adaptable for many species.

4.5.3. *Mechanical disruption of tissues (Neck dislocation, decapitation, maceration)*

Conclusions:

1. Handling and restraint for neck dislocation and decapitation will cause some distress as the animal will be restrained in an unnatural position and will not be free to escape. Anaesthetising the animal first may reduce this distress.

2. After neck dislocation and decapitation electrical activity of the brain may persist for 13 s during which time animals may feel pain due to afferent stimuli from the trigeminal nerve. Cutting of the skin and tissues of the neck may cause some pain for a short period (less than one second).

3. After cervical dislocation, convulsions only occur when separation is made cranial to the fifth thoracic vertebra, while severance caudal to this location results in paralysis in conscious animals.

4. Mouse fetuses *in utero* are not killed within 20 min when the dam has been killed by cervical dislocation or decapitation. The heads of fetal rodents after decapitation may show signs of consciousness and this would be of welfare concern if the fetus had breathed (see Section 2.4).

5. After decapitation signs of consciousness may persist for some time e.g. 13 min in the heads of eels, and hours in reptiles.

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6. If the macerator is overloaded animals may be not be humanely killed.

7. All these mechanical disruption techniques are aesthetically controversial. The interpretation of the electrical activity in the brain after neck dislocation and decapitation is controversial as to what feeling remains, and is still a matter of debate.

8. Anaesthetising animals before decapitation or cervical dislocation will minimise distress and any subsequent pain. This may be required in some cases of maceration where the animal may escape the blades.

9. Tissue damage to the CNS or induced neuronal discharge may affect neuropeptide levels and brain histology.

10. Severance of the spinal cord using a knife does not render the animal immediately unconscious and so it may suffer for some short time.

Recommendations:

1. When using these techniques, cervical dislocation and decapitation, the necessary handling and restraint can be stressful for the animal and anaesthetising them first will minimise distress and eliminate any subsequent pain.

2. A purpose built mechanical device with a sharp blade should be used for decapitation.

3. When pregnant females are killed the fetal forms should be allowed to die *in utero* before being removed, unless they are required for scientific reasons, in which case they should be killed by another method as quickly as possible.

4. Severance of the spinal cord using a knife should not be used.

5. For efficient and effective killing the macerator should not be overloaded.

Future Research: Since there are doubts that some species may not be immediately unconscious after neck-dislocation, alternative techniques should be developed.

4.5.4. *Physical methods*

Conclusions: Focal irradiation of the heads (brain) of restrained small animals with microwaves of 2450 MHz for 1s suggests a rapid loss of consciousness.

Focal heating of the brain by irradiation can only be applied by using a specially and constructed designed microwave oven specific for the species.

Hypothermia is not considered an acceptable method of euthanasia because it prolongs the period of consciousness and does not reduce the ability to feel pain.

Recommendations: Heating the brain focally with appropriately designed microwaves is accepted for use in adult rats and mice by trained operators and can be used for other animals such as guinea-pigs and hamsters when they are less than 300g.

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Cooling down should not be used for any species.

Future Research: For many years, techniques using microwaves have been used for local damage of cells in cancer therapy. These techniques could be adapted to locally damage of brain tissue in a variety of species.

4.5.5. *Gaseous methods*

4.5.5.1. Exposure to carbon dioxide mixtures

Conclusions: CO2 is aversive to all vertebrates used in research that have been tested. Some species find even low (10-20% by volume in air) concentrations aversive, regardless of any additions. It cannot be recommended as a sole method of humane killing for any species. CO2 may be used as a secondary euthanasia procedure on unconscious animals.

Mouse fetuses *in utero* are not killed within 20 min even though the mother has been killed with CO2, but it is possible to kill neonatal forms with CO2.

Recommendation: Carbon dioxide should not be used as a sole agent in any euthanasia procedure unless the animal has first been rendered unconscious, i.e. it should be phased out as soon as possible. It is important that equally effective and non-aversive methods that are already partially developed, be developed further from a practical viewpoint, and that users are given time to change to those more humane gas mixtures.

It would be inappropriate to place a fully conscious animal in a known noxious gaseous environment from which it would be unable to escape.

Future Research: Research on euthanasia of animals should follow the guidelines set out by the International Association for the Study of Pain.

New methods of humane killing of animals using gas mixtures other than those containing CO2 need urgently to be developed.

The time to onset of unconsciousness has usually been determined on the basis of behaviour (e.g. ataxia) but needs to be established more clearly using defined neurophysiological criteria.

An objective method of measuring breathlessness is needed to demonstrate and quantify breathlessness in laboratory animals (especially rodents), which would enable quantification of duration and severity of distress in animals exposed to any gas mixture.

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4.5.5.2. Argon and Nitrogen as inert hypoxia inducing gases

Conclusions: It is suggested that the use of anoxia as a method of killing is humane for pigs and poultry, and probably rodents, although more practical experience is needed. Because of the high affinity for oxygen of haemoglobin in fetal and neonatal animals it may take longer than in mature animals of the same species to kill. However, no studies on time taken or welfare seem to have been carried out. More research is needed on nitrogen.

Recommendations: Research into hypoxic gas mixtures should be carried out as a matter of urgency, especially practical methods for small animals, such as rodents.

Future Research: Investigation is needed into the humaneness of killing with hypoxic and anoxic gas mixtures.

4.5.5.3. Nitrous oxide

Conclusions: Owing to human health and safety concern, nitrous oxide is not suitable for euthanasia.

Recommendations: (see Tables 1- 8)

Future Research: (probably species driven)

4.5.5.4. Carbon monoxide

Conclusions: Owing to human health and safety concern, carbon monoxide has a high risk for killing humans.

Recommendations: Under controlled conditions carbon monoxide can be used for dogs, cats and mink, however it is not recommended due to concerns for human health and safety, and also animal welfare.

4.5.5.5. Overdose of inhalation anaesthetic gases

Conclusion: Overdose of an established inhalational anaesthetic agent at a suitable concentration may cause minor distress in some species, but all such gases may be aversive at high concentrations. However, they have the advantage that restraint for administration is unnecessary.

Mouse fetuses *in utero* are not killed within 20 min even though the dam has been killed with an overdose, but neonatal forms (1-7 do) are killed.

Recommendation: Overdose of an inhalation anaesthetic agent should be considered as a humane way of killing animals providing some of the caveats relating to aversion and concentration are taken into consideration.

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Future Research: Aversion testing may need to be carried out in some species for some agents (e.g. ferrets).

4.5.5.6. Overdose of injectable anaesthetic agents

Conclusion 1: Overdose of any anaesthetic agent may well be acceptable but all agents have some drawbacks in terms of irritancy and necessary restraint for administration. Suitable for mouse neonates (8-14 do) but not fetuses *in utero*.

Conclusion 2: In some member states some chemicals for euthanasia that cause a minimum of pain and distress may not be available.

Recommendation 1: Overdose of an injectable anaesthetic agent should be considered as a humane way of killing animals providing some of the caveats relating to aversion, irritancy and restraint are taken into consideration.

Recommendation 2: Member states should try to ensure that suitable chemicals for euthanasia are available.

4.5.5.7. Lethal injection of non-anaesthetising chemicals including: Neuromuscular blocking agents; Magnesium sulphate; Potassium chloride; Exposure to Hydrogen cyanide (HCN) gas; Ketamine; T-61

Conclusion: the administration of a non-anaesthetising chemical is potentially a major welfare problem.

Recommendation: Lethal injection of non-anaesthetising chemicals should only be administered in unconscious animals.

4.6. Humane killing of cephalopods, cyclostomes, decapods (if accepted)

Decapods include several kinds of crabs, lobsters and crayfish. Neither the number of crustaceans or cephalopods used in research is known and nor the methods of killing them are known. Although humane killing of crustaceans for food is not a statutory requirement in Europe, animal welfare organisations have provided some guidelines, for example, UFAW, RSPCA). In some countries, for example New Zealand, humane killing of some species of crustaceans is covered under the Animal Welfare Act 1999.

Recommendations:

The following methods cause a minimum of pain and distress:

- Chilling in air
- Chilling in ice/water slurry
- Immersion in a clove oil bath
- Electrical methods

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The following methods are likely to cause pain and distress:

- Any procedure involving the separation of the abdomen (tailpiece) from the thorax (tailing) or removal of tissue, flesh or limbs while the crustacean is still alive and fully conscious (including when in a chilled state).
- Placing crustaceans in cold water and heating the water to boiling point.
- Placing live crustaceans into hot or boiling water.
- Placing live marine crustaceans in fresh water.
- Unfocussed microwaves to body as opposed to focal application to the head.

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5. Tables with the recommended methods for the humane killing of animals in the laboratory.

Adapted and modified Tables from Close et al. (1996/1997)

The following tables have been taken from the previous EU Report on euthanasia, and form the basis for methods of killing laboratory animals that involve a minimum level of pain and distress. The data have been largely retained and only a few recommendations have been changed. (These tables in the scientific report are numbered as 7 to 14)

Table 1 - Characteristics of methods for euthanasia of fish

Agent	Rapidity	Efficacy	Ease of use	Operator safety	Aesthetic value	Overall rating (1-5)	Remarks
MS-222	++	++	++	++	++	5	Acceptable
Benzocaine	++	++	++	++	++	5	Acceptable
Etomidate	++	++	++	++	++	5	Acceptable
Metomidate	++	++	++	++	++	5	Acceptable
Electrical	++	+	+	+	++	4	Acceptable for some species
Maceration	++	++	++	++	+	4	Only for fish less than 2 cm in length
Quinaldine	++	++	++	+	++	4	Difficult to obtain in Europe
Concussion	++	+	+	++	-	3 *	Death to be confirmed Acceptable for use by experienced personnel
Sodium pentobarbitone	++	++	-	+	++	3	May be useful for large fish, intraperitoneal injection
Cervical dislocation	++	++	+	++	-	3	Not in large fish. To be followed by destruction of the brain
Halothane	+	+	++	++	++	2	Other methods preferable. Death to be confirmed

Changed from Close et al. * was 4

The following methods may only be used on unconscious fish: pithing, decapitation and exsanguinations

The following methods are not to be used for killing fish: removal from water, whole body crushing, hypothermia, hyperthermia, 2-phenoxyethanol, carbon dioxide, diethyl ether, secobarbital, amobarbital, urethane, chloral hydrate, tertiary amyl alcohol, tribromoethanol, chlorobutanol, methyl pentynol, pyridines, electrical stunning only for some species.

Rapidity: ++ very rapid, + rapid, - slow. **Efficacy:** ++ very effective, + effective, - not effective. **Ease of use:** ++ easy to use, + requires expertise, - requires specialist training. **Operator safety:** ++ no danger, + little danger, - dangerous. **Aesthetic value**: ++ good aesthetically, +acceptable for most people, - unacceptable for many people**. Rating:** 1-5 with 5 as highly recommended

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Table 2 - Characteristics of methods for euthanasia of amphibians

Agent	Rapidity	Efficacy	Ease of use	Operator safety	Aesthetic value	Overall rating (1-5)	Remarks
MS-222	++	++	++	++	++	5	Acceptable
Benzocaine	++	++	++	++	++	5	Acceptable
Sodium pentobarbitone	+	++	-	+	+	4	Involves handling and intravenous or intraperitoneal injection
Concussion	++	++	+	++	- *	3 **	Acceptable for use by experienced personnel
T-61	+	++	-	+	+	3	Involves handling and intravenous injection
Microwave	++	++	-	+	++	3	Only for small amphibians. Not a routine procedure
Electrical stunning	+	+	+	-	-	2	To be followed immediately by destruction of the brain

Changed from Close et al. * was +, ** was 4

The following methods are only to be used on unconscious amphibians: pithing and decapitation

The following methods are not to be used for killing amphibians: hypothermia, hyperthermia, exsanguination, strangulation, carbon dioxide, diethyl ether, chloroform, volatile inhalational anaesthetics, chloral hydrate, ketamine hydrochloride, chlorbutanol, methylpentynol, 2-phenoxyethanol, tertiary amyl alcohol, tribromoethanol and urethane

Rapidity: ++ very rapid, + rapid, - slow. **Efficacy:** ++ very effective, + effective, - not effective. **Ease of use:** ++ easy to use, + requires expertise, - requires specialist training. **Operator safety:** ++ no danger, + little danger, - dangerous. **Aesthetic value**: ++ good aesthetically, +acceptable for most people, - unacceptable for many people**. Rating:** 1-5 with 5 as highly recommended

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Table 3- Characteristics of methods for euthanasia of reptiles

Agent	Rapidity	Efficacy	Ease of use	Operator safety	Aesthetic value	Overall rating (1-5)	Remarks
Sodium pentobarbitone	++	++	++	+	++	5	Acceptable, but involves handling
Captive bolt	++	++	++	+	+	5	Acceptable for large reptiles
Shooting	++	++	++	-	+	4	Acceptable only in field conditions
Concussion	+	+	+	++	-	3**	Acceptable for use by experienced personnel To be followed by destruction of the brain

Changed from Close et al. * was +; was 4

The following methods are to be used on unconscious reptiles only: pithing and decapitation

The following methods are to be used on unconscious reptiles only: pithing and decapitation
The following methods are not to be used for killing reptiles: spinal cord severance, hypothermia, hyperthermia, exsanguination, chloroform, MS-222, ether, halothane, methoxyflurane, isoflurane, enflurane, carbon dioxide, neuromuscular blocking agents, ketamine hydrochloride, chloral hydrate and procaine

Rapidity: ++ very rapid, + rapid, - slow. **Efficacy:** ++ very effective, + effective, - not effective. **Ease of use:** ++ easy to use, + requires expertise, - requires specialist training. **Operator safety:** ++ no danger, + little danger, - dangerous. **Aesthetic value**: ++ good aesthetically, +acceptable for most people, - unacceptable for many people**. Rating:** 1-5 with 5 as highly recommended

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Table 4 - Characteristics of methods for euthanasia of birds

Agent	Rapidity	Efficacy	Ease of use	Operator safety	Aesthetic value	Overall rating (1-5)	Remarks
Sodium pentobarbitone	++	++	+	+	++	5	Acceptable
T-61	++	++	+	+	++	4	Requires expertise: acceptable for small birds only (<250 g)
Inert gases (Ar, N2)	++	++	++	++	+	4	Acceptable. But more research needed for nitrogen
Halothane, enflurane, isoflurane	++	++	++	+	++	4	Acceptable
Maceration	++	++	++	++	-	4	Acceptable for chicks up to 72 h
*Cervical dislocation decapitation	++	++	-	++	- *	3 **	Acceptable for small and young birds (<250 g) if followed by destruction of the brain
Microwave	++	++	-	++	+	3	To be used by experienced personnel only and specific equipment. Not a routine procedure
Concussion	++	++	-	++	-	3	Acceptable
Electrocution	++	++	+	-	-	3	Danger to operator. Use of special equipment Other methods Preferable
Carbon monoxide	+	+	++	-	-	1	Danger to operator

Changed from Close et al. * was +; was 4

The following methods may only be used on unconscious birds: decapitation, pithing, nitrogen, potassium chloride.

The following methods are not to be used for killing birds: neck crushing, decompression, exsanguination, carbon dioxide, nitrous oxide, diethyl ether, chloroform, cyclopropane, hydrogen cyanide gas, trichlorethylene, methoxyflurane, chloral hydrate, strychnine, nicotine, magnesium sulphate, ketamine and neuromuscular blocking agents

Rapidity: ++ very rapid, + rapid, - slow. **Efficacy:** ++ very effective, + effective, - not effective. **Ease of use:** ++ easy to use, + requires expertise, - requires specialist training. **Operator safety:** ++ no danger, + little danger, - dangerous. **Aesthetic value**: ++ good aesthetically, +acceptable for most people, - unacceptable for many people**. Rating:** 1-5 with 5 as highly recommended

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Table 5 - Characteristics of methods for euthanasia of rodents

Agent	Rapidity	Efficacy	Ease of use	Operator safety	Aesthetic value	Overall rating (1-5)	Remarks
Halothane, enflurane, isoflurane	++	++	++	+	++	5	Acceptable
Sodium pentobarbitone	++	++	+	+	++	5	Acceptable
T-61	++	++	-	+	++	4	Only to be injected intravenously
*Inert gases (Ar)	++	+	++	+	+	4	Acceptable
Concussion	++	++	+	++	-	3	Other methods preferred; Acceptable for rodents under 1 kg. Death to be confirmed by cessation of circulation
Cervical dislocation	++	++	+	++	-	3	Other methods preferred; Acceptable for rodents under 150g Death to be confirmed by cessation of circulation
Microwave	++	++	-	++	+	3	To be used by experienced personnel only. Not a routine procedure
Decapitation	+	+	+	++	-	2	Other methods preferred
*Carbon dioxide	+	++	++	+	++	1 if sole agent 5 if animal unconscious	To be used when animal unconscious i.e. overall rating then based on the method to induce unconsciouness
Carbon monoxide	+	+	+	-	++	1	Danger to operator
Rapid freezing	-	+	++	++	-	0	Not acceptable

** Changed from Close et al.*

The following methods may only be used on unconscious rodents: rapid freezing, exsanguination, air embolism, potassium chloride and ethanol

The following methods are not to be used for killing rodents: carbon dioxide (when sole agent, but urgent research need for a replacement), hypothermia, decompression, strangulation, asphyxiation, drowning, nitrogen, nitrous oxide, cyclopropane, diethyl ether, chloroform, methoxyflurane, hydrogen cyanide gas, trichlorethylene, strychnine, nicotine, chloral hydrate, magnesium sulphate and neuromuscular blocking agents

Rapidity: ++ very rapid, + rapid, - slow. **Efficacy:** ++ very effective, + effective, - not effective. **Ease of use:** ++ easy to use, + requires expertise, - requires specialist training. **Operator safety:** ++ no danger, + little danger, - dangerous. **Aesthetic value**: ++ good aesthetically, +acceptable for most people, - unacceptable for many people**. Rating:** 1-5 with 5 as highly recommended

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Table 6 - Characteristics of methods for euthanasia of rabbits

Agent	Rapidity	Efficacy	Ease of use	Operator safety	Aesthetic value	Overall rating (1-5)	Remarks
Sodium pentobarbitone	++	++	++	+	++	5	Acceptable
T-61	++	++	-	+	++	4	Acceptable. Intravenous injection only
Captive bolt	++	++	-	+	+	4	Requires skill. Death to be confirmed by another method
Cervical dislocation	++	++	-	++	-	3	Acceptable for rabbits under 1 kg. Sedation prior to dislocation. Death to be confirmed by cessation of circulation
Concussion	++	+	-	++	-	3	Expertise required. Death to be ensured by another method
Electrical stunning	++	+	++	-	+	3	Death to be confirmed by another method
Microwave	++	++	-	++	+	3	To be used by experienced personnel only on small rabbits. Not a routine procedure
Decapitation	+	+	+	-	-	2	Acceptable for rabbits under 1 kg if other methods not possible
Halothane, enflurane, isoflurane	++	++	++	+	-	2	Rabbits show signs of distress
Carbon monoxide	+	+	++	-	++	1	Danger to operator
Rapid freezing	+	+	++	++	+	1	Only in fetuses under 4 kg. Other methods preferred

Changed from Close et al.: CO2 deleted

The following methods are only to be used on unconscious rabbits: exsanguination, nitrogen, potassium chloride and air embolism.

The following methods are not to be used for killing rabbits: carbon dioxide, hypothermia, decompression, strangulation, asphyxiation, drowning, nitrous oxide, cyclopropane, diethyl ether, chloroform, trichlorethylene, hydrogen cyanide gas, methoxyflurane, chloral hydrate, strychnine, nicotine, magnesium sulphate, hydrocyanic acid, ketamine hydrochloride and neuro-muscular blocking agents.

Rapidity: ++ very rapid, + rapid, - slow. **Efficacy:** ++ very effective, + effective, - not effective. **Ease of use:** ++ easy to use, + requires expertise, - requires specialist training. **Operator safety:** ++ no danger, + little danger, - dangerous. **Aesthetic value**: ++ good aesthetically, +acceptable for most people, - unacceptable for many people**. Rating:** 1-5 with 5 as highly recommended

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Table 7 - Characteristics of methods for euthanasia of dogs, cats, ferrets, foxes

Agent	Rapidity	Efficacy	Ease of use	Operator safety	Aesthetic value	Overall rating (1-5)	Remarks
Sodium pentobarbitone	++	++	-	+	++	5	Acceptable. Intravenous injection
T-61	++	++	-	+	+	4	Acceptable but only by slow intravenous Injectioninjection under sedation
Secobarbital/ dibucaine	++	++	-	+	++	4	Acceptable. Intravenous injection
Halothane, isoflurane, enflurane	++	++	+	+	++	4	Acceptable
*Shooting with a free bullet with appropriate rifles and guns.	++	++	-	-	-	4 *	Acceptable only in field conditions by specialized marksmen when other methods not possible
Captive bolt	++	++	-	++	+	3	To be followed by exsanguination
Electrocution	++	++	-	-	-	3	Use only special equipment.To be followed by exsanguination
Concussion	++	++	+	++	-	2	Only to be used on neonates.To be followed by exsanguination

Changed from Close et al. * was 1

The following methods can be used for unconscious carnivores: exsanguination, neck dislocation and potassium chloride , in order to minimise pain and distress.

The following methods are not to be used for killing carnivores: decompression, decapitation, drowning, strangulation, asphyxiation, inert gases, nitrogen, air embolism, striking chest of cats, carbonmonoxide, carbon dioxide, methoxyflurane, nitrous oxide, trichlorethylene, hydrocyanic acid, diethyl ether, chloroform, hydrogen cyanide gas, cyclopropane, chloral hydrate, strychnine, nicotine, magnesium sulphate and neuromuscular blocking agents

Rapidity: ++ very rapid, + rapid, - slow. **Efficacy:** ++ very effective, + effective, - not effective. **Ease of use:** ++ easy to use, + requires expertise, - requires specialist training. **Operator safety:** ++ no danger, + little danger, - dangerous. **Aesthetic value**: ++ good aesthetically, +acceptable for most people, - unacceptable for many people**. Rating:** 1-5 with 5 as highly recommended

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Table 8 - Characteristics of methods for euthanasia of large mammals

Agent	Rapidity	Efficacy	Ease of use	Operator safety	Aesthetic value	Overall rating (1-5)	Remarks
Sodium pentobarbitone	++	++	-	+	++	5	Acceptable by intravenous injection (all species including primates
Quinalbarbitone/ Nupercaine	++	++	-	+	++	5	Effective for horses intravenously
Captive bolt	++	++	+	+	+	5	To be followed by exsanguination
Free bullet using e.g. appropriate ammunition, rifles and guns	++	++	+	-	+	4 *	Experienced marksman. May need a method to ensure death. In field conditions only.
T-61	++	++	-	+	++	4	Acceptable by intravenous injection
**Inert gases (Ar)	++	++	+	+	+	4	Acceptable for pigs only
Electrical stunning	++	++	+	-	-	4	Use only specialised equipment. To be followed immediately by exsanguination
Concussion	++	+	-	+	+	2	To be followed immediately by exsanguination
Halothane, isoflurane, enflurane	+	+	+	+	+	2	Recommended for lambs and kids

Changed from Close et al. CO2 deleted, * was 5, ** introduced, CO2 deleted

The following methods can be used only on unconscious large mammals: exsanguination, chloral hydrate and potassium chloride, in order to minimise pain and distress.

The following methods are not to be used for killing large mammals: carbon dioxide, carbon monoxide, methoxyflurane, trichlorethylene, strychnine, nicotine, magnesium sulphate, thiopentone sodium, ketamine hydrochloride, neuromuscular blocking agents

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Rapidity: ++ very rapid, + rapid, - slow. **Efficacy:** ++ very effective, + effective, - not effective. **Ease of use:** ++ easy to use, + requires expertise, - requires specialist training. **Operator safety:** ++ no danger, + little danger, - dangerous. **Aesthetic value**: ++ good aesthetically, +acceptable for most people, - unacceptable for many people**. Rating:** 1-5 with 5 as highly recommended

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6. DOCUMENTATION PROVIDED TO EFSA

Letter sent on the 23/07/2004 with ref. DG ENV. C JV/jm D (04) 430238, from Mr Jos Delbeke, from the Directorate-General Environment, Directorate C - Air and Chemicals

Supportive Documents

- The Commission sent, as background information, the EU reference on approved methods for euthanasia (Close *et al*., 1996, 1997).

6.1. REFERENCES

All references are available in the scientific report.

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The declarations of conflicts of interest of all participants in this working group will be available on internet, in the EFSA web site (http://www.efsa.eu.int)

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European Food Safety Authority- AHAW Panel

Annex to the *EFSA Journal (2005) 292, 1-136;* Aspects of the biology and welfare of animals used for experimental and other scientific purposes

SCIENTIFIC REPORT

"Aspects of the biology and welfare of animals used for experimental and other scientific purposes"

EFSA-Q-2004-105

Accepted by the AHAW Panel on 12th and 13th October 2005

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TABLE OF CONTENTS

TABLES, APPENDIXES AND FIGURES 4
ABREVIATIONS 5
DEFINITIONS 6
1. TERMS OF REFERENCE 10
1.1. Background 10
1.2. Mandate 10
1.2.1. Question 1 on the sentience of invertebrate species, and fetal and embryonic forms of both vertebrate and invertebrate species 10
1.2.2. Question 2 on fetal and embryonic forms 11
1.2.3. Question 3 on purpose-bred animals 12
1.2.4. Question 4 on humane methods of euthanasia 13
1.2.5. Supportive Documents 13
1.3. Scope of the Report 13
2. QUESTION ON THE SENTIENCE OF INVERTEBRATE SPECIES, FETAL AND EMBRYONIC FORMS OF BOTH VERTEBRATE AND INVERTEBRATE SPECIES AND ON FETAL AND EMBRYONIC FORMS. 15
2.1. The questions asked and the risks to be considered 15
2.2. How to decide which animals should be protected 15
2.3. Capabilities of invertebrates in relation to the need for protection 18
2.3.1. Cognitive Capacities of Invertebrates 20
2.3.2. Brain Cell Numbers 26
2.3.3. Nociception and Pain in Invertebrates 27
2.3.4. Evidence against invertebrates having the capacity to experience suffering. 32
2.3.5. Summary: 33
2.4. Brief summaries for non-vertebrate groups and recommendations 33
2.4.1. Cyclostomes (lampreys and hagfish). 33
2.4.2. Amphioxus 34
2.4.3. Tunicate 34
2.4.4. Hemichordata such as Balanoglossus 34
2.4.5. Cephalopods (octopods, squid, cuttlefish, nautiloids) 34
2.4.6. Land gastropods 35
2.4.7. Tectibranch and nudibranch molluscs 35
2.4.8. Social insects 35
2.4.9. Other insects 35
2.4.10. Spiders, especially jumping spiders 35
2.4.11. Decapod crustaceans (lobsters, crabs, prawns, etc.) 35
2.4.12. Isopods (woodlice and marine species) 36
2.4.13. Other phyla (e.g. Annelida, Echinodermata, Platyhelminthes, and Nematoda) 36
2.5. Fetal and embryonic animals which might be protected 37
2.5.1. Fetal sentience 37
2.6. Implications for the definition of a “protected animal” 43
3. QUESTION ON PURPOSE-BRED ANIMALS 44
3.1. Introduction 44
3.2. Risk assessment framework 44
3.2.1. Introduction 44
3.2.2. IMPACT ON ANIMAL WELFARE 45

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3.2.3. IMPACT ON SCIENTIFIC QUALITY 51
3.3. SCIENTIFIC CRITERIA THAT COULD BE USED TO DETERMINE WHICH ANIMALS SHOULD BE PURPOSE-BRED 53
3.3.1. Key criteria to be considered for being purpose bred and inclusion in Annex I 54
3.3.2. Assessement in relation to specific species used in research: 55
4. QUESTION ON HUMANE METHODS OF EUTHANASIA 64
4.1. Introduction 64
4.2. Reasons for euthanasia: 65
4.2.1. Scientific reasons: 65
4.3. Education, training and competence of those carrying out humane killing: 65
4.4. Killing animals for their tissues: 66
4.5. The approach, scope and layout in the euthanasia section 66
4.6. Gathering information 67
4.7. Species to be dealt according to the annex sent by DG ENVIR 67
4.8. Methods of euthanasia 67
4.8.1. Electrical stunning 68
4.8.2. Mechanical stunning methods 69
4.8.3. Mechanical disruption of tissues (Neck dislocation, decapitation, maceration) 73
4.8.4. Physical methods 76
4.8.5. Gaseous methods 79
4.9. Humane killing of cephalopods, cyclostomes, decapods (if accepted) 103
4.9.1. Methods inducing the minimum level of pain and distres: 103
4.9.2. Methods likely to cause pain and distress 105
4.10. The following Tables (8-15) give the recommended methods for the humane killing of animals in the laboratory. 106
4.11. References 114
5. ACKNOWLEDGEMENTS 136

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TABLES, APPENDIXES AND FIGURES

Figure 1 - Self-stimulation of areas of the brain by land snails (Balaban and Maksimova, 1993)……. pg. 25

Figure 2 - "Common patterns of plasticity contributing to nociceptive sensitisation in mammals and Aplysia" from Woolf & Walters (1991)……pg. 29

Table 1 - Evidence of higher cognitive capacities in invertebrates……pg. 36

Table 2 - Evidence of the capacity for invertebrates experiencing pain using the criteria of Smith and Boyd (1991). ……....pg. 38

Appendix 1 - Purpose bred criteria / Critical Species Species to be added: Hamsters – Chinese (*Cricetulus griseus)*, Syrian (*Mesocricetus auratus*), European (*Cricetus cricetus*), Djungarian (*Phodopus sungorus*) ……...…..pg. 70

Appendix 2 - Purpose bred criteria / Critical Species Species to be added: Mongolian Gerbil (*Meriones unguiculatus*)…… pg. 71

Appendix 3 - Purpose bred criteria / Critical Species Species to be added: Ferret (*Mustela putorius furo*)……. ……..pg.72

Appendix 4 - Purpose bred criteria / Critical Species Species to be added: Pig (*Sus scrofa*) including Minipig ……pg.73

Appendix 5 - Purpose bred criteria / Critical Species Species to be removed: Quail (*Coturnix cornutix*) ……pg.74

Appendix 6 - Purpose bred criteria / Critical Species Species to be added: Bird (other than quail)……..pg .75

Appendix 7 - Purpose bred criteria / Critical Species Species to be added: Amphibian (focusing on *Xenopus sp*. and *Rana sp*.)……. pg. 76

Table 3 - Mechanical stunning methods…….. pg. 91

Table 4 - Methods causing mechanical disruption of tissues in adult and fetal animals……. pg. 94

Table 5 - Summary of killing methods using hypoxia. ……...pg. 114

Table 6 - Concentrations of agents (% in oxygen) used to test aversion in rodents. ……pg. 118

Table 7 - Overdose of inhalational anaesthetic gases for euthanasia…….…. pg. 120

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Table 8 - Characteristics of methods for euthanasia of fish ……pg 126

Table 9 - Characteristics of methods for euthanasia of amphibians ……pg 127

Table 10 - Characteristics of methods for euthanasia of reptiles…….. pg 128

Table 11 - Characteristics of methods for euthanasia of birds ……..pg 129

Table 12 - Characteristics of methods for euthanasia of rodents…….. pg 130

Table 13 - Characteristics of methods for euthanasia of rabbits……... pg 131

Table 14 - Characteristics of methods for euthanasia of dogs, cats, ferrets, foxes... pg 132

Table 15 - Characteristics of methods for euthanasia of large mammals ……pg 133

ABREVIATIONS

ACTH - Adenocorticotrophic Hormone

AHAW - Panel on Animal Health and Welfare

AR - Aspiration reflex

CA - Carbonic anhydrase

CITES - Convention on International Trade in Endangered Species of Flora and Fauna

CNS - Central nervous system

DG ENV - Directorate-General Environment

DNA - Deoxyribonucleic acid

ECG - Electrocardiogram

EEG - Electroencephalogram

EFPIA - European Federation of Pharmaceutical Industries and Associations

EFSA - European Food Safety Authority

ETS - European Treaty Series

EU - European Union

GAA - Genetically altered animals

H&S - Health and Safety

IPC - Intrapulmonary chemoreceptors

IPCs - Intrapulmonary chemoreceptors

JWGR - Joint Working Group on Refinement

MELs - Maximum exposure levels

mRNA - Messenger RNA

MTL - Maximum tolerated level

NHP - Non-human primates

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NMDA - N-methyl-D-aspartate

NMP - Negative mucosal potential

PB - Purpose Bred

Pers. comm. - personnal communication

PI - Production Index

POMC - pro-opiomelanocortin derived peptides (from the pituitary gland)

REM - Rapid eye movement sleep

RH - Relative Humidity

RI - Reproduction Index

SCAHAW - Scientific Committee on Animal Health and Animal Welfare

SPB - Sodium pentobarbitone

SVC - Scientific Veterinary Committee

TEWG - Technical Expert Working Groups

UK - United Kingdom

WG - Working group

DEFINITIONS

Anoxia: depletion of oxygen in atmosphere or in the blood.

Aspiration reflex: stimulation (chemical, electrical or mechanical) of the pharyngeal branch of glossopharyngeal nerve or trigeminal afferents that evokes a short-duration spasmodic inspiratory sniff- or gasp-like aspiration reflex.

Aversion: a tendency to show behaviour to avoid or to withdraw from a situation which is associated with a noxious stimulus.

Brain centre: A functional set of brain cells that receive and process types of input, for example that from pain receptors and related information. The centre need not be spatially localised.

Consciousness: is the state of awareness of a normal animal when it can perceive stimuli from its external environment and respond in the normal behaviour of an awake individual.

Death: a pathological state of an animal, where respiration and blood circulation have permanently ceased. The main clinical signs seen are absence of respiration (and no gagging i.e. attempts to breathe), absence of pulse and absence of somato-sensory reflexes and presence of pupillary dilation.

Efficacy: The effectiveness of a method to kill in the appropriate manner

Efficiency: The proportion of animals being killed at the first attempt

Electroencephalogram: electrical activity of the brain usually recorded from the surface of the skull using non-invasive techniques.

Electroencephalography: is the neurophysiologic measurement of the electrical activity of the brain by recording from electrodes placed on the scalp, or in the special cases on the cortex.

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Embryo: an animal that is developing from a sexually fertilized or parthenogenetically activated ovum and which is contained within egg membranes or within the maternal body. The embryonic stage ends at the hatching or bird of the young animal.

Euthanasia: gentle death and should be regarded as an act of humane killing with the minimum of pain, fear and distress.

Exposure assessment: consists of describing the conditions which predispose to the hazard occurring. Where appropriate it may describe the biological pathway(s), the probability of the exposure(s) occurring, either qualitatively (in words) or quantitatively (as a numerical estimate) with respect to amount, timing, frequency, duration of exposure, routes of exposure, the number, species and other characteristics of the animal populations exposed.

Fetus: is an embryo from the stage of its development to when its main adult features can be recognised until its birth, normally applied to mammals

Gagging or gasping: rudimentary respiratory activity occurring through mouth (oral breathing).

Generalised epilepsy: a pathological state of the brain, involving both cerebral hemispheres, incompatible with the persistence of consciousness and sensibility.

Genetic fidelity: that the correct genetic line has been maintained.

Genetically altered animals: an animal in which the heritable DNA has been intentionally altered, or the progeny of such an animal or of an animal with a mutation recognised as harmful. This includes animals produced by genetic modification or by induced mutagenesis, or animals created by nuclear transfer procedures, as well as harmful mutant lines arising from spontaneous mutations. This definition excludes animals with changes that are not heritable, such as gene therapy interventions or DNA immunisations.

Hazard: Any thing or action or omission of an action that could potentially harm an animal and as a consequence cause poor welfare or poor science.

Humane killing: a method of killing that causes no avoidable pain, distress or other suffering to the animal(s) concerned.

Hypercapnia: increased blood carbon dioxide levels in the blood or atmosphere.

Hypoxia: decrease in oxygen levels in the atmosphere or blood.

In vitro: literally meaning in glass, and is used to infer experimental techniques that may involve animal organs, tissues and cells taken from dead animals and kept in a nutrient medium.

In vivo: literally meaning experiments involving a living animal with its whole body systems intact in order to study what happens in the body itself.

Insensible: inability to perceive external stimuli and internal stimuli (e.g. pain).

Intrapulmonary chemoreceptors: are CO2-sensitive receptors in lungs of birds that respond to inspired CO2, but not oxygen levels.

Manipulandum: A physical feature, such as a lever or other movable object, whose manipulation by an animal leads to an environmental change and perhaps to learning.

Neck cutting: severing major blood vessels in the neck (skin and vessels cut simultaneously).

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Pain: may be defined as an "aversive sensory experience that elicits protective motor actions, results in learned avoidance and may modify species-specific traits of behaviour, including social behaviour" (Zimmermann, 1986). Use of the word pain implies a conscious awareness of the stimulus and not an unconscious reflex response.

Period: the period of a given electric current frequency (Hz) is expressed in milliseconds and is calculated using the formula 1000 (milliseconds) divided by the frequency (Hz) of current. For example, electric currents of 50, 400 and 1500 Hz sine wave have periods of 20 (1000/50), 2.5 (1000/400) and 0.67 (1000/1500) milliseconds.

Production Index: number of animals/ year

Purpose Bred: means animals specially bred for use in experiments in facilities approved by, or registered with, the competent authority (defined in Article 2 of the Council Directive 86/609/EEC).

Reduction: whether the same objectives can be achieved with fewer animals, for example by improving the experimental design or by reducing variability between animals.

Refinement: whether the amount of pain, suffering, distress or lasting harm, caused to the animals used in the experimental procedure is the least required to achieve the scientific objective, or whether their wellbeing can be improved. Refinement refers the entire lifetime experiences of the animal including breeding, housing and husbandry, and during experimental procedures.

Replacement: Another method that does not involve the use of living protected animals that will achieve the same goal and that is reasonably and practicably available.

Reproduction Index: number of offspring/ breeding female/ annum

Risk assessment means a scientifically based process consisting of a series of steps: hazard identification, hazard characterisation, exposure assessment, risk characterisation, and risk pathways.

Risk: The evaluation of the likelihood that the hazard will occur i.e. hazard and exposure.

Seizure: convulsions that may occur with or without loss of consciousness or pathological electroencephalogram.

Slaughter: in this report, slaughter means the process of bleeding to induce death, usually by severing major blood vessels supplying oxygenated blood to the brain.

Spiking: is a fish killing process whereby a spike or tube is driven into the brain through the top of the head, manually or by using a pneumatically operated pistol. It is similar to captive bolt stunning of red meat species.

Sticking or bleeding: act of severing major blood vessels (also see neck cutting).

Stun or stunning: stunning before slaughter is a technical process subjected to each single animal to induce immediate unconsciousness and insensibility in animals, so that slaughter can be performed without avoidable fear, anxiety, pain, suffering and distress.

Stun/kill or stunning/killing: process of rendering animals unconscious first and then inducing death or achieving these simultaneously.

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Suffering: one or more unpleasant feelings (mental state) such as pain, distress, frustration, boredom, etc., that disturbs the normal quality of life.

Unconsciousness: is a state of unawareness (loss of consciousness) in which there may be temporary or permanent damage to brain function and the individual is unable to respond to normal sensory stimuli, including pain.

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1. TERMS OF REFERENCE

1.1. Background

Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes provides for controls of the use of laboratory animals, it sets minimum standards for housing and care as well as for the training of personnel handling animals and supervising the experiments.

Since 1986, important progress has been made in science and new techniques are now available, such as use of transgenic animals, xenotransplantation and cloning. These require specific attention, which the current Directive does not provide. Nor is the use of animals with a higher degree of neurophysiological sensitivity such as non-human primates specifically regulated. Therefore, Directorate-General Environment (DG ENV) has started revising the Directive.

The revision addresses issues such as compulsory authorisation of all experiments, inspections, severity classification, harm-benefit analysis and compulsory ethical review. Also specific problems relating to the use and acquisition of non-human primates will be tackled.

In 2002, as part of the preparatory work for the revision, DG ENV requested the opinion of the Scientific Committee on Animal Health and Animal Welfare, SCAHAW, on the welfare of non-human primates used in experiments. This Opinion, adopted by SCAHAW on 17 December 2002, was made available to the TEWG. The Opinion already provides some information especially concerning the requirements for purpose-bred animals and the question on gestation for non-human primates.

In 2003, DG ENV organised a Technical Expert Working Group (TEWG) to collect scientific and technical background information for the revision. The experts from Member States, Acceding Countries (which are now the new Member States), industry, science and academia as well as from animal welfare organisations worked through a set of questions prepared by DG ENV. The results of the TEWG provide an important input for the revision of the Directive. However, the TEWG highlighted four specific questions requiring further scientific input. These questions are detailed below. The final reports of the TEWG are provided as background documents.

1.2. Mandate

1.2.1. Question 1 on the sentience of invertebrate species, and fetal and embryonic forms of both vertebrate and invertebrate species

1.2.1.1. Detailed background on invertebrate species

The following definitions are applied in the current Directive:

"'animal' unless otherwise qualified, means any live non-human vertebrate, including free-living larval and/or reproducing larval forms…"

"'experiment' means any use of an animal for experimental or other scientific purposes which may cause it pain, suffering, distress or lasting harm, including any course of action intended, or liable, to result in the birth of an animal in any such

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condition, but excluding the least painful methods accepted in modern practice (*i.e.* 'humane' methods) of killing or marking an animal"

The TEWGs and other experts recommended to enlarge the scope to include **invertebrate species** provided there is sufficient scientific evidence as to their sentience and capacity to "experience pain, suffering, distress or lasting harm". Certain species of invertebrates are already included in the national legislation of some countries, both within and outside the EU (*e.g.* UK, some Scandinavian countries, Australia Capital Territories, New Zealand). The UK currently only includes *Octopus vulgaris* in its national legislation but is considering the inclusion of additional cephalopod species.

1.2.1.2.Terms of reference of question 1

In view of the above, the Commission asks the European Food Safety Authority to issue a scientific opinion on:

- the sentience and capacity to "experience pain, suffering, distress or lasting harm" of all invertebrate species used for experimental purposes.

1.2.2. Question 2 on fetal and embryonic forms

1.2.2.1.Detailed background on fetal and embryonic forms

The definition of 'animal' in the current Directive excludes fetal or embryonic forms.

According to TEWG and other experts, fetal and embryonic forms should be brought under the scope of the Directive in case there is enough scientific evidence on their capacity to "experience pain, distress or lasting harm".

Some Member States have included in their national legislation such forms beyond a certain stage of pregnancy. A criterion for determining the appropriate stage of pregnancy may be the development of the cerebral cortex and when it reaches a stage at which it can register sensory experiences.

The view of several members of the TEWG was that a time limit of half way through the gestation period should be used, at least for all large mammalian species other than rodents. This was based on data relating to sheep and non-human primates whilst providing for a 'safety margin' with regard to the ability of fetuses/embryos of these species to feel pain. However, the TEWG could not reach a consensus on when a rodent fetus or new-born may be capable of suffering, although they suggested that the final 20% of pregnancy may be appropriate for rodent and poultry species.

1.2.2.2.Terms of reference of question 2

In view of the above, the Commission asks the European Food Safety Authority to issue a scientific opinion on:

- The stage of gestation after which the fetus/embryo of the species in question is assumed to be capable of "experiencing pain, suffering, distress or lasting harm",

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- whether a generic rule for a cut-off point for the advancement of gestation can be indicated for those species where insufficient scientific data exist to establish a species-specific cut-off point.

1.2.3. Question 3 on purpose-bred animals

1.2.3.1. Detailed background on purpose-bred animals

Species listed in Annex I to Directive 86/609/EEC are those that must be 'purpose bred' when used in experiments (unless a specific exemption has been obtained). The criteria for inclusion of species in Annex I have not been clearly defined and no information is available on why the various species were originally included.

For example, mini-pigs which have become a widely-used laboratory species, obtained from commercial suppliers where they are bred in a controlled environment similar to that to be experienced at user facilities. According to the TEWG, their inclusion in Annex I would therefore appear logical and in the interest of sound principles of scientific research and welfare. Other species to be considered for inclusion could be ferrets and some hamster species in addition to *Mesocricetus auratus*. Conversely, the current inclusion of quail (*Coturnix coturnix*) should be re-considered.

The TEWG proposed multiple criteria as a basis for species inclusion into Annex I, such as:

- numbers of animals required for procedures;
- the type of procedures (*e.g.* farm animal studies/population studies);
- animal welfare aspects;
- practical and commercial aspects of establishing breeding;
- disease-free requirements;
- specific animal welfare aspects such as social deprivation, confinement and other aspects of sudden involuntary changes of living environment (use of pet or stray animals as experimental animals.)

1.2.3.2. Terms of reference of question 3

In view of the above, the Commission asks the European Food Safety Authority to issue a scientific opinion on:

- the scientific criteria that could be used to determine in which cases animals to be used in experiments should be purpose-bred, in order to safeguard *inter alia* animal welfare, using the proposal of the TEWG. The proposed criteria should also take into account other factors such as current and future needs, practicability or any specific scientific requirements.

- Based on these criteria, determine which species currently used in experiments meet these criteria.

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1.2.4. Question 4 on humane methods of euthanasia

1.2.4.1. Detailed background on humane methods of euthanasia

Some experimental animals are only bred to be euthanised for the purpose of using their tissues and/or organs, e.g. in the development and application of *in vitro* methods. To ensure highest possible animal welfare standards in the EU, it needs to be defined which methods of killing are scientifically the most humane and appropriate for different species of experimental animals.

1.2.4.2. Terms of reference of question 4

In view of the above, the Commission asks the European Food Safety Authority to issue a scientific opinion on:

- the methods of euthanasia which could, on the basis of current scientific knowledge and respecting good animal welfare, be justified as being the most appropriate per type of species.

- To specify these methods and their suitability for different species most commonly used in experiments.

1.2.5. Supportive Documents

- The Commission sent, as background information, the EU reference on approved methods for euthanasia (Close *et al.,* 1996, 1997).

1.3. Scope of the Report

While the principal reason for the Directive 86/609/EEC is to prevent distortions of competition or barriers to trade, it is also clearly to harmonise the implementation of the Three Rs of Replacement, Reduction and Refinement. The last two of reduction and refinement will help to minimise poor welfare in those animals used in experimentation and testing, whilst allowing their use in studies that involve accurate, reproducible, reliable science and are considered justifiable by the competent authorities. There might be any of a variety of possible causes of poor welfare such as injury, disease and various unfulfilled needs. As a consequence, the animals might feel pain, fear or anxiety or show other coping responses involving brain, behaviour or body physiology.

This scientific report comprises 3 parts/Chapters in response to the 4 questions posed by the Commission (see Section 1.2). Questions 1 and 2 overlapped in scope essentially dealing with sentience of both fetal forms and invertebrates, and are addressed in *Chapter 2*. Questions 3 and 4 remain separate and as they are given in the mandate. They cover purpose breeding of animals (*Chapter 3*), and euthanasia of the commonly used species (*Chapter 4*). It was decided that if in Chapter 2, some species were to be able to experience pain and distress, then the report and opinion should also address the question of whether they should be purpose bred in Chapter 3, and how they could be humanely killed in Chapter 4.

Three working groups (WGs) were set up to address these questions with relevant experts being appointed as members. Experts were chosen on the basis of their scientific expertise (significant publications on the topic since 2000) or relevant experience, and the suggestions of the stakeholders group were taken into account. Information was also sought from and provided by scientific experts from several countries outside the EU.

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EFSA's approach to such questions as those being requested is based on a risk assessment, and the WGs have tried to do that in each of the three parts. In each part / Chapter is a short introduction to the question, followed by two basic risk questions:

1) 'What would be the consequences for animal welfare if X happened or did not happen?' And

2) 'What would be the consequences for the scientific outcomes, if X happened or did not happen?'

For example, if animals were not purpose bred, or the method of euthanasia was not ideal for some reason. In such situations the consequences could be poor welfare or impacted adversely on the scientific investigation. For sentience the question was slightly different and Chapter 2 focuses on which groups of animals should be protected by virtue of their welfare being poor and their level of awareness being sufficient for protection to be necessary (see section 2.2). The risk assessment format differed between each part as the hazards are very different and there is no standard form of animal welfare, or scientific impact, risk assessment. It is not within the remit of the Panel on Animal Health and Welfare to cover risks related to ethical, socio-economic, human safety, cultural or religious aspects.

It may seem a paradox, but the interaction between good animal welfare and good science is crucially important. Rephrasing the statement may make it clearer. If the welfare of an animal is poor, there is likely additional variance due to the animal's responses to that form of suffering or other environmental effect. As it may be possible to avoid such suffering, for example by purpose breeding or a humane method of killing, the quality of analysis of the specific scientific factor could be improved by avoiding the confounding of the research data being obtained. Animal disease during an experiment might well affect the scientific outcome, for example by affecting the immune response. Consequently, for those species used in this area of research it is important that such animals are purpose bred in order to ensure a high health status. Any impact of that disease on the welfare of an animal is likely to result in scientific data being inaccurate, unreliable and not reproducible in another laboratory, all of which contribute to poor science, as well as causing avoidable suffering, and hence being labelled as inhumane.

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2. QUESTION ON THE SENTIENCE OF INVERTEBRATE SPECIES, FETAL AND EMBRYONIC FORMS OF BOTH VERTEBRATE AND INVERTEBRATE SPECIES AND ON FETAL AND EMBRYONIC FORMS.

2.1. The questions asked and the risks to be considered

The question to be considered in this part of the report is which animals should be protected. Should the range of animals be limited to vertebrate animals or should it be extended to any of the invertebrate groups? Should protection begin at the point of hatching from an egg, or birth in the case of mammals, or should it begin at some point during fetal or embryonic development. If animals should be protected before hatching or before birth, at what point in development, and how practical would this be, e.g. would it be possible to protect immature forms of some invertebrates? The terminology used in the conclusions to the report has to be relevant to any of the animals considered so cannot refer to gestation length, which is relevant only to mammals. Similarly, reference to the brain will take account of function rather than anatomy because animals vary in the parts of the brain that have complex analytical functions. Whilst some mammals have high-level analysis functions in the cerebral cortex, a comparable high level analysis occurs in areas of the striatum in birds and in a variety of brain regions in fish and cephalopods. Care has also be taken not to be anthropocentric and over-emphasise visual analysis as other senses have a more primary role in the lives of many mammals, fish, etc. (see Gregory, 2004).

There is a general view amongst biologists and amongst the public that there is a threshold level amongst animals above which protection should occur. Very few people would seek to protect protozoans or nematode worms but the vast majority of people would wish to protect Primates so a line has to be drawn somewhere between the two, based on scientific evidence. If protection was limited to a too restricted group of animals, poor welfare could occur in animals used in experimental procedures. Risk assessment of this kind has to change according to the level of development of human knowledge. Our knowledge of the functioning of the brain and nervous system and of animal welfare has advanced rapidly in recent years. New knowledge has tended to show that the abilities and functioning of non-human animals are more complex than had previously been assumed. It is likely that future advances in knowledge will require re-appraisal of the recommendations made as a result of this report.

2.2. How to decide which animals should be protected

As a background to the risk assessment it seems reasonable to look at how human attitudes to animals have changed over recent times. It is noteworthy that people have changed their attitudes to people of different nationality and race, as they have understood more. Most consider that they have obligations to other people and also living beings of other species (e.g. Midgley, 1994) and the range of individuals encompassed by this has been expanding (Broom, 2003, 2005) from those readily recognised as close relatives, to all of those "who know who I am", those who "might have access to the same information that I have" and "sentient beings who share characteristics with me". Evidence which has been used in deciding on the animals for which welfare is an important consideration, in addition to similarity to and utility to humans, has included their ability to experience pain and distress, as well as evidence for the biological basis of suffering and other feelings such as fear and anxiety, and indications of awareness based on observations and experimental studies. Some other

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concerns for living beings do not relate to their sentience but to the fact that they are living animals and able to flourish, that they have highly complex cognitive capacities, and the fact that they are human or some other particular species (Nuffield Council on Bioethics, 1996; 2005). For example, some have the attitude that a human fetus, or fetus of another species, should be preserved and potentially not be harmed or destroyed, even though it may not be in a position to perceive pain or distress. This may be because it has the potential to develop into an adult human or other adult animal. Such a view is much less often held for the immediately earlier step, i.e. the protection of viable ova and sperm, the zygote or embryo.

Animals vary in the extent to which they are aware of themselves (Dawkins, 1992; DeGrazia, 1996) and of their interactions with their environment, including their ability to experience pleasurable states such as happiness and aversive states such as pain, fear and grief. This capacity may be referred to as their degree of sentience. Human opinion as to which individuals are sentient has changed over time to encompass, first all humans instead of just a subset of humans, and then certain mammals which were kept as companions, animals which seemed most similar to humans such as monkeys, the larger mammals, all mammals, all warm-blooded animals, and then all vertebrates. The general public has been ready to accept some guidance about evidence for sentience from biologists who collected information about the abilities and functioning of the animals. Animals which are shown to be complex in their organisation, capable of sophisticated learning and aware are generally respected more than those which are not, and such animals are less likely to be treated badly. However, some people view animals solely on the basis of their effects on, or perceived (extrinsic) value to, humans and have little concern for the welfare of pests, disease carriers or those that cannot be eaten (Broom, 1989, 1999: Serpell, 1989).

Animals are more complex if they have to contend with a varied environment and, as a consequence, have an elaborate motivational system that allows them to think about and then take appropriate decisions. Some kinds of feeding methods demand much brain power, as do aspects of predator avoidance, but the most demanding thing in life for humans and other species is to live and organise behaviour effectively in a social group. Analysis of the degree of complexity of living possible for members of an animal species is a first step in deciding whether such animals are sentient. Without a level of brain functioning that makes awareness possible, an animal could not normally be sentient.

Some degree of learning is possible for simple animals but those animals which can learn more, learn fast and make few errors once they have learned are considered more likely to be sentient. Learning is not, in itself, evidence for awareness but is an indicator that further investigation of cognitive ability might reveal the existence of awareness commensurate with sentience. Comparative studies of learning ability are not easy to carry out because learning situations usually require that an operant, such as pressing a lever, is performed and animals may vary in their physical ability to carry out the operant. Kilgour *et al.* (1991) solved this problem by making mazes with a food reward and the same sequence of decisions for success but with each maze of a size appropriate for the species. They studied various domestic animal species. As a result, they were able to find out that cattle, pigs and sheep are slightly better at learning than dogs and considerably better than horses, cats, rats etc.

Some of those who have sought to compare the cognitive abilities of animals of different species have reported on total brain size or the size of some part of the brain (Hemmer, 1983, Jerison, 1973). However, there are animal species or individuals with very small brains, or with a small cerebral cortex, which can function very well. The brain can

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compensate for lack of tissue or, to some extent, for loss of tissue. There are so many anomalies in relationships between ability and brain size, that no conclusions can be reached, except perhaps within small taxonomic groups when aberrant individuals are excluded (Barton and Dunbar, 1997, Broom, 2003). Studies of complexity of brain function, on the other hand, can give much information about ability as well as about welfare (Broom and Zanella, 2004).

In humans, nociception is considered to be the physiological relay of pain signals which is an involuntary, reflex process not involving the conscious parts of the brain. Pain leads to aversion: behavioural responses involving immediate avoidance and learning to avoid a similar situation or stimulus later. It has a sensory component often related to injury but also requires complex brain functioning of the kind associated with a feeling. Kavaliers (1988), based on the International Association for the Study of Pain 1979 definition, suggested that for non-humans, pain is an aversive sensory experience caused by actual or potential injury that elicits protective motor and vegetative reactions, results in learned avoidance and may modify species specific behaviour, including social behaviour'. Smith and Boyd (1991) considered pain to be the conscious, emotional experience that, in humans, involves nerve pathways in the cerebrum. Hence a definition of pain should refer to the sensory and emotional aspects, and the reference to function and consequences is not needed as it may unnecessarily restrict its meaning. Broom (2001) defined pain as an aversive sensation and feeling associated with actual or potential tissue damage.

A pain system involving receptors, neural pathways and analytical centres in the brain exists in many kinds of animals. The fact that there is rather similar evidence of physiological responses, direct behavioural responses and ability to learn from such experiences so that they are minimised or avoided in future, suggests the existence of feelings of pain in many species. Indeed the feelings will often be an important part of the biological mechanism for coping with, perhaps by avoiding, actual or potential damage. The advantages of pain are that action can be taken when damage occurs, consequent learning allows the minimising of future damage and, where the pain is chronic, behaviour and physiology can be changed to ameliorate adverse effects. Pain systems have been identified by anatomical and physiological investigation and by studies of behavioural responses, particularly with the assistance of analgesic administration as an experimental probe. Species differ in their responses to painful stimuli because different responses are adaptive in different species. The feeling of pain may be the same even if the responses are very different. Other feelings such as fear, anxiety and the various forms of pleasure have also been deduced to exist by careful observation and experiment.

The high level of brain functioning in relation to life events known as awareness can be deduced, with some difficulty, from behaviour in controlled situations. Awareness is defined here as a state in which complex brain analysis is used to process sensory stimuli or constructs based on memory. Awareness has been described using five headings: unaware, perceptual awareness, cognitive awareness, assessment awareness and executive awareness (Sommerville and Broom, 1998). A mother recognising her offspring and an individual responding to a known competitor, ally, dwelling place or food type are showing cognitive awareness. Where the individual is able to assess and deduce the significance of a situation in relation to itself over a short time span, for example vertebrate prey responding to a predator recognised as posing an immediate threat but not directly attacking, it is showing assessment awareness. Executive awareness exists when the individual is able to assess, deduce and plan in relation to long-term intention. This may involve deductions about choices of action available to

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that individual (retroduction) (e.g. Morton, 2000), the feelings of others, imagination, and the mental construction of elaborate sequences of events.

The link between level of awareness and welfare is complex. Welfare concerns how well individuals are able to cope with good or bad environments (Broom and Johnson, 1993). Animals that are sentient would have a wider array of ways in which their welfare could be poor, because the complexity of their brain function is above a threshold level compared with non-sentient animals. We should be concerned about the welfare of all sentient animals. Within this category of sentient animals, some pain can be especially disturbing because the individual concerned uses its sophisticated brain to appreciate that such pain indicates a major threat. However, more sophisticated brain processing will also provide better opportunities for coping with some problems. Humans may have means of dealing with pain that fish do not have. For example, humans may suffer less from pain because they are able to rationalise that it will not last for long. As a consequence, in some circumstances humans who experience a particular pain might suffer more than fish, whilst in other circumstances a certain degree of pain may cause worse welfare in fish than in humans (Broom, 2001). These arguments will also be valid for other causes of poor welfare. In addition to considerations of pain, more complex brains should allow more possibilities for pleasure, which contribute greatly to good welfare. When scientific evidence concerning the functioning of animals is taken into account, it is clear that there are illogicalities in protecting animals because of their similarities to humans or their use to humans.

The next sections review the evidence for relevant functioning, first in non-vertebrates and then in embryos and fetuses. Before referring to the wide range of invertebrate animals, it is important to consider whether or not it is valid to review animal abilities in taxonomic groups. Very little consideration is needed for it to become apparent that there can be very great variation, in this respect, within some taxonomic groups. In general, sessile animals seem to show lower levels of brain function than active, mobile animals but some invertebrates, including non-vertebrate chordates, might be protected. Social animals usually have more sophisticated behaviour and higher levels of learning and awareness than non-social animals (Humphrey, 1976; Broom, 2003).

All invertebrate animals were considered and our recommendations proposed some groups as “protected animals”.

2.3. Capabilities of invertebrates in relation to the need for protection

This section includes information about non-vertebrate chordates and members of all animal phyla other than Chordata. After some general information details are presented about animal groups that may be considered for protection. In the text which follows the term invertebrates includes non-vertebrate chordates.

The Phylum Chordata include: those which are unquestionably Vertebrata, the fish, amphibians, reptiles, birds and mammals; the Cyclostomata (lampreys and hagfish), the Cephalochordata (e.g. amphioxus); the Urochordata (or tunicates such as sea squirts and salps); and the Hemichordata (acorn worms, pterobranchs). Although the lampreys and hagfish do not have a normal vertebral column, most zoologists consider them to be fish. Amongst the clearly non-vertebrate chordates, amphioxus and tunicate larvae are active and free-swimming, salps and other pelagic tunicates are relatively active, whilst the other species are bottom-living or sessile.

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The Phylum Mollusca includes Gastropoda, Cephalopoda and several other Classes not discussed here. Gastropoda are largely snail-like or slug-like and include some actve marine swimming forms like the tectibranchs (e.g *Aplysia*) and the nudibranchs (e.g.*Tritonia*) whose behaviour and nervous systems have been much studied. Cephalopoda is a Class of the Phylum Mollusca which includes the pelagic, shelled nautiloids (e.g. *Nautilus*) and the coleoids which include cuttlefish, (e.g. *Sepia*), squid (e.g. *Loligo*) and octopods (e.g. *Octopus*). They range in adult size from 8mm to the largest invertebrate animal, the giant squid *Architeuthis* which can be over 5m long. All except nautiloids seem to be rather short-lived, often living for only one year but their pace of life is considerable and they live longer in colder conditions. They have little food storage ability. Squid are extremely numerous predators (several million tonnes per year are fished) in shallow or deep water whilst cuttlefish swim in shallow water and most octopods are bottom dwellers. Many squid and cuttlefish species live socially and have some complex social responses. Sophisticated sense organs are described for cuttlefish, squid and octopods. A complex rapid movement system, with giant nerve fibres, is also present in most cephalopods and most have a chromatophore and photophore system for rapidly changing colour and light production from the skin. This is used for communication, as well as subtle camouflage, and a wide range of social signals is reported for some cuttlefish and squid. Cephalopods have touch and pressure receptors including a sophisticated lateral line system good enough for cuttlefish to detect a 1m long fish 30m away and some low frequency sound detection. They have a wide range of chemoreceptors which in *Octopus vulgaris* allow discrimination between solutions at 10-1000 times lower concentrations than humans can. Basil *et al.* (2002) describe the sensory cells in the skin of the tentacles and rhinophore, a specialised organ for detecting waterborne chemicals, of *Nautilus pompilius* that are used for distance and contact chemoreception (i.e., chemical reception. Their eyes are very elaborate and allow discrimination of objects on the basis of brightness, size, orientation, form and plane of polarisation. The brain of all cephalopods is large with those of cuttlefish, squid and octopods being particularly complex (Wells, 1962; Hanlon and Messenger, 1996).

Other Phyla considered here are the Arthropoda, which include Crustacea such as crabs, Insecta such as bees and Arachnida such as spiders, and worms in the Annelida (e.g. earthworms), Playhelminthes (flatworms) and Nematoda (roundworms). The most complex of these animals are the decapod crustaceans, active spiders and many insects, especially those which are social. All of these animals have good sensory ability which could allow them to recognise individuals, respond to close or distant objects and regulate their interactions with their surroundings. Olfactory, auditory and visual signalling and detection systems are well-described. In insects, the compound eye has some discriminatory limitations in image formation but advantages in movement detection. The eyes of many spiders, especially the salticid (jumping) spiders are now known to produce very good images.

In addressing the question of whether invertebrates can experience suffering, the next section of this document presents evidence concerning higher cognitive capacities in invertebrates. Pain is a particularly important form of suffering, therefore, the following section presents evidence for the capacity of invertebrates to experience pain. Fear is another important form of suffering which is not easily recognised in invertebrates, although evidence exists about the widespread nature of some of the physiological changes associated with fear. Many species of invertebrates behave in ways that, if displayed by vertebrates, would be considered to be indicative of higher cognitive capacity (Sherwin, 2001), and these are discussed below and summarised in Table 1.

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2.3.1. Cognitive Capacities of Invertebrates

2.3.1.1.Memory

It is often suggested that invertebrates have little or no memory and this indicates they have a reduced capacity for suffering, however, many studies have clearly shown that some invertebrates have memories that can be complex and long-term.

Slugs can be trained to associate a food with a noxious tasting substance by allowing them to feed on carrots and then transferring them to paper soaked with a quinine-based solution (Yamada *et al.,* 1992). When subsequently tested in a preference apparatus, they remembered the association between the carrot and the quinine, and avoided the carrot. Some slugs learnt this association with just one pairing (a finding also reported by Gelperin, 1975 and Sahley *et al.,* 1981), although others required four pairings. Memory of this association persisted for up to a month, i.e. the slugs had a long-term memory.

Some invertebrates have both short- and long-term memory. When cuttlefish are presented with a shrimp in a glass tube, they initially vigorously attack the shrimp but then quickly learn to inhibit their predatory response (Dickel *et al.,* 1998; Agin *et al.,* 2003). From these studies, Dickel *et al.* (1998) concluded that cuttlefish have a short-term memory of 5 min that is fully operational at 8 days of age, whereas 60 min retention increases progressively between 15 and 60 days of age. Memory has been widely investigated in foraging honeybees which use both transient short-term working memory that is non-feeder specific and a feeder-specific long-term reference memory (Greggers and Menzel, 1993; Menzel, 1993; Wustenberg *et al.,* 1998). Hammer and Menzel (1995) stated that memory induced in a free-flying honeybee by a single learning trial lasts for days and, by three learning trials, for a lifetime. Using cooling-induced retrograde amnesia, Yamada *et al.,* (1992) showed that slugs have a short-term memory of approximately 1min and long-term memory of 1 month. The authors suggested that although it is difficult to make inter-study comparisons because of animal and methodological differences, the short-term memory for slugs (and cited references for other invertebrates) was not unusually short, even compared with vertebrate species such as the rat and goldfish.

As with vertebrates, invertebrates show a decline in the effectiveness of memory and learning as animals get older. Flies trained to suppress the proboscis extension response to sucrose solution all learned the task, but acquisition of the suppression was slower in flies aged 30 and 50 days compared with flies aged 7 days (Fresquet and Medioni, 1993). Tomsic *et al.* (1996) reported similar age-related memory deficits in crabs, and Halm *et al.* (2000) reported that senescent cuttlefish were less able to learn a novel method of handling prey than were sub-adults.

Gherardi and Atema (2005) examined individual recognition amongst hermit crabs. The crabs classified conspecifics into two 'heterogeneous sub-groups', i.e. familiar vs. unfamiliar individuals, but did not discriminate one individual of a group from every other conspecific. One day of interactions with different crabs did not erase the memory of a former rival, suggesting that they use a refined system of social partner discrimination. Memory of individuals lasted up to 4 days. Feld *et al.* (2005) described crabs as having a long-term memory lasting at least a week and an intermediate-term memory that lasts no longer than 3 days.

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2.3.1.2.Learning

It is sometimes argued that invertebrates show only simple forms of learning, indicating a reduced capacity for suffering, however, invertebrates can exhibit several forms of complex learning. As mentioned above, the occurrence of complex learning helps in sentience identification.

Within the Cephalopoda, the most work on learning ability has been carried out in the non-social but visually very competent *Octopus vulgaris.* Attempts to study learning in other *Octopus* species have been less successful. In *Octopus cyanea* this is probably because it is crepuscularcrepuscular and feeds by groping under coral heads etc so visual cues are less relevant. Experimentally, *Octopus vulgaris* has been shown to be able to associate shapes, patterns etc with food rewards, habituate to a variety of stimuli, turn left or right in a maze, and copy a demonstrator octopus which had been trained to attack a particular shape for a food reward. They could also generalise from a stimulus to a class of stimuli and show pattern reversal learning. Tasks which had been learned could be remembered and performed efficiently after a delay of at least two months (Wells, 1962; Hanlon and Messenger, 1996).

Visual discrimination tasks and habituation to a visual response have also been demonstrated to be carried out by five other species of *Octopus*, and three species of cuttlefish and squid. Avoidance learning in natural or semi-natural situations has been reported for the octopod *Eledone moschata* and three species of *Octopus*. The parts of the brain which are involved in storing and setting up memories in *Octopus vulgaris* were found to be present in all of the 62 species of coleoid cephalopods (cuttlefish, squid and octopods) examined by Maddock and Young (1987).

Social Learning

Social learning is said to occur when social interaction facilitates the acquisition of a novel behaviour pattern.

Octopuses watching other octopuses trained to attack balls that differ only in colour, consistently attacked the same coloured ball as the demonstrators (Fiorito and Scotto, 1992; Fiorito and Chichery, 1995). This learning occurred irrespective of the actual colour of the ball, and was more rapid than the learning that occurred during the training of the demonstrator octopus. Some other authors have tried unsuccessfully to replicate these studies.

Among vertebrates, alarm calls or food signals by demonstrators result in facilitation of avoidance or approach behaviour in observers. Suboski *et al.* (1993) suggested a similar form of learning occurred in freshwater snails in that feeding behaviour was regulated by food pheromones. Hungry snails, exposed overnight to effluent from non-observable conspecifics feeding on a novel food, approached or avoided that novel food depending on the density of the feeding snails that produced the effluent. Too few snails (0- 4) produced no preference for the novel food, an intermediate number (8) produced attraction, and too many snails (16) produced aversion. It was claimed that demonstrators responded to the novel food by feeding and modulating their release of feeding pheromone.

Perhaps the best-known example of social learning in invertebrates is the 'waggle dance' of bees in which individuals communicate complex and detailed information

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about the quality, distance and direction of food sources to other members of the hive (e.g. Hammer and Menzel, 1995; Rohrseitz and Tautz, 1999; Weidenmuller and Seeley, 1999). Furthermore, Seeley and Buhrman (1999) and Seeley (2003) described how when a honeybee swarm is about to move nest site, scouts locate potential sites in all directions up to several kilometres away, then return and initially advertise a dozen or more of the sites by dancing in the swarm. Some of the dancers progressively stop dancing and others switch allegiance from one site to another until, eventually, the scouts advertise only one site. Within an hour of unanimity amongst the dancers, the swarm lifts off to the chosen site - this is not necessarily the first one that is advertised to the swarm.

Conditioned suppression

If an animal can be taught to suppress a response, this indicates its behaviour is not rigidly fixed. Several studies have shown that the behaviour of invertebrates can be influenced in this way. Dethier (1964) cited experiments showing that honeybees can be trained to arrive at feeding places at specific times. The bees suppressed flying activity during the normal periods of flying until the appointed hour, indicating they have 'voluntary' control over what most people might consider to be a relatively fixed pattern of behaviour. Balaban (1993) showed that terrestrial snails could be trained to associate the acidity of water with receiving electric shocks and suppressed radular rasping on the substrate. Lukowiak and Syed (1999) showed that aerial breathing attempts by water snails in a hypoxic environment were subject to conditioned suppression by eliciting the pneumostome withdrawal response, and Krasne and Glanzman (1995) cited work showing that the pototaxic response of water snails is amenable to conditioned suppression.

When the foreleg tarsi of a fruit fly contact sucrose, the insect automatically extends its proboscis. It will continue to repeat this time after time for as long as it is hungry. However, if presentation of the sugar is followed immediately by exposure to a solution of quinine, the fly can be conditioned to suppress this response (Fresquet and Medioni, 1993). Smith *et al.* (1991) showed that honeybees learned to discriminate between two odours and could be conditioned to suppress the proboscis extension response when one of the odours was paired with an electric shock.

Discrimination and generalisation tasks

Marshall *et al.* (1996) used an associative learning paradigm to show that stomatopods could visually discriminate between shades of grey and colours. Octopuses are capable of discriminating in both visual (Fiorito and Scotto, 1992; Fiorito and Chichery, 1995) and tactile (Wells & Young, 1969) sensory modalities (also see Mather, 1995). It has been reported that cuttlefish show associative learning, autoshaping and rapid learned aversion (Darmaillacq *et al.,* 2004; Cole and Adamo, 2005).

Giurfa *et al.* (1996) described how honeybees could be trained to discriminate bilaterally symmetrical from non-symmetrical patterns, and that this could be applied to novel stimuli. This shows an ability to detect and generalise the concept of symmetry and asymmetry, and possibly indicates a high level of intelligence when intelligence is defined as the ability to utilise acquired information in a novel situation.

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Reversal learning

Kisch and Erber (1999) fixed honeybees in small tubes such that they could touch one or two small silver plates with their antenna. These touches were sensed by a computer that rewarded the bees by giving them a small drop of sucrose solution. During a 10 minute pre-test period, the mean spontaneous frequency which the antenna touched the plate was recorded. After this, the bee was rewarded if the touching rate exceeded the mean spontaneous frequency by more than one standard deviation. This resulted in an increase in touching of the silver plate. In a second experiment, two plates were put in reach of the antenna. The plate that was spontaneously touched least was then rewarded. This resulted in a higher frequency of touching the 'lesser' plate, indicating the bees were able to discriminate between the two. In a second phase, the alternative plate was reinforced and again the bees learned this, i.e. reversal learning, a type of learning considered to be advanced.

Mather (1995) reports that octopuses can learn reversals, although the study she cites found this was only at the criterion of 70% successful choices, not at the more stringent criterion of 80%. Robertson *et al.* (1995) showed that octopuses can learn reversals of a tactile discrimination task. Mackintosh and Mackintosh (1963) published a paper of which the title suggests that octopuses exhibit reversal learning, but it has not been possible to directly access a copy of this paper.

Development of learning ability

Dickel *et al.* (1998) showed that *Sepia* cuttlefish develop short-term memory within eight days of age, and long-term memory, demonstrated by performance of a learned task 60 minutes after it was taught, develops progressively between 15 and 60 days of age. In a more recent paper (Dickel *et al.,* 2001), the same authors showed a relationship between this development, and the temporal development of particular brain structures, the superior frontal and vertical lobes, relevant to long-term memory.

2.3.1.3.Summary of Memory and Learning in Invertebrates

The memory and learning of invertebrates has been widely investigated. It has been shown that invertebrates are capable of learning in several ways very similar to vertebrates: for example, slugs are capable of first- and second-order conditioning, blocking, one-trial associative learning and appetitive learning (Yamada *et al.,* 1992). In a comprehensive review of invertebrate learning and memory, Carew and Sahley (1986 p. 473) were so impressed by the learning capabilities of invertebrates they were moved to write:

"In fact, the higher-order features of learning seen in some invertebrates (notably bees and Limax) rivals that commonly observed in such star performers in the vertebrate laboratory as pigeons, rats, and rabbits."

2.3.1.4. Spatial awareness and cognitive maps

Some invertebrates appear to have a great awareness of their environment and their spatio- temporal position within that environment, indicating plasticity in behaviour and the ability to monitor and memorise both time and motion.

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Earwigs taken from the wild and removed to the laboratory initially oriented themselves correctly toward their home shore by using the sun and moon as orienting cues. However, after one week this direction had been forgotten, indicating landward direction was learnt and not genetically determined (Ugolini and Chiussi, 1996).

The possible ability of insects to form cognitive maps has received much attention (e.g. Beugnon *et al.,* 1996). Menzel *et al.* (1998) displaced bees caught either at feeders or at the hive entrance. They found that the bees' return journey sometimes included novel shortcuts, indicating formation of a cognitive map, but this was in one direction only (to the hive) and only when the bees had been displaced from the hive, not the feeders.

Some spiders appear to be highly cognisant of their surroundings and their movements in time and space. Wandering spiders have been shown to use highly developed visual systems when locating and chasing prey. Seyfarth *et al.* (1982) painted over the eyes of wandering spiders then placed them into an arena and allowed them to briefly encounter prey. The experimenters then removed the prey and chased the spider away in a straight line to a distance of up to 75cm from where the prey had been encountered. Despite the lack of visual cues, the spiders were able to move back accurately (i.e. within 5cm) to the area where the prey had been caught, at which point they would often commence searching behaviour. More surprisingly, if after encountering prey the spiders were chased through a semicircular corridor, they did not simply retrace this curvilinear route. Rather, they chose a straight, direct path to the site of the prey encounter although there was some bias in starting toward the corridor and the shape of some return paths reflected the curved shape. These experiments showed that spiders use idiothetic orientation, i.e. they memorised information about their previous movements. Seyfarth *et al.* (1982) cited other studies showing that spiders use this ability for egg-sac retrieval and prey recapture in the wild. These indicate that some spiders have not only proprioceptive capabilities, but they also appear to be aware of these in relation to space and time in the form of a simple cognitive map.

Some invertebrates show detour behaviour, in which an animal chooses to take an indirect route to a goal, rather than the most direct route. This is pertinent because it indicates flexibility in behaviour and route planning, and possibly insight learning. Jackson and Wilcox (1993a) reported that jumping spiders in the wild scanned the environment surrounding the web of potential prey before moving to capture the prey, but sometimes chose an indirect route on up to four occasions during a single attack. It is unlikely the spiders were simply wandering away and then inadvertently relocating the web because those spiders that did not scan the environment did not find the web, whereas all those spiders which scanned did find the web. Controlled studies on detour behaviour have been conducted under laboratory conditions. Spiders will successfully navigate an apparatus that requires them initially to move away from a prey item before reversing direction (Tarsitano and Jackson, 1992, 1994; Carducci and Jakob, 2000). Successful navigation was dependent on the presence of a prey item in the goal area, indicating the detour behaviour was not simply aimless wandering by the spiders. The spiders would stop and scan their environment prior to a detour being required, much as if they were planning which route was the next best - possibly indicating a capacity for insight learning. Certainly, such behaviour indicates these spiders have a great ability to comprehend the complex spatial relationship between themselves, their prey and possible routes to a goal.

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Spatial awareness in *Octopus* was demonstrated by Mather (1991*)* and Karson *et al.* (2003) studied maze-learning behaviour in cuttlefish. They concluded that the animals had demonstrated simultaneous discrimination learning and reversal learning. Boal *et al.* (2000) showed that octopus learned the position of a single open escape burrow amongst 6 locations. They retained this information for at least one week and could relearn the position when these were reversed by 180 degrees.

2.3.1.5.Deception

Some spiders use an intriguing method of capturing prey that appears to involve deception (Jackson and Wilcox, 1993b). A hungry *Portia* invades other spiders' webs and then makes a wide range of vibratory behaviours, including twitching its abdomen, and plucking, striking and fluttering movements using virtually any combination of legs and pedipalps at various phases, rates and amplitudes. When the preyed-upon spider moves or performs pull-ups (its normal response to web invasion by a conspecific), the *Portia* repeats the vibration given immediately prior to this until the spider whose web is being invaded moves close enough to be attacked. Jackson and Wilcox (1993b) categorised deception into four levels: Level 1 - mimicry in which deception is effected by appearance; Level 2 - deception is affected by co-ordinated perception followed by action; Level 3 - deception effected by learning; and Level 4 - planned deception. The authors suggest this is an example of level 3 deception.

2.3.1.6.Operant studies

Operant studies are those in which animals operate a manipulandum or change the environment in some way to gain reinforcement or avoid punishment. These indicate flexibility of behaviour, but more than this, they indicate a voluntary act; the animal exerts control over the frequency or intensity of its responses, so the behaviour cannot be based on simple reflexes or complex fixed motor patterns.

An operant learning protocol was developed by Kisch and Erber (1999) in which honeybees were fixed in small tubes such that they could touch one or two small silver plates with their antenna for reinforcement with sucrose solution (see Reversal Learning above).

Balaban and Maksimova (1993) showed that snails would operantly control electrical stimulation of their brains. The snails had fine wire electrodes surgically implanted in two regions of the brain. To receive stimulation, the snail was required to displace the end of a rod, thus closing the switch. Each session began with a 20 min period without reinforcement, and then a 20 min period with reinforcement. When operation of the manipulandum delivered self-stimulation to the parietal ganglion, the frequency of the operant response decreased (Figure 1). However, when operation of the manipulandum delivered self-stimulation to the mesocerebrum, which is involved in sexual activity, there was an increase in the frequency of the operant response. These appear to be typical positive and negative reinforcement responses that we might expect from vertebrates in operant studies.

There are three studies (Dews, 1959; Crancher *et al.,* 1972; Hales *et al.,* 1972) of which the titles indicate that the octopus can be trained in operant studies. Mather (1995) wrote that Crancher *et al.* (1972) conditioned arm extension up to a tube out of water, but attempts by Dews to condition lever pressing were 'less successful'. Fiorito *et al.* (1998) describe how octopuses learn to remove crabs sealed in a jar

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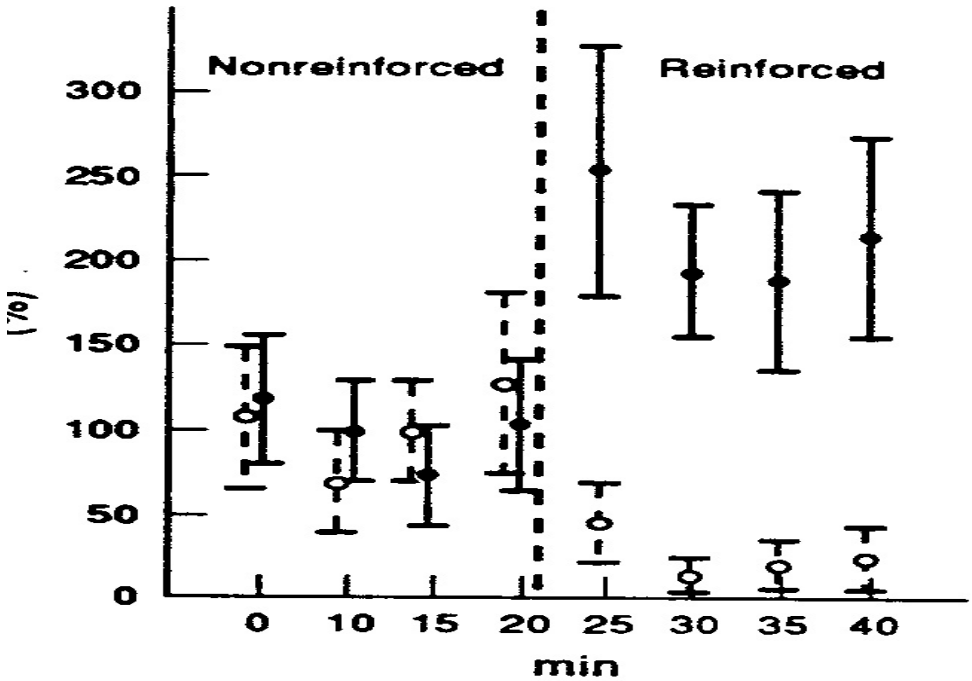
by a plug. In aquaria, octopuses are sometimes given prey items in a screw-lid jar as a form of environmental enrichment. The octopuses readily learn how to grip and twist the lid to open the jar to retrieve the prey. Mather (1994) also described tool use in octopus.

Figure 1 - Self-stimulation of areas of the brain by land snails (Balaban and Maksimova, 1993).



2.3.1.7.Signalling, social and emotional responses

When stressed, cephalopods secrete noradrenaline and dopamine. For example, Malham *et al.* (2002) have shown a 2-2.5-fold increase in these hormones in response to air exposure in *Eledone cirrhosa*. Octopus, cuttlefish and squid have the ability to change colour. These changes in colour are in many instances in response to situations or occurrences that would be associated with an unpleasant emotion in vertebrates, e.g. after fighting or handling. They also play an important role in signalling. Learning is involved in most signalling and the most elaborate signalling and communication systems occur in cuttlefish and squid (Moynihan, 1985). Indeed many of these animals live in social groups and hence may have levels of cognitive ability like those of those vertebrates which have complex social relationships. They demonstrate aggressive behaviour, show rapid colour changes in response to social signals and stop feeding in crowded conditions (e.g., cuttlefish; Boal *et al.,* 1999). In detailed studies of octopod behaviour they appeared to play and to have individual temperaments (Mather and Anderson, 1993, 1999; Wood and Wood, 1999; Sinn *et al.,* 2001).

2.3.2. Brain Cell Numbers

Although, as mentioned above, it is better to judge animal cognition and awareness by their functioning, it is still of interest to consider the numbers of brain cells available for processing. As noted later in relation to spider capabilities, sophisticated

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processing can occur with smaller numbers of cells at the expense of the rate of processing. Spiders may be clever if allowed enough time. The remainder of this section refers solely to number of cells.

Studies of complexity of brain function can give much information about ability as well as about welfare (Broom and Zanella, 2004). One measure of brain complexity is the total numbers of nerve cells present in the central nervous systems, for these cells are the basic elements responsible for neural integration, memory and the generation of behaviour. Nerve cell numbers in central nervous systems vary enormously across different animal groups with around 10^{10} in mammalian brains, 10^{8} in cephalopod brains (Young, 1971), 10^{6} in the nervous systems of social insects such as honey bees (Giurfa, 2003), 10^{5} in other insects (Burrows, 1996), 10^{4} in non-cephalopod molluscs, such as Aplysia, (Kandel, 2001) and less than this in simpler invertebrates, such as leeches, worms and nematodes (Williams and Herrup, 1988). This rank order seems well correlated with the performance ability and behavioural sophistication of the different animal groups.

2.3.3. Nociception and Pain in Invertebrates

Smith and Boyd (1991) suggested seven criteria indicating the capacity for the experience of pain in non-human animals and these are presented in slightly modified form below.

1. Possession of receptors sensitive to noxious stimuli, located in functionally useful positions on or in the body, and connected by nervous pathways to the lower parts of a central nervous system

2. Possession of brain centres which are higher in the sense of level of integration of brain processing (especially a structure analogous to the human cerebral cortex).

3. Possession of nervous pathways connecting the nociceptive system to the higher brain centres.

4. Receptors for opioid substances found in the central nervous system, especially the brain.

5. Analgesics modify an animal's response to stimuli that would be painful for a human.

6. An animal's response to stimuli that would be painful for a human is functionally similar to the human response (that is, the animal responds so as to avoid or minimise damage to its body).

7. An animal's behavioural response persists and it shows an unwillingness to resubmit to a painful procedure; the animal can learn to associate apparently non-painful with apparently painful events

Evidence relating to these criteria in invertebrates is discussed below and summarised in Table 2.

<u>Criterion 1 - Possession of receptors sensitive to noxious stimuli, located in functionally useful positions on or in the body, and connected by nervous pathways to the lower parts of a central nervous system</u>.

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Illich & Walters (1997) stated that nociceptors could be distinguished by 3 characteristics.

(1) They must respond maximally to an injurious stimulus but not to an innocuous one.

(2) They must increase their sensitivity after tissue has been injured to help the animal avoid further injury.

(3) Their rate of firing or sensitivity should be related to the sensitivity of the tissue which they protect – for example, in humans, the nociceptors associated with the eye respond at a pressure 10% less than other nociceptors in the skin of the arm.

Illich & Walters (1997) examined these properties in Aplysia, the sea-hare, which is a marine mollusc. This animal has a complex nervous system, but in particular, large nerves that run from the siphon at the back-end of the animal to the brain in the head. They dissected one of these nerves out, attached a recorder device to the anterior end and then stimulated the siphon with stiff fibres. Not all the fibres caused the nerve to fire. Only the stiffer fibres that put more pressure on the animal's siphon caused the nerves to fire, or when the siphon was pinched with a pair of tweezers, i.e. fulfilling Characteristic 1. They noted that after the nerve had fired, it showed increased sensitivity, i.e. fulfilling Characteristic 2. The mechanical force required to elicit a response in this nerve was approximately 35 g/mm^2, lower than nerves from other tissues in accordance with the soft character of the siphon's tissue, i.e. fulfilling Characteristic 3. Cephalopods have nociceptors in their skin (Wells, 1978).

<u>Criterion 2 - Possession of higher brain centres (especially a structure analogous to the human cerebral cortex).</u>

There can be no doubt that the nervous systems of invertebrates are less complex than vertebrates. Earthworms have bundles of nerve cells called ganglia at intervals along the nerve cord. In the third body segment, several such ganglia are fused together forming a cerebral ganglion. Insects have an anterior ganglion, or 'brain', and a posterior ganglion. The latter controls many functions such as walking and respiration in the absence of any input from the brain, however, the brain is required for the control of feeding in blow-flies and for learning in honeybees (Smith and Boyd, 1991). In cephalopods, the brain: bodyweight ratio exceeds that of most fish and reptiles. For the octopus, its basic movements are controlled by the ganglionated nerve cords of the arms that contain almost three times as many neurones as the brain. The brain weight therefore represents only the more specialised sensory integrative, higher movement control and learning parts of a rather diffuse nervous system. The cephalopod brain shows hierarchical organisation as in vertebrates and might be considered analogous to the cerebrum of higher vertebrates (Russel-Hunter, 1979 cited by Smith and Boyd, 1991).

Sandeman *et al.* (1992) describe in detail the brain structure of several decapod crustaceans and considered that they have a"…brain size and complexity [that] lies somewhere between the octopus and insects. "Learning and subsequent avoidance of putatively painful stimuli in several kinds of invertebrates indicate connections are present from the nociceptors to the nervous tissue associated with learning.

<u>Criterion 3 - Possession of nerve pathways connecting the nociceptive system to the higher brain centres.</u>

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Invertebrates can learn to avoid putatively painful stimuli (See Criterion 7 below). This indicates that there must be nervous pathways between the nociceptors and the higher brain centres, or at least the nervous tissue that is accomplishing the learning process. Woolf and Walters (1991) described the common plasticity of neural pathways associated with nociception in mammals and Aplysia (Figure 2).

Criterion 4 - Receptors for opioid substances found in the central nervous system, especially the brain.

Some invertebrates have many of the neurotransmitters that are involved in vertebrate pain reception and mediation. It has been found that molluscs (Kream *et al.,* 1980 cited by Greenberg and Price, 1983) and insects (Stefano and Scharrer, 1981 cited by Eisemann *et al.,* 1984; Nunez *et al.,* 1983; Zabala *et al.,* 1984 cited by Fiorito, 1986) have opioid binding sites or opioid sensitivity. Certainly, there are many examples of neuropeptides that are involved in vertebrate pain responses being found in invertebrates (Clatworthy, 1996; Stefano *et al.,* 1998), for example, enkephalin and endorphins have been found in platyhelminths, molluscs, annelids, crustaceans and insects (Greenberg and Price, 1983; Fiorito, 1986). As pointed out by Greenberg and Price (1983), the occurrence of vertebrate pain-related neuropeptides in invertebrates does not necessarily mean that invertebrates experience pain; analogous physiological roles in different classes or phyla are not always carried out by homologous peptides, but it does at least indicate that many invertebrates might have the physiological capacity to experience pain or an analogous sensation. In molluscs, naloxone injections (but not other neuroactive substances) into the sites of severed nerves counteract the migration of haematocytes in response to the injury, indicating the involvement of opioid peptides in this response (Clatworthy, 1996). In support of this, injection of a synthetic analogue of metenkephalin induces the directed migration of haematocytes to the site of injection. Furthermore, Clatworthy (1996), in discussing the responses of nociceptors to damaging or potentially damaging stimuli, wrote:

"The enhancement of responsiveness in these sensory neurones following injury or the induction of a foreign body response is therefore functionally similar to hyperalgesia, i.e. a heightened sensitivity to painful stimuli, in mammal(s)".

This may be correct but opioids like metenkephalin whose receptors are blocked by naloxone have roles in a range of physiological processes. Stefano *et al.,* (1998) reported that some invertebrates contain an opioid precursor, proenkephalin. Enkelytin, an antibacterial peptide, is found in proenkephalin, exhibiting 98% sequence identity with mammalian enkephalin. Stefano *et al.* (1998) suggested that the function of enkelytin is to attack bacteria and allow time for the immunocyte-stimulating capabilities of the opioid peptides to emerge. Furthermore, based on the similarity of the biochemical and physiological responses, they proposed that pain itself might be a component of this response. This could be correct but is not proven.

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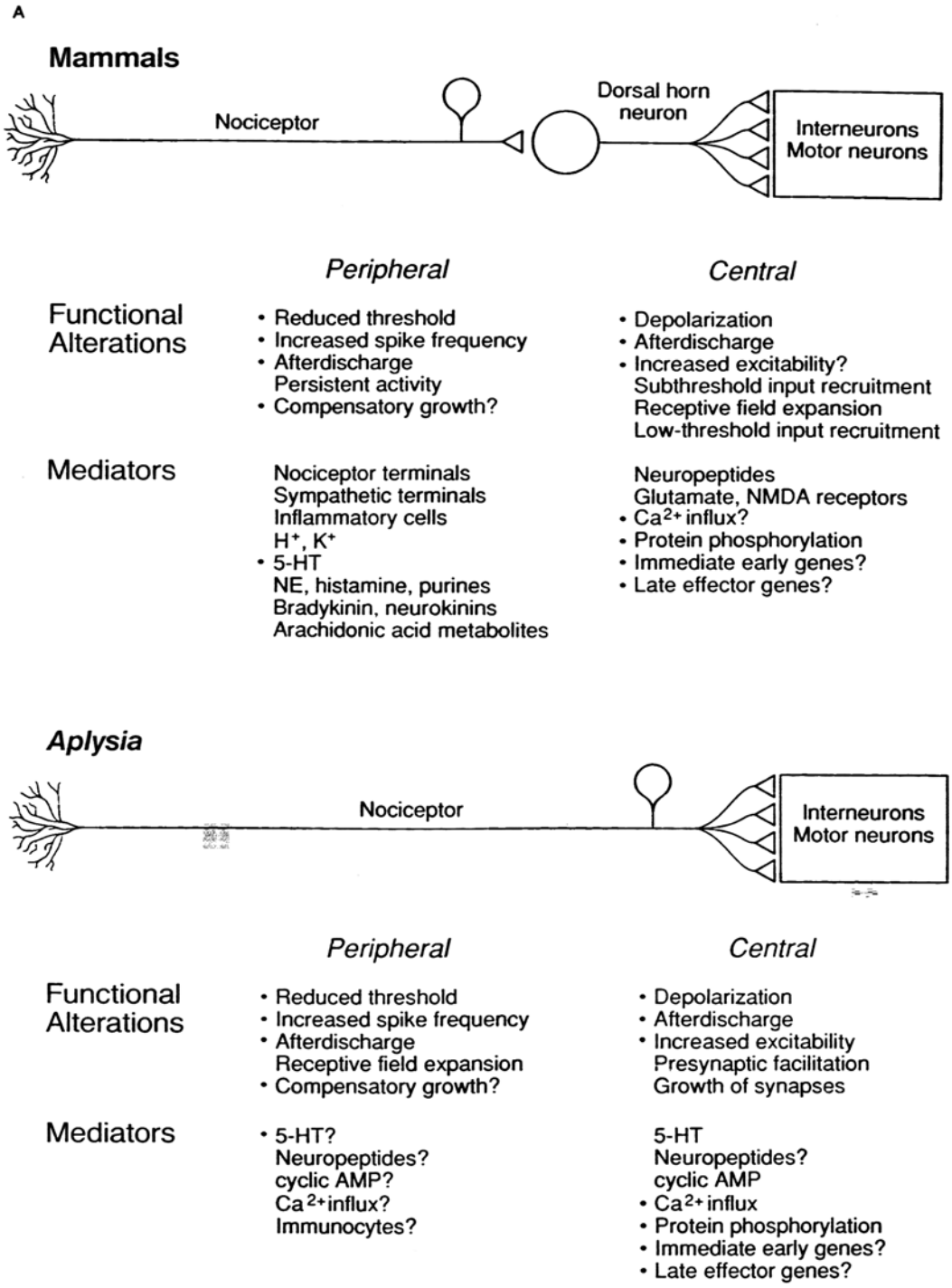
Figure 2 - "Common patterns of plasticity contributing to nociceptive sensitisation in mammals and Aplysia" from Woolf & Walters (1991)



Criterion 5 - Analgesics modify an animal's response to stimuli that would be painful for a human.

Drugs which act as analgesics or counteract analgesia in humans can also influence behavioural responses of invertebrates to putatively painful stimuli. However, in all such studies the other various effects of that analgesic require consideration. For example some mammalian analgesics also have sedative effects and it might be that only the sedative effect occurs in some other types of animals. In consequence, studies of animals in situations that might cause tissue damage are of particular relevance. Injection of morphine produces a dose-dependent decrease in the crabs'

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defensive response to being struck between the eye-stalks (Lozda *et al.,* 1988; Bergamo *et al.,* 1992; also see Fiorito, 1986). Gritsai *et al.* (2004) reported that injections of morphine increased the amount of time that cockroaches and other invertebrates spend on a hot plate. Using a similar technique, Zabala and Gomez (1991) showed that injections of water had no effect on the time that the crickets spent on a hot plate, whereas injections of morphine increased the time crickets spent on there. The effect of morphine was reversed (blocked) by naloxone, an effect we would expect in vertebrates. Nunez *et al.* (1983) placed Africanised wild bees into an apparatus, passed several electric shocks through the animals and recorded how many times the bees performed the stinging response. On separate groups of bees, they examined the effects of injecting various concentrations of morphine and naloxone into the bee 15 minutes before the electric shocks. They noted that there was a very strong dose-dependent effect of morphine, and if they administered naloxone, the morphine dependent response completely disappeared. In a similar experiment, Maldonado and Miralto (1982) examined the effects of morphine on the defence response of the mantis shrimp. They also reported that naloxone completely inhibited the insensitivity effect attributable to morphine. Using snails, Saksida *et al.* (1993) showed dose-dependent analgesic effects of enkephalinase inhibitor, and Kavaliers and Perrot-Sinal (1996) showed that nociceptin increased sensitivity to a hot-plate - a response we would expect from vertebrates administered these drugs.

Dyakonova (2001) reviews the role of opioid peptides in the behaviour of invertebrates. Data were presented indicating that opioids give an apparent analgesic effect in leeches, molluscs, crabs and insects. In many of these, naloxone reversed this analgesic effect. Where locomotion is measured as the main response to a potentially painful stimulus, the direct effects of opioids on locomotion must be taken into account.

Agnisola *et al.* (1996) stated that for octopus, cold water anaesthesia should be considered as having anaesthetic and analgesic properties and this paper cites several other studies investigating analgesia in octopuses.

<u>Criterion 6 - An animal's response to stimuli that would be painful for a human is functionally similar to the human response (that is, the animal responds so as to avoid or minimize damage to its body).</u>

When considering this criterion, it should be remembered that natural selection has acted on many vertebrate species to prevent them from showing pain under some circumstances, e.g. to avoid attracting unwanted attention from predators. Many invertebrates are also prey species and therefore might have evolved the same mechanism of not behaviourally responding to stimuli which might cause pain. However, it is often suggested that the responses of invertebrates to putatively painful stimuli are simple and stereotyped, indicating they are unlikely to feel pain.

Walters *et al.* (2001) examined in great detail the responses of the tobacco hornworm larvae to different levels of noxious stimuli. They pressed the ends of stiff fibres against the legs and body of the caterpillar. They observed that the responses were far from stereotypical. If the fibre was pressed to the posterior of the caterpillar, it would rear its head back and then strike at the source of the stimulus, the fibre. They also noticed that when the caterpillar struck, it opened its mandibles and would sometimes regurgitate. They investigated whether this response was sensitive to the degree of putative pain. They noted that if a stiffer fibre was used which placed more pressure on the caterpillar, the animal would rear back further, and would strike more

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forcefully. In addition, if the fibre was pressed into the animal at the anterior end, there was a completely different reaction - the caterpillar would instead withdraw its head and attempt to avoid the noxious stimulus. So, this response appears to be both sensitive and flexible. They also conducted a similar set of experiments by pinching the caterpillar with a pair of tweezers and got identical results. But they noticed, in this particular case, that if the animal was accidentally wounded by this procedure, it would often move its mouth-parts over the wound-site with the appearance of grooming behaviour, much like a dog would lick a wounded leg.

When vertebrates injure a part of the body, they often move in such a way that they to protect the area. In some species, if a limb or tail is caught, the animal reduces the likelihood of being killed by shedding the limb or the tail, i.e. autotomy (Punzo 1997). Fiorito (1985) reported that crabs exposed to a hot plate show leg autotomy. There is evidence that in spiders, this response might be invoked by a sensation similar to human pain. Orb-weaving spiders undergo autotomy if they are stung in a leg by wasps or bees. Under experimental conditions, when spiders were injected in the leg with bee or wasp venom, they shed this leg. But, if they were injected with only saline, they rarely autotomised the leg, indicating it was not the physical insult or the ingress of fluid *per se* that caused autotomy. Spiders injected with venom components that caused injected humans to report pain (serotonin, histamine, phospholipase A2 and melittin) autotomised the leg, but if the injections contained venom components which do not cause pain to humans, autotomy did not occur (Eisner and Camazine, 1983).

Criterion 7 - An animal's behavioural response persists and it shows an unwillingness to resubmit to a painful procedure; the animal can learn to associate apparently non-painful with apparently painful events.

Many species have been trained to withdraw from or alter their behaviour in response to a conditioned stimulus when this has been previously paired with an electric shock (adult and larval Drosophila: Carew and Sahley, 1986) (snails: Balaban, 1993) (leeches: Sahley, 1995) (locusts: Horridge, 1962) (bees: Smith *et al.,* 1991) (various marine molluscs: Carew and Sahley, 1986) (octopus: Robertson *et al.,* 1995). If a vertebrate species is used in such studies, it is usually taken for granted that the learning process has arisen as the result of the animal experiencing pain or discomfort from the electric shock. Octopods can learn to avoid electric shocks and other painful stimuli as well as to gain rewards, e.g. *O. cyanea* (Papini and Bitterman, 1991). They release stress hormones in response to situations that would elicit pain and distress in humans. Snails learnt to reduce spontaneous operation of a manipulandum (see Figure 1) when this resulted in electrical stimulation of the parietal area of the brain (Balaban and Maksimova, 1993) i.e. the behavioural response persisted and the snails learned to associate apparently non-painful with apparently painful events.

2.3.4. Evidence against invertebrates having the capacity to experience suffering.

Doubt has been expressed that invertebrates (except perhaps the cephalopods) are able to experience suffering or pain, (e.g. Eismann *et al.,* 1984; Wigglesworth, 1980; Varner, 1999), however, there is little empirical 'evidence' that invertebrates are not capable of these experiences. Arguments that invertebrates do not possess these capacities are based on two observations. First, the occasionally noted absence of

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behavioural responses in conditions that we would expect great responsiveness from vertebrates. Second, a lack of central nervous system complexity.

It has been noted that insects will continue to feed whilst being eaten by predators, parasitoids or even in the case of the male praying mantis, their sexual partners (Eismann *et al.,* 1984). However, when invertebrate behaviour is examined in more detail (e.g. Horridge, 1962; Carew and Sahley, 1986; Balaban, 1993; Sahley, 1995; Walters *et al.,* 2001), there is compelling evidence for invertebrates showing avoidance behaviours in response to noxious or putatively painful stimuli. In addition, there may be evolutionary advantages to not showing pain such as avoiding attracting predator attention, or, the male mantid avoiding risking injuring the female he has just mated and is presumably about to become pregnant with his offspring. Furthermore, there are occasions when vertebrates behave as if they are not in pain, e.g. racing horses continuing to run after they have broken a leg, or hens allowing themselves to be severely cannibalised with no indication of experiencing pain. Such exceptions do not lead us to make sweeping statements regarding the capacity for pain sensitivity in vertebrates in such circumstances.

2.3.5. Summary:

In respect to brain and nervous complexity, there is no doubt that invertebrates have simpler nervous systems than vertebrates, but does this mean they are unable to suffer? The cerebral cortex is thought to be the seat of consciousness in humans (Smith and Boyd, 1991). In fact, pain and suffering are sometimes defined in terms of neural activity in the cerebrum, which makes it a rather circular argument to then dismiss the possibility of invertebrates being capable of suffering because they lack such a structure. It is possible that other structures, as yet undetermined, within the brain or elsewhere fulfil a similar function to the cerebrum in terms of processing information related to suffering. Analogous yet disparate structures have evolved throughout the animal kingdom. For example, the compound eye of some invertebrates is strikingly different in form from the mammalian eye, yet they both achieve the same function - they allow the animal to perceive light. Parts of the nervous system of invertebrates that are not the anterior brain are capable of controlling breathing, movement and learning (e.g. octopuses, cockroaches). Possibly, areas of invertebrate nervous tissue have evolved abilities analogous to the cerebrum of mammals and give these animals the capacity to suffer. Above all, we should remember that absence of evidence is not evidence of absence.

2.4. Brief summaries for non-vertebrate groups and recommendations

There are two reasons for recommending inclusion. First, when there is sufficient concern based on scientific evidence that these animals have the ability to experience pain and distress. And secondly, when there is some knowledge but not sufficient to make a case for full inclusion and so they should be protected until it can be shown that they do not experience pain and distress. Whenever scientific knowledge arises that helps to eludicate better whether animals are able to experience pain and distress, so that evidence should be reviewed and, if necessary, that grouping of animals should be reclassified.

2.4.1. *Cyclostomes (lampreys and hagfish).*

These animals have relatively simple brains in comparison with the most complex fish but there are other fish with brains that differ little in complexity. Some of the

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first studies of the pain system in fish were carried out on lampreys. When Martin and Wickelgren (1971) and Mathews and Wickelgren (1978) made intracellular recordings from sensory neurones in the skin and mouth of a lamprey (*Petromyzon*) during heavy pressure, puncture, pinching or burning, the output was like that which would be recorded in a mammalian pain receptor. The conduction velocity was slow relative to other sensory neurones, so they are probably of small diameter. There was no fatigue with repeated stimulation and the receptors were sensitised following local tissue damage. Lamprey behaviour is little studied.

2.4.2. *Amphioxus*

This small animal, usually known by its former scientific name amphioxus, is sometimes called the lancelet because of its shape. Amphioxus can swim and respond to stimuli in a way that is similar to juvenile fish. The neural tissue is localised into a brain which is small and less complex than that of fish. The behaviour is little studied and learning ability largely unknown.

2.4.3. *Tunicate*

Larval tunicates are small tadpole-like animals that appear to respond to stimuli in a way which may be more complex than many larval fish. Most adult tunicates are sessile filter feeders with a much reduced nervous system. However, some marine pelagic tunicates such as salps and possibly also species such as *Oikopleura* may be complex in behaviour and ability to assess their environment.

2.4.4. *Hemichordata such as Balanoglossus*

Balanoglossus, the acorn worm, lives on the bottom in marine environments. There is no indication from its behaviour that it has any sophisticated brain function.

2.4.5. *Cephalopods (octopods, squid, cuttlefish, nautiloids)*

There is evidence that cephalopods have a nervous system and relatively complex brain similar to many vertebrates, and sufficient in structure and functioning for them to experience pain. Notably, they release adrenal hormones in response to situations that would elicit pain and distress in humans, they can experience and learn to avoid pain and distress such as avoiding electric shocks, they have nociceptors in their skin, they have significant cognitive ability including good learning ability and memory retention, and they display individual temperaments since some individuals can be consistently inclined towards avoidance rather than active involvement. Most work on learning ability has been carried out in the non-social but visually very competent *Octopus vulgaris*. All squid, cuttlefish and octopods (coleoid cephalopods) studied have a similar ability to sense and learn to avoid painful stimuli, and many are more complex and more likely to experience pain and distress than *O. vulgaris*. Learning is involved in most signalling and the most elaborate signalling and communication systems occur in cuttlefish and squid that can show rapid emotional colour changes and responses to these. Indeed many of these animals live in social groups and hence may have levels of cognitive ability like those of vertebrates that have complex social relationships. Nautiloids have less complex behaviour than coleoid cephalopods and much less is known about their learning ability. They use odour discrimination to find mates and respond to and track other individuals of their own species (Basil, 2000, 2002) but little is known about their pain system and it is not clear whether they are as capable of suffering as other cephalopods. However, they

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live for a long time and are active pelagic animals so we cannot be sure about their level of awareness.

2.4.6. *Land gastropods*

Snails and slugs can show quite complex learning but the relatively slow locomotion of most of them does not enable them to show rapid escape responses, except for localised movements like eye withdrawal. The case for a substantial degree of awareness would appear to be weak.

2.4.7. Tectibranch and nudibranch molluscs

The most active marine gastropod molluscs are the tectibranchs, such as *Aplysia* and some of the nudibranchs (sea slugs). Much research has been carried out on the nervous system of *Aplysia* and it relatives. Evidence of learning and flexibility of behaviour is considerable but there are also studies showing very rigid responses. Nudibranchs appear to be less flexible than some tectibranchs.

2.4.8. *Social insects*

The social ants and bees, and to a lesser extent the wasps and termites, show considerable learning ability and complex social behaviour. There is evidence of inflexibility in their behaviour but the trend in recent research has been to find more flexibility. The small size of the brain does not mean poor function as the nerve cells are very small. A case might be made for some bees and ants to be as complex as much larger animals. They might be aware to some extent but we have little evidence of a pain system.

2.4.9. *Other insects*

There is a difference in complexity of behaviour between the social and non-social insects. However, learning is clearly possible in these animals. There is little evidence of awareness but few people have looked for it.

2.4.10. *Spiders, especially jumping spiders*

In recent years, dramatic evidence has been produced of the sensory processing, analytical and prediction ability of salticid spiders. The eyes are large and complex and although the brain is composed of a relatively small number of cells, the level of processing is considerable and sophisticated, if rather slow. Evidence for awareness is greater than in any other invertebrates except cephalopods but we have little evidence of a pain system.

2.4.11. Decapod crustaceans (lobsters, crabs, prawns, etc.)

The largest of these animals are complex in behaviour and appear to have some degree of awareness. They have a pain system and considerable learning ability. Little evidence is available for many decapods, especially small species. However, where sub-groups of the decapods, such as the prawns, have large species which have been studied in detail they seem to have a similar level of complexity to those described for crabs and lobsters.

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2.4.12. *Isopods (woodlice and marine species)*

Learning is clearly possible in these animals and some of them live socially. The degree of complexity of functioning is lower than that of the larger decapods or many insects and spiders.

2.4.13. ***Other phyla (e.g. Annelida, Echinodermata, Platyhelminthes, and Nematoda)*** not described above, as well as other Classes, have been considered but are not thought to need protection.

Table 1 - Evidence of higher cognitive capacities in invertebrates.

	Gastropods	**Insects and Arachnids**	**Crustaceans**	**Cephalopods**
Complex memory	Yamada *et al.,* 1992 Fresquet and Medioni, 1993	Greggers and Menzel, 1993 Menzel, 1993 Hammer and Menzel, 1995 Wustenberg *et al.,* 1998	Tomsic *et al.,* 1996 Gherardi and Atema, 2005 Feld *et al.,* 2005	Dickel *et al.,* 1998 Halm *et al.,* 2000 Agin *et al.,* 2003 Darmaillacq *et al.,* 2004
Discrimination and Generalisation	Yamada *et al.,* 1992	Giurfa *et al.,* 1996	Marshall *et al.,* 1996	Wells and Young, 1969 Young, 1991 Fiorito and Scotto, 1992 Fiorito and Chichery, 1995 Robertson *et al.,* 1995
Social learning	Suboski *et al.,* 1993	Hammer and Menzel, 1995 Rohrseitz and Tautz, 1999 Seeley and Buhnnan, 1999 Weidenmuller and Seeley, 1999 Seeley, 2003		Fiorito and Scotto, 1992
Conditioned suppression	Krasne and Glanzman, 1995 Balaban, 1993 Lukowiak and Syed, 1999	Dethier., 1964 Smith *et al.,* 1991 Fresquet and Medioni, 1993		Mather, 1995
Reversal learning		Kisch and Erber, 1999		Mather, 1995 Robertson *et al.,* 1995
Spatial awareness		Seyfarth *et al.,* 1982 Tarsitano and Jackson, 1992, 1994 Jackson and Wilcox, 1993a Ugolini and Chiussi, 1996 Menzel *et al.,* 1998 Carducci and Jakob, 2000		Boal *et al.,* 2000 Karson *et al.,* 2003
Deception		Jackson and Wilcox, 1993b		
Operant studies	Balaban and Maksimova, 1993	Kisch and Erber, 1999		Dews, 1959* Crancher *et al.,* 1972* Hales *et al.,* 1972* Mather, 1994 Fiorito *et al.,* 1998

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Table 2 - Evidence of the capacity for invertebrates experiencing pain using the criteria of Smith and Boyd (1991).

	Gastropods	**Insects and Arachnids**	**Crustaceans**	**Cephalopods**
Nociceptive system	Illich and Walters, 1997			
Complex brain structure perhaps analogous to human cerebral cortex			*Sandeman *et al.,* 1992	*Smith and Boyd, 1991
Nociceptors connected to higher brain structures	Not known	Not known	Not known	Not known
Opioid type receptors or sensitivity	Kream *et al.,* 1980 Greenberg and Price, 1983 Fiorito, 1986	Stefano and Scharrer, 1981 Greenberg and Price, 1983 Nunez *et al.,* 1983 Zabala *et al.,* 1984 Fiorito, 1986		
Responses modified by analgesics	Dyakonova, 2001	Zabala and Gomez, 1991 Dyakonova, 2001 Gritsai *et al.,* 2004	Maldonado and Miralto., 1982 Lozda *et al.,* 1988 Bergamo *et al.,* 1992 Dyakonova, 2001	Agnisola *et al.,* (1996)
Response to noxious stimulus persists	Balaban and Maksimova, 1993	Walters *et al.,* 2001	Kawai *et al.,* 2004	Robertson *et al.,* 1995
Associates neutral with noxious stimuli	Gelperin, 1975 Sahley *et al.,* 1981 Carew and Sahley., 1986 Yamada *et al.,* 1992 Balaban., 1993 Krasne and Glanzman, 1995	Horridge, 1962 Carew and Sahley, 1986 Smith *et al.,* 1991 Krasne and Glanzman, 1995		Robertson *et al.,* 1995

** Information is equivocal*

2.5. Fetal and embryonic animals which might be protected

2.5.1. *Fetal sentience*

2.5.1.1.Some developmental differences

In this section the likelihood of consciousness occurring in fetuses and embryos is considered together with the implications this has for safeguarding their welfare. The key question is at what stage of development is a fetus of a particular species likely to become aware and be able to experience pain and distress. While we have data on some species, in general this is not a well researched area for many of the protected species in the Directive.

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Firstly, it is helpful to distinguish species according to whether they are altricial or precocial at birth. An example of a precocial species is the horse. It is well-developed physiologically and behaviourally at birth, unlike altricial species, which would include marsupials where the joeys are born in less advanced states. These are extreme cases, and there are finer levels of distinction between altricial and precocial species. For example, in avians many duck species are precocial and show strong following behaviour within minutes of hatching, whereas raptors have a relatively long fledging period before they are able to perform well-coordinated walking or flying. It is plausible that there is a greater likelihood of sentience in precocial species than in altricial species. Precocial species depend on greater development and use of sensory faculties from the moment of birth or hatching, whereas this requirement is at a lower level in many altricial species.

Secondly, the differences between oviparous and viviparous species require consideration. The mothers of mammals and other viviparous species could have substantial problems if the fetus or fetuses were too active. A system for the suppression of activity is therefore adaptive in these animals. Such suppression could, but need not, involve suppression of consciousness until independent living, usually associated with the onset of breathing, occurs. However, there may be advantages associated with an ability to respond to and learn from stimuli received in utero and this could require some degree of awareness. Development in an egg, on the other hand, has less constraint on the development of brain function because movement is physically limited by the egg-shell and fetal activity is less risky than it would be in viviparous species. A consequence is that awareness could safely develop earlier and be continuous instead of intermittent in oviparous species. If awareness is the criterion for protection, birds, reptiles, amphibians, fish and cephalopods may, therefore, be more obviously in need of protection pre-hatching than mammals are in need of protection pre-partum.

2.5.1.2.Constraints on late development pre-partum and pre-hatching

Shortly after birth, precocial species are able to stand, walk and run. These activities have to be suppressed whilst the fetus is in the uterus, otherwise they could jeopardise the comfort of the dam, and when violent they could pose a risk of uterine rupture, placental abruption and abortion. Under normal conditions *in utero*, these activities are suppressed through control over fetal oxygenation. Oxygenation in the fetus is normally lower than that in the newborn. If oxygenation is raised artificially, the fetus becomes physically aroused and more active. The situation may, however, be more complex in the case of oviparous species. Some chicks show responses to sounds, touch and light several days before hatching, breathe for many hours before hatching, and there is clicking communication among unhatched chicks ("pipping") which allows synchronisation of hatching in some species (Vince, 1973; Broom, 1981). It may also be that some reptiles develop brain function hours or days before hatching. Whilst most of the data presented in the text which follows concerns mammals, precocial birds and reptiles have many similarities to precocial mammals in development of potential for awareness and altricial mammals have similarities to altricial birds. Most amphibians and fish have larval forms which are not well developed at hatching but develop rapidly with experience of independent life, Those fish and amphibians that are well developed at hatching or viviparous birth and all cephalopods, since these are small but well developed at hatching, will have had a functioning nervous system and the potential for awareness for some time before hatching.

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2.5.1.3.Neural development

In mammalian species such as the human and the rat, sensory pathways in the peripheral nervous system and spinal cord are well-developed by the time the individual is born (Anand and Hickey, 1987; Fitzgerald, 1999). They possess the necessary neural structures, neural connections, and neurotransmitters for afferent sensory and efferent motor activity that serve a range of functions. In the near-term human fetus, there is, however, a limited repertoire of physical movements before birth even though some fetal limb movements may start after 4 to 5 months of pregnancy (quickening). Those movements that do occur are in cycles, usually once every 1 to 10 minutes, and limb movements predominate. The activity cycles are similar to those seen in the supine newborn baby, and in the latter they can occur whilst the baby is asleep as well as awake (Robertson, 1987).

Sensory and neural development in a precocial bird such as the domestic chick is very well advanced several days before hatching. Controlled movements and coordinated behavioural and electrophysiological evoked responses to tactile, auditory and visual stimuli appear three or four days before hatching occurs after 21 days of incubation (Broom, 1981).

The near-term rat fetus is capable of physical reflex responses to noxious cutaneous stimuli, such as pricking a foot with a needle. The responses are generalised whole body movements, rather than the typical limb withdrawal response seen later in the infant pup. The transition from generalised to localised types of response is thought to depend on post-natal maturation of central nervous system pathways and the emergence of descending inhibitory control of the generalised writhing movements.The pattern of the pre-natal generalised responses is often unpredictable, and this has led observers to suspect that the responses are poorly organised centrally. The onset of transition from generalised to localised responses to potentially painful stimuli may vary between species.

2.5.1.4.Pain system development

Evidence on the maturity at birth of afferent nociceptive pathways and the central nervous system gives contrasting impressions. The central nervous system in the human fetus is usually considered as being both structurally and functionally immature at the time of birth (Marsh *et al.,* 1997) and that fetal pain perception is unlikely before the third trimester (Lee *et al.,* 2005). Not only is there poor central organisation in the physical responses to potentially painful stimuli, but there is incomplete development of C-fibre afferent activity (unmyelinated C fibres are an important type of nerve fibre for nociception). Taken together, this indicates that opportunities for perceiving some painful stimuli in the fetus are reduced. However, there are other features that suggest the opposite. For example, the exaggerated NMDA (N-methyl-D-aspartate) induced responses in the substantia gelatinosa, and the reduced descending inhibition along the spinal cord, imply a capacity for heightened afferent activation of nociceptive pathways.

The newborn lamb, foal and calf are, in comparison with the rat and human newborn, relatively well developed neurologically and behaviourally. Neural development is sufficiently advanced at full term in the sheep fetus, to use this species as a model for assessing high levels of risk of perception and suffering before and during delivery. This is fortunate, as the sheep has been the preferred

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species for experimental research into fetal physiology. Corresponding knowledge on arousal is relatively advanced in the sheep fetus.

2.5.1.5.Awareness in the fetus

It may also be helpful to differentiate in the context of sentience the difference between embryos and fetuses. The embryo is the unborn offspring from the zygote until the all major structures are represented. On the other hand, the fetus is the unborn offspring in the post-embryonic period when the major body structures have been outlined (Dorland Medical Dictionary). Only after the development of high level brain functioning would it be possible for a fetus to be capable of being sentient.

Experimental work on conscious awareness in the sheep fetus has been reviewed by Mellor and Gregory (2003) and up-dated by Lyche *et al.* (2005) and Mellor *et al.* (2005). In summary, the work indicates that wakefulness does not occur in the fetus until it breathes air after it has been delivered by natural birth or removed from the womb during the latter stages of development when breathing is possible (Mellor and Gregory, 2003). Consciousness is suppressed *in utero* by a number of endogenous factors including allopregnanolone, pregnanolone, hypoxia, adenosine, prostaglandin D2, and warmth (Mellor *et al.,* 2005). Key steps during birth that provoke wakefulness are oxygenation derived from breathing air, and the effect this has in reducing adenosine concentrations in the bloodstream. Exposure to cold, physical stimulation (e.g. by licking or rubbing) and reduction in blood supply through the umbilicus are also important in initiating breathing, which in turn stimulates the increase in oxygenation that allows consciousness to occur.

It cannot be claimed with certainty that there are no periods of transient or episodic conscious awareness in the fetus *in utero* but, based on the electroencephalogram, no distinct phase of EEG activity has yet been identified that demonstrates the presence of this or any other type of wakefulness. The electroencephalogram of the fetus alternates between two types of sleep state; rapid eye movement (REM) sleep and non-REM (NREM) sleep. The interface between these two states has been discounted as a period when awareness is likely to occur (Mellor *et al.,* 2005). Not all of those who interpret EEG data would express certainty that the data obtained from fetal sheep in the latter stages of pregnancy could never indicate awareness. However, it would be widely accepted that the EEG evidence from the fetal brain, supports the view that consciousness in the fetus is suppressed to some large degree before it breathes air. If there are episodes of conscious awareness in the fetus, they would probably coincide with periods of above-normal fetal oxygenation. Since, in the lamb fetus, the normal level of oxygenation is quite close to the level that is thought to be the interface between consciousness and unconsciousness in the neonatal lamb (Mellor and Gregory, 2003), it is possible that such episodes of consciousness could occur in the fetus. It must be emphasised, however, that such episodes have not been demonstrated or proven to exist, and we are not in a position to estimate either their frequency or their duration.

It has been suggested that consciousness is not an all-or-none phenomenon. Instead, there could be degrees of consciousness, and different depths of unconsciousness (Gregory and Shaw, 2000) often related to the disappearance of somatosensory reflexes. We have no exact words that describe such gradations in consciousness, and until our understanding of the different facets and depths of

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consciousness becomes more developed, we are unable to comment on the likelihood or their potential significance in a fetus.

Reflex responses in the rat fetus are not necessarily signs of true pain experience. Appreciation of pain or distress requires functional maturation of higher brain centres, and it has been suggested that those centres are not sufficiently advanced in the near-term rat or human fetus to support those perceptions (Fitzgerald, 1999). Notwithstanding this, the fetus can show behavioural responses to relatively innocuous stimuli that resemble conscious responses. For example, intra-oral infusion of lemon juice elicits face-wiping behaviour in the rat fetus, whereas milk infusion evokes a stretch response similar to that seen post-natally (Robinson and Smotherman, 1992).

Circumstantial anecdotes, which have considered whether or not human and animal fetuses are conscious peri-natally, produce conflicting impressions. Some comments support the view that the human newborn is not conscious until it "pinks-up", whereas other accounts consider that reflex responses *in utero* are indicative of awareness or an imprecise plane of conscious responsiveness. It might be helpful if a summary of the scientific evidence on the likely presence or absence of conscious awareness in fetuses is made generally available to interested professional parties such as research scientists, animal care staff and veterinarians, as this may help refine future anecdotes about potential awareness and suffering both during delivery and *in utero*.

2.5.1.6.Fetal manipulations

It is possible that there may be some areas of concern where the fetus may be manipulated or experience a treatment that could have immediate or lasting effects. Notwithstanding the evidence which indicates that the fetus is not conscious before birth, there is evidence which indicates that the fetus can be affected by stressors applied to the pregnant mother, and that those stressors can have long term effects on development and behaviour post-natally (Schneider *et al.,* 1992, Janczak *et al.,* 2005). There are two potentially important effects. First, the fetus is capable of associative learning, which can be maintained after birth (Hepper, 1991). This has been shown as aversion when presented with stimuli that were associated with an artificially induced episode of hypoxia *in utero*, and as attraction in the context of the removal of the hypoxia. This, and other evidence, indicates that the fetus has memory for classical conditioning, habituation and exposure learning paradigms, but exact evidence for learning from auditory cues in the human fetus is limited (Hepper, 1996; Moon and Fifer, 2000). Secondly, emotional stress applied to the pregnant mother can result in low birthweight, early feeding difficulties and growth retardation in the young. In extreme cases there may also be feminisation of males *in utero*, early motor retardation post-natally, learning deficits, and undue anxiety when presented with novel situations. Some of these effects will be solely nutritional via the mother but others may be mediated via effects which the fetus experiences. In primates, there has also been a shorter attention span and increased emotional reactivity in future offspring, after treating the mother daily for 14 days with ACTH when she was in mid-pregnancy.

Experimental procedures that involve oxygenating a fetus or allowing a fetus to breathe oxygen-enriched gas mixtures (e.g. maternal anaesthesia) could induce consciousness in the fetus. Special safeguards may be required in terms of anaesthesia and analgesia in these situations.

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Fetuses are unable to mount the complete profile of acute inflammatory responses that are typically seen following injury in the juvenile (Adzik and Longaker, 1991). Nevertheless, they show sufficient responses to raise concern that injury to the fetus during surgery or delivery can result in postnatal inflammatory pain. This aspect warrants further investigation to understand the likelihood of postnatal inflammatory pain arising from experimental surgery in the near-term fetus.

2.5.1.7. Anaesthetics and analgesics for fetuses

We are not in a position to recommend particular anaesthetics or analgesics for use in fetuses. The anaesthetic or analgesic of choice may need to take into account the risks to the fetus in terms of survival, the stage of development of the fetus and the species. It seems safe to assume that as all the anaesthetic agents are very lipid soluble and that the placenta has a high blood flow, so fetal exposure to these drugs is very similar to that of the mother i.e. the fetal and maternal concentrations achieved will be the same. Consequently, if the mother is adequately anaesthetised, then so also will be the fetus and the type of placentation does not seem to be significant in this respect. However, the practice of delivering volatile anaesthetics to the dam in high levels of oxygen might affect fetal awareness due to the higher partial pressure of oxygen in the maternal blood.

2.5.1.8. Fetus removal in abattoirs etc.

The implications of the above points in terms of managing fetuses during the slaughter of pregnant animals in abattoirs, collecting fetal calf blood serum in abattoirs, performing fetotomy as a veterinary procedure, and collecting fetal tissues in abattoirs for human consumption, have been discussed by Mellor and Gregory (2003).

2.5.1.9. Summary for reptiles, birds and mammals

Even though the mammalian fetus can show physical responses to external stimuli, the weight of present evidence suggests that consciousness does not occur in the fetus until it is delivered and starts to breathe air. However, events in utero can influence the behaviour of the individual once it is born, and some of those effects could be important to its subsequent welfare. Precocial oviparous species present much evidence of being conscious at hatching and during the last days before hatching.

2.5.1.10. Fish and amphibians

Fish and amphibians which develop in water utilize the food reserves from the egg and then start to feed independently. It is at this stage of development that brain function and sensory systems, including pain systems, start to be similar to those of adult fish. There is, however, considerable variation amongst fish in the stage of development at which independent feeding starts.

2.5.1.11. Invertebrates

It is not known at what stage the developing forms of invertebrates recommended to be included from the first section of Chapter 2, develop the ability to experience pain and distress, if at all.

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2.6. Implications for the definition of a "protected animal"

While the principal reason for the existence of legislation is to harmonise the implementation of the Three Rs of Replacement, Reduction and Refinement. This would imply that it is important to define the term "protected animal" and other animal forms which are to be protected during experimental and other research work.

When experiments are carried out *in vivo* (literally meaning scientific procedures involving a living animal with its whole body systems intact) a key point is whether the animal is able to experience pain and distress and other forms of suffering. The inclusion, therefore, of invertebrates and fetal forms from certain stages of gestation, as well as vertebrates, based on the information given in Chapter 2, is essential information for risk management. The WG have tried to give guidance on that issue with the criteria used to do so. The use of terms such as free-living, capable of independent feeding etc are fraught with difficulties as they do not allow all animals forms at all stages of development to be clearly distinguished on the basis if their ability to experience pain, distress etc. There are however, some worthwhile analogies that can be made, so that more complex forms are more likely to be sentient than simple forms i.e. independent feeders are more likely to be sentient than sessile free living forms,

The WG is proposing therefore, that three categories be established.

Category 1 - The scientific evidence clearly indicates that those groups of animals are able to experience pain and distress, or the evidence, either directly or by analogy with animals in the same taxonomic group(s), are able to experience pain and distress.

Category 2 - The scientific evidence clearly indicates that those groups of animals are NOT able to experience pain and distress, or the evidence, either directly or by analogy with animals in the same taxonomic group(s), are unable to experience pain and distress.

Category 3 - Some scientific evidence exists that those groups of animals are able to experience pain and distress, either directly or by analogy with animals in the same taxonomic group(s), but it is not enough to make a reasonable risk assessment on their sentience to place them in either Category 1 or 2.

Any such categorisation of animals and their forms will need updating as scientific knowledge accumulates.

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3. QUESTION ON PURPOSE-BRED ANIMALS

3.1. Introduction

The species listed in Annex I to Council Directive 86/609/EEC are those that must be "purpose bred" when used in experiments, unless a specific exemption has been obtained. This list already includes most of the commonly used animals in research. The term "purpose bred animals" means animals specially bred for use in experiments in facilities approved by, or registered with, the competent authority. The Directive defines "Breeding Establishment' as any establishment where animals are bred with a view to their use in experiments, and "Supplying Establishment" as any establishment, other than a breeding establishment, from which animals are supplied with a view to their use in experiments. In short, animals deriving from breeding establishments are considered 'purpose-bred' while those from supplying establishments are not. A discussion of the precise housing and management conditions is outside the scope of this report.

Article 5 of Council Directive 86/609/EEC requires that all animals:

(a) shall be provided with housing, an environment, at least some freedom of movement, food, water and care which are appropriate to their health and well-being;

(b) any restriction on the extent to which an experimental animal can satisfy its physiological and ethological needs shall be limited to the absolute minimum;

(c) the environmental conditions in which experimental animals are bred, kept or used must be checked daily;

(d) the well-being and state of health of experimental animals shall be observed by a competent person to prevent pain or avoidable suffering, distress or lasting harm;

(e) arrangements are made to ensure that any defect or suffering discovered is eliminated as quickly as possible.

Additional guidance on care and accommodation is contained in Annex II of the same Directive.

Article 5 and Appendix A of European Convention ETS 123 also provide guidance on accommodation and care of animals used in scientific procedures.

The principles contained in the Directive and Convention are similar to the 'Five Freedoms' adopted by the UK's Farm Animal Welfare Council (1992) and provide a series of criteria, i.e. an ethical framework, for the achievement of minimum welfare goals.

3.2. Risk assessment framework

3.2.1. Introduction

<u>Risk Questions assessed in this document:</u>

1. What is the probability of the occurrence of adverse welfare effects if non-purpose-bred animals are used for experimental work?

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2. What is the probability of the failure to achieve or confound the required scientific outputs if non-purpose-bred animals are used for experimental work?

Under these risk questions, the risks to be assessed are:

a) The probability of the occurrence of adverse welfare effects

b) The probability of the failure to achieve the required scientific outputs (e.g. accurate, reliable and reproducible data).

Two issues will be considered: animal welfare and scientific quality. For each of them, three steps will be followed:

- *Identification of the hazard(s)*
- *Exposure assessment*
- *Consequence assessment*

When assessing risks for animal welfare, the Three Rs approach (Russell and Burch, 1959) will be used as a general framework. This is now widely accepted by the scientific community as one of the main guiding principles in the use of live animals in research. According to this ethical framework, three issues should be taken into consideration when using live animals in scientific procedures:

1. **Replacement**: Another method that does not involve the use of living protected animals that will achieve the same goal and that is reasonably and practicably available.

2. **Reduction**: whether the same objectives can be achieved with fewer animals, for example by improving the experimental design or by reducing variability between animals.

3. **Refinement**: whether the amount of pain, suffering, distress or lasting harm, caused to the animals used in the experimental procedure is the least required to achieve the scientific objective, or whether their wellbeing can be improved. Refinement refers the entire lifetime experiences of the animal including breeding, housing and husbandry, and during experimental procedures.

Of the Three Rs above, reduction by decreasing variability between animals and refinement by providing appropriate breeding, housing and care conditions are the most relevant to this report.

3.2.2. IMPACT ON ANIMAL WELFARE

Identification of hazards, exposure and consequence assessment:

In the following sections the hazards are first identified and then the likelihood of exposure is discussed. Many factors may compromise animal welfare, and as a consequence may also influence scientific outcomes. The risk of exposure to the identified hazards can be influenced by the degree of control and oversight at local, national and international levels.

Accommodation and Care:

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Hazard 1 -Poor environment: temperature, relative humidity, ventilation, noise (Clough, 1982; Gamble, 1982; Sales *et al.,* 1994), absence or use of inappropriate nesting/bedding materials (Reinhardt, 2004), light (O'Steen *et al.,* 1972) and lack of social and environmental enrichment.

Exposure to inappropriate temperatures.

Some species are very sensitive to changes in temperature, and effects can be seen on behaviour, food and water consumption, and growth rates (Svendsen, 1994). Significant deviations from the thermoneutral zone can result in significant distress, morbidity and even death.

Exposure to inappropriate Relative Humidity.

Although many species will tolerate well variations in relative humidity, for some species extreme variations can adversely affect wellbeing, breeding performance, and, by affecting the rate of heat loss, can affect activity and food intake (Stille *et al.,* 1968; Clough, 1982; 1984).

Inadequate ventilation.

Abnormal behaviour due to poor air quality (e.g. elevated carbon dioxide or ammonia levels); increased susceptibility to respiratory disease (Lipman and Perkins, 2002).

Exposure to inappropriate noise.

Loud, unexpected and unfamiliar sounds, including ultrasound, can disrupt breeding programmes and may cause behavioural disturbances (Gamble, 1982; Sales *et al.,* 1994)

Failure to provide appropriate nesting/bedding materials.

Increased neonatal mortality rates; abnormal/stereotypic behaviours (Hubrecht *et al.,* 1992; Weidenmayer, 1997a; Reinhardt, 2004)

Failure to provide appropriate lighting.

Unsuitable lighting or lighting patterns can disrupt breeding cycles, and can cause retinal changes (O'Steen *et al.,* 1972).

Failure to provide suitable social and environmental enrichment

The aim of environmental enrichment is to improve the quality of the captive environment so that the animal has a greater choice of activity and some control over its social and spatial environment (Newberry, 1995; Stauffacher, 1995; Bayne *et al.,* 2002). When animals are deprived of the possibility to perform species-specific behaviour they may show signs of suffering such as behavioural disorders, chronic stress or other pathological conditions (Würbel *et al.,* 1996). Housing conditions of laboratory animals should provide opportunities for animals to perform their species-specific behavioural repertoire by providing enrichment in the social, nutritional, sensory, psychological and physical environment (Baumans, 1997).

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For example, individual housing has frequently been shown to be stressful for mice. Detrimental effects of individual housing include both, behavioural and physiological abnormalities usually referred to as 'isolation stress' or 'isolation syndrome' (e.g. Baer, 1971; Brain, 1975; Haseman, 1994). There is evidence that subordinate male mice prefer company to being housed individually, even if that companion is dominant (Van Loo and Baumans, 1998). Gerbils develop extensive stereotypic digging if they are not given the chance to dig burrows, or if they are not provided with an artificial burrow (Wiedenmayer, 1997). There is evidence that hamsters housed in non-enriched cages are more stressed than hamsters housed in enriched cages (Kuhnen, 1997) and that enclosure size and stocking densities induce stress responses that may affect health and welfare (e.g. Sørensen DB *et al.,* 2005).

Failure to provide an appropriate environment and social contact has been demonstrated in many species to lead to behavioural problems, stress and physiological abnormalities, including increased susceptibility to disease. Although there remain some concerns over the effects of enriched environments on scientific outcome, there are data to support the view that because an animal can perform more of its species-specific behaviour in enriched environments it may be better able to cope with novel and unexpected changes and thus show a more uniform response (Rose, 1994; Baumans, 1997) and in some areas of research has been shown radically to affect the scientific outcomes (Rose, 2002 presented at the 4th World Congress on Alternatives and personal communication, 2005).

It has also to be borne in mind that the welfare of some research animals in the laboratory may be jeopardised e.g. wild animals, commercial farmed animals.

Hazard 2 - Inadequate breeding management: e.g. breeding immature animals; poor conformation, genetic abnormalities, peri-parturient losses, early weaning losses, no retirement strategies, poor temperament (GV-SOLAS, 1999).

Failure to implement suitable breeding strategies.

This includes failure to select appropriate breeding animals and failure to manage pregnant and lactating females.

Genetic abnormalities, poor conformation, increased mortality rates (including peri-parturient and pre-weaning).

Hazard 3 - Inadequate health management: e.g. no veterinary care or health screening programme, overt clinical disease, poor productivity, deaths (Poole and Evans 1982).

Failure to maintain an appropriate health management programme.

Poor animal health itself is a potentially serious welfare issue and disease can lead to e.g. mortality, morbidity, reductions in growth rate and breeding performance (GV-SOLAS, 1999; FELASA, 2002).

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Hazard 4 -Unsuitable diet: poor growth, nutrient deficiencies.

Failure to provide an appropriate diet for the animals

Disease due to nutrient deficiencies or excesses; effects on growth rates, which may cause retarded growth or obesity; effects on breeding performance (Coates, 1999).

Hazard 5 - Insufficient and inadequately trained staff:

Poor handling practices, inability to detect or correct health or welfare problems; lack of awareness of evolving improvements in welfare and care standards (Kersten *et al.,* 1989).

Exposure to Hazards 1-5 (Accommodation and Care) will depend on the following factors:

1. *Whether animals will be purpose bred* - When giving consideration as to whether or not any species should be "purpose-bred", one important factor is the legislative provision already in place for the animals. In many countries there is no specific welfare legislation to make provision for many of the commonly used laboratory animal species, for example rats and mice. By including the species as "purpose-bred" the requirements of the EU Directive 86/609, in particular Article 5 and Annex II provide for the welfare of these animals.

   European and national legislation on experimental animals is designed to protect animal welfare in breeding, supplying and user establishments. In addition to laws, there are many recommendations and guidelines provided to improve and maintain a normal physiological state and good psychological welfare of the animals. For example, the recommendations on housing and care in the proposals for the revision of the Council of Europe Convention ETS 123 Appendix A strongly encourage group housing and environmental enrichment. These recommendations reflect the improving scientific basis on which welfare and care practices are based, and are currently being revised which is likely to improve the welfare of animals.

2. *Regulatory surveillance* - monitoring of research Institutes by a system of licensing, which may include an element of inspection. The licensing system will generally include consideration of issues such as training and assessment of competence of those that come into contact with animals (Council of Europe, 1993).

3. *Voluntary external surveillance and independent assessors* - there are very few national and international accreditation programmes that provide some independent reassurance that appropriate welfare and care practices are being provided e.g. AAALAC, comprehensive competent authority inspections.

4. *In house control practices and management* - regular reviews of performance and adjustments as necessary. Controls should include training and assessment of competence or personnel (Council of Europe, 1993). Local research establishments should maintain awareness of

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developments in laboratory animal care, and give consideration to early introduction of refined practices that would benefit animal welfare.

5. In addition to the regulatory guidelines, there are a number of comprehensive guidelines on refined husbandry of mice, rabbits, dogs and birds (JWGR, 1993, 2001, 2003, 2004), on pain and distress (FELASA, 1994), on humane endpoints (Hendricksen and Morton, 1999, ILAR 2000) and on refinements for genetically modified animals (JWGR, 2003), all of which can be implemented in breeding establishments.

***Consequence assessment to Hazards 1-5*:** There are good scientific data available to support the view that there are considerable risks to animal welfare should there be a failure to provide appropriate animal accommodation and care.

Although in-house management practices can set suitable standards for housing and care, these can be very variable in implementation between, and even within, establishments.

Independent assurance schemes offer more confidence in the provision of suitable standards, but these have limited power to require that changes or improvements are made to safeguard welfare standards.

As a consequence of the education and training systems in place within approved breeding establishments, and in part due to regulatory oversight, there is generally a good awareness of emerging developments in and benefits of improved welfare and care practices (Council of Europe, 1993). It follows, therefore, that there is a much greater likelihood of high standards of welfare and care being provided in "approved" breeding establishments.

Hazard 6 - Genetically altered animals (GAA): Genetic modification, including cloning, can adversely affect animal welfare by causing or predisposing animals to pain, suffering, distress or lasting harm (Dennis, 2000, 2002; Mertens and Rulicke, 2000; JWGR, 2003). This may be intentional as a result of the genetic modification introduced, or unintentional through the disruption of gene function by random integration of transgene into the genome. Cross-breeding within and between transgenic and mutant lines can also affect welfare. Differences in husbandry and care practices can influence the expression of phenotype, and therefore the welfare of animals as well as the scientific outcomes. Species that have been genetically altered include: mice, rats, sheep, pigs, fowl, fish.

***Exposure*:** A large and increasing number of laboratory animals, most of which are mice, are being genetically manipulated and altered by transgenic technology or by exposure to mutagens.

Consequence assessment – Most GAA are mice and, as such, happen to be already listed in Annex I of the Council Directive 86/609/EEC and so there will be no change. Notwithstanding this, other GAA may not be so listed e.g. farm animals, fish, poultry.

Risk is lower for purpose-bred animals and for those housed in user facilities simply because scientists and animal care staff are aware of possible welfare compromises and any special husbandry and care requirements. Moreover,

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members of the animal care staff have the training, experience and skills to pick up welfare problems at an early stage. Many GAA are routinely kept behind barrier systems to maintain a high health status and this often precludes scientific staff from visiting their animals regularly that has its own disadvantages in making scientists less aware of the consequences of their scientific manipulations which are not always predictable in terms of genetic fidelity or animal welfare as it will depend on the exact genotype involved.

Hazard 7 - Lack of standardisation on animals used (*e.g.* health of animals, genetic fidelity, microbial status, nutrition and environment) leading to an increase in the number of animals that have to be used to obtain results (see below under scientific quality).

Exposure: will depend on inclusion or not in the Annex. Not being included does not mean that animals will not have good health or genetic fidelity. However, it is less likely they will due to the different standards applied to their breeding and maintenance.

***Consequence assessment*:** Risk will be higher for animals that are not purpose bred, but the increase in risk compared with purpose bred animals will vary depending on the availability of non-purpose bred animals that are of high quality, and this may vary between species and genetic strains.

Hazard 8 - Prolonged journey times: It is accepted that transport may cause distress and long distance transport may have more severe effects than short transports (EFSA, 2004, http://www.efsa.eu.int).

Exposure: will depend on the demand for a particular species which is likely to influence the number of breeding establishments.

Consequence assessment: the fewer the number of breeding establishments, the longer the journey times are likely to be.

Hazard 9 - Capture in the wild: leading to high mortality, injuries and severe distress (see transport report for further information). Moreover, hatching of birds from eggs does not eliminate the hazard of poor welfare.

Exposure: only applies to animals for which, when not purpose bred, the only source is capture in the wild (e.g. Non-human primates, wild animals).

Consequence assessment: the risk will be higher for animals that are not purpose bred.

Hazard 10 - Overproduction of animals: birth of considerable numbers of surplus animals may lead to overstocking. If these animals carry some defective gene that causes adverse effects the welfare problems will be increased. It is not at all common for animals on Annex I to go for human consumption, however, if in the unlikely event surplus GAA of the farmed species are produced, their use and disposal would require authorisation under Regulation 1829/2003.

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Hazard 11 - Over exploitation of breeding animals and confinement for long periods: for some species over many years, in the breeding establishment i.e. no retirement programme for breeders

Exposure: will depend on whether the demand is fluctuating and on the number of providers.

Consequence assessment: the risk will be higher for purpose bred animals if demand fluctuates widely and this will be more pronounced if there is a single provider.

The situation for single providers will be a problem if the demand fluctuates (captive markets of minor species). If the demand for animals from a given species or strain to be used in scientific procedures is low or fluctuates widely, matching production with scientific needs can be difficult.

The consequences could be an overproduction of animals or a delay or even a failure to start experimental studies until suitable animals can be reared. Birth of considerable numbers of surplus animals may lead to overstocking and their consequent culling poses ethical issues.

3.2.3. IMPACT ON SCIENTIFIC QUALITY

Identification of hazards, exposure and consequence assessment:

In the following sections the hazards are first identified and then the likelihood of exposure is discussed. Many factors may compromise the scientific outcomes. The risk of exposure to the identified hazards can be influenced by the degree of control and oversight at local, national and international levels.

Hazard 1 - Lack of standardisation on animals used (e.g. health of animals, genetic fidelity, microbial status, nutrition and environment, wild animals).

A major source of variance in some animal studies is contamination or infection with microbial agents; elimination of these agents contributes to the standardization of experiments using animals (Johnston and Nevalainen 2003). In laboratory animals good health status not only means absence of clinical disease, but also absence of numerous specified etiologic agents of disease (consider – absence of agents which may compromise scientific outcomes, for example certain murine viruses which may enhance or compromise the immune response). As an example of the effects on variance, Gärtner (1990) showed that *Mycoplasma pulmonis* increased rat kidney weight considerably. Consequently, when kidney weight was the scientific outcome measure, 5 times as many rats were required to reveal a significant difference. There are health monitoring guidelines for rodents, rabbits, dogs, cats, pigs, sheep, goats and non-human primates (FELASA, 1998-2000), which are widely implemented in breeding establishments of the most common laboratory species in Europe and beyond, but not with other species. Mixing animals of different health status poses a threat to research as well as to animal welfare.

Laboratory animals that are not purpose-bred pose generally a higher risk of uncertainties in quality and background. There is less confidence in the robustness of and confidence in breeding programmes to ensure appropriate genetic status. Genetic contamination is a real risk even with proper colony

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management, and may go undetected unless genetic monitoring schemes are in place (Benavides, 1999). Such schemes are more likely to be maintained effectively in breeding establishments. There are other factors causing 'statistical noise' i.e. increased variance, like habitat, physico-chemical factors, climate, nutrition and the influence of humans, for example during handling, which may cause significant variance in results, and hence should be subjected to stringent control (Davies and Balfour, 1992; ILAR, 2002). These factors are also less likely to be standardized in establishments other than "breeding establishments".

Notwithstanding the above, in some areas of research e.g. studies into the normal biology of a species, commercial strains and veterinary clinical research, purpose breeding in a laboratory could, for example, result in loss of genetic diversity, the generation of large numbers of surplus animals and significant delays in scientific progress. Moreover, breeding wild animals in captivity could be detrimental to their health and welfare and, as a consequence, to the science.

Another advantage of purpose breeding is that animals may be trained or habituated to procedures that will produce better scientific data as well as better welfare.

Exposure: will depend on inclusion or not in Annex I and on whether there are animals that are not purpose bred but have an equally good health status and genetic fidelity.

Consequence assessment: The risk will be higher for animals that are not purpose bred, but the increase in risk compared with purpose bred animals will vary depending on the availability of non-purpose bred animals that are of high quality, and this may vary between species. The potential consequence is an interference with the scientific data and the interpretation. Any increase in the variability among experimental animals may lead to invalid, skewed and other wise unreliable results as well as an increase in the number of animals used.

Hazard 2 - Insufficient animals of suitable quality:

Inability to produce enough animals from a given species or strain within a reasonable period of time will have a negative impact on research. This may be due to a shortage of adequate breeding facilities, which is more likely to be a problem with species that are not used in large numbers in scientific procedures. Similarly, the inclusion of species of low fecundity and lengthy production times, for example horses, would pose particular problems. It could take many years to produce animals of a suitable maturity for use in scientific procedures.

Exposure: will depend on the demand (low / high, fluctuating / constant) and on the fecundity and length of production time.

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Consequence assessment: Risk will be higher for purpose bred animals if the demand is low or fluctuates widely and the increase in risk compared with animals that are not purpose bred will be higher in species of low fecundity and lengthy production times.

Hazard 3 - Extrapolation of data:

If the results of the study are to be applied for example to wild animals in their natural environment, or to commercial production of farm animals, then the requirement for purpose-breeding may not be desirable. This is because the regulations and guidelines which apply to purpose-bred animals for scientific research may significantly differ from those, for example, in commercial livestock production. As a consequence, the results obtained from using animals bred to the standards required for scientific research are likely also to significantly differ from those bred under "commercial farming" conditions and so there may be a problem in extrapolation.

Similar concerns would apply to investigations in certain breed specific disorders or in some areas of veterinary clinical research where a variety of different breeds of animals, such as dogs, may be required, which are not commonly used for other research purposes.

Exposure: Only applies when the results of the study are to be applied in specific circumstances for example to wild animals in their natural environment, or to commercial farm production.

Consequence assessment: Risk will then be higher for purpose bred animals.

3.3. SCIENTIFIC CRITERIA THAT COULD BE USED TO DETERMINE WHICH ANIMALS SHOULD BE PURPOSE-BRED

The criteria for inclusion of species in Annex I have not been clearly defined and no information is available on why the various species were originally included. The criteria suggested by Technical Expert Working Group of DG ENV (2003), established to help in the revision of the Directive, proposed the following criteria.

1. The numbers of animals required for procedures
2. The type of procedures (e.g. farm animal studies/population studies)
3. Animal welfare aspects
4. Practical and commercial aspects of establishing breeding;
5. Disease-free requirements
6. Other welfare/ethical aspects (e.g. in the case of dogs, moving an animal from a street to a laboratory environment)
7. Social/public concerns (e.g. concern that pet cats and dogs might be used)

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Concerning the last two points it is not within the remit of the European Food Safety Authority to consider ethical and public concerns, but only to consider the scientific evidence. In no way is this meant to imply that these issues are not important when considering the provenance and use of animals in research.

3.3.1. Key criteria to be considered for being purpose bred and inclusion in Annex I

The criteria suggested by the TEWG of DG ENV were considered and incorporated into an assessment process against which the inclusion of each of the commonly used laboratory species was reviewed. This included some consideration of the possible addition of certain invertebrate species. The main findings and conclusions for those species suggested by the TEWG in the report to DG ENV and for additional species suggested by this review are included in this report. The main criteria considered for animals to be purpose bred are as follows.

1. Does legislation already exists to protect animal welfare?

Some animals used in scientific procedures may be protected by animal welfare legislation other than that for laboratory animals (Council Directive 86/609/EEC) and this is the case, for example, with farm animals. Although the protection provided by this legislation may differ from that afforded by the laboratory animal legislation, basic elements of welfare and care are usually included. Therefore, absence of any relevant animal welfare legislation is a reasonable criterion for inclusion into Annex

2. Are the animals genetically altered (GAAs)?

Genetically altered animals are being increasingly used in research and it is important that their phenotype is monitored throughout the animals' lives. Genetic modification and cloning can compromise animal welfare by causing or predisposing animals to pain, suffering, distress or lasting harm (JWGR, 2003). By monitoring the phenotype carefully in a laboratory situation, appropriate care can be given according to the clinical signs observed. Moreover, if animals have adverse effects steps can be taken to establish humane endpoints and to prevent surplus stock being produced. All these factors are likely to be better controlled if the animals are purpose bred in a controlled and regularly inspected environment.

3. Health and genetic fidelity of animals.

For some species, animals that are not purpose bred may still be of high health and genetic fidelity (farmed animals). In other species, however, this may not be the case e.g. rodents and lagomorphs, due to difficulties in disease control.

4. Demand.

When demand for animals of a given species or strain is low, and the animals are to be purpose bred, there are likely to be fewer breeding establishments which will result in animals having to be transported over long distances, which is often a welfare concern.

Furthermore, if the demand is low or widely fluctuates, or the breeding may be seasonal, matching demand with supply may be difficult. This can lead to overstocking of breeding animals as well as overproduction with high cull rates, all of which are welfare and ethical concerns.

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Note: In some cases, as data on actual use of some species of animals are not available, the number of citations in scientific databases has been used to obtain rough estimates of numbers used.

5. Extrapolation of results to farming or to wild populations.

Research on some species or strains may be geared to obtaining results meant for farming or wildlife conditions (e.g. rodent control, ecological studies). If this is the case, purpose bred animals cannot provide a representative sample of the target population.

6. Capture from the wild.

Primates: Purpose breeding may in some cases be the only alternative source to capture in the wild.

The TEWG on scope discussed at length the pros and cons of taking primates for research from F1 or F2 generations. The SCAHAW Report of December 2002, on the welfare of primates in research, suggested it should initially mainly be from the F2, so that future breeding stock could be taken from the F1 generation. As it is still common practice in some overseas breeding establishments to replace breeding stock with wild-caught animals, the only way to reduce this dependence on wild caught animals, is to use the F1 generation. It is likely, therefore, to take some considerable time before F2 generation animals are available in sufficient numbers to meet research needs.

Other species: For other species e.g. for the study of wild life such as birds, fish and mammals, capturing them from the wild may be the only source, but the welfare aspects of free-living animals confined to captivity in a laboratory and the substantive change in their environmental conditions cannot be overlooked. Even taking eggs, as in the case of some reptiles and birds, may not avoid the natural instincts of the animals hatched in captivity (e.g. migratory urges, certain behaviours to hunt). Removal of wild animals from their ecological niche may also disturb that niche and make it difficult to release them back there at the end of an experiment.

3.3.2. Assessement in relation to specific species used in research:

Hamsters

Syrian hamsters are the most commonly used of all the 'hamster types' and, at present, are included in Annex I. However, from an analysis of scientific papers through PUBMED, Chinese hamsters are also commonly used, and only very few European and Djungarian hamsters.

Arguments against inclusion of all hamster species: The small numbers of European and Djungarian hamsters used would make difficulties to match supply and demand leading to delays in scientific programmes

Arguments for inclusion of all hamster species: It would be likely that there would be an improved and more uniform health quality. Moreover no other welfare legislation exists.

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Gerbils

The commonest gerbil used in research is the Mongolian *(Meriones unguiculatus)* which is not in Annex I.

Arguments against inclusion: Difficulties to match supply and demand that may lead to some delays in scientific programmes;

Arguments for inclusion: Better and more uniform health quality; improved accommodation leading to reduced behavioural abnormalities; no other suitable welfare legislation

Quail

Arguments for inclusion: There may possibly better protection for quail if listed in Annex I, through improved accommodation and care practices.

Arguments against inclusion: Small numbers of *Coturnix coturnix* used. Few breeding establishments – difficult to match supply and demand.

Xenopus species (laevis and tropicalis), Rana species (temporaria and pipiens)

Arguments against inclusion: Wide range of species but for many species only small numbers are used. Production of the less commonly used species, *e.g.* newts, salamanders (including axolotls) may not be practicably viable due to the very small numbers used. The purpose breeding of *Xenopus laevis* and *tropicalis* may prove to have economies of scale that make it viable. Potentially high cull rates, difficulties to match supply and demand leading to delays in scientific programmes, lack of information on husbandry and care practices.

Arguments for inclusion: better and more uniform health quality, increasing numbers of some species, no other welfare legislation, elimination of zoonotic diseases, no animals taken from wild.

Invertebrates such as cephalopods, cyclostomes, decapods.

The recommendation from Chapter 2 is for these phyla to receive protection during experimental work due to their potential to experience pain and distress.

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Appendix 1

Purpose bred criteria / Critical Species

Species to be added: Hamsters – Chinese (*Cricetulus griseus*), Syrian (*Mesocricetus auratus*), European (*Cricetus cricetus), Djungarian (Phodopus sungorus)*

CRITERIA	Assessment compared with not purpose-bred
Hazards	
Accommodation & Care	Inadequate temperature control can affect breeding performance, growth rates and can induce hibernation. Enclosure sizes and stocking densities have been shown to induce stress responses in hamsters which may affect health and welfare.
GAA	No known GAA available.
Quality of animals *Microbiological / genetic quality / physicochemical aspects* *Reduction aspects* *Impact on research*	Susceptible to range of infectious agents that can affect welfare and science. PB means use of adequate health and genetic (if applicable) monitoring, good colony management, proper environment, and consequently more uniform animals, which allows use of fewer animals. All this has positive effects on research, and serves purposes of reduction
Transport	Many of the smaller species are used in photoperiodicity studies - transport can have a profound effect.
Exposure assessment	
Welfare of animals *Pertinent legislation & guidelines*	Guidelines available on humane endpoints and euthanasia and the European Convention on the Protection of Pet Animals. The **Syrian/Golden Hamster** (*Mesocricetus auratus*) is already included in Council Directive 86/609/EEC "Hamster" guidelines in revised App A of ETS 123 may be considered inappropriate for **Chinese//European/ Djungarian hamsters** as these vary in size significantly from the Syrian hamster.
Availability of suitable quality animals *Refinement aspects* *Breeding issues*	Difficult to get very high health status animals - problems with re-derivation and embryo transfer. Health monitoring difficult due to lack of standards/tests, often basing results on cross reactivity to murine kits. Outbred animals - but using variety of sources may increase variability, and affect animal numbers. **Syrian hamsters** are the most commonly used and have a good PI (Production Index) - 50/annum Few **European and Djungarian hamsters** are used. **Chinese hamsters** are considered poorer breeders than the Syrian.
Research programme *Extrapolation of data*	Majority of use is fundamental / biomedical, with little research conducted for the benefits of the species.
Additional considerations	
Number *of all animals used in EU*	52000 Syrian Hamsters used in 2002, no information available on numbers of other species used but citations in PUBMED search suggests similar numbers of Chinese hamsters used (17000 for Syrian, 10000 for Chinese , 350 for European and 773 Djungarian).
Supply/demand status	In Europe, inbred and outbred **Syrian** and **Chinese hamsters** available from commercial breeders No breeders for **European or Djungarian hamsters** as laboratory animals Limited/unknown demand
Surplus animals	Surplus could be used as reptile/raptor food.
Impact on research	Reasonable health quality animals should be available without incurring significant delays to research.
CONCLUSIONS	**Syrian hamsters** are the most commonly used and are included at present in Annex I. From analysis of scientific papers through PUBMED, **Chinese hamsters** are also commonly used, but very few **European** and **Djungarian** hamsters are used. <u>Arguments against inclusion :</u> Small numbers of European and **Djungarian** hamsters, difficulties to match supply and demand leading to delays in scientific programmes <u>Arguments for inclusion:</u> improved and more uniform health quality; no other welfare legislation
RECOMMENDATION	**Retain Syrian hamsters, include Chinese hamsters. No compelling need to include any other hamster species.**

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Appendix 2

Purpose bred criteria / Critical Species

Species to be added: Mongolian Gerbil (*Meriones unguiculatus*)

CRITERIA	Assessment compared with not purpose-bred
Hazards	
Accommodation & Care	High RH can cause problems, for example facial dermatitis or greasy coats. Poor noise control may induce epileptiform seizures. In barren/non-enriched enclosures, high incidence of stereotypies noted, in particular digging and bar chewing.
GAA	None commercially available - may be in future.
Quality of animals *Microbiological / genetic quality / physicochemical aspects* *Reduction aspects* *Impact on research*	Susceptible to wide range of infectious agents that can affect welfare and scientific outcomes. Genetic status important due to susceptibility to epilepsy.
Transport	No specific transport issues.
Exposure assessment	
Welfare of animals *Pertinent legislation & guidelines*	Guidelines available on humane endpoints and euthanasia and the European Convention on the Protection of Pet Animals for pet gerbils. Guidance on housing, husbandry and care included in revised App A in ETS 123. There is no other specific legislation that offers protection to the Mongolian gerbil.
Availability of suitable quality animals *Refinement aspects* *Breeding issues*	Mainly purpose-bred animals used from commercial breeders, bred and housed in accommodation more reflective of the laboratory conditions under which they would be used. Reasonable health quality available. Outbred colonies. Good reproduction Index- 25 offspring/ breeding female/ annum. PB means use of adequate health and genetic (when applicable) monitoring, good colony management, proper environment, and consequently more uniform animals, which allows use of fewer animals. All this has positive effects on research, and serves purposes of reduction. Purpose-breeding also serves refinement and hence has a positive effect on research
Research programme *Extrapolation of data*	Animals used mainly in neuroscience / epilepsy / immunology research. Few studies conducted for the benefit of the species.
Additional considerations	
Number *of all animals used in EU*	UK references: 7500 gerbils used. No EU specific references for gerbils. Impossible to extract from statistics, but likely to account for a significant % of the 60K "other rodents" returned in 2002 EU statistics.
Supply/demand status	Fluctuating demand can lead to high cull rates
Surplus animals	Surplus animals should not be an issue - reptile/raptor food.
Impact on research	Commercial breeders available, reasonable PI - there should not be any significant delays & difficulties in obtaining suitable animals.
CONCLUSIONS	The commonest gerbil used in research is the Mongolian (*Meriones unguiculatus*) which is not in Annex I. Arguments against inclusion: Difficulties to match supply and demand which may lead to some delays in scientific programmes; Arguments for inclusion: Better and more uniform health quality; improved accommodation leading to reduced behavioural abnormalities; no other suitable welfare legislation
RECOMMENDATION	**Include Mongolian gerbils *(Meriones unguiculatus*)**

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Appendix 3

Purpose bred criteria / Critical Species

Species to be added: Ferret *(Mustela putorius furo)*

CRITERIA	Assessment compared with not purpose-bred
Hazards	
Accommodation & Care	Susceptible to heat stress; loud, unfamiliar noise and vibration can cause stress-related disorders. Abnormal & stereotypic behaviours without complex and stimulating environment. Specialist dietary needs - inadequate diets can lead to poor growth rates, poor reproductive performance, and pregnancy toxaemia.
GAA	No GAA available.
Quality of animals *Microbiological / genetic quality / physicochemical aspects* *Reduction aspects* *Impact on research*	Ferrets are susceptible to a range of infectious diseases which can have high morbidity and mortality e.g. Distemper. Ferrets are also susceptible to human influenza. Clinical episodes of Aleutian disease can be precipitated by stress, such as undergoing scientific procedures, and infection may influence immune responses.
Transport	Few breeders - transport distances likely to be significant, but no particular transport issues
Exposure assessment	
Welfare of animals *Pertinent legislation & guidelines*	98/58/EC on the welfare of farm animals covers fur animals. 93/119/EC deals with the killing of farmed species and specifies the permitted methods for individual species. ETS 87 (1976) and the recommendations concerning fur animals (CoE Standing Committee 1999) provide husbandry, housing and care guidelines for ferrets. Proposals for ETS 123 (1986) Appendix A revision also gives guidance - during technical discussions concerns were raised re inadequacies of enclosure dimensions for fur-farmed animals. Ferrets kept as pets protected under the European Convention for the Protection of Pet Animals ETS 125 (1987), but no detailed care recommendations provided and welfare and science likely to be better if included in Council Directive 86/609 Annex.
Availability of suitable quality animals *Refinement aspects*	Purpose-bred likely to provide better health quality. Ferrets are available as conventional outbred - small colony breeding could create risk of inbreeding. Purpose-bred ferrets have a more uniform genetic and microbial quality which has both refinement and reduction impact on research.
Breeding issues	Seasonal breeders - can be manipulated by light-dark cycles to give a RI of 12 offspring/ female/ annum. Single housing of males during breeding season generally necessary due to risk of fighting/ injury. Careful management of non-breeding females essential to avoid oestrous-associated anaemia.
Research programme *Extrapolation of data*	Majority of use is in fundamental research/ drug development with few studies conducted to gather data on ferrets in commercial production or in ecological studies.
Additional considerations	
Number *of all animals used in EU*	**2000 animals used in Europe in 2002.**
Supply/demand status	Single sex preference, can lead to high cull rates in males. Single housing during breeding season.
Surplus animals	Few outlets for surplus animals.
Impact on research	Few commercial breeders
CONCLUSIONS	
	Arguments against inclusion : Small numbers; Potentially high cull rates, other welfare legislation, difficulties to match supply and demand leading to delays in scientific programmes Arguments for inclusion: better and more uniform health quality, increasing numbers
RECOMMENDATION	**No compelling need to include ferrets in Annex I**

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Appendix 4

Purpose bred criteria / Critical Species

Species to be added: Pig (*Sus scrofa*) including Minipig

CRITERIA	Assessment compared with not purpose-bred
Hazards	
Accommodation & Care	Inappropriate standards of accommodation and care can have profound effects on welfare and science. Extensive review in Report of SVC on The Welfare of Intensively kept Pigs (1997).
GAA	Genetic altered pigs have been produced (e.g. for xenografts) - but not mini-pigs.
Quality of animals *Microbiological / genetic quality / physicochemical aspects* *Reduction aspects* *Impact on research*	Pigs are susceptible to a range of infectious and other diseases that can adversely impact on science and welfare.
Transport	Transport covered by EU Transport regulations, but including pigs in the annex with a small numbers of commercial breeders, could result in animals having to be transported over long distances, which could become a welfare concern.
Exposure assessment	
Welfare of animals *Pertinent legislation & guidelines*	General EU Farming Council Directive 98/58/EC. Also covered by Council Directive 91/630/EEC that has been amended by Council Directives 2001/88/EC and Commission Directive 2001/93/EC. It includes aspects related to housing and training of personnel. Council Directives 64/432/EEC and 93/119/EC and Council Regulation 1255/97 have been amended by Council Regulation 1/2005 on the transport of animals. CoE Conventions and recommendations apply (new pigs enters into force on 02/06/05). **No specific guidelines on minipigs.** Much discussion by Groups of Expert for ETS 123 App A are definition of mini-pig, and eventually provided a single document. Note - present EU recommendations differ between farm and laboratory animals. Mini-pigs kept as pets protected by the European Convention for the Protection of Pet Animals, but standards of housing and care likely to be higher and more uniform if included in the annex.
Availability of suitable quality animals *Refinement aspects* *Breeding issues*	Defined health status pigs are readily available, but there are few commercial breeders of mini-pigs - however those which are available are of high health status, line bred, and some are reared in full barriers.
Research programme *Extrapolation of data*	There is a demand for "conventional" and "mini" pigs in fundamental and drug development studies. For some surgical preparations, long-term studies and toxicology studies where availability of compound is limited, the "mini-pig" is often preferred due to the growth rates and adult body weights. There are many studies conducted related to commercial pig production.
Additional considerations	
Number *of all animals used in EU*	Minipigs are classified as *Sus scrofa domestica* as are normal pigs. They originated by selective breeding for miniature size in the 1950s and 60s. Minipigs bred for scientific procedures are essentially all purpose-bred, while other pigs are not.
Supply/demand status	61,000 pigs used in 2002 - no split between minipigs and pigs. High PI. Many commercial breeders for farm Pigs with a few breeders for minipigs.
Surplus animals Impact on research	Surplus animals could go for slaughter for meat production.
CONCLUSIONS	<u>Arguments for inclusion</u>: None <u>Arguments against inclusion</u>: Other welfare legislation, extrapolation of results to farming conditions and high health status animals available Minipigs are already specifically bred for research purposes and the limited market demand and economies of scale means this is likely to remain the case.
RECOMMENDATION	**Difficult to foresee benefits of inclusion of pigs in Annex I on either welfare or scientific grounds**

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Appendix 5

Purpose bred criteria / Critical Species

Species to be removed: Quail (*Coturnix cornutix*)

CRITERIA	Assessment compared with not purpose-bred
Hazards	
Accommodation & Care	Aggressive feather pecking has been reported as a problem in intensive husbandry conditions, and following mixing of birds in established groups. Appropriate enclosure design is necessary to minimise head injuries (caused by vertical flight response) and foot problems. High post-hatch mortalities can occur without good temperature control and suitable feeding practices.
GAA	No genetic altered quail available.
Quality of animals *Microbiological / genetic quality / physicochemical aspects* *Reduction aspects* *Impact on research*	Quail are susceptible to a range of bacterial, fungal and parasitic diseases. Respiratory disease has been associated with poor ventilation. Potential zoonoses include *Salmonella* and *Campylobacter*.
Transport	Low demand could result in few breeding establishments and animals having to be transported over long distances, which could become a welfare concern.
Exposure assessment	
Welfare of animals *Pertinent legislation & guidelines*	Council Directive 98/58/EC on the protection of animals kept for farming purposes and Council Regulation 1/2005 on the protection of animals during transportation. ETS Convention 87 on welfare of farm animals. Revised App A of ETS123 has guidance on quail. Quail is already included in Annex I of Council Directive 86/609/EEC *Coturnix coturnix* is protected by the Wild Birds Directive (79/409/EEC), but is not CITES listed. No EU legislation or widely accepted guidelines on housing and care of quail that are not purpose bred Therefore, welfare likely to be better if included in the Annex I.
Availability of suitable quality animals *Refinement aspects* *Breeding issues*	Conventional "clean" animals available - not barrier bred. Purpose bred animals could be more uniform from the microbial and genetic standpoints and this serves purposes of reduction. Also, rearing conditions could be more uniform in breeding establishments. Reasonable productivity.
Research programme *Extrapolation of data*	
Additional considerations	
Number *of all animals used in EU*	A number of "Quail" used - *Coturnix coturnix* (European quail) - 13000 in 2002 (EU). A search on Pubmed revealed 4000 *Coturnix coturnix* citations, *Coturnix japonicum* (Japanese quail) – 3200, *Colinus virginianis* (Bobwhite quail) – 135, *Lophortyx californica* (Californian quail), *Excalifactoria chinensis* (Chinese painted quail) - most commonly used *C. japonicum* (Note - figures above do not reflect this - one problem is that nomenclature is not clear - in a taxonomic review it is argued that the European quail is *Coturnix coturnix coturnix* , and the Japanese quail is *Coturnix coturnix japonicum.* Most UK institutes use *Colinus virginianis* sourced from hatcheries rearing game birds for other purposes. The Bobwhite is used most commonly used as this is the species used in ecotoxicology worldwide.
Supply/demand status	May be difficult to match supply and demand; inbreeding in small colonies can reduce productivity. Very few (if any) commercial breeders for scientific purpose.
Surplus animals	
Impact on research	
CONCLUSIONS	Arguments for inclusion: There may possibly better protection for quail if listed in Annex I, through improved accommodation and care practices. Arguments against inclusion; Small numbers of *Coturnix coturnix* used. Few breeding establishments – difficult to match supply and demand.
RECOMMENDATION	**No compelling need to retain *Coturnix coturni* in Annex I nor to include any other species of quail.**

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Appendix 6

Purpose bred criteria / Critical Species

Species to be added: Bird (other than quail)

CRITERIA	Assessment compared with not purpose-bred
Hazards Accommodation & Care	The effects of inappropriate accommodation and care practices are largely dependent on species and may be affected by the source of the animal. For example the domestic fowl is susceptible to lameness and other musculoskeletal disorders, and in broilers cardiovascular disease can cause high mortality rates. Nutrition and housing practices are important considerations in managing these problems. Wild-caught birds can be highly stressed in captivity and exhibit a range of abnormal behaviours.
GAA	Genetic altered fowl are available.
Quality of animals *Microbiological / genetic quality / physicochemical aspects* *Reduction aspects* *Impact on research*	Birds are susceptible to a wide range of viral, bacterial, fungal and parasitic diseases, some of which have zoonotic potential, for example Salmonellosis in poultry and Psittacosis in passerine birds.
Transport	This category potentially includes many different species. Including each of them in the annex could result in animals having to be transported over long distances, which could become a welfare concern. Wild-caught birds to be considered also.
Exposure assessment Welfare of animals *Pertinent legislation & guidelines*	Council Directive 99/74/EC for laying hens is in addition to general welfare legislation (98/58/EC). Council of Europe Farming Convention has adopted recommendations on domestic ducks (*Anas platyrhynchos*), domestic fowl (*Gallus gallus*) and turkeys (*Melleagris gallapavo*). Revised ETS 123 Appendix A has detailed guidance on a number of commonly used species – ducks, geese, fowl, turkeys, pigeons, zebra finch. There is no other legislation or widely accepted guidelines for other birds, except for wild birds (CITES, Convention on the Conservation of European Wildlife and Natural Habitats).
Availability of suitable quality animals *Refinement aspects* *Breeding issues*	High health status fowl, ducks and turkeys are available from commercial livestock producers. Genetics are generally known. Rearing conditions could be more uniform in breeding establishments. The same applies to any other species of bird - purpose bred animals could also be more uniform from the microbial and genetic standpoints. Uniformity and quality likely to be better for any species if included in the Annex I.
Research programme *Extrapolation of data*	Much research on food-producing animals is applied and performed under commercial conditions. Many diverse species are used in ecological studies in wild birds.
Additional considerations Number *of all animals used in EU*	Difficult to extract numbers of each species used from statistics. <u>EU reference</u>: 520,000 birds used in 2002. <u>Pubmed citation</u>: Chicken – 93000, Turkey – 15000, Pigeon – 9500, Duck – 7700, Geese – 1600, Zebra finch – 500.
Supply/demand status	Commercial farm species are generally available and are of a good health quality. Zebra finches often used on breeding/production studies using in-house colonies, and in various other basic research studies.
Surplus animals	
Impact on research	
CONCLUSIONS	<u>Arguments against inclusion :</u> For the most common used species there is other welfare legislation, extrapolation of data - much research is applied and performed under commercial farming conditions – and work in the wild ; availability of high health quality animals (farm species). <u>Arguments for inclusion:</u> Better and more uniform health quality (other than farm species), no other welfare legislation (other than farm species); accommodation and care provisions proposed in revised Appendix A would seem to offer higher standards than found in commercial units. - Birds farming species – chicken, geese, turkeys and ducks – other legislation applies to these species. - Pigeon – much of research is applied; like surveys for pathogenic microbes and behaviour in the wild. Basic research would benefit of inclusion, but since no breeders found, not feasible to include. - Zebra finches – few numbers used and so difficulties to match supply and demand, no need to be included
RECOMMENDATION	**There is no compelling need to include any other birds species**

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Appendix 7

Purpose bred criteria / Critical Species

Species to be added: Amphibian (focusing on *Xenopus sp. and Rana sp.*)

CRITERIA	Assessment compared with not purpose-bred
Hazards	
Accommodation & Care	Susceptible to changes in water quality and temperature. Inappropriate stocking densities can influence growth rates and behaviour. Inappropriate nutrition can cause health problems and will affect egg quality.
GAA	GAA being used – *X. tropicalis* is much quicker maturing (5mths vs 2 yrs). *Xenopus laevis* was the first vertebrate animal to be cloned (Gurdon *et al.* 1975), and recent years have seen an upward trend in their use with the advent of further developments of genetic technologies. They are now one of the most widely used vertebrate species in developmental, cell and molecular biology research (Gurdon 1996).
Quality of animals *Microbiological / genetic quality / physicochemical aspects* *Reduction aspects* *Impact on research*	Wild caught animals are commonly used - these have unknown health status - zoonotic risk; parasitic and bacterial infections are common. Variability in quality and sourcing is likely to result in increased numbers, and adversely effect quality of research.
Transport	Due to small number of breeding establishments there may be lengthy journey times. Amphibians do require special care during transportation but no real issues if done competently.
Exposure assessment	
Welfare of animals *Pertinent legislation & guidelines*	Convention on International Trade in Endangered Species of Flora and Fauna (CITES) 1973, Convention on the Conservation of European Wildlife and Natural Habitats 1979, for some wild species, and European Convention on the Protection of Pet Animals, for amphibians kept as pets, provide protection. Little specific protection for non-CITES listed species. Some guidance on accommodation and care in proposals for revision of Appendix A of ETS 123.
Availability of suitable quality animals *Refinement aspects* *Breeding issues*	Purpose bred amphibians could be produced to a higher/more uniform health and genetic status and could be free of zoonotic infections. As a consequence, scientific variables could be reduced, and potential health risks to animal care staff and research workers reduced.
Research programme *Extrapolation of data*	Captive bred amphibians could be provided with complex environmental enrichment to mimic natural conditions - however little is known about the requirements of some species of amphibians. Purpose bred amphibians could be produced to a higher health status and a more uniform genetic background thus reducing scientific variables. Better quality animals will give better science - oocyte quality is a particular issue in research using Xenopus spp.
Additional considerations	
Number *of all animals used in EU*	EU references: 60000 amphibian in 2002; Pubmed references: 25000 *Xenopus sp*., 22500 *Rana sp.*, 5200 *Bufo sp.*, 2200 *Ambystome sp.*. Few statistics, but recent analysis of experimental papers illustrates growing use of *Xenopus*, in particular *laevis* and *tropicalis*. However, the Class *Amphibia* includes three orders of which two, *Urodela* and *Anura* contain species used in research. *Urodela* contains the salamanders and newts in 358 species; and *Anura* contains 3494 species.
Supply/demand status	Wild-caught animals are still used due to lack of suitable purpose-bred animals. Estimated that one third of *X. laevis* used may be wild-caught. Few commercial sources - suppliers often source wild-caught. USA centre for *X. laevis*. There is a commercial source for *Xenopus* within EU. High production index - but slow maturity in captivity a potential problem with *X. laevis*.
Surplus animals	Possible risk of over production from purpose breeding Increasing use of GAA may reduce demand for conventional amphibians and therefore reduce the numbers required and therefore the economies of scale for the production of purpose bred amphibians may disappear.
Impact on research	Improved health quality, nutrition is likely to have significant impact on quality of welfare and science.
CONCLUSIONS	Arguments against inclusion: Huge range of species and for many species there are only small numbers used. Production of the less commonly used species, *e.g.* newts, salamanders (incl. axolotls) may not be practicably viable due to the very small numbers used. The purpose breeding of *Xenopus laevis* and *tropicalis* may prove to have economies of scale that make it viable. Potentially high cull rates, difficulties to match supply and demand leading to delays in scientific programmes, lack of information on husbandry and care practices. Arguments for inclusion: better and more uniform health quality, increasing numbers of some species, no other welfare legislation, elimination of zoonotic diseases, no animals taken from wild.
RECOMMENDATION	**For the majority of the species there is no good justification to include. For certain *Xenopus* species (*laevis* and *tropicalis*) and *Rana* (*Rana temporaria* and *R. pipiens*) there are likely to be scientific and welfare benefits for inclusion as purpose bred**

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4. QUESTION ON HUMANE METHODS OF EUTHANASIA

4.1. Introduction

Nearly all laboratory animals are killed at the end of an experiment because their tissues are needed for further scientific analysis, or because they cannot be found homes (especially the commonly used species like rodents), or cannot be re-used in another research protocol, or cannot be returned to stock, or cannot be released into the wild. Other reasons for killing animals include: when they are surplus to requirement, or sick (experimental and stock animals), or in an emergency (e.g. fire alarm sounding in the middle of an experiment), and for reasons related to maintaining a high health status. Occasionally it may be possible to re-use an animal rather than killing it, but this is relatively uncommon. Animals may also be killed for their tissues to be used in *in vitro* research.

As emphasised in the mandate, it is important that all animals are killed using humane methods and this report looks specifically at the methods used to kill animals used in research and testing (though to the animal its use by humans is somewhat irrelevant). These methods are used with the aim of providing good scientific data and so the objectives may be somewhat different compared with other uses of animals. This report deals with new data that has arisen since the publication of 3 earlier reports: 1) the Scientific Report related to welfare aspects of animal stunning and killing methods of the main commercial species of animals (EFSA, 2004, http://www.efsa.eu.int); 2) Close *et al.* 1996/1997 (endorsed by the EU for the humane killing of laboratory animals); and 3) the AVMA Report (2000) dealing with methods for all animals. Our report does not repeat what is already dealt with in detail in those reports but we have included a section dealing with new data for each method where applicable, and some conclusions and recommendations are retained. The section on the use of gaseous agents is in some considerable detail as it is both contentious and also the subject of much new data, with more than 20 new papers in the past 10 years, many of them dealing with the commonest laboratory animals. The recommended methods for each species are given in Tables 7 to 14 at the end of this section but, in general, we have adopted the recommendations given in the existing EU Guidance (Close *et al.,* 1996/97) except where stated. The WG suggested that these methods could be varied but only with a scientific justification and appropriate authority, *i.e.* the recommended methods represent the default position.

The section on the use of gaseous agents is in some considerable detail as it is both contentious and also the subject of much new data, with more than 20 new papers in the past 10 years, many of them dealing with the commonest laboratory animals.

The aims of humane killing are to ensure a humane death for a good reason. Thus an ideal method from an animal's viewpoint should be painless, minimally invasive, cause a rapid loss of consciousness, and should not cause distress in any way e.g. through being aversive or causing fear or anxiety. From a user perspective one has to consider whether the method is safe for humans (this is outside EFSA's remit and so such issues are not addressed in this report), is aesthetic, easy to perform, can be repeated without loss of effectiveness and efficiency and, for an experimental animal, will be appropriate for the scientific outcomes being measured. Finally, when pregnant animals are killed it is likely to be more humane if the young are left to die in uterus than removing and killing separately.

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4.2. Reasons for euthanasia:

The reasons for killing animals have also to be considered, as some methods may cause more pain and distress than others. For example, breeding more animals than are required simply to have them available on demand, and then killing those that have not been used. This is especially true for animals that have a painful harmful defect caused for example by a genetic alteration. Sometimes killing of surplus is inevitable as in the breeding of some transgenic or mutant animals as only a particular genotype is wanted, and uses cannot be found for the surplus animals. On other occasions, breeding strategies can avoid having to kill such large numbers, but can also increase the numbers that have to be killed due to a balance between inducing adverse effects in all animals as opposed to just some. Archiving (freezing down) rodent strains that are currently unwanted is a way of reducing the number of animals to be culled, as is accurately forecasting the number of animals to be used.

4.2.1. Scientific reasons:

Occasionally, after considering all available methods, animals may have to be killed using methods that do not meet the animal welfare criteria set out for a humane method of killing for scientific reasons e.g. using some of the recognised methods may interfere with the scientific outcome. In a choice between two or more methods of humane killing, pilot studies may be carried out to determine the method that is most suitable for the scientific purpose and for the animals concerned. This may not always be the traditional method as new methods come along, or more information is gained on old methods questioning its humaneness, or its impact on the animal, its scientific validity and, therefore, its suitability. If animals are killed using less than ideal methods then that should be justified and taken into account when carrying out the harm (cost) benefit analysis. Some methods are listed in the report that cannot be considered humane, and are identified as such. For others, where there is a lack of information, that is addressed in future research.

Because the numbers of animal killed at any one time can range from one to several hundred, the method should be appropriate to dealing with both ends of the scale, again with the minimum distress to the animals as well as to the human operators.

4.3. Education, training and competence of those carrying out humane killing:

It is important that those carrying out such methods of killing are suitably trained and are deemed competent in that method (Council of Europe, 1993). As nearly all methods require an element of restraint, it is equally important that they are competent in handling animals humanely. We suggest that a training plan, particularly for the use of physical methods that require a measure of manual skill, such as cervical dislocation or concussion, should incorporate a progression from the use of freshly killed animals, to anaesthetised animals, before going on to kill conscious animals. In that way there is less chance of poor welfare and poor science due to poor technique.

The attitude of persons carrying out humane killing is important as over-sensitivity or a lack of care is more likely to result in poor welfare for the animals concerned. Killing animals in research establishments has been described as a kind of "initiation right" for animal care staff, and appropriate help and guidance should be available to guide young persons who are asked to do it (Arluke, 1993, 1996). If senior staff members treat animals without sufficient respect, habits which lead to poor welfare may be formed in

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younger staff members. No-one should be coerced to kill animals, so scientists and others should be sensitive to the fact that those looking after animals did not enter this area of work to kill them; it is seen as an unavoidable, unpleasant aspect of animal care in research.

4.4. Killing animals for their tissues:

Killing animals to retrieve tissues for *in vitro* work is outside the existing EU Council Directive (86/609/EEC), but such a use of animals is included in some countries (e.g. The Netherlands, Germany), and the number of animals used is counted giving an indication of the level of *in vitro* research by the scientific community. By including those animals killed for their tissues, the total annual number of animals used in research in those countries increased by 10 to 15%. Even though this use of animals is outside the Directive, there is EU and other national guidance on the ways by which animals should be humanely killed under laboratory conditions. Consequently, at present, research work involving killing animals by a recognised and approved method would permit, for example, researchers to kill 100 chimpanzees or dogs for a research purpose, without a licence, without oversight, and without any ethical or scientific approval. As death can be considered to be a lasting harm, it is debatable as to what level of licensing and scrutiny is required, and whether killing should be classified as a regulated procedure. In that case, animals killed for their tissues would receive the same level of care during euthanasia as an experimental animal and the staff would receive appropriate training and be certified competence as for any regulated procedure. Killing sick or injured stock animals could be exempted or encompassed.

4.5. The approach, scope and layout in the euthanasia section

The approach in the report is to carry out a risk assessment in order to identify the hazard and the likely exposure, and the possibility of poor welfare occurring in a particular species for a given method of killing. This is quantified where possible. For each method the type of hazard is identified and, where possible, some idea is given of how often that hazard may occur. The impact of causing these inadvertent adverse states to an animal has also to be considered in the light of producing reliable scientific data with low variance that can be accurately interpreted, and can be reproduced in another laboratory. Consequently the potential scientific impact for each method is also given.

Poor welfare itself may vary in degree and duration when an identified hazard occurs. For example, an animal given an inadequate dose of anaesthetic to kill it may be unconscious and so the hazard in that case involves no extra suffering. On the other hand, an animal killed by a method e.g. stunning or dislocation, in the event of a mis-stun may suffer poor welfare until it is rendered unconscious by a successful stun, and the degree of pain (intensity) in the intervening interval (duration) has to be taken into account e.g. the pain of a tissue damage of the mis-stun, or having a bruised and inadequately dislocated neck. So for each method the consequences of it going wrong in terms of animal welfare (intensity and duration of suffering) are evaluated and, in addition, the number of times it may go wrong (efficiency). With some methods the number of animals being killed by that method has also to be taken into account. For example an inadequate dose of an aversive inhalational agent given to a batch of animals on one occasion, would rate an incidence of 1, but in terms of animals suffering hundreds of animals may have been exposed, and that also has to be factored into the risk assessment. This is dealt with under the heading of "Consequences of inappropriate administration" for each method, and as there are few data on which to base an

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assessment, the experience and opinion of the working group was taken into consideration.

4.6. Gathering information

In order to know how often poor welfare occurs during euthanasia, we need to have quality control procedures and document when things go wrong and why, and what measures have been taken to stop it happening again. It is also important to know how often the method is used successfully so that an overall picture can be gained. This will then inform future risk assessments. At present this sort of information is not available, as it is in abattoirs in some countries.

4.7. Species to be dealt according to the annex sent by DG ENVIR

Mice	*Mus musculus*
Rats	*Rattus norvegicus*
Guinea-Pigs	*Cavia porcellus*
Hamsters	*Mescocricetus*
Other Rodents	Other *Rodentia*
Rabbits	*Oryctolagus cuniculus*
Cats	*Felis catus*
Dogs	*Canis familiaris*
Ferrets	*Mustela putorius furo*
Other Carnivores	Other *Carnivora*
Horses, donkeys and cross-breds	*Equidea*
Pigs	*Sus*
Goats	*Capra*
Sheep	*Ovis*
Cattle	*Bos*
Prosimians	*Prosimia*
New World Monkeys	*Ceboidea*
Old World Monkeys	*Cercopithecoidea*
Apes	*Hominidae*
Other Mammals	Other *Mammalia*
Quail	*Coturnix coturnix*
Other birds	Other *Aves*
Reptiles	*Reptilia*
Amphibians	*Amphibia*
Fish	*Pisces*

4.8. Methods of euthanasia

General comments applying to all methods

The WG suggested that the recommended methods could be varied but only with a scientific justification and appropriate authority, i.e. the recommended methods represent the default position.

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When pregnant animals are killed, the fetuses should be allowed to die *in utero* before being removed, unless they are required for scientific reasons, in which case they should be considered as neonates and killed by another method that is appropriate for the species and that causes a minimum of pain and distress.

4.8.1. Electrical stunning

Basic Effect: Electrical disturbance of the CNS.

Description of the method: This is a commonly used method for farmed species but not for the commonest laboratory species (*i.e*. rats, mice, guinea-pigs), or necessarily for farmed species kept in a laboratory. It involves electrical stimulation of the brain by placing electrodes on either side of the head, or on the head and body so that current passes through the brain and heart. It is important that an adequate voltage is used to drive sufficient current through the animal. This stimulation of the brain causes the equivalent of a generalised epileptiform brain activity accompanied by seizures indicative of unconsciousness and insensibility (Roos and Koopman, 1940; Lopes da Silva, 1983). The expected outcome is either stunning (when applied to the head only) or death (when applied to head and body). Electrocution (application head to body) causes death by cardiac fibrillation or arrest, whereas electro-anaesthesia (head only) involves stunning without death and the animal is subsequently killed by another procedure *e.g*. exsanguination.

In the case of some species of fish (*e.g.* salmon, tilapia, trout), the fish are placed in a water tank with electrode plates on either side. However, the brain mechanisms associated with the induction of unconscious by electrical stunning in fish have not been clearly elucidated (i.e. criteria applied to mammals are also used in fish). Other fish such as eel and catfish are difficult to kill using this method and electrocution has to be followed by another method to ensure death occurs (e.g. chilling, exsanguination).

This method is not normally applied to amphibia and reptiles.

Fetal and immature forms have not been investigated but if the dam is killed by electrocution or electrical stunning followed by bleeding, then the fetus dies through a lack of an oxygenated blood supply.

New Data: The EFSA report is the most comprehensive on this method. Recent work has extended our knowledge in fish and shown that species vary in their susceptibility to electrocution. The duration of unconsciousness decreases with increasing current frequency in poultry and fish. In general, the efficacy of electrical stunning depends upon the waveform, frequency, and amount of current. (Raj *et al.,* 2005a, b, c).

Advantages and Disadvantages - Animal Welfare: The overall hazard is that of poor welfare. In the case of electrocution, the hazard can be recognised by the animal remaining conscious with some intact reflexes. There are also secondary factors that directly contribute to poor welfare because of the consequences.

Failure of the equipment

1. The recommended current levels given below come from the slaughter of food animals at a high throughput rate and little is known about the incidence under laboratory conditions. Poor stunning can occur through incorrect placement of the

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electrodes; inadequate electrical current delivered to the brain due to wrong waveform (electrical) frequency, voltage or current. There is always some uncertainty in outcome due to equipment, and to varying resistances between animals.

The recommended current levels for achieving for humane stunning is at least 100 mA per broiler (Gregory, 1986), 1.3 A for slaughter pigs (Wotton *et al.,* 1992), 1.3A for cattle (Cook *et al.,* 1986), 1A for lamb (Velarde *et al.,* 2002), 0.3A for rabbits (Anil *et al.,* 1998), and 1.6 A/dm2 for fish (Kestin *et al.,* 2002; Lambooij *et al.,* 2002a). If the current is lower it may be that an animal is not rendered immediately unconscious and therefore it experiences electric shocks, and even cardiac arrest which in a conscious animal may be painful. Electrical stimulation of muscle causes contractions that can be painful in a conscious animal (Croft, 1952).

2. In the case of small mammals (e.g. rodents and mink) electrocution is not recommended as it is difficult to induce a permanent cardiac arrest.

3. The time interval between rendering an animal unconscious and inducing death through bleeding out is critical in the head only stunning situation, as it may recover consciousness before it is dead. Figures show that 10% of broiler chickens in mechanised high throughput abattoirs may recover consciousness (EFSA, 2004, http://www.efsa.eu.int).

4. Killing itself is not risk free, as during stunning/electrocution may give rise to skin burns that will be painful in conscious animals and may even further prevent the passage of an adequate current to induce unconsciousness. The incidence of this is increased when the currents are too low due to a high electrical resistance and inadequate voltage. There is no good data on the overall incidence of skin burns.

5. Handling and restraint will cause stress as, at the outset, the animal will not be free to escape the pressure of the electrodes (which may be sharp). The change of environment, mixing in different groups, social isolation from the cage/pen/flock mates have also to be taken into consideration.

6. Time to unconsciousness: With good equipment and ideal conditions it should be less than 0.2sec and should always be less than 1sec.

The **disadvantages** are that it is a risk to humans when using high voltages i.e. more than 110v in a wet environment.

Advantages and Disadvantages - Scientific Impact: Electrical currents through the brain will alter the biochemistry of the brain, brain neuro-peptide levels, and also may affect the biochemistry of other tissues e.g. muscle. Extravascular haemorrhages may occur in muscle, connective tissue and fat, and it may cause muscle fibre ruptures and broken bones (Gregory *et al.*, 1991).

Requirements for Optimal Operation: Ensure electrical equipment is well maintained and calibrated prior to its application to live animals. Animals should be adequately restrained

4.8.2. Mechanical stunning methods

Basic effect: The tissue of the CNS is traumatically disturbed to produce a state of unconsciousness

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Description of the metod:

4.8.2.1.Penetrative methods

Captive bolt and free bullet

The method is commonly used for ruminants to stun them for slaughter and after stunning death is caused through exsanguination before the animals regain consciousness. The method can be used for the bigger experimental animals such as guinea-pigs, rabbits and dogs. The ideal shooting position is frontally on the head.

The commercially available captive bolt for ruminants can be used for the biggest animals and the "Goldhase Schusz Apparat" for smaller animals. Cartridges with gunpowder, compressed air or a spring under tension are used to drive bolts (missiles) against or through the skull of farm animals.

In general, penetration of a missile into the brain, depending on its velocity and shape, can cause injury in the following three ways: by laceration and crushing (< 100 m/s), by shock waves (about 100 to 300 m/s and by temporary cavitation (> 300 m/s) (Hopkinson and Marshall, 1967). In captive bolt stunning methods, the most important factor is to cause an epileptiform seizure and a rapid and large sub-dural or sub-arachnoidal bleeding at the base of the brain where the arterial vessels enter. The shock waves and cavitation cause the arteries to rupture and heavy bleeding. This leads to a levelling out of the arterial pressure with the intracranial pressure that substantially reduces the cerebral perfusion pressure and so cell function ceases.

Missiles used for stunning and killing of animals are a bullet, a bolt, water jet and air pressure. Immediately after stunning the animals express a tonic spasm for approximately 10 s prior to relaxation, however, excessive convulsions may occur (Eichbaum *et al.,* 1975). Immediately after shooting major changes are seen on the EEG (delta and theta waves tending to an iso-electric line) it is assumed that the animal is unconscious by analogy to similar EEG changes described in man (Lopes da Silva, 1983; EFSA, 2004, http://www.efsa.eu.int).

Water jet

High pressure water jets are used for cutting and drilling in solid materials and experiments to explore the suitability of water jets for stunning and killing purposes were conducted under laboratory conditions with post mortem materials (pig heads) and live slaughter pigs. Immediate unconsciousness as determined by EEG, was initiated by a rapid penetration of the skin and skull. Destruction of the brain occurred in 0.2 to 0.4 s. The water jet should be injected frontally on the head into the brain cavity at the intersection of the imaginary lines from the ear to the opposite eye (Schatzmann *et al.,* 1990).

Air jet

The development of captive bolt air stunning is not used in most species due to an inability to prevent post-stun convulsions. Recently, a captive needle stunning method for broilers has been developed, in which air pressure is injected into the brain and partly directed to the spinal cord. The latter may prevent the convulsions. In broilers the air pressure stunning reduced post-stun convulsions to less than 13%

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of the level of convulsions. The captive bolt stunning method for broilers has been modified, in which air pressure was used to block post stun convulsions. To improve the method for practical application a commercial air staple gun was modified. The plunger of the original design was replaced by two needles, which penetrate the skin and skull at an angle of 15 degrees in a caudal direction. Both needles were provided with small holes, which allow air through in different directions. The stunning position was at the intersection of two imaginary lines drawn from the ear on one side to the inner corner of the eye on the other side. A trigger starts the injection of compressed atmospheric air when the needles penetrate the skull, and the duration of air injection was electronically controlled. The duration of injection as well as the air pressure was adjusted to a shooting pressure of 8 bar and an air injection of 3 bar for 1.5 s. It is hypothesised that in the captive needle pistol the compressed atmospheric air administered through the needle, placed more anterior on an animal's head, damages higher brain regions to cause unconsciousness, while the other needle damages the upper spinal cord to prevent post stun convulsions.

The captive needle pistol was adapted for guinea-pigs, eels and cat fish regarding the length and shape of the needle. Only one cone shaped needle of 16 mm was used, which pressed the air in 3 directions radial 120 degrees, where one direction was caudally towards the spinal cord. For correct positioning on the head an adapter was placed at the barrel of the pistol (Lambooij *et al.,* 2002).

4.8.2.2.Non-penetrative methods (including concussive blows)

Cerebral concussion is generally agreed to be a traumatically induced derangement of the nervous system, resulting in an instantaneous diminution or loss of consciousness without gross anatomical changes in the brain (EFSA, 2004, http://www.efsa.eu.int). Irrespective of the type of force which produces the traumatic depolarisation of the cell membrane there is now evidence that powerful pressure waves are provoked within the cranial cavity such a blow on the head and that the frequency and force of the waves vary in different parts of the brain. It has been suggested that it is not the pressure as such developed by these waves that is the important factor, but the rapid oscillations in this pressure (Nilsson and Nordstrom, 1977).

It should be noted that many investigators consider blood flow impairment as being primarily responsible for the electrical changes in the brain, although the immediate changes in the brain cannot be explained by this theory.

A blow on the head with a blunt instrument (or sometimes swinging the animal itself against a solid surface) can be used to kill animals, and is a not uncommon method in laboratory rodents and lagomorphs. The blow acts in a similar way to the non-penetrating captive bolt (these methods are reported in Table 1).

New Data: The EFSA (2004, http://www.efsa.eu.int) report is the most comprehensive on these methods.

The water jet apparatus and needle captive bolt are just in an experimental stage and are not commercially available (Lambooij *et al.,* 2001).

Advantages and Disadvantages - Animal Welfare: The velocity of a bolt of a captive bolt gun used for stunning farm animals is about 100 m/s in air. At this

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relatively low velocity the shape of the bolt should crush the cortex and deeper parts of the brain either by the bolt itself or by the generation of forward shock waves. Captive bolt stunning is widely used for red meat farm animals.

1. The use of a free bullet is not recommended due to the hazard of injuring other animals including the operator(s) as a free bullet has a low controllability.

2. Brain particles are found in the blood, lungs, heart and muscle after penetrative stunning methods. Brain particles are visually observed in up to 33%, 12% and 1% of the carcasses after captive bolt stunning with air injection afterwards, bolt penetration using air pressure, and cartridges with gunpowder, respectively (Anil et al.,1999; Schmidt *et al.,* 1999).

3. A problem with the water jets could be convulsions, which may appear after the use of this stunning method. Whenever an animal is de-cerebrated, convulsions of the carcass, and muscle spasms mainly of the hind limbs occur, caused by stimuli evoked by the medulla oblongata.

<u>*Time to unconsciousness*</u>: With good equipment and ideal conditions it should be instantaneous which means less than 1 second.

4. The percentages of cattle stunned with 1 shot from a captive bolt stunner were 100% in 12% of processing plants, 99% in 24%, 95 to 98% in 54% of the plants and < than 95% in 10% of plants in the USA in 1999. All cattle where the first shot missed were immediately re-stunned (Grandin, 2001).

5. The disadvantages are that it is a risk to humans when using apparatuses that are not maintained in an appropriate way.

6. After these stunning procedures the animals may not die immediately depending on the degree of injury to the brain. Therefore, it is recommended to kill the animal by exsanguination, delivering compressed air into the cranium, or pithing to damage the deeper parts of the brain and to prevent convulsions.

7. When concussion by a blow to the head is incorrectly performed the animal may be injured and not either stunned or killed. The method is used in small animals such as rodents, rabbits and small birds.

8. For fish, mechanical stunning methods, such as percussion, spiking and decapitation are used. Both percussive stunning and spiking could be mechanized. However, it is essential that the blow is delivered correctly to ensure that consciousness is lost immediately. Mechanical methods are recommended for use under practical conditions (Boyd *et al.,* 1984; Robb *et al.,* 2000).

Advantages and Disadvantages - Scientific Impact: It is generally known that the removal of inhibitory influences from higher centres of the brain (e.g. damage by captive bolt), before the spinal cord becomes anoxic, results in convulsive activity and enhancement of some spinal reflexes. This may affect research on muscle and brain.

Most investigations concerning the mechanism of concussion have been performed using laboratory animals (i.e. rats, cats, primates). It is evident from these investigations that concussion does not always cause an immediate loss of consciousness. In humans, amnesia after the blow occurs. Successive severe blows

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results in prolonged loss of reflex activity and cause almost complete disappearance of all frequencies i.e. an isoelectric line on the EEG (Nilsson and Nordstrom, 1977)

Requirements for Optimal Operation: Ensure that equipment is well maintained and calibrated prior to application to live animals. Animals should be adequately restrained.

Table 3 – Mechanical stunning methods

	Penetration	Non-penetrative	Concussive blow	Fetus Intrauterine	Remarks
Guinea-pigs	A		A	Not killed	
Hamsters	A		A	Not killed	
Other rodent			A	Not killed	
Rabbits	A	A		Not killed	Exsanguination /pithing required
Dogs	CA	CA		Not killed	Exsanguination /pithing required
Horses, donkeys and cross-breds	A			Not killed	Exsanguination /pithing required
Pigs	CA			Not killed	Exsanguination /pithing required
Goats	A			Not killed	Exsanguination /pithing required
Sheep	A	CA		Not killed	Exsanguination /pithing required
Cattle	A	CA		Not killed	Exsanguination /pithing required
Quail			A		
Other birds	A	A	CA		Exsanguination /pithing required
Reptiles			CA		
Amphibians	A				Pithing required
Fish		A	CA		Exsanguination /pithing required

A – Causes a minimum level of pain and distress; CA – May cause more than a minimum level of pain and distress but for various reasons can be used. Both have to be given appropriate design and skilled persons.

4.8.3. Mechanical disruption of tissues (Neck dislocation, decapitation, maceration)

Basic Effect: Dislocation of the cervical vertebrae with the consequence that the CNS is disturbed by a neural shock.

Decapitation causes death through anoxia of the CNS due to blood loss.

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Maceration destroys all body tissues including the brain.

Description of the method:

Neck dislocation

This method is commonly used in small animals such as rodents, rabbits and birds. There are several ways in which to dislocate the neck of rodents. One way is to place the thumb and index finger on either side of the neck at the base of the skull and to pull the hind limbs or tail away from the head. Another is to place a blunt instrument at the base of the neck before pulling on the based of the tail. A rapid pull induces separation of the vertebrae, mainly the cervical but sometimes the thoracic vertebrae are separated (Keller, 1982). In birds the head may be twisted and extended dorsally while stretching. Due to turning and stretching, blood vessels may be damaged and bleeding may occur at the site of vertebral separation; sometimes decapitation may also occur.

Severing the spinal cord with a knife (puntilla)

This method is used in animals such as cattle but is not used in laboratory animals. The spinal cord is destroyed by thrusting a knife between the head and the 1st or 2nd cervical vertebra. After dislocation or cutting a tonic cramp occurs which changes to paralysis after 5 to 10 s. Inhibition of the contact between brain and spinal cord causes apnoea and loss of sensation from the body, excluding the face via the trigeminal (5th cranial) nerve, thus causing spinal shock.

Decapitation

This method is used in small animals such as rodents, rabbits and birds. The head is separated from the body normally using a purpose built mechanical device with a sharp blade, i.e. guillotine, so that separation of the head from the body is achieved swiftly in the first and only attempt. Because the animal is decapitated bleeding will occur from the neck blood vessels although there may be some recoil in the arteries that will reduce blood loss. In pre-born and neonatal animals decapitation may be carried out with scissors or a sharp knife.

Maceration (maceration leading to fragmentation)

This method is used in small animals such as day old chicks and unhatched eggs. However, there is a risk that for birds that can fly there will be a tendency to keep high up in the macerator, this can be prevented by fast flow through rates or by killing these animals another way. Instantaneous fragmentation in high-speed rotating blades will kill a small animal within one second. Macerators with rotating blades with a speed of at least 2800 revolutions or more (recommended 6000) per min at a power of 4KW are effective.

New Data: The EFSA report (2004, http://www.efsa.eu.int) is the most comprehensive on these methods.

Advantages and Disadvantages - Animal Welfare:

1. Handling and restraint for neck dislocation and decapitation will cause some distress as the animal will be restrained in an unnatural position and will not be free to escape. Anaesthetising the animal first may reduce this distress.

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2. After neck dislocation and decapitation electrical activity of the brain may persist for as long as 13 s in some mammals and birds (Mikeska and Klemm, 1975; Gregory and Wotton, 1990) during which time animals may feel pain due to afferent stimuli from the trigeminal nerve. If decapitation is a powerful pain stimulus and if the EEG activation (low voltage, fast activity) commenced at the instant of decapitation, it would only be perceived for 2.7 sec at which time the decapitated rat brain would be unconscious due to lack of oxygen (Derr, 1991). Moreover, Vanderwolf (1988) found that atropine-resistant forms of cerebral activation were virtually absent following decapitation, which suggests that decapitation is not painful. However, the cutting the skin and tissues of the neck may cause some for a short period (less than one second).

3. After cervical dislocation, convulsions only occur when separation is made cranial to the fifth thoracic vertebra, while severance caudal to this location results in paralysis of conscious animals (Eichbaum, 1975).

4. Mouse fetuses *in utero* are not killed within 20 min when the dam has been killed by cervical dislocation or decapitation. The heads of fetal rodents after decapitation may show signs of consciousness and this would be of welfare concern if the fetus had breathed (see Section 2.5.1.6.). (Klaunberg *et al.,* 2004).

5. It is concluded from observations after decapitation that signs of consciousness (visually evoked brain activity) may persist for some time e.g. 13 min in the heads of eels (Vis *et al.,* 2002), and hours in reptiles (Warwick, 1986, Close *et al.,* 1996/1997). The head of fetal neonatal rodents after decapitation may also show signs of consciousness and this would be of welfare concern if the fetus had breathed (see Section 2.5.1.6). Fetal forms of rodents in utero are not killed within 20 min when the dam has been killed by decapitation (Klaunberg *et al.,* 2004).

6. If the macerator is overloaded animals may be not be humanely killed.

7. All these mechanical disruption techniques are aesthetically controversial. The interpretation of the electrical activity in the brain after neck dislocation and decapitation is controversial as to what feeling remains, and is still a matter of debate (Gregory and Wotton, 1990; Hollson, 1992).

8. Anaesthetising animals before decapitation or cervical dislocation will minimise distress and any subsequent pain. This may be required in some cases of maceration where the animal may escape the blades.

Advantages and Disadvantages - Scientific Impact: Any tissue damage to the CNS or induced neuronal discharge may affect neuropeptide levels and brain histology.

Requirements for Optimal Operation: When using these techniques of dislocation and decapitation the necessary handling and restraint can be stressful for the animal and anaesthetising the animal before it is killed can mitigate this stress and any subsequent pain.

The operator using decapitation should be aware the danger of this apparatus and should take adequate precautions to prevent personal injury.

Severance of the spinal cord using a knife does not render the animal immediately unconscious and so it may suffer for some short time.

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Table 4: Methods causing mechanical disruption of tissues in adult and fetal animals

Method Species	Neck dislocation	Decapitation and bleeding	Fragmentation	Fetus
Mice	CA	CA		Not killed
Rats	CA	CA		Not killed
Guinea-pigs	CA	CA		Not killed
Hamsters	CA	CA		Not killed
Other rodent	CA	CA		Not killed
Rabbits	CA	CA		Not killed
Quail	CA	CA	CA	
Other birds	CA	CA	CA	
Fetal and neonatal forms: reptiles and amphibians, quail and other birds			A	

A – Causes a minimum level of pain and distress; CA – May cause more than a minimum level of pain and distress but for various reasons can be used. Both have to be given appropriate design and skilled persons. Effective killing may also depend on maturity of the bred e.g. cervical dislocation in small mature animals (e.g. rabbits) is more difficult than young animals of the same weight. Cervical dislocation can be difficult in mammalian fetal forms.

4.8.4. Physical methods

Basic Effect: Inactivation of the enzymes in the brain.

Description of the method:

Raising the brain temperature

Since the end of the 19th century high frequency electric currents have been used to heat tissues. Long wave diathermy, using frequencies in the order of 1 MHz required the use of electrodes to be in direct contact with the skin and consequently the risk of burning was high. Later frequencies known as short wave diathermy were introduced with the advantage that it was not necessary for the electrodes and the skin to be in contact.

After irradiation of heads of rats with microwaves of 2450 MHz for 1 s the temperature in the brain increased up to 75-90C within 1 s. Consequently brain enzymes are inactivated very rapidly, and this may have advantages in some neurochemical investigations. It is observed that an increase of about 10C in the brain resulted in a clinical state of unconsciousness using 2450 MHz (6kW) within 2s. A change of 6.4C at a depth of 3 mm could cause a stunning effect using 915

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MHz. After showing seizures, rats lay in an unconscious state for 4 to 5min (Wiegant *et al.,* 1979; Guy and Chou, 1982).

High-energy microwave irradiation results in rapid heating of the brain and it has been widely used in laboratories for inactivating brain enzymes of small animals *in vivo* (Delaney and Geiger, 1996; Takahashi, et. al, 1997). The literature suggests that the power outputs normally employed to kill rats without inducing structural changes in the brain are 1.33, 3.5, 6.0 or 10.0 kW delivered with 2450 MHz. In general, the duration of irradiation is decreased as the power output is increased. It is worth noting that these parameters have been implemented on the basis of achieving an end point temperature of 85C *in situ* rather than being appropriate to protect animal welfare during euthanasia of laboratory animals. In this regard, Delaney and Geiger (1996) used a power level of 10 kW for 1.25s, 6.0 kW for 2s or 3.5 kW for 3.25s. From an animal welfare point of view, a disconcerting finding by Delaney and Geiger (1996) is that, among the three microwave irradiation regimens, only the 10kW irradiation treatment seems to have denatured the rat brain enzymes completely and quickly as determined from the regional levels of adenosine in the brain. This implies that a rapid inactivation of enzymes cannot be achieved if the microwave energy is not sufficiently high for the species. However, the energy requirement would vary according to the size or volume of the brain to be irradiated, type and configuration of antennae and the adaptation of irradiation device to suit the head of the animal or bird.

At an internal temperature of 85C, most of the enzymes will be denatured in the brain and therefore it can be assumed that the brain is incapable of receiving and processing signals. It has been reported that a 10 kW, 2450 MHz instrument operated at a power of 9 kW will increase the brain temperature of 18 to 28g mice to 79C in 330 milliseconds, and the brain temperature of 250 to 420g rats to 94C in 800 milliseconds (Ikarashi *et al.,* 1984). It is highly likely that animals become unconscious when the temperature in the brain exceeds 45C as judged from studies on malignant hyperthermia in anaesthetised pigs. However, Zeller *et al.* (1989) concluded that this procedure should only be carried out in small laboratory animals such as amphibians, birds, mice, rats and small rabbits (less than 300g). From the animal welfare viewpoint, the main concern would appear to be the use of restraint necessary to hold the head and focus the microwave beam and ensuring correct positioning of the beam, although some animals will naturally follow down the length of a dark cone. Another concern is that high-energy microwave irradiation of rats at a given power creates a non-uniform heating throughout the brain with the ventral brain being hotter than the dorsal, and cerebellum and brain stem cooler than the cerebral cortex (Delaney and Geiger, 1996). The reason for this non-uniformity is unclear.

It has been suggested in a later study that a magnetic field concentrated on the head of the rat helped to achieve an even distribution of microwave heating with maximum certainty of inactivating brain enzymes (NJE, Model 2603, New Japan Radio Company, Tokyo, Japan; Takahashi et. al., 1997). Although the scientific rationale for the magnetic stimulation and the power of the magnetic field employed in this study were not presented in this report, it may be inferred that the magnetic stimulation could have reduced the movement of the head induced by the stress associated with the restraint and, possibly, microwave irradiation per se (e.g. local heating effect and hyperthermia of the brain).

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With regard to the stunning and slaughter of farm animals, high energy microwave irradiation has been tested in pigs. The only article published in a trade journal (which was not peer reviewed - Lambooij *et al.* (1990) involved irradiation of pigs' heads (obtained post mortem) with a power output of 6kW delivered using 2450 MHz for 1.5 to 2.5 s. The results of this study, although they showed a maximum temperature increase of 22C, replicated the results of a previous report on rats regarding the uneven distribution of temperature within the brain. Nevertheless, Lambooij *et al.* extrapolated the results of experiments with rats and concluded that an output of 45 to 70 kW would be necessary to kill pigs humanely. Presumably, owing to the lack of a device, which will put out such a high energy, no further experimental evidence on farm animals has been reported to substantiate this recommendation.

Laboratory experiments with focal microwave irradiation of chicken heads with 4 - 5 kW delivered with 2450 MHz seem to indicate that this procedure leads to immediate brain death (Zeller, 1986). However, owing to the lack of head restraining and fixing devices that do not cause distress, Zeller *et al.* (1989) attempted to irradiate the whole chicken and found that the birds became unconscious only after 5 to 9 s of irradiation. The prolonged irradiation required to kill chickens is possibly due to the very low power output used, which was inappropriate for the species, and to the absorption of microwave energy by body tissues other than the brain, which resulted in a cooked appearance in the extremities of chicken carcasses. These authors reported that the birds showed signs of excitation and pain before they became unconscious, and therefore, condemned the method of whole body irradiation on welfare grounds.

A United States Patent (Method serving the stunning of animals for slaughter; No. 3,973,290 granted in 1976) claims that very quick heating without contact can be achieved in particular with microwaves in a frequency range of between 100 and 10,000 MHz, preferably ranging between 500 and 2,000 MHz. It was also suggested that the skull be irradiated from the side in the temporal area and thus, for example in pigs, a temperature rise of 10C was achieved in the skull with a microwave beam of 955 MHz and 5kW power applied over a time of 5 seconds. It was also claimed that, since microwave irradiation leads to increased heart rate and it remains so for a period, this procedure will give enhanced bleed out. However, since microwave energy is hazardous even at low levels, extremely good safety measures will be needed to operate such a device under commercial conditions. In any event the increased heart rate could be a sign of extreme pain. An example of safety concerns using radiation is the recent controversy over the use of GSM (global system for mobile communication) cellular phones which mostly operate with carrier frequencies from 0.9 to 1.8 Gigahertz range.

Cooling down

The current pre-slaughter process used for fish consists of live chilling to immobilise them prior to evisceration. Assessment of live chilling revealed that this method is stressful as vigorous activity of the animals and an irregular heart rate were observed. Responses to pain stimuli disappeared at a body temperature of approximately 8 to 10C, which occurred after 10 to 15 min, which suggests that consciousness is lost (Lambooij *et al.,* 2002b). During live chilling, theta and delta waves appeared on the EEG traces and responses to pain stimuli disappeared after 10 – 15 min. Occurrence of theta and delta waves and no response to pain stimuli, both on the EEG and in

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behaviour, supports the assumption that the fish were unconscious and insensible as gauged by analogy with similar EEG changes in other animals, including humans.

New Data: The EFSA report is the most comprehensive on this method. Recent work on cooling down has extended our knowledge in fish.

Advantages and Disadvantages - Animal Welfare: A patented alternative method of stunning and killing eel consists of cooling them down gradually to death. According to the patent description the eels should ideally stay at least for 10min in a medium with a temperature below –20C, but brine at –15C may also be used. In addition, eels should be stunned prior to killing by cooling down the body temperature to between 0° and 5°C (Lambooij *et al.,* 2002b). Placing eels in brine at –18C is an effective method to kill eel. However, it cannot be recommended to place conscious eels in cold brine, because it takes more than 27 s before consciousness is lost.

Advantages and Disadvantages - Scientific Impact: In fish, stressors activate the hypothalamo-pituitary-inter-renal-system and the subsequent increased release of pro-opiomelanocortin (POMC)-derived peptides from the pituitary gland induces cortisol release from the corticosterone producing cells of the head kidney. For example, exposure of carp *(Cyprinus carpio)* to a rapid drop in temperature of 9°C resulted in a time-dependent cortisol response and induced a differential expression of both the POMC and mRNAs. Plasma cortisol levels increased up to 6 times the control level 20min after the start of the experiment, and remained high until the end of the temperature shock (Arends *et al.* 1998). Increased plasma cortisol levels were also observed for Atlantic salmon *(Salmo salar)* after live chilling compared to percussive stunning (Robb *et al.* 2000).

Requirements for Optimal Operation: Heating the brain can only be applied by using a special designed microwave oven.

4.8.5. Gaseous methods

In general, exposure of animals to gas mixtures and inhalation anaesthetics, unlike some other methods of euthanasia, does not produce immediate loss of consciousness in animals. Therefore, it is important to research for gas mixtures that are non-aversive and do not induce distress or pain prior to loss of consciousness (Close *et al.,* 1996; 1997). Gaseous methods also have the distinct advantage that animals do not have to be restrained in any way which is good from both scientific and animal welfare viewpoints.

Some species of animals are tolerant of hypercapnia or hypoxia / anoxia. Moreover, neonatal animals may take longer to kill because their haemoglobin has a high affinity for oxygen (Pritchett *et al.,* 2005, unpublished). Furthermore, diving or aquatic species, amphibians, burrowing animals and reptiles survive these conditions by either compensatory cardiovascular mechanisms, holding their breath or due to having very slow rate of breathing, for example, fresh water turtle genus *Chrysemys* (painted turtle), fishes such as carp, African lungfish and amphibians such as *Rana temporaria*, Australian frog (*Cyclorana platycephala*) or American spadefoot toad (*Scaphiopus couchii*). Tadpoles of some species, the naked Kenyan mole rat (*Hetercephalus glaber*), embryonic and neonatal *Rattus norvegicus*, and hibernating mammals such as the ground squirrels are also tolerant of anoxia (Bickler and Donohoe, 2002).

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4.8.5.1. Exposure to carbon dioxide mixtures

Can be used in all vertebrates and is probably the most commonly used method for killing laboratory rodents at present.

Description of the method: Carbon dioxide induces acidosis and inhibition of neurones that leads to a loss of consciousness, insensibility, and finally death. It is a commonly used method for culling surplus rodents in breeding establishments, for the procurement of tissues for *in vitro* studies, and for killing animals at the end of a study. It is particularly useful when large numbers of animals are to be killed.

Carbon dioxide is denser than air (relative density 1.6) and therefore can be easily contained in a chamber. Air breathing (terrestrial and aquatic) animals are exposed to atmospheres in a chamber of CO2 at varying concentrations. Restraint, other than inability to escape from euthanasia apparatus or cage, is therefore minimal. Concentrations may be rising from 0 to 100% or they may be exposed to a lethal concentration from the start i.e. (e.g. depending on the species 40%), or there may be staged rises in concentration (e.g. from 20 to 40 to 60 to 90%).

Aquatic species: some species of farmed fish are killed by immersion in water saturated in CO2, (EFSA, 2004, http://www.efsa.eu.int) but there is little specific information on the common methods used in laboratories.

Fetal, neonatal and immature forms are tolerant of anoxia and hypercapnia and therefore exposure to carbon dioxide is considered to be inappropriate. Although it is effective in neonatal mice it is ineffective for fetuses *in utero*.

New Data: Two animal welfare issues emanate from scientific literature: (1) the risk of compromising animal welfare is high and inherent to CO2; and (2) the method of administration of the gas itself could further confound or exacerbate this risk.

The high risk of compromising animal welfare due to the inherent properties of CO2 has been addressed in a considerable number of papers involving aversion tests, behavioural observations and physiological responses.

It is widely accepted that some animals experience subjective feelings of pleasure in the presence of rewards or positive reinforcers, and that they experience distress and / or pain in the presence of punishments or negative reinforcers. Aversion studies, i.e. passive or active avoidance tests, reveal the animals' experience of pleasure or fear, distress and pain. Therefore, the outcome of avoidance tests / aversion studies is less likely to be influenced by the scientists' emotions, perceptions or interpretations. Additionally, such studies also quantitatively reveal the severity or magnitude of distress and pain, independent of other biological variables, i.e. how distressing is the stimulus when presented to a particular species, breed, genotype or population. For example, a potentially distressing or painful stimulus is likely to elicit a uniform behaviour in all, i.e. 100% of the population avoid it (i.e. uniformity in response). Where as, a moderately distressing or painful stimulus would elicit avoidance behaviour in 50% of the animals (statistically significant variation in response) and at least distressing or painful stimulus may not elicit avoidance behaviour at all (ideal scenario). Some other experiments have looked at the strength of the 'aversion' in terms of tempting the animal to remain in the gas mixture through diet restriction and

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feeding a desirable nourishment or titbit, as a sort of 'economic demand' experiment. The willingness of an animal to stay in the gas mixture can then be assessed in relation to its hunger, i.e. its motivation to feed.

Clearly, avoidance behaviour gives a better indication of welfare than physiological measures, as an animal's spontaneous behaviour (avoidance) is the definitive response of an animal to its environment. If an animal has the option to choose to leave a gaseous mixture or to spend time in it, or to re-enter it, it is a positive choice. However, if an animal is unable to, or not provided with an opportunity to, leave that environment, then it does not have that choice and any welfare assessment on the behaviour of animal is left to human interpretations. Numbers could also be assigned to behavioural manifestations (e.g. behavioural index) and quantitatively analysed, but that too has still to be interpreted subjectively by the scientists. Some scientists believe that physiological measures are better evidence of aversion than behavioural measures. But, simply because a number or score can be assigned to, e.g. a hormone level, and subjected to statistical analysis does not make the interpretation of that data any more reliable or secure from animal welfare point of view. Physiological measures may be important as adjunctive evidence, but sometimes the nature of the gas mixture itself will induce or even mask a physiological stress response. For example, carbon dioxide will depress heart rate and so an elevated heart rate cannot be used to determine fear, distress or pain. Mixtures of gases that are not inert are even more difficult to interpret e.g. carbon dioxide and oxygen (see e.g. Johnson, 2005). In addition, the inability to manifest instinctive escape behaviour or inhibition of natural behaviour repertoire of a particular species can lead to different conclusions by human observers. For example, the freezing behaviour of some species in response to a strange, even aversive, environment from which it cannot escape, can be interpreted as that animal tolerating, not minding, even enjoying that gas mixture, as well as not showing any aversion. Owing to these reasons, the results of behavioural and physiological studies have been interpreted in different ways giving conflicting conclusions on the acceptability of carbon dioxide for killing animals as well as its use as an anaesthetic.

The scientific literature concerning the three methods of exposure to CO2, especially rodents, reveals contradictory views among scientists (the users) regarding the relative animal welfare merits of each method. Most of the contradiction seems to be due to variations in the choice of parameters (aversion, behaviour or physiology), experimental protocols and interpretation of data.

Finally, regardless of the animals' responses, the uniformity of response as evidenced by a low standard deviation of the response is significant. The greater the variance the more legitimate would be the argument that it is a matter of the individual animal's response. A narrow variance would indicate some common, even fundamental, response that was vital for an animal's survival, even for survival of the species.

It is for these reasons that in the following sections we have given more weight to those experimental designs in which the animal can leave a gas mixture at any time under its own volition, and less weight to those situations where the animal is forced to remain in the gas mixture and it is up to the observer to interpret the animals responses rather than the animal itself. Moreover, the interpretation of data from EEGs, blood pressure, heart rates, estimated time to loss of consciousness have all to be deduced, partly from human studies and their

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experiences, but are also dependent on observations and recording of animal behaviour for their subtle effects.

CO2 Chemosensitivity and potential for suffering in animals

Phylogenic studies reveal that peripheral CO2 chemoreception evolved prior to central CO2 chemoreception during vertebrate evolution but the mechanism of chemoreception varies between species and their stages of development (Milsom, 2001). Fish have peripheral CO2 chemoreceptors primarily in the gills innervated by the glossopharygeal and vagus nerves, whereas air-breathing primitive (holostean) and modern (teleost) fish have central CO2 chemorecoprors. Central CO2 chemoreceptors are not present in young amphibian tadpoles but develop over time, and these receptors initially stimulate gill ventilation but transfer to lung ventilation when the aquatic larval stage develops into an air breathing adult. Recent research suggests that there is a biphasic developmental pattern of CO2 chemosensitivity in rats. After birth, rats display a neonatal pattern of chemosensitivity that decreases during the first week of life and is replaced after the second week by a more adult form of chemosensitivity. Therefore, it is reasonable to assume that all vertebrates have well developed chemoreception to detect and respond to CO2. The responses to inhalation of CO2 could vary according to species of animals from freezing (remaining motionless), through various behavioural reactions indicative of distress (defecation, urination, shivering, rearing and agitation in mammals and birds, and vigorous swimming in fish), to attempting to escape from the CO2 atmosphere. A search on the Internet revealed that fumigation of active burrows with carbon dioxide (as dry ice, delivered from a fire extinguisher or cylinder) is used in the United States to drive rodents out of their burrows.

Aversive reactions due to inhalation of CO2 in animals

The results of experiments on several species of farm animals (chickens, turkeys, pigs and mink) (Raj, 1996; Raj and Gregory, 1995; 1996; Cooper, Mason and Raj, 1998) have shown that they perceive carbon dioxide as being extremely aversive. This aversion to CO2 has been reported to be more overwhelming than motivation to feed (in a CO2 atmosphere) after overnight fasting in pigs and poultry. However, Jongman *et al.* (2000) found evidence that it was less aversive than an electric prodder. It is very likely that burrowing animals, including rabbits, would find this gas even more aversive (Hayward and Lisson, 1978) as it may alert them to a lack of air.

Euthanasia of rodents has been studied using aversion tests in rats and mice (Leach *et al.,* 2002a; 2002b; 2004; Kirkden *et al.,* 2005a and 2005b; Niel *et al.,* 2005; Niel and Weary, 2005). In these animals, the aversion to carbon dioxide, regardless of whether this gas was presented in a pre-filled chamber, or as a rising concentration, or whether it was humidified, or combined with an inert gas, or with oxygen, the aversion was always found to be far greater than that shown to other commonly used gaseous agents for anaesthesia or euthanasia (Leach *et al.,* 2004; Niel and Weary, 2005). These papers show that gas mixtures containing CO2 are aversive to some extent depending on the concentration but even 10-20% has been found to induce aversion. Some anaesthetic gases have also been shown to be aversive but none of them was as aversive as CO2. Furthermore, the consistency of aversion with CO2 (regardless of what other gases were in the mixture) produced significant differences that would only have occurred by chance of less

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than 1 in 10,000 as the variance was so low, i.e. all animals responded in the same way (aversion), unlike all the other gaseous agents tested suggesting an unusual uniformity of response.

More recently, aversion to carbon dioxide in rats was ascertained by comparing the motivation to avoid carbon dioxide with motivation to feed on Cheerios (a highly attractive food for rats) after 24 hours of food deprivation (Kirkden *et al.,* 2005a). In this study, rats were required to choose between leaving a chamber that was gradually filling with carbon dioxide or remaining in the chamber to consume food, at various levels of food deprivation. Seven male Wistar rats, aged 15 months, were housed individually in an apparatus consisting of two cages, one above the other, joined by a tube. In a series of training sessions, the rats descended in the tube for a reward of 20 Cheerios in the lower cage, while air flowed into the cage. In the experimental sessions, rats performed the same response, but the test gas flowed into the lower cage at a fixed rate of 16.5% cage volume/min. The gas was turned on at the moment the rat started eating the Cheerios, so the concentration increased steadily from zero. Subjects were tested at seven levels of food restriction, defined as percentages of estimated ad libitum intake given in the preceding 24 hours: 0%, 17%, 33%, 50%, 67%, 83% and ad libitum, in a Latin square design. Test trials were separated by a two day wash-out period with ad libitum feeding and an air re-training trial. Statistical (General Linear Model; GLM) analysis of data indicated that there was no linear effect of food deprivation upon the time taken to stop eating, the time to leave the lower cage, the CO2 concentrations at which these events occurred, or the number of Cheerios consumed. The main finding was that, regardless of food deprivation level, all subjects left the lower cage before the carbon dioxide rendered them unable to do so. They generally left at around the time of the onset of ataxia. This indicated that the rats were strongly averse to even the moderate carbon dioxide concentrations (16.6 ±3.0%) experienced when they chose to leave the chamber.

Another study investigated whether the maintenance of a high oxygen concentration, using a mixture of a 70% carbon dioxide and 30% oxygen, would reduce the aversiveness of carbon dioxide to rats during a gradual fill procedure (Kirkden *et al.,* 2005b). In this study, eight male Wistar rats, aged 8-10 months, were housed individually in an apparatus consisting of two cages, one above the other, joined by a tube. In a series of training sessions, subjects descended the tube for a reward of 20 Cheerios (a familiar treat item) in the lower cage, while air flowed into the cage. In experimental sessions, rats performed the same response, but gas flowed into the lower cage at a fixed rate. The gas was turned on at the moment the rat started eating the Cheerios, so the concentration increased steadily from zero (a gradual fill procedure). There were four treatments: (1) 100% carbon dioxide at 14.5% cage volume/min; (2) gas mix at 14.5%/min; (3) gas mix at 21.0%/min, which delivered carbon dioxide at approximately 14.5%/min; and (4) air. Each treatment was presented twice to each subject in a Latin square design and the entire experiment was run twice with the same subjects. Statistical (GLM) analysis of data indicated that there was a small but significant difference between treatments 1 and 3 in the latency to stop eating Cheerios (36.4s±3.4 vs. 42.5s±2.0; $P<0.05$), in the latency to leave the lower cage (44.4s±3.7 vs. 51.5s±2.4; $P<0.05$) and in the number of Cheerios eaten (3.2±0.4 vs. 3.8±0.2; $P<0.01$). Although the results suggest that the addition of oxygen slightly reduced the aversiveness of carbon dioxide when delivered at a fixed rate in a gradual fill procedure, it failed to eliminate the aversiveness of this gas.

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More recently, some observations by Flecknell and others (2005, Pers. comm.) support the notion that immersion in 100% CO2 is indeed painful, but seem to suggest that the effects of a gradual fill of CO2 of 100% over 5 minutes in a chamber that the animals are unable to escape from, were"modest" and were "no greater than what would have been expected from the known physiological effects of elevated CO2 concentrations". We are not able to evaluate the methods used, and their conclusion over gradual fill conflicts with those of detailed behavioural studies reported below.

There is also evidence to suggest that, in addition to aversion, animals unable to escape from an environment containing carbon dioxide experience distress and even pain before loss of consciousness (Ambrose *et al.,* 2000; Leach *et al.,* 2002a; 2002b; 2004). In particular, lung oedema, and lung haemorrhaging occur prior to loss of consciousness regardless of whether carbon dioxide alone or a mixture of carbon dioxide and oxygen is used to kill mice (Danneman *et al.,* 1997; Ambrose *et al.,* 2000). On the other hand, according to the EFPIA submission to EFSA to this Report, there are seldom reports from toxicologists of lung haemorrhages after carbon dioxide euthanasia in pathology studies for regulatory toxicology. A possible reason for this contradiction could be differences in the methodology used (e.g. temperature, humidity, rate of administration), strain of animals involved and the interactions between the variables.

CO2 induced distress and breathlessness in animals

It is worth noting that hypercapnia is a more potent respiratory stimulant than hypoxia or anoxia. Regardless of the method of exposure to carbon dioxide gas and species of animal involved, a common phrase used in the literature to describe the initial reaction in animals upon contact with this gas is 'sniffing', which seems to have been inappropriately used in this instance as a synonym of, or harmless act of, 'sampling the air' or 'familiarisation' with the environment. By contrast, the scientific literature suggests that stimulation (chemical, electrical or mechanical) of the pharyngeal branch of glossopharyngeal nerve evokes a short-duration spasmodic inspiratory effort termed sniff- or gasp-like aspiration reflex (AR). Similar responses are elicited by stimulation of the whole pharyngeal cavity, upper pharynx or nasal septum (Benacka and Tomori, 1995). Physiological studies revealed that this reflex is activated from the sub-epithelial free-nerve endings with rapidly adapting discharge properties, conveyed centrally in myelinated glossopharyngeal and possibly trigeminal afferents, and results in short latency strong excitation of various bulbar inspiratory neurones. The central drive leads to recruitment of nearly all spinal inspiratory motor units, which fire at rates in excess of any other respiratory activity (Jodkowski *et al.,* 1989). The sniff- like AR is believed to play a vital role in the establishment of eupneic respiratory rhythmogenesis (i.e. establishing normal breathing pattern) and is considered to be a very practical method to test resuscitation effects and treatment of apnoeic disorders in humans. The animal welfare implications are that sniffing has a physiological purpose to the survival of the animals and, more importantly, the inhalation of carbon dioxide induces sniff-like AR as a direct result of stimulation by CO2 of nerve endings in the nasal epithelium. It is also possible to suggest that 'sniffing' or AR is the earliest sign of respiratory distress or breathlessness as the sensory input from the nerve endings would not be compensated by the central motor output to re-establishing normal rhythmic breathing.

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Intrapulmonary chemoreceptors (IPC) are CO2-sensitive, but insensitive to hypoxia, sensory neurons that innervate the lungs of birds, help control the rate and depth of breathing, and require carbonic anhydrase (CA) for normal function. However, the CO2 stimulus detected by IPC varies during the breathing cycle, under the influence of inspired PCO2, venous PCO2, pulmonary ventilation and perfusion, and metabolism (Hempleman *et al.,* 2000). It is interesting to note that IPC action potential discharge rate has an inverse relationship with the inspired PCO2 (inhaled concentration of carbon dioxide) but its capability to detect and respond to the venous PCO2 seems to be direct (see, Hempleman *et al.,* 2000). Overall, stimulation of the IPCs depresses respiration (via the vagus nerve). Tschorn and Fedde (1974) referred to these IPCs as intrapulmonary carbon dioxide-sensitive receptors and reported that a unidirectional ventilation of chickens with carbon dioxide induced apnoea. In adult birds (chickens), the effect of carbon dioxide on the IPCs is independent of the effect of this gas on arterial and central chemoreceptors and the pH of the blood (Fedde, Nelson and Kuhlmann, 2002). In addition, in mammals and birds, inhalation of carbon dioxide leads to stimulation of arterial and central (brain) chemoreceptors. There is a positive relationship between the discharge rate and PCO2 in carotid bodies (Iturriaga *et al.,* 1991; Hempleman *et al.,* 1992) and in CO2-sensitive neurons in the mammalian medulla (Erlichman and Leiter, 1997; Richerson, 1995). Some mammalian CO2-sensitive laryngeal mechanoreceptors have an inverse CO2 sensitivity, like that of avian IPC (Coates *et al.,* 1996), as does a subset of mammalian medullary CO2 chemoreceptors (Richerson, 1995).

It is possible to suggest that the distressing feeling of breathlessness and suffocation reported by humans (see human experience) could be very similar to the apnoea induced in conscious birds by Tschorn and Fedde (1974). This could be one of the reasons why birds and other animals completely avoid or rapidly leave an atmosphere containing high concentrations of carbon dioxide. A mixture of 40% by volume carbon dioxide, 30% by volume of oxygen and 30% by volume of nitrogen and a mixture of 30% by volume of carbon dioxide and 60% by volume of argon in air are used to stun or stun/kill poultry under commercial conditions. Lambooij *et al.* (1999) reported that broiler chickens showed some degree of reaction (head shaking and gasping) in all the gas mixture containing carbon dioxide. It is not certain whether the addition of oxygen to carbon dioxide or humidification of mixtures containing carbon dioxide reduces the effect of this gas on the IPCs and other CO2-sensitive receptors, and thus, benefit bird welfare. On the other hand, Raj (1996) found that turkeys did not avoid an atmosphere containing a mixture of 30% by volume of carbon dioxide and 60% by volume of argon in air (Raj, 1996). The times to loss of consciousness and onset of death have been reported previously in a document dealing with stunning or killing (EFSA, 2004, http://www.efsa.eu.int).

Barbaccia *et al.* (1996) used CO2 to elicit a stress response in rats in order to examine the effects of acute stress on brain steroid concentrations and GABA-A receptor function. A combination of 35% CO2 and 65% O2 inhaled from gas cylinders for just 1 min caused a sufficient stress response for the study. It was concluded that exposure to CO2 is correlated with an increase in various brain neuroactive steroid concentrations. Cook (1999) exposed male laboratory rats (N = 40) to 80% CO2 by delivering the gas to cages at a flow rate of 3 litres/minute and measured spontaneous behaviour, brain neurotransmitter levels, EEG and ECG. The results showed that (a) gas delivery noise (N=10) increased movement, defecation and startle behaviour and prolonged time to onset of lethargy when

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compared with 'no' delivery noise (N=30); (b) in both situations, respiratory gasping occurred in conscious animals for up to 40 s (when the animals were presumed to be unconscious); (c) high amplitude, high frequency EEG activity (EEG arousal) occurred initially after exposure in both situations however the occurrence of this EEG pattern was more frequent in noisy CO2 delivery situation; (d) heart rate increased upon CO2 delivery and the magnitude of increase was more in the noisy CO2 delivery; (e) short latency (<12 ms, presumed subcortical origin) auditory evoked potentials were present until approximately one minute after ventricular fibrillation but long latency (>12 ms, presumed cortical in origin) evoked potentials were absent at the time of ventricular fibrillation; (f) brain GABA levels increased under both situations, but more so under noisy CO2 delivery, until unconsciousness as determined subjectively using behaviour; and (g) glutamate and aspartate levels increased in all animals.

These results indicate that distress occurred during exposure of rats to 80% CO2, as evidenced by EEG arousal and increases in brain neurotransmitter levels, and is exacerbated by the noise of CO2 delivery.

Many species of animals (fish, poultry, rodents and pigs) are known to avoid atmospheres containing high concentrations of CO2 or evacuate from atmospheres containing low concentrations of CO2 as soon as they start to experience breathlessness induced by elevated blood CO2 levels. For example, Raj and Gregory (1995) reported that pigs withdrew their heads from a CO2 atmosphere as soon as they began to hyperventilate (noted from abdominal breathing pattern). Niel *et al.* (2005) demonstrated, using rats and exposure to static CO2 concentrations ranging from 0 to 20% by volume, that the dwell time in CO2 atmosphere decreases in a non-linear manner, starting at a concentration of about 10% by volume. In humans, inhalation of 7 to 8% by volume of CO2 has been reported to be 'breathable' but very unpleasant experience due to the occurrence of dyspnoea (Banzett *et al.,* 1996). Air hunger can be a frightening experience that strongly motivates escape behaviour (Banzett and Moosavi, 2001), which provides an explanation to why rats escape from an atmosphere containing as low as 10% by volume of CO2 (Kirkden *et al.,* 2005c). Niel and Weary (2005) found that with gradual fill CO2 escape behaviours such as activity, rearing, touching the nose to the chamber lid and vocalization, all increased.

The human experience of dyspnoea (shortness of breath, difficult or laboured breathing, breathlessness or air hunger).

Trials involving human exposure to carbon dioxide indicated that it induces a sense of breathlessness and / or suffocation prior to loss of consciousness, and 36 out of 40 persons reported adverse sensations at concentrations of 50% by volume (i.e. a level similar to those used in animal anaesthesia and exceeded when carbon dioxide is used for euthanasia). The sensation of breathlessness or dyspnoea in humans is believed to originate from a direct activation of cerebral cortical sensory systems involved with respiration (i.e. conscious awareness of efferent motor command corollary discharge). It is also known that dyspnoea occurring during inhalation of carbon dioxide is caused by activation of vascular chemoreceptors from increases in blood carbon dioxide levels (hypercapnia) and resulting in increased respiratory motor activity.

People suffering from acute asthma, compared with chronic asthma patients, have a rapid rate of increase in blood CO2 levels owing to low buffering capacity in their

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blood and, as a consequence, suffer panic attacks. Although genetic predisposition seems to determine the panic attack, many human patients report helplessness, fear and anxiety (American Thoracic Society – Consensus on dyspnoea, can be accessed at http://www.olivija.com/dyspnea). The possibility that animals also experience these mental states cannot be ruled out because, given a free choice, they escape from CO2 atmospheres.

CO2 as a stimulus to cause distress and pain

The aversive nature of carbon dioxide should perhaps not be surprising since concentrations similar to those used for animal euthanasia have been used as noxious stimuli in human and animal pain research (Thurauf *et al.,* 1991; Komai and Bryant 1993; Peppel and Anton 1993; Danneman *et al.,* 1997). In addition, old data that was available but was not mentioned previously shows that humans find CO2 aversive (Gregory *et al.,* 1990; Dannemann *et al.,* 1997) and that the gas has even been used as a noxious stimulus to test for the analgesic properties of new drugs. For example, Thurauf *et al.* (1991) exposed rats to various concentrations of CO2 (up to 90% by volume) and measured evoked potentials in electroencephalograms (EEGs) in order to determine the origin of negative mucosal potential (NMP—negative potential recorded from the respiratory mucosa following painful stimulation with CO2). Local anaesthetics eliminated NMPs and EEG cortical responses, signifying that the pain response had ceased. When no local anaesthetic was administered, the result was increased NMPs, indicating an increased nociceptive response. Banzett and Moosavi (2001) while reviewing scientific literature concerning pain and dyspnoea stated that 'dyspnoea and pain alert the conscious brain to a disturbed physiological state' and highlighted the commonality of central neural processing of these two very unpleasant sensations in humans.

It is therefore reasonable to assume, based on current understanding of comparative respiratory anatomy and physiology, that laboratory animals can also experience similar feelings to humans. The cumulative stress associated with the induction of unconsciousness is a serious welfare concern. In this regard, exposure to low concentrations of carbon dioxide causes distress and higher concentrations cause pain.

Some argue that euthanasia of animals with CO2 does not cause significant pain and distress (Wood, 2005, pers. comm., and response by Leech *et al.,* 2005) but his criteria have been challenged particularly on the basis that behaviour of animals in an escapable situation is more discriminating. Wood suggests that placing animals in a novel gas simply stimulates horizontal movements and so they are more likely to exit a chamber, however, this does not account for the very narrow variance in response movement and time., Wood raises the lack of a dose response effect, but CO2 is so aversive at low concentrations that it is difficult to do that experiment. Nevertheless, dose response curves have, in effect, been shown through the use of rising concentration experiments, where rats seem to tolerate only 15-20% CO2 before escaping (e.g. Leach *et al.,* 2002a, 2002b, Kirkden *et al.,* 2005a; Niel and Weary, 2005). Several groups working with rats have concluded that, on animal welfare grounds, although exposure to CO2 is a practical method of euthanasia there are significant welfare concerns associated with this procedure, and therefore, alternatives should be developed. (Leach *et al.,* 2002b; 2004; Kirkden *et al.* 2005c; Niel *et al.* 2005; Niel and Weary, 2005)

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Advantages and Disadvantages - Animal Welfare:

Advantages:

Carbon dioxide may be administered in home cages or in a specialized compartment and may be used to kill individuals or small groups of animals and so minimal stress due to handling and restraint are involved. Administration of CO2 in home cages however eliminates stress associated with the handling. Mixing of unfamiliar groups of animals should be avoided.

Other advantages are that it is cheap, readily available and can be easily administered.

Disadvantages:

Poor welfare can be induced through irritation of the mucous membranes, breathlessness. Breathlessness can defined as an unpleasant sensation that occurs due to a mismatch between peripheral sensory input (e.g. from chemoreceptors) and resultant motor output (e.g. to respiratory muscles) from the brain. It would appear that the cumulative distress and suffering outweighs any welfare advantages of using this gas in home cages. Animals given the choice do not enter or remain in high concentrations of CO2 (e.g. for rodents 100% of animals at concentrations above 16%), and indeed they make active attempts to escape lethal concentrations. Lung oedema, and lung haemorrhaging, may induce a sense of breathlessness or drowning, prior to loss of consciousness regardless of whether carbon dioxide alone or a mixture of carbon dioxide and oxygen is used to kill mice. Exposure to carbon dioxide results in increased brain neurotransmitter, especially dopamine, levels in rats, which also occurs during distress.

These effects of CO2 exposure are likely to occur in all animals exposed to the gas. From a human safety viewpoint, especially while delivering carbon dioxide from a source of 100%, MELs (Maximum Exposure Levels) are 1.5% acute (15 minutes), and 0.5% for MTL (Maximum Tolerated Level) over an 8 hr day would be built up very quickly in the vicinity. Therefore, the operatives as stipulated by the local UK H&S guidelines must wear calibrated personal protection equipment, i.e. CO2 monitors.

Carbon dioxide delivered using a liquid or solid source must be vaporised, using specialised electrical heaters, prior to administration to avoid cold shock in animals as the temperature can be reduced to as low as minus 78°C. Large capacity vaporisers require a 3-phase electrical supply, which may not be readily available.

Owing to their resistance to hypercapnia, neonates would require a secondary killing procedure, e.g. decapitation or destruction of brain to prevent recovery after exposure to CO2, which would incur additional labour and costs.

Time to lose consciousness:

While low concentrations can kill, it may take a long time before the animals lose consciousness (e.g. rats 30 min at 40%; and 2 min at 95%). Feng *et al.* (1990) have reported that administration to Wistar rats (600-900g) of a mixture of 20% by volume of carbon dioxide, 30% by volume of oxygen and 50% by volume of nitrogen for 10 minutes decreased the amplitude of evoked electrical activity in the brain (elicited by single constant current pulses of 0.5ms duration delivered to the

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olfactory nerve through bipolar stainless steel electrodes) by 25% of the control. In contrast, administration of 100% by volume of nitrogen for one minute that induced hypocapnic anoxia resulted in complete abolition of the evoked electrical activity. In comparison with anoxia alone, there was no apparent rate or magnitude of evoked potential depression due to the administration of a mixture of 20% by volume of carbon dioxide and 80% by volume of nitrogen (hypercapnic anoxia).

Induction of unconsciousness with a prolonged exposure to lower concentrations of carbon dioxide has been known to induce nasal haemorrhage indicative of irritation and acute inflammation. Therefore, not surprising to note that aversion to carbon dioxide was found to be very similar at 25, 35 or 50% by volume (in air) in rats, and at 28, 36 or 53% by volume (in air) in mice (Leach *et al.,* 2002a). The average time to withdrawal from 50% carbon dioxide was found be 0.7 s, which was not significantly altered when carbon dioxide gas was humidified or presented with up to 30% by volume of oxygen (Leach *et al.,* 2004). In view of these reports and the fact that significantly high concentrations of carbon dioxide are required for euthanasia, it is apparent that animals would be subjected to distress for a considerable period of time (up to 2 minutes) before induction of unconsciousness or death, and that this could not be achieved without causing distress and suffering. The time to loss of consciousness is prolonged by addition of oxygen to any amount of carbon dioxide, thus prolonging the duration of suffering; furthermore, humidification of gas mixtures did not alleviate this problem (Leach *et al.,* 2004).

In contrast with these, some other reports dealing with euthanasia of rats and mice using carbon dioxide concluded that the procedure is humane (Hackbarth *et al.,* 2000). It has been suggested that introducing carbon dioxide after animals have been placed in the chamber (gradual induction) is a potentially less distressing alternative to the common pre-filling method (Hewett *et al.,* 1993), as exposure to increasing concentrations is suggested to cause animals to begin to lose consciousness before being exposed to concentrations that cause pain and distress. However, recent research indicated that aversion to carbon dioxide occurs at low concentrations, which inevitably will be reached relatively rapidly, even with slowly increasing concentration (Leach *et al.,* 2002b). In view of the fact that all animals have biological predisposition to detect and respond to elevated CO2 levels in the atmosphere and in their blood, the assertion that it is humane seems to have been based on the interpretation of behaviours. This view is supported by the fact that each individual and species may have a different coping strategy and pain threshold and tolerance (Leach *et al.,* 2002b; 2004). Leach *et al.* (2004) described animals that leave and do not re-enter the CO2 atmosphere as 'escapers' and animals that leave the CO2 atmosphere but re-enter as 'searchers'. The literature concerning euthanasia with CO2 indicates that some animals cannot or do not seem to try to escape from this gas and therefore it possible to suggest that these animals probably showed a freezing behaviour (motionless and possibly sniffing) due to fear, which is an escape strategy common to many species of animals. This interpretation is supported by Cook (1999) who found that regardless of the behaviour of rats during exposure to 80% CO2 all the animals showed the same EEG, ECG and neurotransmitters responses. The welfare implication of these reports is that the absence of behavioural responses such as escape attempts should not be interpreted as evidence to lack of suffering in animals that are exposed to CO2.

In addition, despite the circadian breathing patterns in animals, the hyperventilatory responses to hypercapnia remain constant throughout the day, at least, in rats

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(Mortola and Seifert, 2001). Therefore, the time of day during which CO2 is administered is unlikely of any benefit to animal welfare.

The time to loss of consciousness during exposure to carbon dioxide of other species of animals and the welfare concerns associated with the induction phase have been covered in other reports (Close *et al.,* 1996, 1997; EFSA, 2004, http://www.efsa.eu.int). The exact times may vary as many of the studies estimated the concentration of CO2 rather than measuring it directly, and also used polythene containers that are likely to have been porous to some degree which may have prolonged times depending on the flow rates of incoming gases.

Administration of CO2 as the killing method in a 2 stage procedure

Owing to the animal welfare concerns associated with the use of CO2 as a sole euthanasia agent, it has been suggested that anaesthesia should be induced with a least aversive inhalation anaesthetic agent prior to killing with a lethal concentration of CO2. In this regard, Leach *et al.* (2004) recommended that anaesthesia should be induced in rats with about 3–4% halothane and in mice with about 5% enflurane, since these concentrations produce rapid and effective induction of anaesthesia with minimal distress and aversion as shown in separate studies (Leach *et al.,* 2002a, 2004). Use of inhalation anaesthetic agents at low aversive concentrations prior to euthanasia with CO2 would cause only a minimum level of pain and distress. Anaesthetic agents are particularly advantageous because they require minimal handling of the animals and larger numbers of animals can be killed simultaneously. The personnel safety concern about the use of volatile anaesthetics could be overcome by the use of commercially available portable scavenging units that would allow for safe use of both the inhalation anaesthetic and CO2. Therefore, induction of unconsciousness in animals with anaesthetic agents and subsequently killing them with carbon dioxide seems to be the best option from an animal welfare point of view.

Consequences of Inappropriate Administration: Poor welfare is caused by an irritation of the mucous membranes, and also breathlessness and lung haemorrhaging. If loss of consciousness is prolonged animals will suffer these avoidable adverse effects for longer than necessary. They will be unable to escape from these atmospheres and so any pain or distress will be unremitting. In addition to these effects when CO2 is delivered as dry ice, or administered straight from a liquid source without a vaporiser, then as it sublimates or evaporates so the temperature will fall drastically. This itself is a welfare problem as inhalation of dry cold carbon dioxide is likely to be painful, leading to freezing of tissues and cold burns, and nasal bleeding. If CO2 is delivered by a fire extinguisher, the same events occur this time from a dry ice 'snow', notwithstanding the noise if the animals are in the chamber at the time. The intensity of pain and distress is likely to be high, and may persist for several minutes (for example at 95% CO2, unconsciousness may take up to 2 minutes). Furthermore, as animals are frequently killed in batches by this method, this level of suffering might have to be endured by hundreds of animals when large numbers are being killed at one time (Leach *et al.,* 2004; Ambrose *et al.,* 2000).

Advantages and Disadvantages - Scientific Impact: Inhalation of CO2 leads to altered neurotransmitters in the brain. CO2 may cause activation of the Hypothalamic-Pituitary-Adrenal Cortex system and cause a release of corticosteroids prior to death. Lung haemorrhage may affect histological studies,

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although affected areas may be avoided by judicious sampling. CO2 is known to inhibit muscle glycolytic enzymes and retard onset of rigor mortis. Accumulation of blood in vital organs such as liver and spleen, and hence, discolouration, has been reported in poultry.

Requirements for Optimal Operation: Ensure competence of those placing animals or cages into the chamber, and that the chamber is purpose built and relatively gas tight. Concentrations should be appropriate to the species and should be monitored until death.

4.8.5.2.Argon and nitrogen as inert hypoxia inducing gases

Hypoxia and anoxia are not routinely used for killing laboratory animals, although that is one consequence of using 100% carbon dioxide. Inert gases are used to maintain general anaesthesia during airway laser surgery (see, EFSA, 2004, http://www.efsa.eu.int) and have been studied extensively in farm animals where argon is used for stunning and killing of poultry intended for human consumption. Indeed, argon is recommended as a welfare friendly alternative to using carbon dioxide for stunning / killing pigs (FAWC, 2003; EFSA, 2004, http://www.efsa.eu.int).

Fetal and immature forms: The mammalian brain metabolism is transformed from predominantly anaerobic at birth to aerobic with maturation. For example, newborn rabbits could survive 30-35 minutes of anoxia whereas adult rabbits withstand anoxia for only 3-5 minutes. It has been shown that succinic dehydrogenase and cytochrome oxidase activities are very low at birth, but gradually increase until 15-18 days post-natally when adult levels are attained (Cassin and Herron, 1961). Rats are reported to be resistant to anoxia immediately after birth, but show a gradual decline in resistance during post-natal development and between 12-15 days after birth they are like adults. In contrast, neonatal and adult guinea-pigs are equally susceptible to anoxia. Investigation into systemic and cerebral metabolic responses to acute anoxia (exposure to 100% nitrogen for 20-40 minutes at 37C) in the Wistar rat fetus at term (18-24h prior to expected delivery) and neonates (one day old) suggests that total cerebral energy consumption during anoxia is significantly lower in fetuses than in neonates, and severe hypercapnia superimposed on anoxia in fetuses decreased cerebral metabolic demands, thus prolonging survival (Vannucci and Duffy, 1976). However, it is not certain whether survival of brain during exposure to anoxia causes suffering during euthanasia of fetuses and neonates. The welfare merits of anoxia in comparison with the other commonly used euthanasia methods for fetuses and neonates warrant investigation. Pritchett *et al.* (2005, unpublished) showed prolonged survival times in hypoxic mixtures using CO2, in inbred and outbred mice. Klaunberg *et al.,* (2004) showed that CO2 (gradual fill 20%/min) or halothane (5%) overdose killed fetal mouse pups *in utero*, within a 20min period (nor did barbiturates or cervical dislocation or potassium chloride), whereas in neonates (1-7do) CO2 was very effective killing in 4-5min unlike halothane where they still had a good heart beat after 20min.

Nitrogen-induced hypoxia has been investigated in some species of laboratory animals (e.g. dogs and cats; see, Close *et al.* 1997). However, the use of hypoxia/anoxia as a euthanasia agent for other species of animals is novel and only limited published information is available regarding its suitability and the humaneness of exposure to anoxic agents such as argon or nitrogen. The available

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published information indicates that anoxia is significantly less aversive than carbon dioxide, and therefore, the method would seem to be a potential alternative to using carbon dioxide.

Description of the method:

Nitrogen

Placing air-breathing animals in a compartment that has been pre-filled with a minimum of 98% by volume of N2 induces unconsciousness and death by hypoxemia. Since nitrogen is lighter than air (relative density 0.97), specialised equipment is necessary to administer this gas.

Argon

This gas is denser than air (relative density 1.38) and therefore can be contained easily. Placing air-breathing animals in a compartment that has been pre-filled with a minimum of 98% by volume of argon induces unconsciousness and death by hypoxemia.

New Data: Animals, including birds, do not have intrapulmonary chemoreceptors to detect inert gases and therefore, do not show any aversion during initial exposure to hypoxia induced with nitrogen, argon or their mixtures. However, diving, aquatic and burrowing animals may show aversion to resultant hypoxemia (low blood oxygen levels) and some are also extremely tolerant. For example, mink did not show aversion to enter a hypoxic atmosphere (<2% by volume of oxygen in argon) but evacuated from it on average at 20 seconds, when given a free choice, and the dwell time in argon atmosphere is similar to previously reported dive durations (Raj and Mason, 1999). In laboratory rodents (rats and mice), aversion to hypoxia (argon) was reported to be less than that observed with hypercapnia (elevated carbon dioxide levels) (Niel and Weary, 2005 – but only to 14% O2 hypoxia) but significantly greater than that shown to anaesthetic agents such as halothane, enflurane, sevoflurane, desflurane and isoflurane (Leach *et al.,* 2004). Niel and Weary (2005) found that escape behaviours were less with argon (14% O2 which will not kill) than with gradual fill CO2. In view of the fact that the use of a procedure involving single euthanasia agent is simple and operator friendly and that argon-induced anoxia is less aversive than carbon dioxide, Leach *et al.* (2004) suggested that anoxia induced with argon is more humane than using CO2. It is known that the peripheral and central chemoreceptors respond to changes in pH (blood and intracellular) that accompany hypoxia. However, the onset of unconsciousness is believed to precede blood pH changes. This is probably the reason that hypoxia has been described as a euphoric way of losing consciousness in humans and is probably why certain mammals do not show aversion. Further research is needed in this area to determine species-specific requirements.

Advantages and Disadvantages - Animal Welfare:

<u>Advantages:</u>

Since the principle of using hypoxia and anoxia for euthanasia of laboratory animals is new, the welfare advantages are not clearly established.

The exposure time necessary to kill with hypoxia / anoxia is not known for all the species of animals. However, farm animal studies have indicated that exposure

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time required to kill pigs with anoxia (2% residual O2 by volume; 7 min) is slightly longer than required to killing them with 90% by volume of CO2 (5 min) (EFSA, 2004, http://www.efsa.eu.int). Since exposure to anoxia is not aversive to pigs, the slightly longer exposure time required to killing them with anoxia is not considered to be important on animal welfare grounds. It can also be argued that the cumulative distress and pain induced in conscious animals with an exposure to high concentration of carbon dioxide is likely to be more than that would be experienced with anoxia; hence, exposure to anoxia is better than exposure to CO2. This view is supported by the fact that humans inhaling anoxic agents, without CO2, experience no breathlessness, instead reported it to be a euphoric way of losing consciousness (see, Close *et al.,* 1997). Therefore, other species of mammals used in research may not suffer during the induction unconsciousness but this finding needs to be confirmed.

Nitrogen

Needs further research to elucidate welfare advantages in laboratory animals. Research involving poultry indicated that nitrogen-induced hypoxia is not aversive.

Argon

Argon-induced hypoxia is not aversive to pigs and poultry (see EFSA, 2004, http://www.efsa.eu.int). Further research is needed in other species of laboratory animals although Niel & Weary showed that the use of argon (hypoxia of 14% residual oxygen) was less aversive in terms of escape behaviours than CO2.

It is suggested that argon-induced hypoxia may be administered in familiar cages or in a specialised compartment and may be used to kill individuals or small groups of pigs or poultry, and possibly rodents. Euthanasia of animals in home cages would eliminate the need for handling. Mixing of unfamiliar groups of animals should be avoided.

Hazards to personnel are minimal as nitrogen and argon are naturally occurring gases and are chemically inert. The volume of gases required to depleting oxygen levels in a working environment from 20% by volume to a critical level to 18% by volume in air is also too large, which could improve H&S risk assessments.

Disadvantages:

Poor welfare can be caused in some species, which perceive hypoxemia aversive. Maintaining residual oxygen level below 2% by volume is critical. In some animals (i.e. hypoxia tolerant species), however, the time to onset of death could be prolonged due to the reason that the brain stem survival time is longer than the cerebral cortex. Hypoxic convulsions occurring in unconscious animals are aesthetically unpleasant.

Time to lose consciousness

The average time to loss of consciousness in mice during exposure to nitrogen has been reported to be 55 seconds (Lawson *et al.,* 2003). Feng *et al.* (1990) have reported that administration to Wistar rats (600-900 g) of 100% by volume of nitrogen for one minute resulted in complete abolition of the evoked electrical activity (elicited by single constant current pulses of 0.5ms duration delivered to the olfactory nerve through bipolar stainless steel electrodes). In comparison with

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anoxia, there was no apparent rate or magnitude of evoked potential depression due to the administration of a mixture of 20% by volume of carbon dioxide and 80% by volume of nitrogen (hypercapnic anoxia).

Given a free choice, mice and rats evacuated an anoxic atmosphere (99% by volume of argon) after an average of 3 seconds (Leach *et al.,* 2004; Neil *et al.,* 2005). Therefore, less aversive alternatives should be sought.

Consequences of inappropriate administration:

The concentration of residual oxygen in nitrogen or argon necessary to kill all laboratory animal species and the time to onset of unconsciousness are not known. However, it is possible to suggest that welfare of animals that perceive hypoxia and anoxia aversive could be compromised.

Advantages and Disadvantages - Scientific Impact: Hypoxia and anoxia would alter brain neurotransmitter and metabolite levels. It would also affect blood and muscle biochemistry.

Requirements for Optimal Operation: Ensure competence of those placing animals in which anoxia is not aversive into the chamber, and that the chamber is purpose built and relatively gas tight. The concentration of oxygen should be kept below 2% by volume, or as appropriate for the species, and be monitored until death. Induction of unconsciousness in animals with anaesthetic agents and subsequently killing them with anoxia may be a better option from an animal welfare point of view.

Table 5 – Summary of killing methods using hypoxia:

Mice	*Mus musculus*	CA	More data needed
Rats	*Rattus norvegicus*	CA	More data needed
Guinea-Pigs	*Cavia porcellus*	No data	
Hamsters	*Mescocricetus*	No data	
Other Rodents	Other *Rodentia*	No data	
Rabbits	*Oryctolagus cuniculus*	No data	
Cats	*Felis catus*	More humane alternatives available	
Dogs	*Canis familiaris*	More humane alternatives available	
Ferrets	*Mustela putorius furo*	No data	
Other Carnivores	Other Carnivora	No data	
Horses, donkeys and cross breds	Equidea	More humane alternatives available	
Pigs	Sus	A	
Goats	Capra	No data	

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Sheep	Ovis	No data	
Cattle	Bos	More humane alternatives available	
Prosimians	Prosimia	More humane alternatives available	
New World Monkeys	Ceboidea	More humane alternatives available	
Old World Monkeys	Cercopithecoidea	More humane alternatives available	
Apes	Hominidae	More humane alternatives available	
Other Mammals	Other Mammalia	More humane alternatives available	
Quail	*Coturnix coturnix*	A	
Other birds	Other Aves	A	
Reptiles	Reptilia	More humane alternatives available	
Amphibians	Amphibia	More humane alternatives available	
Fish	Pisces	More humane alternatives available	

A – Causes a minimum level of pain and distress; CA – May cause more than a minimum level of pain and distress but for various reasons can be used. Both A and C methods have to have due regard for an appropriate design and experienced persons.

4.8.5.3.Nitrous oxide

Not commonly used alone.

Description of the method: Placing air-breathing animals in a compartment that has been pre-filled with 100% by volume of nitrous oxide induces unconsciousness and death by hypoxemia. Can also be used as a mixture with inhalation anaesthetics.

Fetal, neonatal and immature forms: Not appropriate.

New Data: None

Advantages and Disadvantages - Animal Welfare:

Advantages:

Nitrous oxide may be administered in familiar cages or in a specialised compartment and may be used to kill individuals or small groups of animals. Euthanasia of animals in home cages would eliminate the need for handling. Mixing of unfamiliar groups of animals should be avoided.

Disadvantages:

Poor welfare can be caused in hypoxia tolerant species. Maintaining 100% by volume of nitrous oxide is critical.

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Human exposure is 25ppm. Nitrous oxide also supports ignition.

Time to lose consciousness:

Is very similar to anoxia induced with argon or nitrogen, as the mode of action is also similar.

Consequences of inappropriate administration

Welfare of anoxia tolerant animals will be seriously compromised. Exposure to inadequate concentration may prolong onset of unconsciousness.

***Advantages and Disadvantages -Scientific Impact**:* Very similar to anoxia induced with argon and nitrogen.

Requirements for Optimal Operation: Ensure competence of those placing animals into the chamber, and that the chamber purpose built and is relatively gas tight. Concentration of nitrous oxide should be kept as close as possible to 100% by volume and be monitored until death.

4.8.5.4. Carbon monoxide

The most toxic effect of the gas is probably mediated through its action on the circulation: a generalized vascular dysfunction due to extensive vasodilatation accompanied by haemorrhages caused by blood vessels ruptures and diapedesis (Von Oetingen, 1941).

The method is rarely used for euthanasia of laboratory animals.

Description of the method: Carbon monoxide binds to haemoglobin in the red blood cells, with an affinity 250 times that of oxygen. This results in a markedly and cumulative reduced oxygen-carrying capacity and altered delivery of oxygen to cells. The most pronounced recorded symptoms in humans involving the nervous system are an initial headache with occasional nausea, followed by deep unconsciousness. During this stage of unconsciousness, muscular convulsions and spasms occur because of stimulation by carbon monoxide of the motor centres in the brain, where local bleeding may result in paralysis. In the event of recovery in humans amnesia usually occurs (Von Oetingen, 1941).

Hypoxia - the reduction of oxygen supply to the tissues - leads to unconsciousness and death. Death occurs rapidly at CO concentrations of 4 to 6%. Carbon monoxide concentrations greater than 2% are sufficient to cause loss of consciousness within minutes.

An efficient exhaust or ventilation system is essential to prevent accidental exposure of humans. CO is highly explosive in high concentrations (above 10%). Death during sleep in humans is the result of prolonged low levels of CO.

Fetal and immature forms: inappropriate.

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New Data: None

Advantages and Disadvantages - Animal Welfare:

Advantages:

Administration of CO in home cages would eliminate the need for handling animals.

Mixing of unfamiliar groups of animals should be avoided.

Disadvantages:

Poor welfare can be caused in hypoxia tolerant species.

The operators' health and safety is a major concern.

Convulsions were observed in humans, dogs, cats and mink after they had reached complete unconsciousness (Von Oetingen, 1941; De Vries *et al.,* 1976; Carding, 1977; Lambooy *et al.,* 1985), but this was not the case in piglets (Lambooy and Spanjaard, 1980).

Exhaust gases from motor vehicles contain several elements e.g. particulates that cause irritation to the mucous membranes and a considerable degree of excitation (Carding, 1977) and should not be used.

Time to lose consciousness:

Highly variable and could take up to 2 minutes.

Consequences of inappropriate administration

Rapid administration of carbon monoxide or too high a level could lead to distress and convulsions.

Advantages and Disadvantages - Scientific Impact: Changes to blood and muscle biochemistry.

Requirements for Optimal Operation: Ensure competence of those placing animals into the chamber, and that the chamber is purpose built and relatively gas tight. CO should be delivered as a pure gas and the concentration of CO should be gradually raised to as close as possible to 6% by volume and be monitored until death.

4.8.5.5. Overdose of inhalation of anaesthetic gases

Several fluorinated hydrocarbons are in common use as anaesthetic agents, and an overdose of them can be used to kill animals, as well as to render them unconscious before the use of other methods that in the conscious animal may cause poor welfare. Halothane, isoflurane, sevoflurane, desflurane, and enflurane are commonly used in air breathing vertebrates, including neonates, but their cost can be prohibitive. However, in relation to the total real costs involved to take an experimental animal to that stage, the costs may be relatively low.

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Halothane induces anaesthesia rapidly and is the most effective inhalant anaestheticanesthetic for euthanasia. Isoflurane should induce anaesthesia more rapidly than halothane, however it has a slightly pungent odour. Sevoflurane does not have an objectionable odour and anaesthesiaanaesthesia can be rapidly achieved. Desflurane is quite pungent and may be slow to induce anaesthesia and subsequent euthanasia. Enflurane is similar to halothane in rate of induction.

Older agents such as ether and chloroform are not commonly used as they pose a safety hazard for human operators (e.g. ether forms explosive mixtures in air; chloroform is hepatotoxic) or cause poor animal welfare (ether is irritant to mucous membranes).

Description of the method: Anaesthetic concentrations of vapour in oxygen or air (or nitrous oxide) are delivered into a purpose built chamber containing individual or an appropriate number of small animals. Vaporisers are commonly used as they deliver a known concentration of agent and a gas scavenging system should be used. These anaesthetic gases can also be administered with a facemask in large animals, but it is less easy to avoid human exposure.

New Data: Based on aversion tests in an escapable situation, the relative animal welfare merits, of using inhalation agents for anaesthesia / euthanasia purposes have been studied in rats and mice (see Table 5; Leach *et al.,* 2004).

Table 6 - Concentrations of agents (% in oxygen) used to test aversion in rodents.

Agent	Wistar Rats			BALB/c Mice		
Halothane	1.8	3.9	7.4	2.0	3.5	8.5
Isoflurane	1.7	3.7	7.2	1.3	3.6	8.0
Enflurane	2.7	4.7	8.1	3.1	5.2	8.5
Sevoflurane	1.8	3.2	7.2	1.8	3.2	7.2
Desflurane	3.5	5.5	11.6	3.5	5.5	7.2

The results showed that some degree of aversion was evident in all the agents at some level, but that the degree of aversion depended upon the type and concentration of the agent, and there were considerable variations between animals compared with CO2. The least aversive effective concentrations for euthanasia were about 3-4% by volume of halothane in rats and around 5% by volume of enflurane in mice. In view of the need to kill animals fairly quickly (as a matter of human expediency and not animal welfare as the animals would be unconscious), Leach *et al.* (2004) suggested that these least aversive inhalation agents at an appropriate low aversion concentration should be used to anaesthetise rodents prior to killing them by another method (e.g. decapitation, exposure to anoxia, CO2). Conlee *et al.* (2005) also suggested that the use of pre-anaesthetic should be used for the euthanasia of animals not involved in research protocols (e.g. surplus to requirements) or where the effects of the anaesthetic gas is not a problem for the science.

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Advantages and Disadvantages - Animal Welfare:

Advantages:

Administration of anaesthetic agents in home cages would eliminate the need for handling animals. Mixing of unfamiliar groups of animals should be avoided. Certain types of fish can also be anaesthetised by using water bubbled with the inhalation agents.

Fetal and neonatal rodent forms: not very effective.

Disadvantages:

Halothane and other halogenated compounds are not suitable for animals suspected of genetic susceptibility to malignant hyperthermia (a skeletal muscle hypermetabolic state). Not suitable for reptiles (due to breath holding) and amphibians.

Regarding human exposure to inhalant anaesthetics, the concentrations of halothane, enflurane, and isoflurane should be less than 2 ppm (0.0002%), therefore, a scavenging apparatus is required.

Time to lose consciousness:

Can be rapid with very little reaction in animals.

Consequences of inappropriate administration

It is probably not a major concern in species of animals that find these gases least aversive among the known gaseous methods.

Advantages and Disadvantages - Scientific Impact:

Advantages

The consistent low level stress with the use of these gases may contribute to a reduction in variance for any subsequent tissue analysis.

Disadvantages

Reports demonstrate that in humans anaesthetic concentrations of some of these anaesthetic agents can produce hepatic injury, ranging from mild transient increases of liver enzymes to fatal hepatic necrosis in very rare instances. Isolated cases of increased carboxyhaemoglobin have been reported with the use of halogenated inhalation agents with a $-CF_2H$ moiety (i.e. desflurane, enflurane and isoflurane). No clinically significant concentrations of carbon monoxide are produced in the presence of normally hydrated absorbents. Care should be taken to follow manufacturers' instructions for CO2 absorbents.

If tissues are to be used for *in vitro* work some validation may be necessary to compare with previous data. A wash-out period may also be required to remove residual anaesthetic gas.

Requirements for Optimal Operation: Ensure competence of those placing animals into the chamber, and that the chamber purpose built and is relatively gas

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tight. Concentration of agents should be achieved rapidly and kept as appropriate to the species and be monitored until death. Ensure uniform distribution of agents throughout the chamber. The use of a vaporiser is required to avoid animals being exposed to excessively high concentrations of gas, or being in contact with the liquid form.

Table 7 – Overdose of inhalational anaesthetic gases for euthanasia

Mice	*Mus musculus*	A
Rats	*Rattus norvegicus*	A
Guinea-Pigs	*Cavia porcellus*	A
Hamsters	*Mescocricetus*	A
Other Rodents	*Other Rodentia*	A
Rabbits	*Oryctolagus cuniculus*	CA
Cats	*Felis catus*	A
Dogs	*Canis familiaris*	A
Ferrets	*Mustela putorius furo*	A
Other Carnivores	*Other Carnivora*	A
Horses, donkeys and cross breds	*Equidea*	ALT
Pigs	*Sus*	ALT
Goats	*Capra*	ALT
Sheep	*Ovis*	ALT
Cattle	*Bos*	ALT
Prosimians	*Prosimia*	ALT
New World Monkeys	*Ceboidea*	ALT
Old World Monkeys	*Cercopithecoidea*	ALT
Apes	*Hominidae*	ALT
Other Mammals	*Other Mammalia*	ALT
Quail	*Coturnix coturnix*	A
Other birds	*Other Aves*	A
Reptiles	*Reptilia*	NA
Amphibians	*Amphibia*	NA
Fish	*Pisces*	CA

A – Causes a minimum level of pain and distress; CA – May cause more than a minimum level of pain and distress but for various reasons can be used. Both have to be given appropriate design and skilled persons. NA = it is inherent that such methods cause more than a minimum of pain and distress. ALT: more humane alternatives are available.

4.8.5.6. Overdose of injectable anaesthetic agents

Can be used in all vertebrates but the route may vary according to size and temperament of the animals. There may be some restriction on the availability of certain chemicals in some member states.

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Description of the method: Before using anaesthetic agents for euthanasia, the operator should consult the manufacturer's information leaflet with regard to dosage and route of injection.

Barbiturates are the most widely used and accepted agents for euthanasia for most animals, with sodium pentobarbitone being commonly considered the most suitable agent. They act by depression of the central nervous system and cause cardiac and respiratory arrest. Sodium pentobarbitone (SPB) is generally used either by intravenous or intraperitoneal injection. Intravenous injection results in quicker death but the intraperitoneal route may be simpler to perform in small species, thus reducing the stress caused by restraint for an intravenous injection. However, sodium pentobarbitone may cause irritation of the peritoneum due to its alkalinity (pH 10-13), which can be avoided by diluting the drug, or by combining it with a local anaesthetic.

SPB causes rapid euthanasia with minimal discomfort, depending on the dose of the agent and route of injection (intravenous is preferred as it is quickest).

Excitable and vicious animals should be sedated prior to intravenous administration. Trained personnel are essential for using these methods for suitable restraint. The importance of good restraint and positioning of the animal for intravenous injection cannot be overemphasised.

It can be used on fetal and immature forms.

New data: None

Advantages and Disadvantages - Animal Welfare:

Advantages

Intravenous injections induce rapid loss of consciousness with minimum of discomfort to animals. This effect depends on the dose, concentration, route, and rate of the injection. Barbiturates are less expensive than many other euthanasia agents.

Disadvantages

The restraint necessary for the administration can be distressing and requires well-trained and competent personnel.

Intraperitoneal injection takes longer to act and may cause pain and distress due to irritation. Commercially prepared ‘euthanasia solutions’ are very alkaline and cause irritation of the peritoneum and pain (seen as writhing) prior to unconsciousness. Dilution of sodium pentobarbitone and combining it with lignocaine has been found to reduce the severity of stress reactions in rats (Ambrose *et al.,* 2000).

Although intra-cardiac administration itself is not considered to be painful, penetration of needle through the skin, muscle and accidentally hitting a rib, or injection into the cardiac muscle (rather than into a ventricle or atrium) is painful in humans. In addition, accurate penetration of a heart chamber is not always successful on the first attempt and therefore the intra-cardiac route is not recommended except in unconscious animals.

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Time to lose consciousness:

Intravenous injection produces rapid loss of consciousness (a few seconds) with minimum of adverse reactions. Intraperitoneal injection may take several minutes 5-10min).

Consequences of inappropriate administration

Perivascular administration can be very irritant as can intraperitoneal injection.

4.8.5.7. Lethal injection of non-anaesthetising chemicals

There is very little new information for these groups of chemicals, so only a short summary will be given below with a general recommendation for all such chemicals. However, there may be some restriction on the availability of certain chemicals in some member states.

4.8.5.7.1. *Neuromuscular blocking agents*

Neuromuscular blocking agents and other agents that do not induce loss of consciousness prior to death are not to be used for euthanasia in conscious laboratory animals.

4.8.5.7.2. *Magnesium sulphate*

This has been used with or without sodium pentobarbitone at 80 mg/kg. It is a neuromuscular blocking agent and myocardial depressant, not a central nervous system depressant. Large volumes are required and the animals may exhibit muscle spasms, convulsive seizures, vocalization, gasping breaths and defecation before death. The animal remains conscious until the brain succumbs to anoxaemia. It lacks analgesic or anaesthetic effects and therefore causes more than minimal pain and distress.

4.8.5.7.3. *Potassium chloride*

KCl has a toxic effect on the heart muscle stopping it in diastole and it is sometimes administered intravenously to kill unconscious animals.

4.8.5.7.4. *Exposure to Hydrogen cyanide (HCN) gas*

Hydrogen cyanide gas blocks oxygen uptake by the red blood cells, causing respiratory difficulties and violent convulsions before the onset of unconsciousness and death. It is also very dangerous to the operator. It causes more than minimal pain and distress.

4.8.5.7.5. *Ketamine*

Large volumes would be necessary to kill animals. Convulsions and vocalisation occur in rabbits which makes it aesthetically unpleasant, but it may be used in combination with other agents that have rendered an animal unconscious.

4.8.5.7.6. *T-61*

Used in small vertebrates.

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Description of the method: Slow rate of intravenous injection, according to manufacturers instructions, induces unconscious and death.

Fetal and immature forms: Not appropriate.

New Data: None

Advantages and Disadvantages - Animal Welfare:

Advantages

Intravenous injection according to manufacturers' instructions induces rapid loss of consciousness and death with minimum of distress.

Disadvantages

Animal welfare may be compromised due to peri-vascular injection (high pH and so is irritant) or a too rapid injection rate as there has been concern that the drug may cause cessation of respiratory activity before the onset of unconsciousness.

Animals may need to be sedated prior to administration of T-61.

Time to lose consciousness:

Rapid when administered intravenously according to the rate as prescribed by the manufacturer.

Consequences of inappropriate administration:

Perivascular administration of T61 is reported to be painful in some animals (Close *et al.,* 1997). Inappropriate rate of administration could lead to potentially distressing respiratory arrest prior to loss of consciousness.

4.9. Humane killing of cephalopods, cyclostomes, decapods (if accepted)

Decapods include several kinds of crabs, lobsters and crayfish. Neither the number of crustaceans or cephalopods used in research is known, nor the methods of killing them. Although humane killing of crustaceans for food is not a statutory requirement in Europe, animal welfare organisations provide some guidelines, for example, UFAW, RSPCA, to humanely killing crustaceans. In some countries, for example New Zealand, humane killing of some species of crustaceans is covered under the Animal Welfare Act 1999.

4.9.1. Methods inducing the minimum level of pain and distres:

4.9.1.1. Chilling in air

Crustaceans are cold-blooded animals and therefore chilling them with an air temperature of 4C or below induces a state of torpor. Sufficient reduction of their body temperature by air chilling renders them unconscious and insensible, however, they will have to be killed by splitting or spiking to destroy their nervous

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system. Splitting involves cutting through the midline of the head and thorax where the nervous system is located in some species. Spiking involves insertion of a knife into the head, especially crabs, to destroy the main nerve centres. However, spiking is not an appropriate method of killing lobsters as they have several nerve centres.

The length of time required to rendering crustaceans unconscious by air chilling will vary, for example, according to species, size and metabolic state of animals and the rate of chilling. Chilling in air temperatures below 15C for a minimum of 30 min will result in eventual death of crustaceans.

4.9.1.2. Chilling in ice/water slurry

Tropical species of marine crustaceans that are susceptible to cold temperatures may be rendered unconscious by this method. The length of immersion time required to inducing unconsciousness will vary according to species and size, but as a guide crustaceans will have to be immersed in ice slurry for a minimum of 20 min before being killed. The ratio of ice to water should be 3:1 and the temperature should be maintained at or below minus 1C until the animals are rendered unconscious.

Temperate species of crustaceans should not be chilled in this way, as their welfare will be adversely affected by osmotic shock caused by the drop in salinity of the water by dilution with melting ice. However, proper control of salinity of the water would help to overcome this potential welfare problem.

4.9.1.3. Immersion in a clove oil bath

Immersion in a clove oil bath has been found to be an effective and humane method of killing crustacean, especially crabs. Eugenol (4-allyl-2-methoxy-phenol), the active compound made up 90 - 95% in clove oil, has only recently been considered as a compound for anaesthesia in fish. Clove oil has been used in fish at concentrations of 25-100 mg/L, depending on species and degree of anaesthesia.

4.9.1.4. Electrical methods

A prototype electrical stunner has been developed recently (known as Crustastun; UTEK-Pax Ltd., Berkhamstead, UK) to stun or kill lobsters, crabs, crayfish and other crustaceans (<www.uteck.co.uk> for details). This process involves application of a 100V electric current through the body of crustaceans and the duration of current application can be varied to cause a stun or stun/kill. Reports suggest that a monophasic pulsed direct current is more effective than a biphasic alternating current but this needs further investigation.

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4.9.2. Methods likely to cause pain and distress

4.9.2.1. Any procedure involving the separation of the abdomen (tailpiece) from the thorax (tailing) or removal of tissue, flesh or limbs while the crustacean is still alive and fully conscious (including when in a chilled state).

4.9.2.2. Placing crustaceans in cold water and heating the water to boiling point.

4.9.2.3. Placing live crustaceans into hot or boiling water.

4.9.2.4. Placing live marine crustaceans in fresh water as they die from severe osmotic shock.

4.9.2.5. Unfocussed microwaves to the body as opposed to focal application to the the head.

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4.10. The following Tables (8-15) give the recommended methods for the humane killing of animals in the laboratory.

Adapted and modified Tables from Close et al. (1996/1997)

The following tables have been taken from the previous EU Report on euthanasia, and form the basis for methods of killing laboratory animals that involve a minimum level of pain and distress. The data have been largely retained and only a few recommendations have been changed.

Table 8 - Characteristics of methods for euthanasia of fish

Agent	Rapidity	Efficacy	Ease of use	Operator safety	Aesthetic value	Overall rating (1-5)	Remarks
MS-222	++	++	++	++	++	5	Acceptable
Benzocaine	++	++	++	++	++	5	Acceptable
Etomidate	++	++	++	++	++	5	Acceptable
Metomidate	++	++	++	++	++	5	Acceptable
Electrical	++	+	+	+	++	4	Acceptable for some species
Maceration	++	++	++	++	+	4	Only for fish less than 2 cm in length
Quinaldine	++	++	++	+	++	4	Difficult to obtain in Europe
Concussion	++	+	+	++	-	3*	Death to be confirmed Acceptable for by experienced personnel
Sodium pentobarbitone	++	++	-	+	++	3	May be useful for large fish, intraperitoneal injection
Cervical dislocation	++	++	+	++	-	3	Not in large fish. To be followed by destruction of the brain
Halothane	+	+	++	++	++	2	Other methods preferable. Death to be confirmed

Changed from Close et al. * was 4

The following methods may only be used on unconscious fish: pithing, decapitation and exsanguinations

The following methods are not to be used for killing fish: removal from water, whole body crushing, hypothermia, hyperthermia, 2-phenoxyethanol, carbon dioxide, diethyl ether, secobarbital, amobarbital, urethane, chloral hydrate, tertiary amyl alcohol, tribromoethanol, chlorobutanol, methyl pentynol, pyridines, electrical stunning only for some species.

Rapidity: ++ very rapid, + rapid, - slow. **Efficacy:** ++ very effective, + effective, - not effective. **Ease of use:** ++ easy to use, + requires expertise, - requires specialist training. **Operator safety:** ++ no danger, + little danger, - dangerous. **Aesthetic value**: ++ good aesthetically, +acceptable for most people, - unacceptable for many people**.** **Rating:** 1-5 with 5 as highly recommended

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Table 9 - Characteristics of methods for euthanasia of amphibians

Agent	Rapidity	Efficacy	Ease of use	Operator safety	Aesthetic value	Overall rating (1-5)	Remarks
MS-222	++	++	++	++	++	5	Acceptable
Benzocaine	++	++	++	++	++	5	Acceptable
Sodium pentobarbitone	+	++	-	+	+	4	Involves handling and intravenous or intraperitoneal injection
Concussion	++	++	+	++	-*	3 **	Acceptable for use by experienced personnel
T-61	+	++	-	+	+	3	Involves handling and intravenous injection
Microwave	++	++	-	+	++	3	Only for small amphibians Not a routine procedure
Electrical stunning	+	+	+	-	-	2	To be followed immediately by destruction of the brain

Changed from Close et al. * was +, ** was 4

The following methods are only to be used on unconscious amphibians: pithing and decapitation

The following methods are not to be used for killing amphibians: hypothermia, hyperthermia, exsanguination, strangulation, carbon dioxide, diethyl ether, chloroform, volatile inhalational anaesthetics, chloral hydrate, ketamine hydrochloride, chlorbutanol, methylpentynol, 2-phenoxyethanol, tertiary amyl alcohol, tribromoethanol and urethane

Rapidity: ++ very rapid, + rapid, - slow. **Efficacy:** ++ very effective, + effective, - not effective. **Ease of use:** ++ easy to use, + requires expertise, - requires specialist training. **Operator safety:** ++ no danger, + little danger, - dangerous. **Aesthetic value**: ++ good aesthetically, +acceptable for most people, - unacceptable for many people**.** **Rating:** 1-5 with 5 as highly recommended

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Table 10 - Characteristics of methods for euthanasia of reptiles

Agent	Rapidity	Efficacy	Ease of use	Operator safety	Aesthetic value	Overall rating (1-5)	Remarks
Sodium pentobarbitone	++	++	++	+	++	5	Acceptable, but involves handling
Captive bolt	++	++	++	+	+	5	Acceptable for large reptiles
Shooting	++	++	++	-	+	4	Acceptable only in field conditions
Concussion	+	+	+	++	-*	3**	Acceptable for use by experienced personnel To be followed by destruction of the brain

Changed from Close et al. * was +; was 4

The following methods are to be used on unconscious reptiles only: pithing and decapitation

The following methods are to be used on unconscious reptiles only: pithing and decapitation The following methods are not to be used for killing reptiles: spinal cord severance, hypothermia, hyperthermia, exsanguination, chloroform, MS-222, ether, halothane, methoxyflurane, isoflurane, enflurane, carbon dioxide, neuromuscular blocking agents, ketamine hydrochloride, chloral hydrate and procaine

Rapidity: ++ very rapid, + rapid, - slow. **Efficacy:** ++ very effective, + effective, - not effective. **Ease of use:** ++ easy to use, + requires expertise, - requires specialist training. **Operator safety:** ++ no danger, + little danger, - dangerous. **Aesthetic value**: ++ good aesthetically, +acceptable for most people, - unacceptable for many people**.** **Rating:** 1-5 with 5 as highly recommended

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Table 11 - Characteristics of methods for euthanasia of birds

Agent	Rapidity	Efficacy	Ease of use	Operator safety	Aesthetic value	Overall rating (1-5)	Remarks
Sodium pentobarbitone	++	++	+	+	++	5	Acceptable
T-61	++	++	+	+	++	4	Requires expertise: acceptable for small birds only (<250 g)
Inert gases (Ar, N2)	++	++	++	++	+	4	Acceptable. But more research needed for nitrogen
Halothane, enflurane, isoflurane	++	++	++	+	++	4	Acceptable
Maceration	++	++	++	++	-	4	Acceptable for chicks up to 72 h
*Cervical dislocation decapitation	++	++	-	++	- *	3 **	Acceptable for small and young birds (<250 g) if followed by destruction of the brain
Microwave	++	++	-	++	+	3	To be used by experienced personnel only and specific equipment. Not a routine procedure
Concussion	++	++	-	++	-	3	Acceptable
Electrocution	++	++	+	-	-	3	Danger to operator. Use of special equipment Other methods Preferable
Carbon monoxide	+	+	++	-	-	1	Danger to operator

Changed from Close et al. * was +; was 4

The following methods may only be used on unconscious birds: decapitation, pithing, nitrogen, potassium chloride.

The following methods are not to be used for killing birds: neck crushing, decompression, exsanguination, carbon dioxide, nitrous oxide, diethyl ether, chloroform, cyclopropane, hydrogen cyanide gas, trichlorethylene, methoxyflurane, chloral hydrate, strychnine, nicotine, magnesium sulphate, ketamine and neuromuscular blocking agents

Rapidity: ++ very rapid, + rapid, - slow. **Efficacy:** ++ very effective, + effective, - not effective. **Ease of use:** ++ easy to use, + requires expertise, - requires specialist training. **Operator safety:** ++ no danger, + little danger, - dangerous. **Aesthetic value**: ++ good aesthetically, +acceptable for most people, - unacceptable for many people**.** **Rating:** 1-5 with 5 as highly recommended

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Table 12 - Characteristics of methods for euthanasia of rodents

Agent	Rapidity	Efficacy	Ease of use	Operator safety	Aesthetic value	Overall rating (1-5)	Remarks
Halothane, enflurane, isoflurane	++	++	++	+	++	5	Acceptable
Sodium pentobarbitone	++	++	+	+	++	5	Acceptable
T-61	++	++	-	+	++	4	Only to be injected intravenously
*Inert gases (Ar)	++	+	++	+	+	4	Acceptable
Concussion	++	++	+	++	-	3	Other methods preferred; Acceptable for rodents under 1 kg. Death to be confirmed by cessation of circulation
Cervical dislocation	++	++	+	++	-	3	Other methods preferred; Acceptable for rodents under 150g Death to be confirmed by cessation of circulation
Microwave	++	++	-	++	+	3	To be used by experienced personnel only. Not a routine procedure
Decapitation	+	+	+	++	-	2	Other methods preferred
*Carbon dioxide	+	++	++	+	++	1 if sole agent 5 if animal unconscious	To be used when animal uncouscious i.e. overall rating then based on the method to induce unconsciouness
Carbon monoxide	+	+	+	-	++	1	Danger to operator
Rapid freezing	-	+	++	++	-	0	Not acceptable

** Changed from Close et al.*

The following methods may only be used on unconscious rodents: rapid freezing, exsanguination, air embolism, potassium chloride and ethanol

The following methods are not to be used for killing rodents: carbon dioxide (when sole agent, but urgent research need for a replacement), hypothermia, decompression, strangulation, asphyxiation, drowning, nitrogen, nitrous oxide, cyclopropane, diethyl ether, chloroform, methoxyflurane, hydrogen cyanide gas, trichlorethylene, strychnine, nicotine, chloral hydrate, magnesium sulphate and neuromuscular blocking agents

Rapidity: ++ very rapid, + rapid, - slow. **Efficacy:** ++ very effective, + effective, - not effective. **Ease of use:** ++ easy to use, + requires expertise, - requires specialist training. **Operator safety:** ++ no danger, + little danger, - dangerous. **Aesthetic value**: ++ good aesthetically, +acceptable for most people, - unacceptable for many people**.** **Rating:** 1-5 with 5 as highly recommended

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Table 13 - Characteristics of methods for euthanasia of rabbits

Agent	Rapidity	Efficacy	Ease of use	Operator safety	Aesthetic value	Overall rating (1-5)	Remarks
Sodium pentobarbitone	++	++	++	+	++	5	Acceptable
T-61	++	++	-	+	++	4	Acceptable. Intravenous injection only
Captive bolt	++	++	-	+	+	4	Requires skill. Death to be confirmed by another method
Cervical dislocation	++	++	-	++	-	3	Acceptable for rabbits under 1 kg. Sedation prior to dislocation. Death to be confirmed by cessation of circulation
Concussion	++	+	-	++	-	3	Expertise required. Death to be ensured by another method
Electrical stunning	++	+	++	-	+	3	Death to be confirmed by another method
Microwave	++	++	-	++	+	3	To be used by experienced personnel only on small rabbits. Not a routine procedure
Decapitation	+	+	+	-	-	2	Acceptable for rabbits under 1 kg if other methods not possible
Halothane, enflurane, isoflurane	++	++	++	+	-	2	Rabbits show signs of distress
Carbon monoxide	+	+	++	-	++	1	Danger to operator
Rapid freezing	+	+	++	++	+	1	Only in fetuses under 4 kg. Other methods preferred

Changed from Close et al.: CO2 deleted

The following methods are only to be used on unconscious rabbits: exsanguination, nitrogen, potassium chloride and air embolism.

The following methods are not to be used for killing rabbits: carbon dioxide, hypothermia, decompression, strangulation, asphyxiation, drowning, nitrous oxide, cyclopropane, diethyl ether, chloroform, trichlorethylene, hydrogen cyanide gas, methoxyflurane, chloral hydrate, strychnine, nicotine, magnesium sulphate, hydrocyanic acid, ketamine hydrochloride and neuro-muscular blocking agents.

Rapidity: ++ very rapid, + rapid, - slow. **Efficacy:** ++ very effective, + effective, - not effective. **Ease of use:** ++ easy to use, + requires expertise, - requires specialist training. **Operator safety:** ++ no danger, + little danger, - dangerous. **Aesthetic value**: ++ good aesthetically, +acceptable for most people, - unacceptable for many people**.** **Rating:** 1-5 with 5 as highly recommended

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Table 14 - Characteristics of methods for euthanasia of dogs, cats, ferrets, foxes

Agent	Rapidity	Efficacy	Ease of use	Operator safety	Aesthetic value	Overall rating (1-5)	Remarks
Sodium pentobarbitone	++	++	-	+	++	5	Acceptable. Intravenous injection
T-61	++	++	-	+	+	4	Acceptable but only by slow intravenous Injection under sedation
Secobarbital/ dibucaine	++	++	-	+	++	4	Acceptable. Intravenous injection
Halothane, isoflurane,enflurane	++	++	+	+	++	4	Acceptable
*Shooting with a free bullet with appropriate rifles and guns.	++	++	-	-	-	4*	Acceptable only in field conditions by specialized marksmen when other methods not possible
Captive bolt	++	++	-	++	+	3	To be followed by exsanguination
Electrocution	++	++	-	-	-	3	Use only special equipment.To be followed by exsanguination
Concussion	++	++	+	++	-	2	Only to be used on neonates.To be followed by exsanguination

Changed from Close et al. * was 1

The following methods can be used for unconscious carnivores: exsanguination, neck dislocation and potassium chloride, in order to minimise pain and distress.

The following methods are not to be used for killing carnivores: decompression, decapitation, drowning, strangulation, asphyxiation, inert gases, nitrogen, air embolism, striking chest of cats, carbonmonoxide, carbon dioxide, methoxyflurane, nitrous oxide, trichlorethylene, hydrocyanic acid, diethyl ether, chloroform, hydrogen cyanide gas, cyclopropane, chloral hydrate, strychnine, nicotine, magnesium sulphate and neuromuscular blocking agents

Rapidity: ++ very rapid, + rapid, - slow. **Efficacy:** ++ very effective, + effective, - not effective. **Ease of use:** ++ easy to use, + requires expertise, - requires specialist training. **Operator safety:** ++ no danger, + little danger, - dangerous. **Aesthetic value**: ++ good aesthetically, +acceptable for most people, - unacceptable for many people**.** **Rating:** 1-5 with 5 as highly recommended

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Table 15 - Characteristics of methods for euthanasia of large mammals

Agent	Rapidity	Efficacy	Ease of use	Operator safety	Aesthetic value	Overall rating (1-5)	Remarks
Sodium pentobarbitone	++	++	-	+	++	5	Acceptable by intravenous injection (all species including primates)
Quinalbarbitone/ Nupercaine	++	++	-	+	++	5	Effective for horses intravenously
Captive bolt	++	++	+	+	+	5	To be followed by exsanguination
Free bullet using e.g. appropriate ammunition, rifles and gun	++	++	+	-	+	4*	Experienced marksman. May need a method to ensure death. In field conditions only.
T-61	++	++	-	+	++	4	Acceptable by intravenous injection
**Inert gases (Ar)	++	++	+	+	+	4	Acceptable for pigs only
Electrical stunning	++	++	+	-	-	4	Use only specialised equipment. To be followed immediately by exsanguination
Concussion	++	+	-	+	+	2	To be followed immediately by exsanguination
Halothane, isoflurane, enflurane	+	+	+	+	+	2	Recommended for lambs and kids

Changed from Close et al. CO2 deleted, * was 5, ** introduced, CO2 deleted

The following methods can be used only on unconscious large mammals: exsanguination, chloral hydrate and potassium chloride, in order to minimise pain and distress.

The following methods are not to be used for killing large mammals: carbon dioxide, carbon monoxide, methoxyflurane, trichlorethylene, strychnine, nicotine, magnesium sulphate, thiopentone sodium, ketamine hydrochloride, neuromuscular blocking agents

Rapidity: ++ very rapid, + rapid, - slow. **Efficacy:** ++ very effective, + effective, - not effective. **Ease of use:** ++ easy to use, + requires expertise, - requires specialist training. **Operator safety:** ++ no danger, + little danger, - dangerous. **Aesthetic value**: ++ good aesthetically, +acceptable for most people, - unacceptable for many people**.** **Rating:** 1-5 with 5 as highly recommended

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5. ACKNOWLEDGEMENTS

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Questions 1&2 - Chairman Prof. Donald Broom**:** Dr Chris Sherwin, Prof. Neville Gregory and Dr Roddy Williamson

Question 3 – Chairman Dr Xavier Manteca: Prof Stefano Cinotti, Dr David Anderson and Prof. Timo Nevalainen

Question 4 - Chairman Prof David Morton: Dr Mohan Raj and Dr Bert Lambooij

The declarations of conflicts of interest of all participants in this working group are available on internet, in the EFSA web site (http://www.efsa.eu.int)

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